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The role and clinical significance of programmed cell death- ligand 1 expressed on CD19$^+$ B-cells and subsets in systemic lupus erythematosus

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ABSTRACT

Background: Programmed cell death-1 (PD-1) and programmed death-ligand 1 (PD-L1)-targeted therapies have enhanced T-cell response and demonstrated efficacy in the treatment of multiple cancers. However, the role and clinical significance of PD-L1 expression on CD19$^+$ B-cells and their subsets, with particular reference to systemic lupus erythematosus (SLE), have not yet been studied in detail.

Objective: The present study aimed to investigate PD-L1 expression on CD19+ B-cells and their subsets, in addition to exploring its possible role in Tfh-cell activation and B-cell differentiation in SLE.

Methods: Frequencies of CD19$^+$ B-cells, their subsets, PD-L1 and Tfh cells in the peripheral blood of SLE patients and healthy controls (HCs) were determined using cytometry. The clinical data of SLE patients were recorded in detail, and the correlation between their laboratory parameters, clinical parameters and disease activity indices was statistically analyzed. CD19$^+$PD-L1$^+$B-cells and CD19$^+$PD-L1$^-$ B-cells were sorted and cultured with a stimulant, following which the supernatants were collected for immunoglobulin G and anti-double stranded DNA detection via enzyme-linked immunosorbent assay.

Results: In SLE patients, CD19$^+$B-cells and partial subgroups were enriched in peripheral blood. Also, the observed increase in the frequency of CD19$^+$PD-L1$^+$B-cells was significantly associated with a higher disease activity index. An in vitro culture test demonstrated that the amounts of anti-dsDNA and immunoglobulin G secreted by the CD19$^+$PD-L1$^+$B-cells of SLE patients and HCs were vastly different. In addition, a strong correlation existed between the frequencies of CD19$^+$PD-L1$^+$B-cells and defined Tfh cells of SLE patients.

Conclusion: This study demonstrated that the expression of CD19$^+$PD-L1$^+$B-cells in the peripheral blood of SLE patients was abnormal, and that disease-related laboratory parameters and clinical indicators were correlated. CD19$^+$PD-L1$^+$B-cells were enriched and played a critical role in activating the pathogenic T-cell and B-cell responses in patients with SLE.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that may lead to multiorgan damage. SLE is characterized by immunological abnormalities, which include a deficient innate immune response and aberrant activation of autoreactive T- and B-cells. These abnormalities may lead to the production of pathogenic autoantibodies against cell nuclear components, resulting in end-organ injury [1–3]. However, the exact pathogenesis of SLE remains unclear. B-cells play a pivotal role in the initiation and perpetuation of autoimmune diseases, such as SLE, by producing autoantibodies. It is believed that abnormal activation of susceptible genes in B-cells produces autoreactive B-cells which regulate T-cell activation through antigen presentation, production of cytokines and costimulatory molecules, and recruitment of T-cell subsets and dendritic cells [4]. In particular, antigen-antibody immune complexes deposited in tissues may damage various focal organs and cause chronic inflammation, resulting in the loss of organ function. In the SLE model, antigen-specific B-cells have been identified as important antigen-presenting cells that deliver antigens to corresponding antigen-specific T-cells [5]. Therefore, SLE is considered a B-

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cell-mediated disease at least in part [6].

Programmed death-ligand 1 (PD-L1), also known as B7 homolog 1 or cluster of differentiation 274, is the ligand of programmed death-1 (PD-1), which is also known as cluster of differentiation 279. PD-L1, which belongs to the B7 superfamily, was first discovered by Dong et al. [7], in 1999, in the placenta complementary DNA library. It is widely expressed in activated T-cells, B-cells, dendritic cells, macrophages, mesenchymal cells, and cultured bone marrow-derived mast cells [8–10]. PD-L1 expression in nonlymphoid tissue indicates that PD-L1 may regulate self-reactive lymphocytes in peripheral tissue. Additionally, some studies have shown that only B7 family costimulatory molecules [11] transmit signals to T-cells from antigen-presenting cells (APCs). The role of PD-L1 in the pathogenesis of SLE is not clear. Therefore, a systematic study of its role in the pathogenesis of SLE as well as an understanding of the mechanisms underlying its function may have a theoretically and clinically significant impact on the search to identify potential targets for SLE treatment.

Abnormal activation of autoantibodies is a sign of SLE. The production of antibodies requires T-cells. The expression of $CXCR5^+$ and $CD4^+$T-cells in human tonsils induce B-cells to secrete immunoglobulin (Ig). These cells, subsequently termed follicular helper T-cells (Tfh), belong to the $CD4^+$T- cell group which contains subgroups such as Tfh1, Tfh2, and Tfh17 [12]. The Tfh cell surface molecule, PD-1, is necessary for the formation of plasma cells and for the selection and survival of germinal center B-cells [13–14]. Some studies have shown that interleukin (IL)-21 secreted by Tfh cells plays a key role in helper B-cell development [15–18]. Therefore, it may be important to clarify the relationship between $CD19^+PD\text{-}L1^+$B-cells and Tfh cells.

It is well-known that SLE is molecularly as well as clinically heterogeneous. This heterogeneity effectively prevents the development of targeted therapies [19]. To address this, we used flow cytometry, enzyme-linked immunosorbent assay (ELISA) and cell culturing, to detect $CD19^+$B-cells and their subsets and to analyze PD-L1 expression in each subgroup in the peripheral blood of SLE patients. Additionally, we analyzed the correlation between the functional characteristics of $CD19^+PD\text{-}L1^+$B-cells and the clinical indices of SLE patients, as well as that between Tfh and $CD19^+PD\text{-}L1^+$B-cell expression.

2. Material and methods

2.1. Experimental objective

Fifty patients with SLE, first diagnosed between April 2017 and August 2017, were enrolled from the Rheumatism Department of the First Affiliated Hospital of Bengbu Medical College in Bengbu, China. Of these, 2 were males and 48 were females and the average age was 33.6 ± 12.3 years. All patients were clinically diagnosed according to criteria established by the American College of diagnosis. Activity was scored using the University of Toronto SLE Disease Activity Index (SLEDAI) scoring system [20,21], by which patients were divided into a low-disease activity category (SLEDAI < 10, $n = 21$) and an active disease category (SLEDAI ≥10, $n = 29$). Patients were further stratified according to presence ($n = 15$) or absence ($n = 35$) of lupus nephritis, where patients diagnosed in the second half of the year were not receiving either hormone or immunosuppressant treatment. At the same time, 40, age- and sex-matched healthy individuals were included as controls (HCs). Of these, 3 were males and 37 were females, and the average age was 34.6 ± 11.2 years. All individuals underwent physical examinations in May of 2017. Clinical and laboratory data of the SLE patients were recorded in detail. The study was approved by the Human Ethics Committee of the First Affiliated Hospital of Bengbu Medical College. Written informed consent was obtained from all subjects. Baseline characteristics of the patients are shown (Table 1).

2.2. Cell staining and flow cytometric analysis

Venous blood samples (5 mL) from patients and healthy controls were collected in tubes containing EDTA. Peripheral blood mononuclear cells (PBMCs), isolated from whole peripheral blood using the Ficoll gradient centrifugation protocol, were divided equally, placed in flow tubes and washed with staining buffer. Lymphocytes were detected in the PBMCs of HCs and SLE patients using 8-color flow cytometry (BD FACS Verse™; BD Biosciences, San Jose, USA). $CD19^+$B-cells and their subsets were detected using the following monoclonal antibodies: APC-cyanine 7 anti-human CD19 (catalog no.302217; BioLegend, San Diego, USA), FITC anti-human IgD (catalog no.348206; BioLegend, San Diego, USA), APC anti-human CD27 (catalog no.302810; BioLegend, San Diego, USA), PE anti-M-IgG2b (catalog no.400314; BioLegend, San Diego, USA), PE/Cy7 anti-human CD38 (catalog no.356608; BioLegend, San Diego, USA) and PE anti-human PD-L1 (catalog no.124307; BioLegend, San Diego, USA). Additionally, Tfh cells were detected using the following monoclonal antibodies: FITC anti-human CD4 (catalog no.344604; BioLegend, San Diego, USA); PE anti-human CXCR5 (catalog no.356904; BioLegend, San Diego, USA) and APC/Cy7 anti-human PD-1 (catalog no.329922; BioLegend, San Diego, USA). Gating of the lymphocytes in forward scatter plots, (FSC)-A and FSC-H, and setting gates for detecting $CD19^+$ B-cell and subsets, $CD19^+$ $PD\text{-}L1^+$ B-cells and Tfh cells, were completed according to the manufacturer's suggestion, while flow data were analyzed using FlowJo vX0.7 (Becton Dickinson, Franklin Lakes, NJ, USA).

2.3. Cell sorting and culture

For the purpose of B-cell sorting, 25 mL samples of peripheral blood were obtained from all subjects. Next, 2.5×10 [7] of PBMCs were separated using the Ficoll gradient centrifugation protocol and incubated for 30 min with antibodies, followed by washing with phosphate-buffered saline (PBS). $CD19^+PD\text{-}L1^+$B-cells and $CD19^+PD\text{-}L1^-$B-cells were then sorted using flow cytometry (BD FACS Aria™ IIU; BD Biosciences, San Jose, CA, USA) with APC/Cy7 anti-CD19, PE anti-M-IgG2b, and PE anti-PD-L1 staining. Sorted B-cells were further assessed by flow cytometric analysis for.

> 95% purity. $CD19^+PD\text{-}L1^+$B-cells and $CD19^+PD\text{-}L1^-$B-cells isolated from SLE patients and HCs were incubated in a 96-well plate with 200 μL of Roswell Park Memorial Institute (RPMI) and 1640 complete medium (Gibco, Billings, MT, USA) supplemented with IL-2 and IL-10 (10 ng/mL), both of which were obtained from PeproTech, Rocky Hill, NJ, USA., and stimulated with CpG2006 oligonucleotide (2.5 μg/mL; Oligos Etc., Inc., Wilsonville, OR, USA). After 1-day, 3-day, 5-day, and 7-day of culturing, the supernatants were harvested for anti-double stranded DNA (anti-dsDNA) and IgG measurement.

2.4. B-Cell proliferation assay

Peripheral blood $CD19^+PD\text{-}L1^+$B-cells and $CD19^+PD\text{-}L1^-$B-cells from patients and controls were isolated via flow cytometry and RPMI 1640 was added to obtain a 1×10^6/mL cell suspension. The cells were then incubated for 10 min at 37 °C with 5 μL of carboxy fluorescein succinimidyl ester (CFSE; catalog no. C34554; Invitrogen, Carlsbad, USA) in PBS. Labeling was terminated after adding an equal volume of bovine serum albumin to the cells. The cells were washed three times before being stored in the dark with gentle shaking. The two groups of labeled cell types were cultured for 7 days in a 96-well plate with 200 μL of RPMI 1640 complete medium containing IL-2 and IL-10 (10 ng/mL; both from PeproTech, Rocky Hill, NJ, USA) and stimulated with CpG2006 oligonucleotide (2.5 μg/mL, Oligos Etc., Inc., Wilsonville, OR, USA). Then, proliferation of the two groups of cell types was defined based on the proportion of CFSE labeled cells, as indicated by flow cytometry-based studies of cell division.'

Table 1
General information characteristics of patients with SLE and healthy control. ($\bar{x}$ ± SD).

Groups	Sex (male/female)	Age	Disease duration	SLEDAI	Immunosuppressive applications
Healthy control	3/37	34.6 ± 11.2(1)	/	/	/
SLE patients	2/48	33.6 ± 12.3(1)	8.91 ± 2.62	11.44 ± 6.61	/
Hyperactive SLE	2/27	34.1 ± 11.7(1)	9.39 ± 2.49(2)	15.66 ± 5.51(3)	/
Low-level active SLE	0/21	35.6 ± 10.3(1)	8.24 ± 2.77	5.62 ± 2.33	/
Lupus nephritis	1/14	34.4 ± 10.9(4)	9.20 ± 2.78(5)	16.80 ± 7.02(6)	/
Non-lupus nephritis	1/34	35.3 ± 11.8	8.75 ± 2.56	9.14 ± 5.08	/

Note: (1) Compared between the four groups, P > 0.05; (2) Compared with low-level active SLE group, P > 0.05; (3) Compared with low-level active SLE group, P < 0.001; (4) Compared with non-lupus nephritis group, P > 0.05; (5) Compared with non-lupus nephritis group, P > 0.1; (6) Compared with non-lupus nephritis group, P < 0.0001;

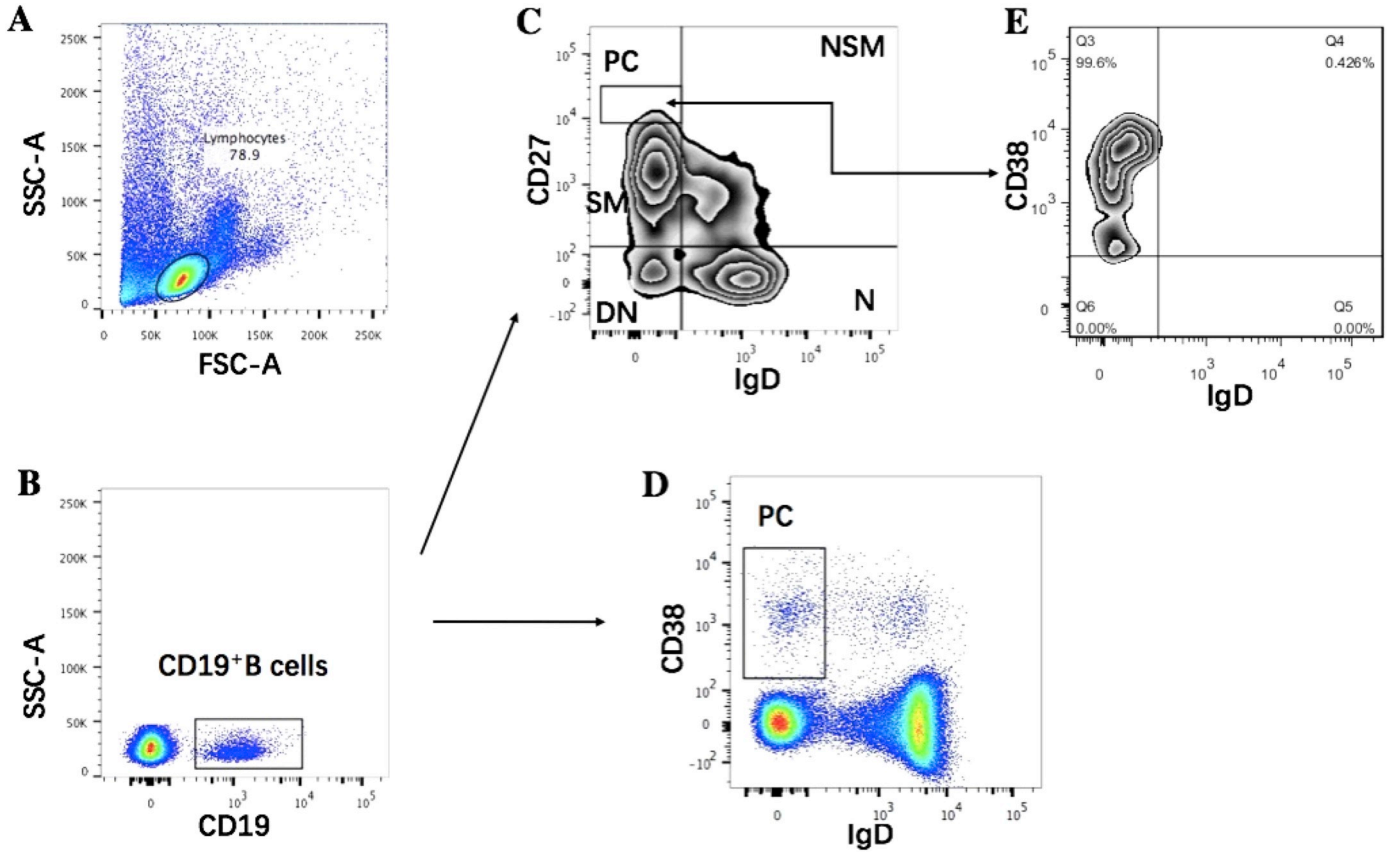

Fig. 1. Expression of CD19$^+$B cells and five subgroups in peripheral blood. A: Healthy PBMCs (from a side-scatter SSC-Avsa forward-scatter FSC-A gate) were analyzed. B-C: CD19 + B-cells were analyzed using eight-color flow cytometry protocol. The distribution of peripheral blood B-cell subsets was identified through surface expression of IgD and CD27 (SM, switched memory; NSM, non-switched memory; DN, double negative; N, naive; PC, plasma cells). D-E: Detection and verification of plasma cells. Interestingly, both CD27$^+$ memory cells and DN cells are almost universally CD38 dull.

2.5. ELISAs

Commercially available ELISA kits, specifically, the anti-dsDNA ELISA kit (catalog no. orb339621; Biorbyt, Cambridge, UK) and the IgG ELISA kit (catalog no. ab100547; Abcam, Cambridge, UK), were used to measure anti-dsDNA and IgG levels the in supernatants.

2.6. Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences version 16.0 (IBM Corp., Armonk, NY, USA). The independent samples *t*-test was used to analyze differences between two groups, while the ANOVA for continuous measures was used to analyze differences among three groups. All values were expressed as mean ± standard deviation. Pearson correlation analysis (*r*) was applied to determine the correlation between numerical data. Statistical significance was set at $p < .05$.

3. Results

3.1. CD19$^+$B-cell and partial subset were increased in patients with SLE

PBMCs were categorized as CD19$^+$B-cells, based on their CD19 expression. The proportion of CD19$^+$B-cells and subsets were determined based on IgD and CD27 expression on the cellular surface. The CD19$^+$B-cells were categorized into the following five subgroups: SM, switched memory; NSM, non-switched memory; DN, double negative; N, naive; PC, plasma cells. A comparison of the percentage of CD19$^+$B-cells and their subgroups among the three groups is shown (Figs. 1, 2).

The percentage of CD19$^+$B-cells was significantly elevated in the

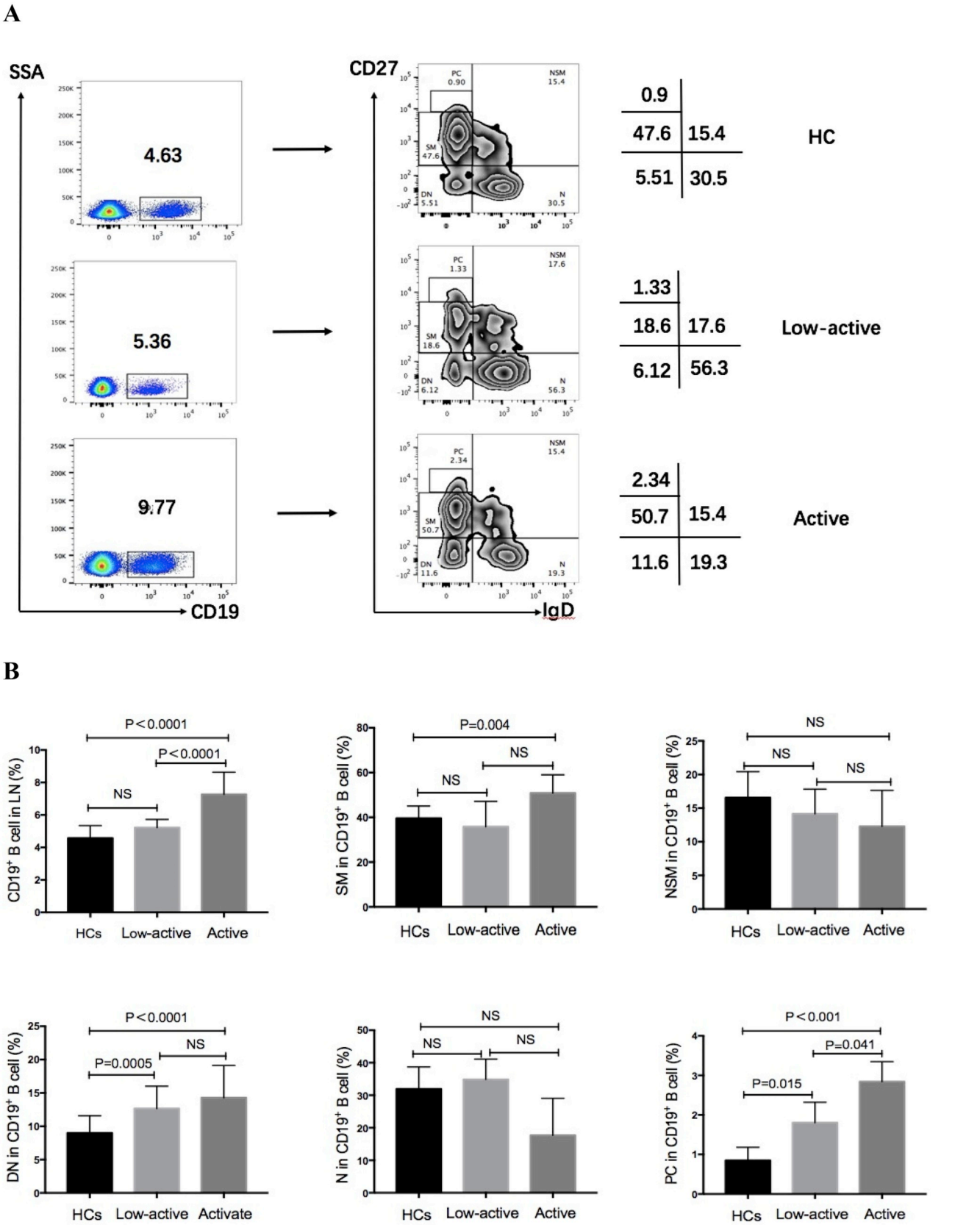

Fig. 2. Expression of CD19$^+$B-cells and subgroups in patients with SLE. A: Representative examples of the profiles observed in HCs and SLE patients (Active and Low-active); the expression of CD19$^+$B-cells and five subsets are shown. The numbers attached to corresponding quadrants represent the percentage of the annotated subset among all CD19 $^+$B-cells. B: The frequencies of CD19$^+$B-cells in the LN, SM, NSM, DN, N, and PC subsets were compared between 50 patients (29 active and 21 stable) and 40 age-matched HCs. NS, no significance.

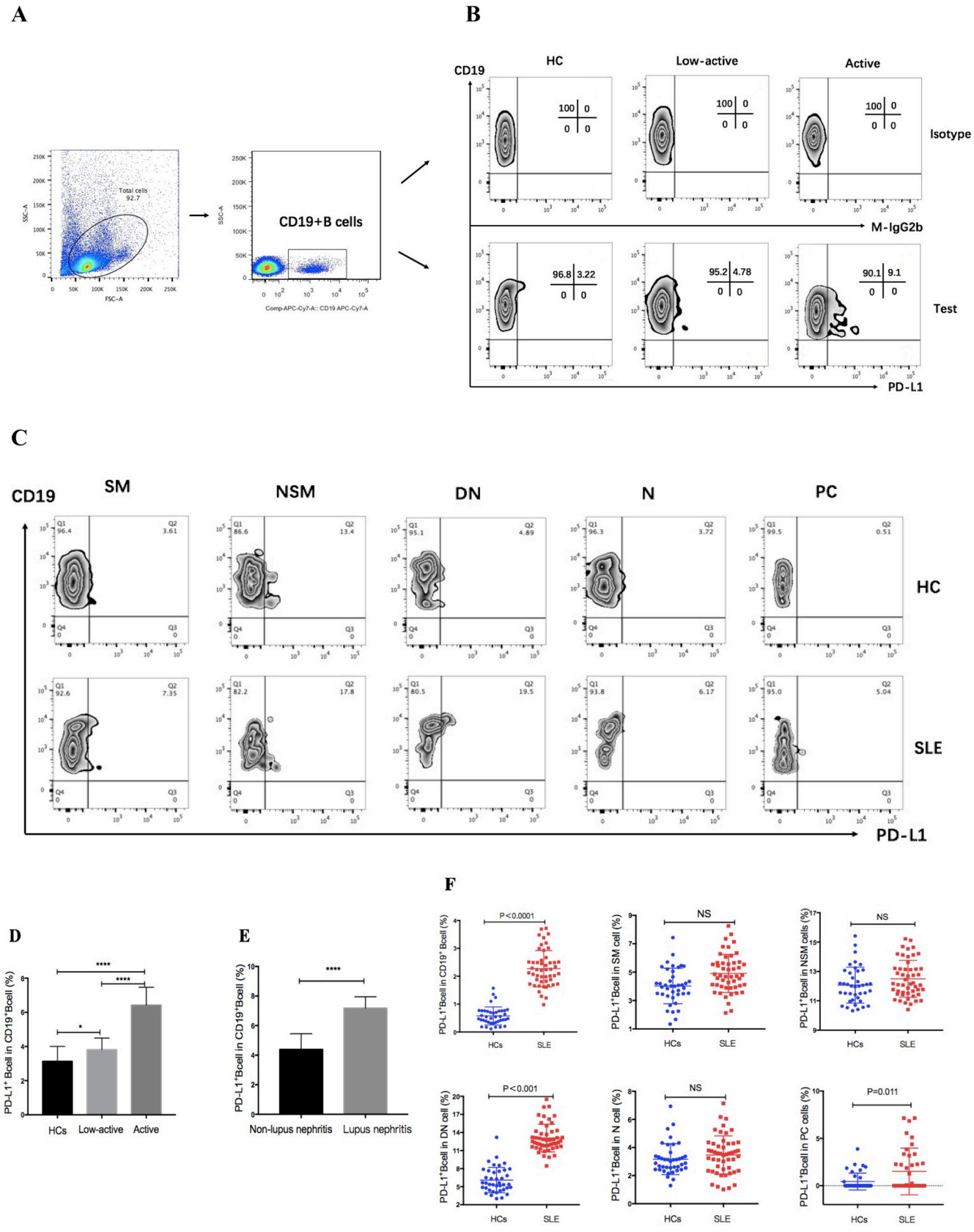

Fig. 3. PD-L1 is differentially expressed on CD19$^+$B-cells. A: PD-L1 expression on CD19$^+$B-cells from the lymphocytes of isotype and test groups. B: Flow cytometry analysis of CD19$^+$PD-L1$^+$B-cell populations within the three groups according to either isotype or test treatment. C: Flow cytometry analysis of five subsets of CD19$^+$PD-L1$^+$B-cell populations between SLE patients and HCs. D: Frequencies of PD-L1$^+$B-cells in CD19$^+$B-cells within the three groups. E: Comparison of CD19$^+$PD-L1$^+$B-cells in lupus nephritis group and the non-lupus nephritis group. F: Frequencies of PD-L1$^+$B-cells in CD19$^+$ B-cells, SM, NSM, DN, N, and PC subsets were compared between 50 patients and 40 age-matched HCs. * = $P < .05$, ** = $P < .01$, *** = $P < .001$, **** = $P < .0001$, NS, no significance.

Table 2
The relationship between laboratory and clinical parameters of SLE patients and the expression levels of CD19$^+$B-cells and CD19$^+$P-L1$^+$B-cells in peripheral blood ($\bar{x}$ ± SD, %).

Laboratory test parameters and clinical manifestations		Cases	CD19$^+$B-cells (%)	*P* value	CD19$^+$ PD-L1$^+$B-cells (%)	*P* value
Anti-dsDNA	+	36	6.85 ± 1.49	0.045	5.45 ± 1.68	0.024
	−	14	5.91 ± 1.29		4.84 ± 1.48	
C3↓	Yes	43	6.87 ± 1.48	0.022	5.52 ± 1.59	0.010
	No	7	5.40 ± 0.94		3.82 ± 1.06	
IgG↑	Yes	27	7.17 ± 1.59	0.002	5.37 ± 1.78	0.675
	No	23	5.90 ± 1.02		5.17 ± 1.48	
IgM↓	Yes	20	7.18 ± 1.78	0.020	5.37 ± 1.70	0.750
	No	30	6.19 ± 1.12		5.22 ± 1.62	
Lupus nephritis	Yes	15	7.34 ± 1.64	0.016	6.86 ± 0.93	0.000
	No	35	6.26 ± 1.31		4.60 ± 1.38	
Raynaud's phenomenon	Yes	8	7.56 ± 2.23	0.043	6.16 ± 1.83	0.096
	No	42	6.40 ± 1.26		5.11 ± 1.56	
Hair loss	Yes	14	7.36 ± 1.87	0.020	5.25 ± 1.65	0.925
	No	36	6.28 ± 1.21		5.29 ± 1.65	
NPSLE	Yes	2	7.10 ± 0.45	0.610	6.85 ± 0.32	0.006
	No	48	6.54 ± 1.53		5.22 ± 1.64	

Note:Anti-dsDNA Ab, anti-double-strand DNA antibody; C3, complement 3.

Table 3
The relationship between laboratory and clinical parameters of SLE patients and the expression levels of SM, DN, PC B-cells and DN PD-L1$^+$B-cells, and PC PD-L1$^+$B-cells in peripheral blood ($\bar{x}$ ± SD, %).

Laboratory test parameters		Cases	SM B-cells (%)	*P* value	DN B-cells (%)	*P* value	PC B-cells (%)	*P* value	DN PD-L1$^+$ B = cells (%)	*P* value	PC PD-L1$^+$ B-cells (%)	*P* value
Anti-dsDNA	+	36	45.55 ± 10.83	0.251	14.25 ± 04.69	0.404	2.52 ± 0.74	0.004	13.19 ± 1.98	0.569	1.48 ± 2.46	0.939
	−	14	41.35 ± 12.99		13.05 ± 04.02		1.86 ± 0.58		12.77 ± 3.05		1.54 ± 2.56	
Anti- Chrom	+	29	46.37 ± 10.47	0.151	14.48 ± 04.77	0.297	2.56 ± 0.79	0.013	13.31 ± 2.51	0.409	1.65 ± 2.65	0.626
	−	21	41.62 ± 12.52		13.12 ± 04.09		2.03 ± 0.59		12.75 ± 2.00		1.30 ± 2.23	
Anti-SSA	+	11	44.25 ± 12.93	0.939	15.47 ± 04.53	0.009	2.26 ± 0.76	0.471	13.04 ± 2.31	0.924	1.85 ± 2.84	0.306
	−	39	44.51 ± 09.99		12.22 ± 03.89		2.42 ± 0.75		13.11 ± 2.35		1.12 ± 1.97	
Anti-SSB	+	26	43.98 ± 11.43	0.899	13.61 ± 04.94	0.804	2.66 ± 0.90	0.105	14.44 ± 2.58	0.024	0.96 ± 1.78	0.421
	−	24	44.49 ± 11.66		13.99 ± 04.44		2.25 ± 0.69		12.69 ± 2.10		1.65 ± 2.36	
C3↓	Yes	43	43.92 ± 11.16	0.491	14.34 ± 04.47	0.094	2.40 ± 0.76	0.117	13.34 ± 2.27	0.043	1.55 ± 2.55	0.748
	No	7	47.19 ± 14.06		11.26 ± 04.01		1.92 ± 0.63		11.45 ± 1.92		1.22 ± 1.98	
IgA↑	Yes	45	47.82 ± 10.54	0.020	13.22 ± 04.70	0.241	2.50 ± 0.75	0.092	13.26 ± 2.13	0.539	1.61 ± 2.68	0.031
	No	29	40.33 ± 11.47		14.73 ± 04.22		2.14 ± 0.72		12.85 ± 2.52		1.15 ± 1.87	
IgM↓	Yes	34	47.45 ± 11.30	0.124	12.91 ± 04.67	0.202	2.33 ± 0.54	0.948	13.25 ± 0.91	0.037	1.68 ± 3.17	0.495
	No	40	42.33 ± 11.35		14.58 ± 04.34		2.34 ± 0.88		12.22 ± 2.77		1.21 ± 1.58	
Lupus nephritis	Yes	15	48.63 ± 07.87	0.044	14.35 ± 05.56	0.654	2.71 ± 0.76	0.022	12.77 ± 1.81	0.544	1.92 ± 2.87	0.310
	No	35	42.55 ± 12.40		13.72 ± 04.05		2.18 ± 0.71		13.20 ± 2.50		1.78 ± 2.07	
Sphenoid erythema	Yes	11	48.33 ± 07.95	0.229	13.53 ± 05.41	0.771	2.26 ± 0.61	0.607	13.54 ± 2.35	0.000	2.21 ± 3.71	0.384
	No	39	43.72 ± 11.77		13.98 ± 04.31		2.40 ± 0.81		11.55 ± 1.07		1.17 ± 1.77	
Arthritis	Yes	21	43.87 ± 13.93	0.796	12.94 ± 04.38	0.198	2.35 ± 0.77	0.947	13.28 ± 2.48	0.600	2.21 ± 2.78	0.035
	No	29	44.74 ± 09.62		14.61 ± 04.54		2.33 ± 0.76		12.93 ± 2.20		0.81 ± 1.78	
Serositis	Yes	13	45.13 ± 02.20	0.714	11.99 ± 06.00	0.452	3.33 ± 0.70	0.017	12.64 ± 0.66	0.739	3.44 ± 1.62	0.119
	No	37	44.33 ± 11.86		14.03 ± 04.45		2.27 ± 0.72		13.10 ± 2.37		1.27 ± 2.32	
NPSLE	Yes	2	52.04 ± 07.45	0.341	09.65 ± 01.89	0.174	3.46 ± 0.94	0.031	11.72 ± 0.24	0.402	4.45 ± 1.57	0.058
	No	48	44.06 ± 11.58		14.09 ± 04.50		2.29 ± 0.72		13.13 ± 2.33		1.27 ± 2.28	

Table 4
Correlation between each CD19$^+$ B cell subset frequencies and clinical parameters in SLE.

Laboratory test parameters	Case	Total B cells		SM B cells		PC B cells		PD-L1$^+$B cells		PC PD-L1$^+$B cells	
		r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
SLEDAI	50	0.1991	0.0012	0.1462	0.0061	0.1744	0.0026	0.1169	0.0152	0.4165	0.0021
24-h urine protein (g/24 h)	33	0.0436	0.2436	0.0048	0.7006	0.0426	0.2492	0.1452	0.0287	0.0831	0.1037
C3↓ (g/L)	43	−0.0021	0.7703	0.0100	0.5229	−0.0198	0.3684	−0.1059	0.0332	0.0091	0.5427
C4↓ (g/L)	26	−0.0687	0.1957	−0.0700	0.1913	−0.1506	0.0401	−0.0500	0.2720	−0.0384	0.3373
ESR↑	50	0.1700	0.0029	0.0849	0.0400	0.1442	0.0065	0.0232	0.2908	0.0128	0.4345
CRP↑	50	0.0092	0.9152	0.0307	0.2234	0.1724	0.9808	0.1189	0.0142	0.0070	0.5661
IgG (g/L)	50	0.0243	0.2799	0.0152	0.3938	0.0920	0.0321	0.0149	0.6178	0.0608	0.0844

Note: SLEDAI Systemic Lupus Erythematosus Disease Activity Index. ESR, erythrocyte sedimentation rate. CRP, C-reactive protein.
Spearman's correlation coefficient (r) was applied to detect correlations between two numerical data.

Table 5
Multivariate linear regression analysis results.

Model		Unstandardized Coefficients		Standardized Coefficients	F	t	P
		B	Std. Error	Beta			
a	(Constant)	5.547	0.377		5.927	14.715	0.000
	SLEDAI	0.103	0.035	0.483		2.951	0.005
	ESR↑	−0.004	0.010	−0.061		−0.372	0.712
b	(Constant)	36.760	2.994		4.369	12.278	0.000
	SLEDAI	0.505	0.278	0.305		1.813	0.046
	ESR↑	0.059	0.078	0.128		0.763	0.449
c	(Constant)	1.225	0.324		4.643	3.780	0.000
	SLEDAI	0.042	0.018	0.382		2.260	0.029
	C4↓	0.095	0.461	0.028		0.206	0.838
	ESR↑	0.003	0.005	0.093		0.531	0.598
	IgG↑	0.037	0.017	0.017		2.177	0.035
d	(Constant)	3.918	0.875		6.342	4.479	0.000
	SLEDAI	0.010	0.033	0.043		3.312	0.023
	24 h urine protein (g/24 h)	0.621	0.139	0.586		4.449	0.000
	C3↓	0.652	1.020	0.086		2.640	0.031
	CRP↑	0.001	0.006	0.024		0.186	0.853

Note: a. Dependent Variable: Total B cells. b. Dependent Variable: SM B cells. c. Dependent Variable: PC B cells. d. Dependent Variable PD-L1$^+$B cells.

SLE active group compared to the SLE stability group and HCs, respectively: (Active: 7.27% ± 1.37% vs Low-active: 5.22% ± 0.51%, $P < .0001$); (Active: 7.27% ± 1.37% vs HCs: 4.58% ± 0.77%, $P < .0001$). In the subgroups, SLE patients exhibited a significantly higher percentage of SM (SLE: 44.38% ± 11.50% vs. HCs: 39.59% ± 5.48%, $P < .05$), DN (SLE: 13.91% ± 4.50% vs. HCs: 8.39% ± 2.35%, $P < .001$) and PC (SLE: 2.34% ± 0.76% vs. HCs: 0.85% ± 0.33%, $P < .001$). There were no significant differences between the 2 sub-groups, NSM and N, in each of the 3 groups (Fig.2).

3.2. PD-L1 is differentially expressed on CD19$^+$B-cells and subsets

After circling CD19$^+$B-cells from lymphocytes, the surface PD-1 phenotype of CD19$^+$B-cells was detected via flow cytometry. Simultaneously, an isotype control of PD-L1 and HCs was established in both SLE patient groups (Fig. 3A, B). In order to confirm whether PD-L1 was differentially expressed in the B-cell subsets of patients and normal subjects, PD-L1 expression on the surface of the five B-cell subpopulations was analyzed using flow cytometry (Fig. 3C). In addition, we divided 50 patients into an SLE disease stable group ($n = 21$) and an SLE disease active group ($n = 29$). Furthermore, according to the presence or absence of kidney damage, the patients were divided into a lupus nephritis group ($n = 35$) and a non-lupus nephritis group ($n = 15$).

We analyzed the PD-L1$^+$B-cells in 21 SLE stable and 29 SLE active patients, as well as in healthy control group. The number of PD-L1$^+$B-cells was significantly higher in SLE patients compared to that of HCs. Furthermore, the number of PD-L1$^+$B-cells in the SLE disease active group was significantly higher than that in the SLE disease stable group. The number of PD-L1$^+$B-cells were significantly higher in the lupus nephritis group compared to the non-lupus nephritis group (Fig. 3D, E). Interestingly, we found that in two subsets of the three subgroups with abnormally high levels of expression, PD-L1 expression was also increased. They were double negative B-cells (DN, CD19$^+$ IgD$^-$ CD27$^-$ B-cells) and plasma cells (PC, CD19$^+$ IgD$^-$ CD27^{++} B-cells). These results suggest that PD-L1 may be involved in the regulatory mechanisms of B-cell subsets (Fig. 3F).

3.3. Clinical correlation data

To explore the relationship between the abnormal distribution of peripheral blood cells and clinical indices in SLE patients, correlation analysis of clinical manifestations and laboratory parameters of CD19$^+$B-cells, SM, DN, PC, CD19$^+$ PD-L1$^+$ B-cells, DN PD-L1$^+$ B-cells, and PC PD-L1$^+$ B-cells in peripheral blood was performed (Tables 2–5).

By comparing laboratory parameters, we found that the expression of CD19$^+$B-cells and CD19$^+$PD-L1$^+$B-cells in anti-dsDNA (+) SLE patients was higher than that of anti-dsDNA (−) SLE patients. The decrease in complement C3 levels in the patient group was greater than that in the normal group; and the expression of CD19$^+$B-cells in the peripheral blood of the high-IgG and low-IgM groups was higher than that of the corresponding normal groups. A comparative analysis of related clinical manifestations indicated that the expression of peripheral blood CD19$^+$B-cells and CD19$^+$PD-L1$^+$B-cells was significantly higher in lupus nephritis patients than in non-lupus nephritis patients. CD19$^+$B expression in patients with Raynaud's phenomenon and alopecia was higher than that in patients without clinical manifestations. Furthermore, CD19$^+$PD-L1$^+$B-expression in patients with psychotic lupus erythematosus was significantly higher than that in those without psychotic lupus (Table 2).

A comparative evaluation of subgroups indicated that the percentage of SM B-cells in the elevated IgA group and lupus nephritis group was higher than that in HC and non-lupus nephritis groups, respectively. Additionally, the expression of DN B-cells in the anti-SSA (+) group was higher than that of the (−) group. The number of PC cells in the peripheral blood of the anti-dsDNA (+) and anti-Chrom (+) groups were higher than that in the corresponding (−) groups. In particular, PC cells in the peripheral blood of patients with lupus nephritis, serous inflammatory disease, and mental nervous performance were higher in number compared to the corresponding no expression groups. The expression of DN PD-L1$^+$B-cells in the anti-SSB (+) group, complement C3 and IgM descending group, and sphenoid erythema group was higher than that in the corresponding HC group. Furthermore, the PC PD-L1$^+$B-cell expression in peripheral blood of the elevated IgA group and the associated arthritis group was significantly higher than that of the HC group (Table 3).

The correlation between laboratory parameters and streaming data was analyzed. Our results indicated that the expression of CD19$^+$B-cells and SM B-cells in the peripheral blood was positively correlated with the SLEDAI score (CD19$^+$B-cells: $r = 0.1991$, $P = .0012$ and SM B-cells: $r = 0.1462$, $P = .0061$) and the increased erythrocyte sedimentation rate (ESR) (CD19$^+$B-cells: $r = 0.1700$, $P = .0029$ and SM B-cells: $r = 0.0849$, $P = .0400$). The percentage of PC cell expression was positively correlated with the SLEDAI score ($r = 0.1744$, $P = .0026$), increased ESR ($r = 0.1442$, $P = .0065$) and IgG ($r = 0.0920$ $P = .0321$), and negatively correlated with the decrease in complement C4 ($r = -0.1506$, $P = .0401$). The percentage of PD-L1$^+$B-cells in the peripheral blood of patients was positively correlated to the SLEDAI score ($r = 0.1169$, $P = .0152$), 24-h urine protein quantification ($r = 0.1452$, $P = .0287$) and C-reactive protein (CRP) elevation ($r = 0.1189$, $P = .0142$), and negatively correlated to the decrease in complement C3 ($r = -0.1059$, $P = .0332$). Finally, the expression of PC PD-L1$^+$B-cell expression was positively correlated to the SLEDAI score ($r = 0.4165$, $P = .0021$). No significant correlations between DN B-cells and DN PD-L1$^+$B-cells, the laboratory parameters and linear correlation diagram were found (Table 4 and Fig. 4). Furthermore, multivariate linear regression analysis results show that SLEDAI score was influenced by multiple B cell populations, CD19$^+$PD-L1$^+$B-cells were associated with multiple laboratory parameters in this study. Detailed results were shown in (Table 5).

3.4. Correlation between CD19$^+$PD-L1$^+$B-cells and Tfh

Tfh cells are the most important T-cell subsets of adjuvant B-cell-producing antibodies. Recent studies have confirmed that Tfh cells were involved in the pathogenesis of lupus rats. Therefore, we evaluated the expression of CD4$^+$ cells and Tfh cells in peripheral blood using flow

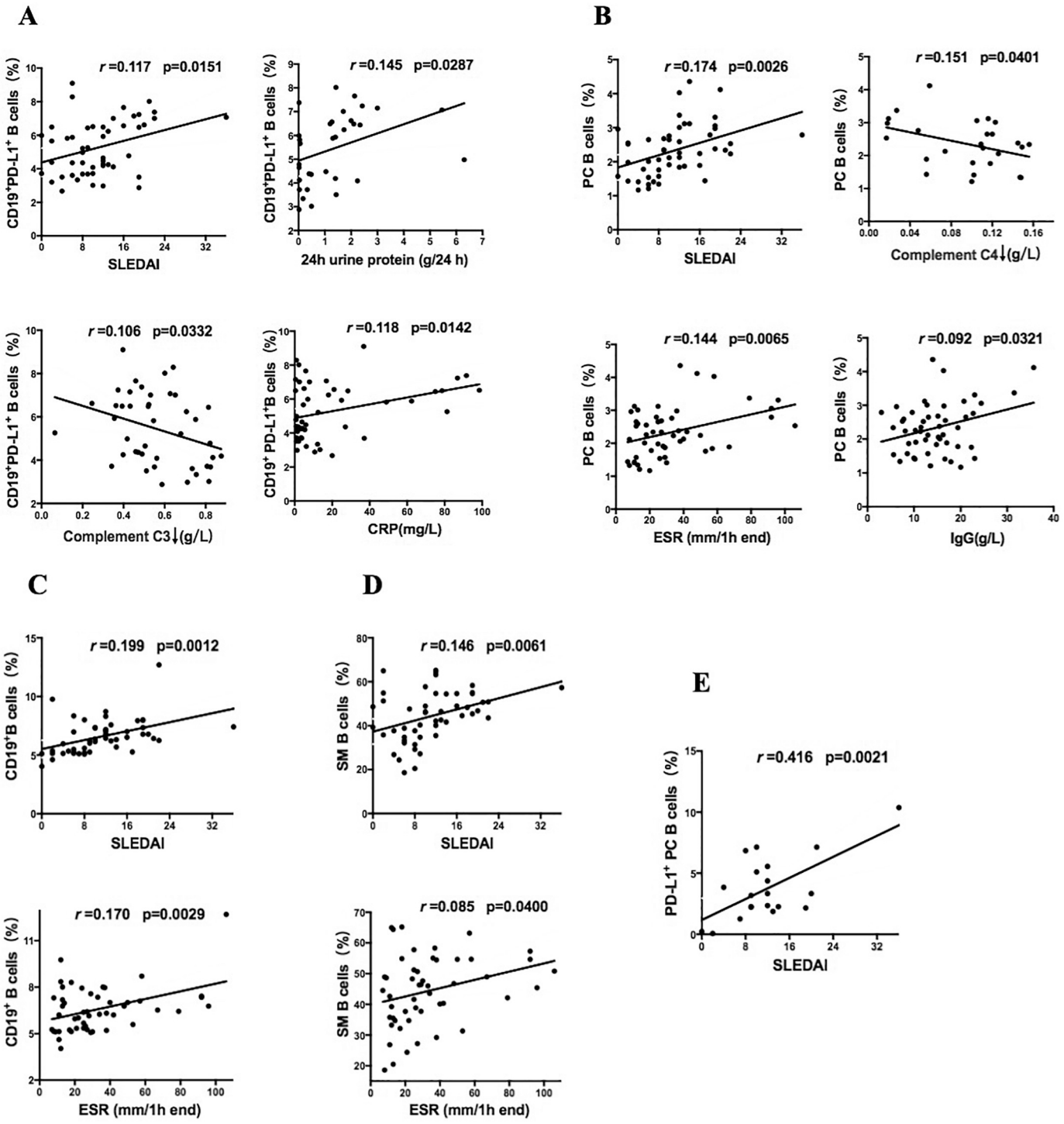

Fig. 4. Clinical correlation diagram. A: Linear correlation between CD19$^+$PD-L1$^+$B-cells and SLEDAI score, 24-h proteinuria, complement C3 decrease, and CRP increase in the peripheral blood of patients with SLE. B: Linear correlation between PC B-cells and SLEDAI score, complement C4 decrease, ESR, and IgG increase in the peripheral blood of patients with SLE. C: Linear correlation between CD19$^+$ B-cells and SLEDAI score and ESR in the peripheral blood of patients with SLE. D: Linear correlation between SM B-cells and SLEDAI score and ESR. E: Linear correlation between PD-L1$^+$ PC B-cells and SLEDAI score increase in the peripheral blood of patients with SLE. $P < .05$ is statistically significant.

cytometry, and analyzed the correlation between CD19$^+$PD-L1$^+$B-cell expression and Tfh in the peripheral blood of patients. Statistical analysis indicated that CD4$^+$T-cell expression in the peripheral blood of SLE patients was higher in the SLE active group than that in the SLE stable group and the HC group, while that in the SLE stable group was higher than that in the HC group. Additionally, the Tfh cell expression in the SLE active group was significantly higher than that in the SLE stable group and the HC group, but there was no significant difference between levels in the SLE stable group and the HC group. Additionally, linear regression analysis revealed that the percentages of CD19$^+$ PD-L1$^+$B-cells were positively correlated to CD4$^+$T-cells ($r = 0.3021$, $P < .0001$) and Tfh cells ($r = 0.1117$, $P = .0177$) in the peripheral blood of SLE patients (Fig. 5).

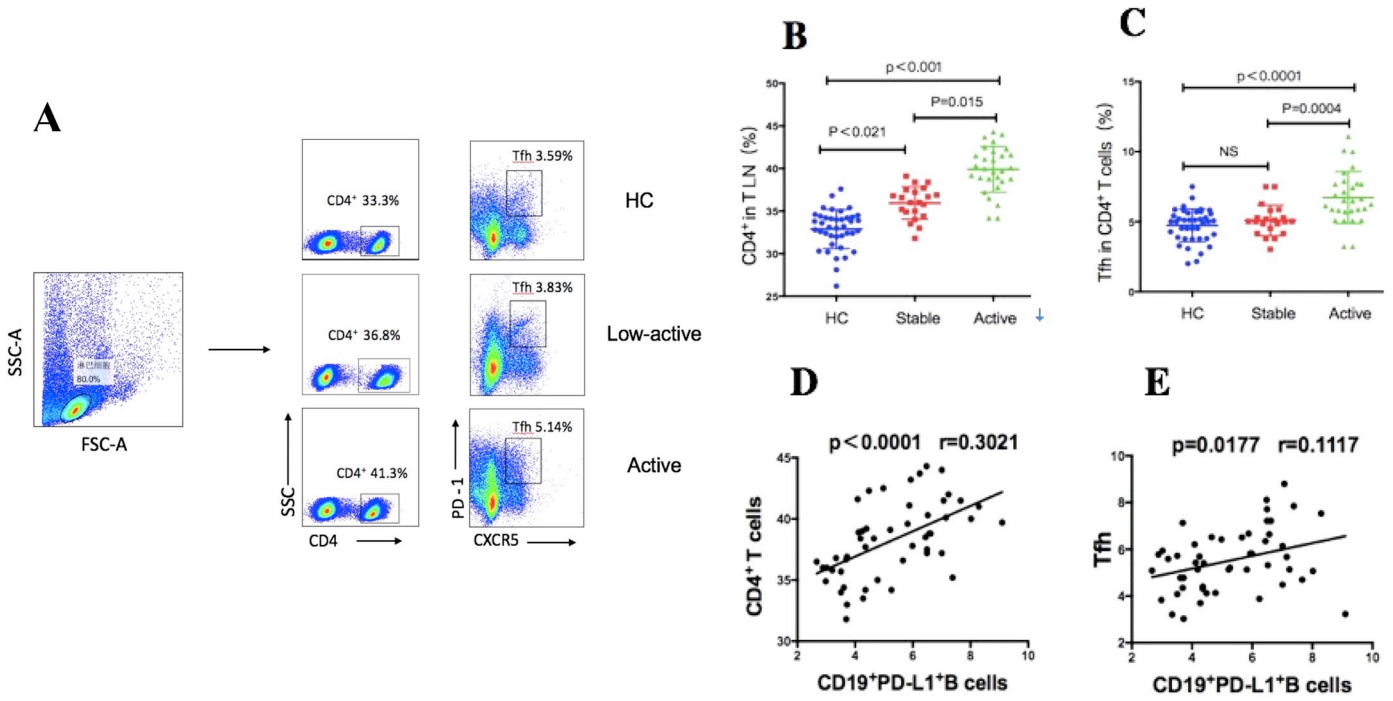

Fig. 5. Tfh cell population was positively correlated with $CD19^+PD\text{-}L1^+$B-cell population. A: Gating strategy of $CD4^+$T-cells and $CD4^+CXCR5^+PD\text{-}1^+$Tfh cells in lymphocytes. B: Frequency of $CD4^+$T-cells in lymphocytes. C: Frequency of Tfh cells in $CD4^+$T-cells. D: Linear regression analysis between $CD4^+$T-cells and $CD19^+PD\text{-}L1^+$B-cells in patients with SLE. E: Linear regression analysis between Tfh cells and $CD19^+PD\text{-}L1^+$B-cells in patients with SLE.

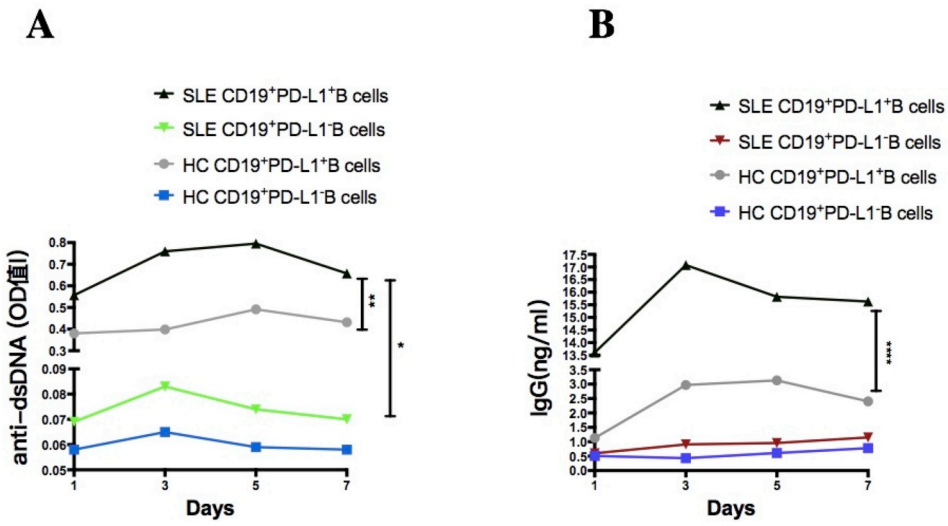

Fig. 6. Functional Analysis of $CD19^+PD\text{-}L1^+$B-cells. A: The secretion of anti-dsDNA by $CD19^+PD\text{-}L1^+$B-cells and $CD19^+PD\text{-}L1^-$B-cells was compared in SLE patient and HC patient peripheral blood samples at 1, 3, 5, and 7 days after culturing in vitro. B: The secretion of IgG by $CD19^+PD\text{-}L1^+$B-cells and $CD19^+PD\text{-}L1^-$B-cells was compared in SLE patient and control patient peripheral blood samples at 1, 3, 5, and 7 days after culturing in vitro. $^*P < .05$, $^{**}P < .01$, $^{****}P < .0001$, no statistical significance with no * sign.

3.5. The production of IgG and anti-dsDNA from $CD19^+PD\text{-}L1^+$B-cells in patients

To address the functional importance of the differential PD-L1 expression in B-cells, we extracted $CD19^+PD\text{-}L1^+$B-cells and $CD19^+PD\text{-}L1^-$B-cells from the peripheral blood of SLE patients and HC patients using a flow sorting technique, followed by in vitro culturing with a stimulating agent. The supernatant was collected on days 1, 3, 5, and 7 of cultivation, respectively. Finally, ELISA was used to detect secretion of IgG and anti-dsDNA by the two cell types. Result indicated that the amount of IgG and anti-dsDNA secreted by $CD19^+PD\text{-}L1^+$B-cells in the peripheral blood of SLE patients was significantly higher than that in the HC group ($P < .01$), while the amount of anti-dsDNA secreted by $CD19^+PD\text{-}L1^+$B-cells in the peripheral blood of SLE patients was also significantly higher than that secreted by the $CD19^+PD\text{-}L1^-$B-cells ($P < .05$) (Fig. 6).

4. Discussion

PD-L1, a PD-1 receptor, is located on human chromosome 9p24 and mouse chromosome 19B. PD-1/PD-L1, an important negative costimulatory molecule active in the combination, proliferation, differentiation, and cytokine production processes of T- and B-cells, was inhibited by several immune regulatory processes, including tyrosine phosphorylation in the ITSM structure domain [[22–24]]. However, some studies suggest that PD-L1 may have an ambiguous second receptor [25] and that the combination of PD-L1 and this second receptor may stimulate the activation and proliferation of T- and B- cells leading to a positive immune regulation effect. Therefore, the combination of PD-L1 and different receptors may exert both positive and negative immune regulation, thereby allowing PD-L1 to act as a molecule with a bidirectional regulation function. Animal experiments demonstrated that PD-1 gene-deficient C57BL/6 mice showed autoimmune-enlarged

cardiomyopathy, lupus-like glomerulonephritis, and arthritis [26–28]. Therefore, it has been suggested that PD-L1 and its negative receptor PD-1 may be highly involved in maintaining immune balance and preventing autoimmune diseases.

B-cells reportedly induce autoimmune disease through multiple antibody-independent mechanisms and therefore play a pivotal role in the initiation and perpetuation of SLE by producing autoantibodies [29–30]. In particular, related studies have indicated that plasmablasts may play a central role in the pathogenesis of SLE, through the production of the anti-dsDNA antibody [31]. Clinical and experimental data indicate a close relationship between IgD^-CD27^-B cells (DN) and SLE pathology [[32–34]], while IgD^-CD27^-B cells expressing mutated BCRs have been identified both in the tonsils and in very small numbers in the peripheral blood of healthy donors [35–36]. Our results indicated that, the expression of $CD19^+$B cells and their partial subsets (SM, DN, and PC) was significantly higher in the peripheral blood of SLE patients compared to that in the HCs. $CD19^+PD\text{-}L1^+$B cells were also more abundant in SLE patients than in the HCs, while their levels in the activity group were also higher than in the stable group. Notably, these abnormal cell distributions were correlated to the disease activity index. Statistical analysis demonstrated that PD-L1 expression in the three B-cell subsets of SM, DN, and PC, were also increased in comparison to that in the HC group. In particular, the percentage of $CD19^+PD\text{-}L1^+$B-cells and PC B-cells were correlated to a variety of disease activity indices (Fig. 3A, B). This suggests that $CD19^+$B-cells and their subsets, as indicated by their PD-L1 expression, may be involved in the pathogenesis and progression of SLE.

$CD4^+$T-helper-cells play an important role in disease progression and pathology [37]. Moreover, Tfh-cells, which are an essential helper subset for B-cell maturation [38], are elevated in the peripheral blood of lupus patients [39]. Therefore, we used a flow technique to detect $CD4^+$T-cells and Tfh cells in the peripheral blood of SLE patients, and our results showed that the expression $CD4^+$T-cells and Tfh cells in the SLE active group were significantly higher than in the SLE stable group and the HC group, respectively. Notably, this is consistent with previous research results. Interestingly, our study indicated that $CD19^+PD\text{-}1^+$B-cells showed a significant, positive correlation with $CD4^+$T-cells and Tfh cells. The abnormal distribution of $CD19^+PD\text{-}L1^+$B-cells in the peripheral blood of SLE patients is correlated with the activity index of the disease, and a positive correlation was present between Tfh cells that produce antibodies and helper B-cells. Based on current results, we hypothesized that the $CD19^+PD\text{-}L1^+$B-cells in the peripheral blood of SLE patients were a type of B-cell with a certain function and nature. In order to further clarify the regulatory function of these cells in the peripheral blood of SLE patients, an in vitro study was subsequently conducted.

The in vitro culture results indicated that the secretion of anti-dsDNA and IgG by $CD19^+PD\text{-}L1^+$B-cells was significantly higher in the SLE patient group, compared to that by $CD19^+PD\text{-}L1^+$B-cells in the peripheral blood of the HC group. $CD19^+PD\text{-}L1^+$B-cell-secreted anti-dsDNA was also significantly higher than that secreted by $CD19^+PD\text{-}L1^-$B-cells. The above findings suggested that $CD19^+PD\text{-}L1^+$B-cells may belong to a class of abnormal activated B-cells, or B-cell subsets, that initiate a pathogenic effect in the peripheral blood of SLE patients.

There were some limitations and deficiencies in our experiment. The first issue of note was the small sample size used. Second, the investigation did not include a coculture of $CD19^+PD\text{-}L1^+$B-cells and Tfh cells. Third, the experiment did not consider $CD19^+PD\text{-}L1^+$B-cells within the serum of patients and HC for coculture in order to exclude the environmental effects on SLE patients. Finally, lack of hormonal interference was not taken into consideration. However, despite these limitations, our results, based on the production of antibodies and cytokines as well as the presence of significant correlation with disease activity indices, indicate that $CD19^+PD\text{-}L1^+$B-cells may be a class of overactive B-cells which are potentially involved in the pathogenesis of SLE. Therefore, we propose conducting further studies in order to investigate the mechanisms operating at the protein and gene level to further verify our current results.

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Disclosures

The authors have no financial conflict of interest.

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