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 andplasmablast 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 in 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 [1]. 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 [2–5].

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[6–8]. In addition, increasing evidence indicates that the PD–1/PD-L1/2 axis is crucial in preventing autoimmune disorders[9–11]. Experimental models of autoimmunity demonstrated that the altered function of PD–1 and its ligands is associated with several human autoimmune conditions [12–14]. Multiple studies have implicated the PD–1/PD-L1 pathway in immune system homeostasis and a variety of autoimmune diseases[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[17–19]. PD-L1-Ig treatment in New Zealand F1 mice significantly delayed the onset of proteinuria and prolonged their lifetime[20]. Nevertheless, the expression of PD–1/PD-L1 in SLE still remains unclear.

It has been reported that the proportion of IgDCD27^{+} class-switched memory B cells were found to be significantly high in patients with SLE, antigen-experienced, and resistant to immunosuppressive therapy[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[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 [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 [24]. Although IgDCD27 B cells expressing mutated BCRs have been detected in the tonsils and peripheral blood of healthy donors[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[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[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[29, 30]. 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, 2×10^{4}/well$PD-1^{+}$ or $PD-1^{-}$ B 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 $CD27^{high}$ 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[31]. B-cell phenotypes and their subsets are changed in patients with SLE[32, 33]. 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[27]. The DN2（CD19$^+$IgD$^-$CD27$^-$CXCR5$^-$） B cells were expanded in SLE patients[34, 35]. Kubo et al[36] 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[36]. 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[7, 37]. CD8$^+$ PD−1$^+$ T cells were exhausted in mice with viral infection and in humans with chronic viral infection[38, 39].But Petrelli et al [40] 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[17]. A subpopulation of PD−1+CD4+ T cells were found to expand in lupus blood and provide B cell help by interleukin −10[41]. PD−1 inhibits the phagocytic potency against tumor cells in tumor-associated macrophages[42]. 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[43]. The inhibition of PD−1 pathways leads to the increase in activation and proliferation of B cells and the production of inflammatory cytokines [43]. The disruption of the PD−1 pathways significantly increases the proliferation and production of IgG by pneumococcal capsule-specific B cells[44]. 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[45] 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[43]. 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

C3 :	complement 3
C4 :	complement 4
dsDNA:	double-stranded DNA
IgM:	immunoglobulin M
IgA:	immunoglobulin A
IgG:	immunoglobulin G
LN:	lupus nephritis
N	naïve
NSM	non-switched memory
PC	plasmablast cells
PD-1	programmed death 1
PD-L1	programmed death ligand 1
Sm:	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	Cases	PC B cells *r*	SM B cells *r*	NSM B cells *r*	DN B cells *r*	N B cells *r*
SLEDAI	74	0.03501	0.08304*	0.01213	0.00436	-0.0845*
Amounts of proteinuria (g/24 h)	69	-0.01255	0.01846	0.09926*	-0.02297	-0.03198
IgG (g/L)	74	0.02445	0.07461*	0.08158*	0.05188	-0.2087*
IgA (g/L)	74	0.06296	0.03587	0.1280	-0.1827	-0.04001
IgM (g/L)	74	0.09914*	-0.02337*	-0.07387*	0.005707	-0.02223
C3 (g/L)	74	0.1162*	0.006449	0.03364	0.001436	-0.06764
C4 (g/L)	74	-0.1857	0.1340	0.09826	-0.1698	0.00045

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 Bcells (%)	*P* value	SM B cells (%)	*P* value	NSM B cells (%)	*P* value	DN B cells (%)	*P* value	NB cells (%)	*P* value
Anti-dsDNA	+	44	7.799 ±0.9774	0.300	19.54 ±1.208	0.896	16.43 ±2.100	0.641	12.04 ±1.612	0.084	50.35 ±3.049	0.198
	-	30	6.449 ±0.626		19.88 ±2.527		14.97 ±2.196		43.99 ±3.049		50.35± 3.880	
Anti-histones	+	26	8.536 ±1.167	0.138	24.15 ±2.613	0.007	17.52 ±3.652	0.420	13.90 ±2.231	0.017	35.85 ±4.009	0.001
	-	48	6.556 ±0.739		17.26 ±1.169		14.92 ±1.297		8.729 ±1.002		52.37 ±2.692	
Anti-smD1	+	45	7.919 ±0.929	0.193	20.32±1.549	0.522	16.31 ±2.045	0.702	11.07 ±1.399	0.537	44.20 ±3.164	0.225
	-	29	6.216 ±0.718		18.68 ±2.082		15.10 ±2.293		9.735 ±1.587		50.23 ±3.669	
Anti-U1snRNP	+	35	7.856 ±1.176	0.371	20.30 ±1.854	0.641	14.95 ±1.662	0.586	11.82 ±1.717	0.253	44.87 ±3.594	0.508
	-	39	6.709 ±0.5865		19.13 ±1.684		16.63 ±2.497		9.406 ±1.255		48.09 ±3.269	
Anti-nucleo	+	35	7.625 ±0.954	0.571	18.42 ±1.839	0.326	14.12 ±2.425	0.277	12.28 ±1.807	0.108	47.52 ±3.960	0.704
	-	39	6.898 ±0.853		20.87 ±1.675		17.46 ±1.879		8.904 ±1.075		45.67 ±2.870	
Anti-SSA60	+	41	7.151 ±0.679	0.832	21.38 ±1.710	0.062	16.74 ±2.194	0.424	11.60 ±1.399	0.173	53.01 ±3.811	0.049
	-	33	7.437 ±1.323		16.54 ±1.428		14.17 ±1.570		8.596 ±1.458		43.08 ±2.999	
Anti-SSA52	+	20	7.444±1.179	0.856	24.30 ±3.274	0.022	23.26 ±4.333	0.02	8.155 ±1.205	0.167	50.18 ±2.671	0.013
	-	54	7.180 ±0.759		17.9 ±1.131		13.09 ±1.172		11.43 ±1.356		36.82 ±4.695	
Anti-SSB	+	15	6.865 ±1.446	0.761	21.24 ±3.548	0.530	22.85 ±5.868	0.019	9.406 ±1.879	0.587	39.57 ±6.199	0.145
	-	59	7.350 ±0.713		19.28 ±1.283		14.05 ±1.137		10.83 ±1.231		48.34 ±2.559	
Anti-P0	+	30	7.653 ±1.066	0.605	17.26 ±1.560	0.108	12.82 ±1.146	0.103	11.78 ±1.774	0.334	50.24 ±3.656	0.210
	-	44	6.978 ±0.791		21.33 ±1.767		17.90 ±2.408		9.704 ±1.287		44.06 ±3.176	
C3↓	Yes	58	6.754 ±0.629	0.137	19.30 ±1.492	0.565	15.82 ±1.900	0.986	11.03 ±1.293	0.378	46.93 ±2.885	0.774
	No	16	9.055 ±1.831		21.05 ±1.961		15.89 ±1.641		8.773 ±1.229		45.23 ±4.000	
C4↓	Yes	51	7.002 ±0.696	0.562	20.67 ±1.651	0.237	14.69 ±1.595	0.266	11.61 ±1.369	0.132	45.97 ±2.924	0.717
	No	23	7.806 ±1.358		17.48 ±1.543		18.38 ±3.407		8.188 ±1.398		47.88 ±4.341	
IgA↑	Yes	45	8.055 ±0.912	0.116	19.95 ±1.570	0.788	14.74 ±1.960	0.376	11.00 ±1.398	0.589	46.20 ±3.194	0.853
	No	29	6.005 ±0.751		19.26 ±2.055		17.54 ±2.440		9.836 ±1.592		47.13 ±3.710	
IgG↑	Yes	44	7.766 ±0.9582	0.344	19.54 ±1.545	0.895	14.07 ±1.681	0.174	12.44 ±1.533	0.032	46.02 ±3.142	0.791
	No	30	6.538 ±0.730		19.87 ±2.076		18.29 ±2.774		7.917 ±1.200		47.33 ±3.815	
Lymphopenia ($<1.5 \times 10^9$ L^{-1})	Yes	52	7.637 ±0.743	0.354	20.86 ±1.629	0.145	16.62 ±1.917	0.433	10.60 ±1.231	0.940	44.26 ±2.948	0.142
	No	22	6.340 ±1.221		16.89 ±1.502		13.98 ±2.427		10.42 ±2.045		52.03 ±4.006	
Thrombocytopenia ($<100 \times 10^9$ L^{-1})	Yes	28	7.591 ±1.174	0.712	17.01 ±1.524	0.103	15.50 ±2.814	0.922	11.20 ±1.892	0.645	48.45 ±4.029	0.581
	No	46	7.097 ±0.755		21.23 ±1.774		15.81 ±1.807		10.18 ±1.276		45.66 ±3.086	
LN	Yes	36	6.569 ±0.657	0.299	20.07 ±2.042	0.761	17.65 ±2.414	0.250	10.70 ±1.567	0.889	44.98 ±3.355	0.525
	No	38	7.898 ±1.067		19.31 ±1.474		14.12 ±1.886		10.40 ±1.425		48.07 ±3.481	
Raynaud′s phenomenon	Yes	20	7.667 ±1.343	0.694	23.49 ±2.857	0.061	12.44± 1.317	0.178	11.30 ±2.043	0.665	45.02 ±4.616	0.699
	No	54	7.098 ±0.721		18.27 ±1.298		17.10 ±2.013		10.26 ±1.233		47.14 ±2.849	
Malar rash	Yes	24	9.554 ±1.404	0.011	25.54 ±2.717	0.001	15.96 ±2.656	0.957	10.92 ±1.755	0.807	38.00 ±4.333	0.013
	No	50	6.146 ±0.607		16.87 ±1.111		15.78±1.883		10.36 ±1.316		50.68 ±2.743	
Fever	Yes	35	7.655 ±0.949	0.551	19.129 ±1.798	0.674	13.78 ±1.890	0.204	11.89 ±1.827	0.226	47.50 ±43.699	0.715
	No	39	6.890 ±0.860		20.18 ±1.733		17.68 ±2.330		9.339 ±1.119		45.72 ±43.188	
Oral ulcer	Yes	12	8.600 ±2.188	0.354	21.22 ±3.628	0.588	17.25 ±3.906	0.687	11.34 ±2.879	0.740	41.56 ±4.945	0.365
	No	62	6.991 ±0.634		19.38 ±1.316		15.56 ±1.669		10.39 ±1.132		47.53 ±2.712	
Arthritis	Yes	26	6.391 ±0.694	0.322	18.61 ±2.247	0.529	13.95 ±1.137	0.367	8.935 ±1.429	0.261	52.08 ±3.650	0.093
	No	48	7.718 ±0.902		20.26 ±1.488		16.86 ±2.267		11.42 ±1.415		43.58 ±3.089	
Serositis	Yes	18	6.234 ±1.018	0.368	18.57 ±2.465	0.615	11.14 ±1.003	0.081	9.713 ±1.915	0.655	54.32 ±4.281	0.068
	No	56	7.579		20.04		17.35±		10.81		44.07	

			±0.772		±1.445		1.953		±1.250		±2.812	
Neurological disorder	Yes	14	6.553 ±1.309	0.599	19.11 ±2.648	0.826	16.32 ±2.251	0.880	9.207 ±2.197	0.541	48.26 ±5.325	0.736
	No	60	7.415 ±0.725		19.81 ±1.410		15.73 ±1.814		10.86 ±1.193		46.17 ±2.720	
Interstitial lung	Yes	13	6.985 ±1.018	0.848	20.25 ±2.737	0.834	11.78 ±1.163	0.222	9.840 ±2.703	0.758	51.09 ±4.689	0.390
	No	61	7.308 ±0.742		19.56 ±1.397		16.70 ±1.819		10.70 ±1.145		45.60 ±2.748	

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

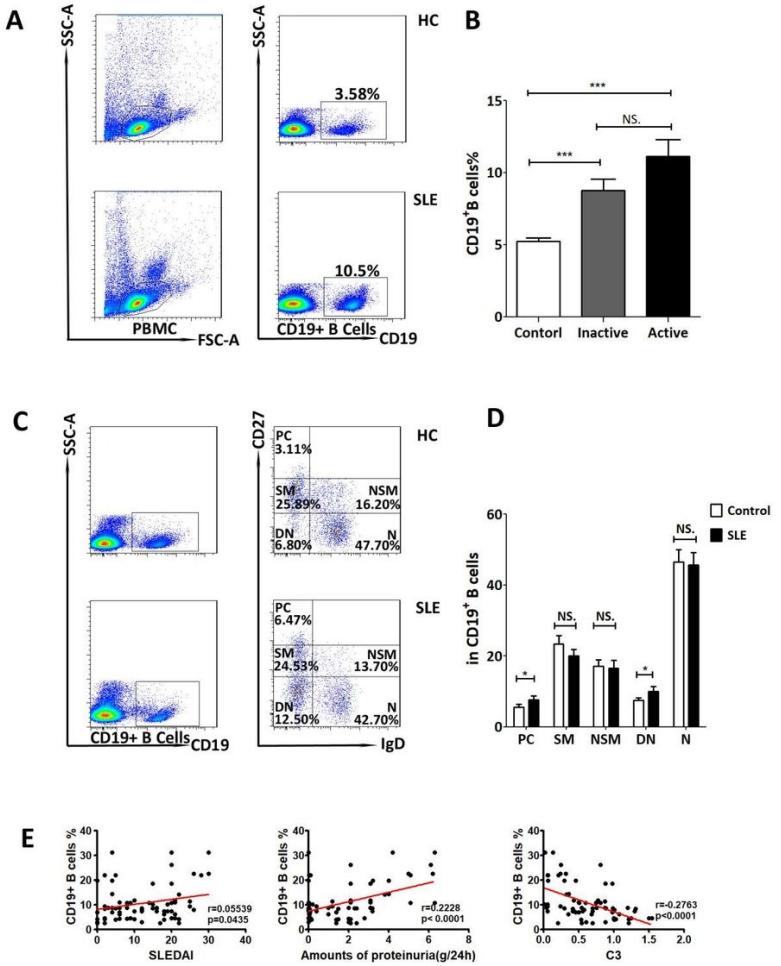

Figure 1

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.

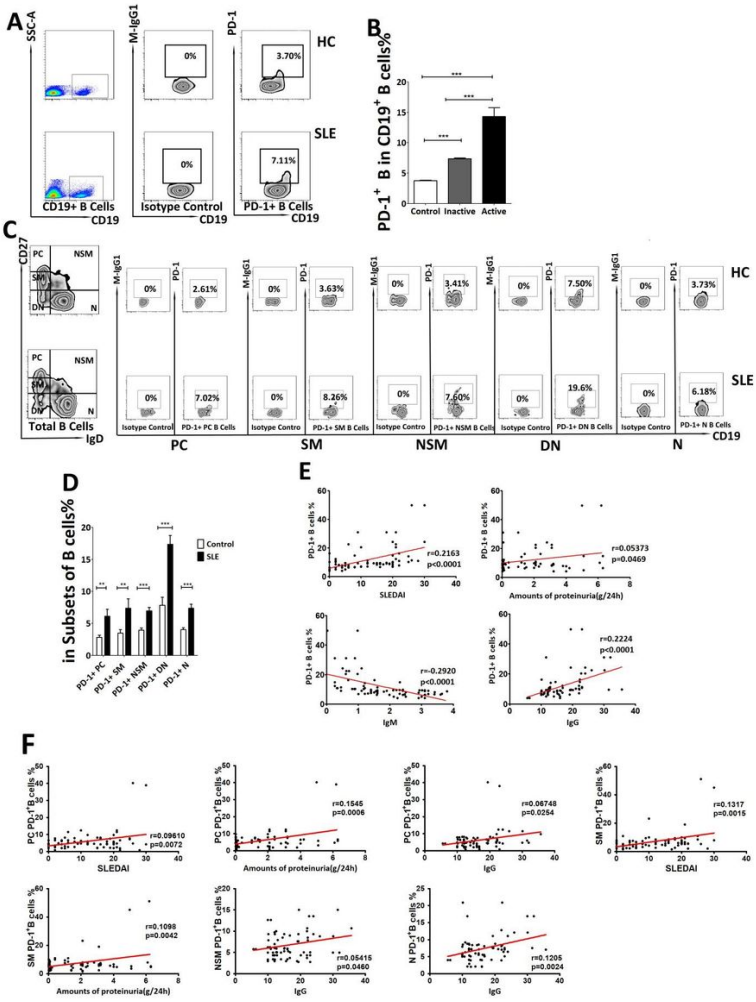

Figure 2

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.

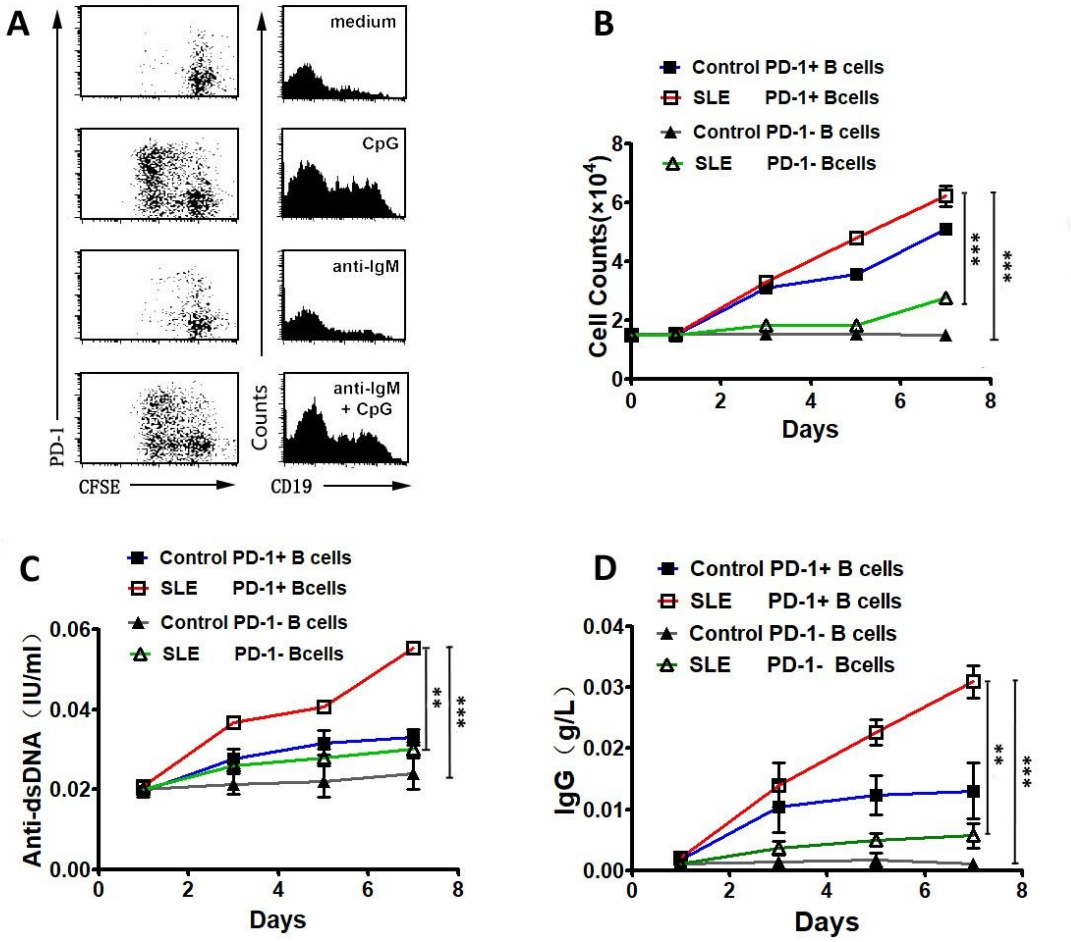

Figure 3

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 2×104/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.