Aberrant expression of PD-1 on B cells and their subpopulations in systemic lupus erythematosus and association with clinical parameters

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Abstract

Background: The binding of programmed death 1 (PD-1) with its ligands inhibits the T cell activation and proliferation. But role of the PD-1 pathway on B cells is unclear. In present study, we aimed to evaluate the expression of PD-1 on B cells and their subpopulations and association with clinical parameters in systemic lupus erythematosus (SLE).

Results: The frequency of B cells increased significantly in patients with active SLE compared with healthy controls and patients with inactive SLE. The proportions of CD19+ IgD- CD27- cells and plasmablast cell among total B cells were significantly higher in patients with SLE compared with controls. The percentage of PD-1+ B cells was higher in patients with active SLE than in healthy controls. The proportion of PD-1+ B cells was correlated with lupus nephritis, complement components, IgG, SLE Disease Activity Index, and autoantibodies. PD-1+ B cells from SLE showed a high proliferative response. The levels of IgG and anti-dsDNA secreted by PD-1+ B cells from SLE patients was higher after 7 days compared with that by PD-1- B cells from patients with SLE and healthy controls.

Conclusions: The expression of PD-1 on B cells and their subpopulations was aberrant and was associated with clinical parameters in SLE.

KEY WORDS: PD-1; B cells; subpopulation; systemic lupus erythematosus

Background

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease triggered by the breakdown of self and non-self discrimination by the immune system and the resultant immunological abnormalities and excessive inflammatory reactions in a wide range of organs. The etiology and pathogenesis of SLE are not fully understood. Nevertheless, it is well known that T and B cells are involved in the development of SLE.

Recent studies demonstrated that programmed death 1 (PD-1) and its ligands, PD-L1 and PD-L2, are involved in the process of T-cell activation, tolerance and immune-mediated tissue damage. In addition, increasing evidence indicates that the PD-1/PD-L1/2 axis is crucial in preventing autoimmune disorders. Experimental models of autoimmunity demonstrated that the altered
function of PD-1 and its ligands is associated with several human autoimmune conditions\textsuperscript{12–14}. Multiple studies have implicated the PD-1/PD-L1 pathway in immune system homeostasis and a variety of autoimmune diseases\textsuperscript{15–17}. Blockade with anti-PD-L1 or anti-PD-1 in mice models of SLE has been shown to ameliorate lupus-like symptoms by decreasing the inhibition of T cells\textsuperscript{17–19}. PD-L1-Ig treatment in New Zealand F1 mice significantly delayed the onset of proteinuria and prolonged their lifetime\textsuperscript{20}. Nevertheless, the expression of PD-1/PD-L1 in SLE still remains unclear.

It has been reported that the proportion of IgDCD27\textsuperscript{+} class-switched memory B cells were found to be significantly high in patients with SLE, antigen-experienced, and resistant to immunosuppressive therapy\textsuperscript{2, 21}. Of note, the peripheral memory B-cell receptor (BCR) repertoire in SLE is formed by abnormal selection, which deteriorates somatic hypermutation and increases receptor editing\textsuperscript{22}. These memory B cells have lower activation thresholds, leading to a higher risk of autoimmunity. Moreover, these cells can be rapidly activated in a non-antigen-specific way by linking with Toll-like receptor agonists, B-cell-activating factor, or a combination of cytokines\textsuperscript{23}. Another memory B-cell subset particularly high in patients with SLE has been identified and is composed of B cells that do not express CD27\textsuperscript{24}. Although IgDCD27 B cells expressing mutated BCRs have been detected in the tonsils and peripheral blood of healthy donors\textsuperscript{25, 26}, a large number of IgDCD27 memory B cells were found in the peripheral blood of patients with SLE, and the highest levels were associated with high disease activity and active renal disease\textsuperscript{27}. Nevertheless, the relation between PD-1/PD-L1 and B cells in SLE is still not fully understood.

Therefore, the present study aimed to explore the importance of the expression of PD-1 on B cells in SLE.

**Methods**

**Patients**

SLE was diagnosed according to the 1997 American College of Rheumatology revised criteria\textsuperscript{28}. A total of 74 patients with SLE and 54 healthy controls (all of Asian origin) were enrolled at the
Department of Rheumatology of the First Affiliated Hospital of Bengbu Medical College, China. Data including age, gender, blood cell counts, 24-h proteinuria excretion, anti-dsDNA antibody, anti-nucleosome antibody, anti-Smith antibody (anti-Sm antibody), anti-Sjogren syndrome A antibody (anti-SSA antibody), anti-Sjogren syndrome B antibody (anti-SSB) antibody, complement component 3 (C3), complement component 4 (C4), IgG, IgM, IgA, and erythrocyte sedimentation rate (ESR) were collected from the medical records. The disease activity was scored using the SLE Disease Activity Index (SLEDAI) and classified as inactive SLE (SLEDAI <10) and active SLE (SLEDAI ≥10).

**Flow cytometry**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood (3 mL) using Ficoll-Hypaque density gradient centrifugation. The gating strategy was based on previous studies. Then, FITC-anti-CD3, PE-anti-CD3, APC-anti-CD3, APC-Cy7-anti-CD19, FITC-anti-IgD, APC-anti-CD27, and PE-anti-PD–1 (all from Biolegend, 9727 Pacific Heights Blvd, San Diego, CA 92121, USA, 1:1000) were incubated with the PBMCs. The samples were assayed using a FACS Verse flow cytometer (BD Bioscience, San Jose, CA 95131, USA). The data were analyzed using the Flowjo software (Version X; Tree Star, Ashland, OR, USA).

Venous blood samples (20 mL) were collected from all subjects who were on an empty stomach in the morning. Peripheral blood mononuclear cells (PBMCs) from patients with SLE and healthy controls were isolated from heparinized blood using Ficoll–Hypaque density gradient centrifugation. And then CD19⁺PD–1⁺ or CD19⁺PD–1⁻ B cells were purified using flow cytometry (BD FACSaria II, BD Biosciences, 2350 Qume Drive, San Jose, CA 95131, 877.232.8995, USA) based on CD19 and PD-1 staining. The isolated cells were further evaluated using flow cytometric analysis with purity >95% for CD19⁺ PD–1⁺ or CD19⁺PD–1⁻ B cells.”

The isolated PD–1⁺ or PD–1⁻ B cells were first incubated with 5.0 M carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Waltham, MA, USA) in phosphate-buffered saline/0.1% bovine serum albumin at 37°C for 10 min. Then, 2 × 10⁴/wellPD–1⁺ or PD–1⁻ cells were cultured for 7 days in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum, 10
ng/ml of IL-2 and IL-10 (both from PeproTech Rocky Hill, NJ, USA), and stimulated with 2.5 µg/mL CpG2006 oligonucleotide (Invivogen, San Diego, CA, USA) in the presence or absence of 2.5 µg/ml goat F(ab)2 anti-human IgM. Afterwards, the cells were stained with anti-PD-1 for flow cytometry analysis.

**Enzyme linked immunosorbent assay (ELISA)**

The culture medium of the isolated PD-1⁺ or PD-1⁻ B cells was changed every 2 days, and the anti-dsDNA and IgG levels in the supernatant were tested at 1, 3, 5, and 7 days by ELISA (Biorbyt, San Francisco, CA, USA).

**Statistical analysis**

All data were presented as mean ± standard deviation. The significance of the differences between groups was analyzed using one-way analysis of variance, and that between two groups was evaluated using the two-tailed Student t test. The Spearman correlation coefficient or Pearson correlation coefficient with two-tailed P value was used to analyze correlations. A P value <0.05 was considered statistically significant. All data were analyzed using SPSS 16.0 (IBM, Armonk, NY, USA).

**Results**

**Alteration of B cells and their subpopulations in patients with SLE**

Table 1 presents the characteristics of the patients. Compared with the healthy controls, the CD19⁺ B-cell population was significantly higher in patients with SLE (P<0.05). The ratio of B cells was slightly higher in active SLE patients than in inactive SLE patients, but showed no statistical significance (Fig 1A-B). Next, we evaluated the frequency of B-cell subpopulations in patients with SLE. As shown in Fig 1C-D, the percentage of CD19⁺ IgD CD27 cells and CD19⁺ IgD CD27high cells (plasmablast cells) was significantly higher in patients with SLE compared with healthy controls. Furthermore, no correlations were found between the frequency of B cells and clinical manifestations or autoantibodies (Data not show), but the percentage of B cells was positively associated with SLEDAI and 24-h urinary protein, and negatively associated with the levels of C3 (Fig 1E). The percentage of plasmablast cells was positively associated with the levels of IgM and C3 (Table 2). The percentage of
switched memory cells was positively associated with SLEDAI and the levels of IgG, and negatively associated with the levels of IgM. Moreover, the percentage of non-switched memory B cells was positively associated with 24-h urinary protein and the levels of IgG, and negatively associated with the levels of IgM. The percentage of naïve B cells was negatively associated with SLEDAI and the levels of IgG. The results demonstrated that the proportion of switched memory and naïve B cells was increased in patients with positive anti-histones, anti-SSA52 and malar rash, and that the proportion of NSM B cells was elevated in patients with positive anti-SSB and anti-SSA52 (Table 3). The association between the percentages of PC, SM, NSM, DN, and N in B cells and other clinical manifestations and laboratory test parameters in SLE patients were not found (Table 3).

**Differential expression of PD–1 on B cells**

Previous studies indicated that PD-1 is an important co-inhibitory receptor in SLE. Therefore the expression of PD-1 on B cells was determined. As indicated in Fig. 2A-B, the percentage of CD19⁺ PD–1⁺ cells (PD–1⁺ B cells) was higher in patients with active SLE than in patients with inactive SLE and healthy controls. The percentage of CD19⁺ PD–1⁺ cells (PD–1⁺ B cells) was higher in patients with inactive SLE than in healthy controls. The expression levels of PD–1 on plasmablast cells, CD19⁺ IgD CD27⁺ (switched memory) cells, CD19⁺ IgD⁺ CD27⁺ (non-switched memory) cells, CD19⁺ IgD CD27 cells, and naïve cells were higher in patients with SLE (Fig. 2C-D). These findings indicated that the levels of PD–1 on B cells and their subpopulations were obviously higher in patients with SLE.

The correlation of PD–1-positive B cells with clinical data was assessed in patients with SLE. The percentage of PD–1⁺ B cells was found to be positively correlated with SLEDAI (Fig. 2E). Moreover, the levels of PD–1⁺ B cells were positively correlated with 24-h urinary protein and serum levels of IgG and inversely with IgM (Fig. 2E). The frequency of PD–1⁺ B cells was also higher in patients with anti-dsDNA (+) Abs \(P = 0.040\), anti-histone (+) Abs \(P = 0.025\), and anti-SSA52 (+) Abs \(P = 0.048\) (Table 4). The frequency of PD–1⁺ B cells was significantly higher in patients with lupus nephritis \(P <0.0001\) and oral ulcer \(P = 0.05\) (Table 4). No significant differences were observed between
hematological manifestations, arthritis, and serositis. The association of PD-1+ in B-cell subsets with clinical manifestations and auto-antibodies in SLE was found in patients with SLE (Table 5). The percentage of PD-1+ PC B cells was positively associated with SLEDAI, 24-h urinary protein, and the levels of IgG (Fig. 2F). The percentage of PD-1+ SM B cells was positively associated with SLEDAI and 24-h urinary protein. The percentage of PD-1+ NSM B cells was positively associated with the levels of IgG. The percentage of PD-1+ N B cells was positively associated with the levels of IgG. These findings indicated that PD-1+ B cells seemed to be associated with disease activity in SLE.

Function of PD-1+ B cells in vitro

The isolated B cells (PD-1+ or PD-1- B cells) were significantly stimulated with CpG DNA (Fig. 3A). PD-1+ B cells from patients with SLE obviously augmented after 7 days compared with PD-1B cells from patients with SLE and PD-1+ or PD-1- B cells from healthy controls in response to CpG DNA stimulation (Fig. 3B). The levels of anti-dsDNA secreted from PD-1+ or PD-1- B cells had no statistical significance at 1, 3, and 5 days among patients with SLE and controls (Fig. 3C), The levels of anti-dsDNA produced by PD-1+ B cells from SLE patients at 7 day were significantly higher compared with PD-1 B cells from patients with SLE or PD-1- B cells from healthy controls ($P < 0.01$, $P < 0.001$, respectively, Fig. 3C). Furthermore, the amount of IgG secreted by PD-1+ B cells from patients with SLE was higher after 7 days compared with that by PD-1 B cells from patients with SLE and PD-1+ or PD-1- B cells from healthy controls ($P = 0.0261$; Fig. 3D). These results showed that the abilities to proliferate and secrete IgG in PD-1+ B cells from SLE patients were high.

Discussion

The present study showed that the frequency of CD19+ B cells and the SM and DN subsets were higher in patients with active SLE, but no association was found between B cells in patients with SLE and clinical manifestations and experimental data. Furthermore, PD-1+ B cells in SLE had a good correlation with clinical data, suggesting that these cells might be associated with disease activity.
and activated B cells. *In vitro* cell stimulation and proliferation experiments further proved that these cells might be activated in SLE.

B cells are crucial in the initiation and development of SLE by producing autoantibodies, inflammatory cytokines, and presenting antigen. B-cell phenotypes and their subsets are changed in patients with SLE. The increase in CD19+ IgD CD27 (DN) B cells has been obviously associated with a higher disease activity index, SLE-related autoantibodies such as anti-dsDNA, and anti-Sm. The DN2 CD19+IgD−CD27−CXCR5− B cells were expanded in SLE patients. Kubo et al. found that the proportions of DN B cells and plasmablasts were higher in patients with SLE than in healthy controls, which was consistent with the present study. The percentages of DN B cells and plasmablasts in this study were found to be significantly higher in patients with SLE compared with healthy controls. The frequency of some B-cell subsets in SLE has been associated with autoantibodies and clinical manifestations. The present study also showed that some B-cell subsets in SLE were associated with autoantibodies and clinical manifestations. Although the proportion of CD19+B cells in PBMC in patients with SLE was elevated, this had no significant association with laboratory parameters and clinical manifestations.

The binding of PD–1 by its ligands inhibits T-cell activation and expansion by inhibiting TCR-induced activating effects and modulating T-regulatory cell suppression. CD8+ PD–1+ T cells were exhausted in mice with viral infection and in humans with chronic viral infection. But Petrelli et al. found that PD–1+CD8+ T cells were enriched at inflammation site and were antigen-experienced, augmented clonal expanding effector T cells. Nevertheless, CD4+ PD–1+ T cells are pro-inflammatory cells and secrete high levels of interferon-γ in NZB/W F1 mice. A subpopulation of PD–1+CD4+ T cells were found to expand in lupus blood and provide B cell help by interleukin –10. PD–1 inhibits the phagocytic potency against tumor cells in tumor-associated macrophages. Therefore, different subsets of T cells and macrophages with PD–1 expression have distinct functions in different diseases. PD–1 is also expressed on human B cells and is differentially expressed on B-cell subpopulations.
The inhibition of PD-1 pathways leads to the increase in activation and proliferation of B cells and the production of inflammatory cytokines\(^4^3\). The disruption of the PD-1 pathways significantly increases the proliferation and production of IgG by pneumococcal capsule-specific B cells\(^4^4\). These studies demonstrated that the PD-1 signaling pathway have an impact on the function of B cells.

Nevertheless, little is known about the effect of PD-1 on B cells in SLE. The present study revealed that the proportion of CD19\(^{+}\) PD-1\(^{+}\) B cells in patients with active or inactive SLE was high. Moreover, the frequency of CD19\(^{+}\) PD-1\(^{+}\) B cells was correlated with SLEDAI, IgG, C3, lupus nephritis, and autoantibodies. In the present study, proliferation and levels of production of IgG and anti-dsDNA in CD19\(^{+}\) PD-1\(^{+}\) B cells were high in vitro. This study indicated that CD19\(^{+}\) PD-1\(^{+}\) B cells might be activated and secreted antibodies in patients with SLE, but this is in contradiction to a previous study\(^4^5\) that demonstrated that PD-1 restrained B cell activation. This might be explained by: 1) B cells were activated by some factors in SLE and PD-1 was a marker of these abnormally activated B cells; 2) the expression of PD-1 on activated B cells was increased in SLE, and then PD-1 signal inhibited further activation of B cells by negative feedback.

PD-1 was differentially expressed on human B subpopulations. The expression of PD-1 on IgM memory (IgD\(^{+}\) CD27\(^{+}\)) B cells was higher than that on naïve and SM B cells\(^4^3\). In this study, all B-cell subsets showed a higher expression of PD-1 in SLE patients compared with healthy controls, but only the expression of PD-1 on some of the B-cell subpopulations was associated with autoantibodies and some laboratory parameters. The reason why the expression of PD-1 is increase in some B-cell subpopulations remains to be determined, as well as the function of these PD-1-expressing B cell subsets.

In summary, this study showed that B cells and their subgroups were abnormal in patients with SLE. The expression of PD-1 on B cells and their subtypes was found to be altered in these patients. CD19\(^{+}\)PD-1\(^{+}\) B cells in these patients had a higher potency to produce anti-dsDNA or IgG.

Nevertheless, this study had some limitations. First, the interaction of T cells and CD19\(^{+}\)PD-1\(^{+}\) B cells
was not investigated. Secondly, the molecular mechanisms underlying the dysregulation of PD-1 pathway in CD19^+PD-1^+ B cells were not investigated.

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| C3           | complement 3 |
| C4           | complement 4 |
| dsDNA        | double-stranded DNA |
| IgM          | immunoglobulin M |
| IgA          | immunoglobulin A |
| IgG          | immunoglobulin G |
| LN           | lupus nephritis |
| N            | naive |
| NSM          | non-switched memory |
| PC           | plasmablast cells |
| PD-1         | programmed death 1 |
| PD-L1        | programmed death ligand 1 |
| S^m           | smith |
| SSA          | Sjögren syndrome antigen A |
| SLE          | systemic lupus erythematosus |
| SLEDAI       | systemic lupus erythematosus disease activity index |
| SM           | switched memory |
| SSB          | Sjögren syndrome antigen B |
| U1snRNP      | U1 small nuclear ribonucleoprotein |

Declarations

Availability of data and materials

The data are owned by Changhao Xie. All data are available from the corresponding author on reasonable request.

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Conflict interest

The authors declare no financial interests.

Ethical approval and informed consent

All participants provided informed written consent. This study was approved by the institutional
review board of the First Affiliated Hospital of Bengbu Medical College.

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Tables

Table 1. Characteristics of patients with SLE and healthy controls (mean ± SD, %).

| Characteristic                | SLE          | Control      | P value |
|------------------------------|--------------|--------------|---------|
| Cases                        | *N* = 74     | *N* = 54     |         |
| Number of males/females      | 3/71         | 3/51         | NS      |
| Age (year)                   | 31.42 ± 12.02| 27.1 ± 8.1   | NS      |
| Disease duration (month)     | 49.13 ± 9.331| -            |         |

Clinical features

- Lupus nephritis: 48%
- Raynaud’s phenomenon: 27%
- Malar rash: 32%
- Fever: 47%
- Oral ulcer: 16%
- Arthritis: 35%
- Serositis: 32%
- Neurological disorder: 18%
- Interstitial lung: 17%

Laboratory findings

- Anti-dsDNA (+): 54%
- Anti-SmD1 (+): 60%
- Anti-U1snRNP (+): 47%
- Anti-SSA60 (+): 55%
- Anti-SSA52 (+): 27%
- Anti-SSB (+): 25%
- C3↓: 78%
- C4↓: 68%
- IgA↑: 60%
- IgG↑: 59%

Except where indicated otherwise, values are number (%) of patients.

NS, No significance; SLE, systemic lupus erythematosus. Anti-SmD1, Anti-Smith D1; Anti-dsDNA, Anti-double-stranded DNA; Anti-U1snRNP, Anti-U1 small nuclear ribonucleoprotein; Anti-SSA, Anti-Sjögren syndrome antigen A; Anyi-SSB, Anti-Sjögren syndrome antigen B; C3/C4, complement component 3/4; IgG/IgM/IgA, immunoglobulin G/M/A.

Table 2. Correlation between frequencies of PC, SM, NSM, DN, or N cells in B cells with SLEDAI or laboratory findings in patients with SLE.

| Laboratory test parameters | SLEDAI     | Cases | PC B cells | SM B cells | NSM B cells | DN B cells | N B cell |
|----------------------------|------------|-------|------------|------------|-------------|------------|----------|
| Amounts of proteinuria (g/24 h) | SLEDAI     | 74    | 0.03501    | 0.08304*   | 0.01213     | 0.00436    | -0.0845  |
| IgG (g/L)                  | SLEDAI     | 69    | -0.01255   | 0.01846    | 0.09926*    | -0.02297   | -0.0319  |
| IgA (g/L)                  | SLEDAI     | 74    | 0.02445    | 0.07461*   | 0.08158*    | 0.05188    | -0.0207  |
| IgM (g/L)                  | SLEDAI     | 74    | 0.06296    | 0.03587    | 0.1280      | -0.1827    | -0.0400  |
| C3 (g/L)                   | SLEDAI     | 74    | 0.09914*   | -0.02337*  | -0.07387*   | 0.005707   | -0.0222  |
| C4 (g/L)                   | SLEDAI     | 74    | 0.1162*    | 0.006449   | 0.03364     | 0.001436   | -0.0676  |

C3/C4, Complement component 3/4; DN, double negative; IgG/A/M, immunoglobulin G/A/M; N, naïve;
NSM, non-switched memory; PC, plasmablast cells; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index; SM, switched memory.

*P 0.05.

Table 3. Association between the percentages of PC, SM, NSM, DN, and N in B cells and clinical manifestations and laboratory test parameters in patients with SLE (mean ± SD, %).

| Parameters     | Cases | PC B cells (%) | P value | SM B cells (%) | P value | NSM B cell (%) |
|----------------|-------|----------------|---------|----------------|---------|----------------|
| Anti-dsDNA     | + 44  | 7.799 ±0.9774  | 0.300   | 19.54 ±1.208   | 0.896   | 16.43 ±2.100   |
|                | - 30  | 6.449 ±0.626   |         | 19.88 ±2.527   |         | 14.97 ±2.196   |
| Anti-histones  | + 26  | 8.536 ±1.167   | 0.138   | 24.15 ±2.613   | 0.007   | 17.52 ±3.652   |
|                | - 48  | 6.556 ±0.739   |         | 17.26 ±1.169   |         | 14.92 ±1.297   |
| Anti-smD1      | + 45  | 7.919 ±0.929   | 0.193   | 20.32±1.549    | 0.522   | 16.31 ±2.045   |
|                | - 29  | 6.216 ±0.718   |         | 18.68 ±2.082   |         | 15.10 ±2.293   |
| Anti-U1snRNP   | + 35  | 7.856 ±1.176   | 0.371   | 20.30 ±1.854   | 0.641   | 14.95 ±1.662   |
|                | - 39  | 6.709 ±0.5865  |         | 19.13 ±1.684   |         | 16.63 ±2.497   |
| Anti-nucleo    | + 35  | 7.625 ±0.954   | 0.571   | 18.42 ±1.839   | 0.326   | 14.12 ±2.425   |
|                | - 39  | 6.898 ±0.853   |         | 20.87 ±1.675   |         | 17.46 ±1.879   |
| Anti-SSA60     | + 41  | 7.151 ±0.679   | 0.832   | 21.38 ±1.710   | 0.062   | 16.74 ±2.194   |
|                | - 33  | 7.437 ±1.323   |         | 16.54 ±1.428   |         | 14.17 ±1.570   |
| Anti-SSA52     | + 20  | 7.444±1.179    | 0.856   | 24.30 ±3.274   | 0.022   | 23.26 ±4.333   |
|                | - 54  | 7.180 ±0.759   |         | 17.9 ±1.131    |         | 13.09 ±1.172   |
| Anti-SSB       | + 15  | 6.865 ±1.446   | 0.761   | 21.24 ±3.548   | 0.530   | 22.85 ±5.868   |
|                | - 59  | 7.350 ±0.713   |         | 19.28 ±1.283   |         | 14.05 ±1.137   |
| Anti-P0        | + 30  | 7.653 ±1.066   | 0.605   | 17.26 ±1.560   | 0.108   | 12.82 ±1.146   |
|                | - 44  | 6.978 ±0.791   |         | 21.33 ±1.767   |         | 17.90 ±2.408   |
| Condition                  | Yes | No  | Mean (±SD)       | Mean (±SD)       | Mean (±SD)       | Mean (±SD)       |
|----------------------------|-----|-----|------------------|------------------|------------------|------------------|
| C3↓                        | Yes | 58  | 6.754 ±0.629     | 4.655 ±3.125     | 8.20   ±1.900    | 5.580 ±1.567     |
|                           | No  | 16  | 9.055 ±1.831     | 15.184 ±2.568    | 7.580 ±1.567     | 15.89 ±1.641     |
| C4↓                        | Yes | 51  | 7.002 ±0.696     | 20.67 ±1.651     | 18.38 ±3.407     | 14.69 ±1.595     |
|                           | No  | 23  | 7.806 ±1.358     | 17.48 ±1.543     | 19.81 ±1.543     | 15.38 ±3.047     |
| IgA↑                       | Yes | 45  | 8.055 ±0.912     | 19.95 ±1.570     | 16.14 ±1.960     | 14.74 ±1.960     |
|                           | No  | 29  | 6.005 ±0.751     | 19.26 ±2.055     | 17.54 ±2.440     | 14.07 ±1.681     |
| IgG↑                       | Yes | 44  | 7.766 ±0.9582    | 19.54 ±1.545     | 14.74 ±1.960     | 15.50 ±2.814     |
|                           | No  | 30  | 6.538 ±0.730     | 19.87 ±2.076     | 18.29 ±2.774     | 16.62 ±1.917     |
| Lymphopenia (<1.5 × 10⁹ L⁻¹) | Yes | 52  | 7.637 ±0.743     | 20.86 ±1.629     | 15.98 ±2.427     | 13.98 ±1.917     |
|                           | No  | 22  | 6.340 ±1.221     | 16.89 ±1.502     | 15.98 ±2.427     | 13.98 ±1.917     |
| Thrombocytopenia (<100 × 10⁹ L⁻¹) | Yes | 28  | 7.591 ±1.174     | 17.01 ±1.524     | 15.50 ±2.814     | 15.78 ±1.883     |
|                           | No  | 46  | 7.097 ±0.755     | 21.23 ±1.774     | 15.81 ±1.807     | 17.65 ±2.414     |
| LN                         | Yes | 36  | 6.569 ±0.657     | 20.07 ±2.042     | 14.12 ±1.886     | 12.44 ±1.317     |
|                           | No  | 38  | 7.898 ±1.067     | 19.31 ±1.474     | 17.10 ±2.013     | 15.96 ±2.656     |
| Raynaud’s phenomenon       | Yes | 20  | 7.666 ±1.343     | 23.49 ±2.857     | 15.78 ±1.883     | 15.78 ±1.883     |
|                           | No  | 54  | 7.098 ±0.721     | 18.27 ±1.298     | 12.44 ±1.317     | 15.96 ±2.656     |
| Malar rash                 | Yes | 24  | 9.554 ±1.404     | 25.54 ±2.717     | 15.78 ±1.883     | 15.78 ±1.883     |
|                           | No  | 50  | 6.146 ±0.607     | 16.87 ±1.111     | 15.78 ±1.883     | 15.78 ±1.883     |
| Fever                      | Yes | 35  | 7.655 ±0.949     | 19.129 ±1.798    | 13.78 ±1.909     | 17.68 ±2.330     |
|                           | No  | 39  | 6.890 ±0.860     | 20.18 ±1.733     | 17.25 ±3.096     | 15.56 ±1.669     |
| Oral ulcer                 | Yes | 12  | 8.600 ±2.188     | 21.22 ±3.628     | 13.95 ±1.137     | 16.86 ±2.267     |
|                           | No  | 62  | 6.991 ±0.634     | 19.38 ±1.316     | 15.98 ±2.814     | 15.78 ±1.883     |
| Arthritis                  | Yes | 26  | 6.393 ±0.694     | 18.61 ±2.247     | 15.98 ±2.814     | 15.78 ±1.883     |
|                           | No  | 48  | 7.718 ±0.902     | 20.26 ±1.488     | 15.98 ±2.814     | 15.78 ±1.883     |
| Serositis                  | Yes | 18  | 6.234 ±1.018     | 18.57 ±2.465     | 11.14 ±1.003     | 17.35 ±1.953     |
|                           | No  | 56  | 7.579 ±0.772     | 20.04 ±1.445     | 17.35 ±1.953     | 15.38 ±1.953     |
| Neurological disorder      | Yes | 14  | 6.553 ±1.309     | 19.11 ±2.468     | 16.32 ±2.251     | 15.73 ±1.814     |
|                           | No  | 60  | 7.415 ±0.725     | 19.81 ±1.410     | 16.32 ±2.251     | 15.73 ±1.814     |
| Interstitial lung          | Yes | 13  | 6.985 ±1.018     | 20.25 ±2.373     | 11.78 ±1.163     | 16.70 ±1.819     |
|                           | No  | 61  | 7.308 ±0.742     | 19.56 ±1.397     | 11.78 ±1.163     | 16.70 ±1.819     |
Anti-SmD1, anti-Smith D1; Anti-dsDNA, Anti-double-stranded DNA; Anti-U1snRNP, Anti-U1 small nuclear ribonucleoprotein; Anti-nucleo, Anti-nucleosomes, Anti-SSA, Anti-Sjögren syndrome antigen A; Anyi-SSB, Anti-Sjögren syndrome antigen B, Anti-P0, anti-ribosomal P0 antibody, C3/C4, complement component 3/4; DN, double negative; IgG/IgM/IgA, immunoglobulin G/M/A; LN, lupus nephritis; N, naïve; NSM, non-switched memory; PC, plasmablast cells; SLE, systemic lupus erythematosus; SM, switched memory.

Figures
B cells and their subsets were abnormal in patients with SLE. All samples were collected from fresh blood. PBMCs were isolated from these samples. (A) Lymphocyte representative dot plots in PBMC showing the gating strategy based on SSC-A and SSC-A. The CD19+B cells were based on SSC-A and CD19 (one patient and one control donor). (B) Comparison of the
percentage of CD19+B cells in PBMC among controls, patients with inactive SLE (SLEDIA <10), and patients with active SLE (SLEDIA ≥10); ***P < 0.00001 (one-way analysis of variance). (C) Distribution of peripheral blood B-cell subsets stained by their surface expression of IgD and CD27. Representative flow charts of B-cell subsets are shown. PC, plasma cells (CD27high IgD- B cells); SM, switched memory (CD27+ IgD- B cells); NSM, non-switched memory (CD27+ IgD+ B cells); DN, double negative (CD19+ IgD- CD27- cells); N, Naïve (CD27- IgD+ B cells) (one patient and one control donor). (D) Comparison of the proportion of B-cell subsets in B cells in controls and patients with SLE (x ± s, %); *P<0.05 (Student t test). (E) Correlation of CD19+ B cells with SLEDAI (SLEDAI: SLE Disease Activity Index), amounts of proteinuria, C3, 24-h urinary protein in patients with SLE. All data were expressed as mean ± standard deviation.
PD-1+ was differentially expressed on B cells. All samples were collected from fresh blood. PBMCs were isolated from these samples. The gating strategy was based on the literatures.
29, 30. (A) Representative flow charts of isotype control in CD19+B cells showing the gating strategy based on M-IgG1 and CD19. PD-1+B cells representative flow charts in CD19+B cells showed the gating strategy used based on PD-1 and CD19 (one patient and one control donor). (B) Comparison of the percentage of PD-1+ B cells in B cells in patients with active or inactive SLE and controls; ***P < 0.0001 (one-way analysis of variance). (C) Representative flow charts for gating strategy of isotype and PD-1+ B cells in PC, SM, NSM, DN, and N B cells (one patient and one control donor). (D) Comparison of the percentage of PD-1+ B cells in B-cell subsets in controls and patients with SLE; **P < 0.001, ***P < 0.0001 (mean ± SD, %; Student t test). (E) Correlation of PD-1+ B cells with SLEDAI (SLEDAI, SLE Disease Activity Index), 24-h urinary protein and serum IgG and IgM in patients with SLE. All data were expressed as mean ± standard deviation. (F) Correlation between the percentages of expression of PD-1 on PC, SM, NSM, DN, or N B cells in subsets of B cells with SLE and SLEDAI or laboratory findings. All data were expressed as mean ± standard deviation.
Proliferation of PD-1+ or PD-1- B cells in response to CpG DNA stimulation. PBMCs were isolated from peripheral blood (20 mL) in patients with SLE (n=5) and peripheral blood (40 mL) in healthy controls (n=5). Then PD-1+ or PD-1-B cells were sorted by flow cytometry based on CD19 and PD-1 staining. The gating strategy was based on the literature 29, 30. The proliferation was assessed with carboxyfluorescein diacetate succinimidyl ester (CSFE). B-cell proliferation was defined as CFSE low population. The 2×10^4/well PD-1+ or PD-1- B cells were cultured for 7 days in 24-well cell culture plates with RPMI 1640 medium containing 10% fetal calf serum, 10 ng/ml of IL-2 and IL-10, and stimulated with 2.5 µg/mL CpG2006 oligonucleotide, and in the presence or absence of 2.5 µg/ml goat F(ab)2 anti-
human IgM. Medium was changed every 2 days. (A) Proliferation of B cells was observed with CpG DNA or anti-IgM stimulation. Proliferation of B cells was obvious with CpG DNA stimulation or CpG DNA/anti-IgM. (B) Proliferation of PD-1+ or PD-1- B cells in patients with SLE or healthy controls in response to CpG DNA stimulation after 1, 3, 5, and 7 days; The number of cells was assessed using flow cytometry with CSFE. The level of (C) anti-dsDNA and (D) IgG was assessed using ELISA in four groups (control PD-1+, SLE PD-1+, control PD-1-, and SLE PD-1- B cells) in response to CpG DNA stimulation after 1, 3, 5, and 7 days. All data were expressed as mean ± standard deviation. *P < .05, **P < .01, ***P < .001, no statistical significance with no * sign. One-way analysis of variance followed by a Newman–Keuls post hoc test.