

# Dysfunction of CD27+IgD+B cells correlates with aggravated systemic lupus erythematosus

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September 11, 2020

## Abstract

Apoptotic signaling pathway is obviously disordered in systemic lupus erythematosus (SLE). The contemporaneous occurrence of enhanced apoptosis and impaired phagocytosis lead to the cumulative exposure to autoantigens, resulting in autoantibody production and autoimmunity. Natural IgM (nIgM) plays a key role in the clearance of apoptotic cells and prevents them from inducing abnormal autoimmunity. B-1 cells and innate-like B cells (ILBs) are proved to be the major producer of natural IgM. Human CD27+IgD+B cells, also termed as un-switched memory B cells, are recently proposed to be a kind of ILBs. However, functional features and characteristics of these cells in SLE remain poorly understood. In this study, we find that in SLE patients the frequencies of CD27+IgD+B cells are significantly decreased. Moreover, these cells are functionally impaired in producing natural antibody-like IgM. These CD27+IgD+B cells are negatively correlated with SLE patient clinical and immunological features. After effective therapy with disease remission in SLE, the frequencies of these cells could be recovered. Taken together, our results suggest that the dysfunction of CD27+IgD+B cells potentially contribute to the exacerbation of SLE, and modulating the features of these cells might provide therapeutic target for this persistent disease.

## Dysfunction of CD27+IgD+B cells correlates with aggravated systemic lupus erythematosus

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## Summary

Apoptotic signaling pathway is obviously disordered in systemic lupus erythematosus (SLE). The contemporaneous occurrence of enhanced apoptosis and impaired phagocytosis lead to the cumulative exposure to autoantigens, resulting in autoantibody production and autoimmunity. Natural IgM (nIgM) plays a key role in the clearance of apoptotic cells and prevents them from inducing abnormal autoimmunity. B-1 cells and innate-like B cells (ILBs) are proved to be the major producer of natural IgM. Human CD27<sup>+</sup>IgD<sup>+</sup>B cells, also termed as un-switched memory B cells, are recently proposed to be a kind of ILBs. However, functional features and characteristics of these cells in SLE remain poorly understood. In this study, we find that in SLE patients the frequencies of CD27<sup>+</sup>IgD<sup>+</sup>B cells are significantly decreased. Moreover, these cells are functionally impaired in producing natural antibody-like IgM. These CD27<sup>+</sup>IgD<sup>+</sup>B cells are negatively correlated with SLE patient clinical and immunological features. After effective therapy with disease remission in SLE, the frequencies of these cells could be recovered. Taken together, our results suggest that the dysfunction of CD27<sup>+</sup>IgD<sup>+</sup>B cells potentially contribute to the exacerbation of SLE, and modulating the features of these cells might provide therapeutic target for this persistent disease.

**Keywords:** systemic lupus erythematosus, innate-like B cells, CD27<sup>+</sup>IgD<sup>+</sup> B cells, natural IgM

## Introduction

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease that develops in genetically susceptible individuals in response to environmental factors(1). The pathogenesis remains to be fully elucidated. Autoantibodies are key mediators in determining the clinical manifestations of SLE. The mechanisms by which antibodies may be harmful to self-tissues encompass complement mediated inflammation, cell apoptosis and immune-complexes mediated damage. However, the precise cooperation of antibodies in SLE have not been unraveled so far(2).

Apoptosis is an energy-dependent process that leads to the programmed destruction of cells. It is tightly regulated by the expression of cell surface molecules such as Fas and intracellular protooncogenes including Bcl-2 and the Bax family members(3). It has been proved that apoptotic signaling pathway is obviously disordered in SLE patients. Abnormal expression of a large number of apoptotic signaling molecules such as TNF-related apoptosis inducing ligand (TRAIL), TNF-like weak inducer of apoptosis (TWEAK) and death ligand FasL (Fas ligand) leads to abnormal increase of apoptotic cells(4, 5). In addition, the body of SLE patients also has obstacles to the removal of apoptotic cells. Cell death, including apoptosis, necrosis and NETosis (special cell death of neutrophil through NETs), is the major potential resource of self-dsDNA which activates the immune system and leads finally to autoimmune disease(6).

Natural immunity is responsible for identification and elimination of damaged and apoptotic cells. Meanwhile, it also complete the intricate regulation of inflammatory response and enhance immune tolerance. Natural IgM(nIgM) is an important component of the body's innate immune system. nIgM is an evolutionarily conserved molecule and reacts with a variety of epitopes expressed on both self-and non-self antigens (7). nIgM deficiency is associated with an increased tendency toward the development of autoimmune disease. It plays a key role in the clearance of apoptotic cells and prevents apoptotic cells from inducing abnormal autoimmunity.

B-1 cells and innate-like B cells (ILBs) are proved to be the major producer of natural IgM. Early transfer experiments showed that more than 80% of serum natural IgM is derived from B1 cells(8). ILBs are heterogeneous populations of unconventional B cells with innate sensing and responding properties. In mice, ILBs are composed of B1 cells, marginal zone (MZ) B cells and other related B cells. CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>+</sup>B cells which are also termed as un-switched memory B cells have been proposed to be a kind of human ILBs(9). ILBs maintain natural IgM levels at steady state, and they can rapidly acquire immune regulatory activities through the secretion of natural IgM and IL-10 after innate activation(10). Our previous study indicated that CD27<sup>+</sup>IgD<sup>+</sup>B cells were impaired in rheumatoid arthritis (RA) with dysfunctional features, which might contribute to the disease perpetuation(11). Nevertheless, the characteristics of CD27<sup>+</sup>IgD<sup>+</sup> B

cells and their potential role in SLE are largely unknown.

In this study, we determined the frequencies and natural antibody-like IgM-producing capacity of CD27<sup>+</sup>IgD<sup>+</sup>B cells in SLE patients. In addition, we also analyzed their clinical associations and revealed their tendency after therapy.

## Materials and Methods

### Patients and specimens

50 SLE patients and 50 healthy controls (HC) were enrolled in the study. SLE patients were recruited from the First Affiliated Hospital of Baotou Medical College who fulfilled the American College of Rheumatology criteria for the classification of SLE(12). The main characteristics of SLE patients are listed in Table 1. Disease activity was measured by the modified Systemic Lupus Erythematosus Disease Activity Index (SLEDAI-2K) (13), and was divided into low-disease activity (LDA) (SLEDAI 0-4, n = 34) and active disease (SLEDAI > 4, n = 16). This study was approved by Institutional Medical Ethics Review Board of Baotou Medical College, and all the participants provided written informed consent.

Twelve SLE patients were follow-up study. All patients had received immunosuppressant and achieved remission. Blood samples were obtained before the initiation of treatment and after 4 weeks of treatment. The characteristics of the patients before and after treatment are shown in Table 2

### Flow cytometry analysis

For CD27<sup>+</sup>IgD<sup>+</sup> B cell detection, 2ml blood was collected from SLE patients and healthy controls, and 100 $\mu$ l fresh whole blood cells was extracted and stained with the following antibodies: APC-CY7-conjugated anti-CD19 (Biolegend, San Diego, CA, USA), APC-conjugated anti-CD27 (eBioscience, San Diego, CA, USA), and FITC-conjugated anti-IgD (eBioscience). Then the red blood cells were lysed using the RBC lysis buffer (MultiSciences, Hangzhou, China) and the left cells were analyzed on FACS Aria II. Dead cell exclusion was performed by scatter profiles and 7-AAD staining during all the flow cytometric analyses.

### Cell sorting and Culture

Fluorescence-activated cell sorting (FACS) were used to purify CD27<sup>+</sup>IgD<sup>+</sup> B cells, 10ml blood was collected from SLE patients and healthy controls, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples using Ficoll density-gradient centrifugation, and then were stained as described above. After that, the aimed cells were harvested into the collection solution (RPMI 1640 supplemented with 10% FBS and 2% antibiotics) using FACS Aria II flow cytometry sorter according to the manufacturer's instructions. The purified cells were further analyzed after sorting, the purity of which was 95–99%.

### Quantitative real-time RT-PCR analysis

Total RNA was extracted from CD27<sup>+</sup>IgD<sup>+</sup> B cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), and reverse transcribed into cDNA with the RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according to the manufacturer's instructions. The resulting cDNA was subjected to real-time PCR analyses. Gene expression was quantified relative to the expression of the housekeeping gene GAPDH as well as  $\beta$ -actin, and was normalized to control by standard  $2^{-\Delta\Delta T}$  calculation(11).

### ELISPOT analysis

ELISPOT was performed using the ELISpotPLUS Human IgM Kit (MABTECH AB, Sweden). Briefly,  $1 \times 10^4$  flow cytometry-sorted CD27<sup>+</sup>IgD<sup>+</sup> B cells as well as other B cell subsets from healthy individuals or SLE patients were subjected to IgM detection in the presence of anti-CD40 (3  $\mu$ g/ml, eBioscience) and CpG (10  $\mu$ g/ml, Invivogen, San Diego, CA, USA) for 24h. The results were analyzed on an ImmunoSpot Analyzer (Cellular Technology Ltd., Shaker Heights, OH, USA)

### Statistics analysis

All statistical calculations were performed using the statistical software program SPSS 17.0 (SPSS, Chicago, IL, USA). Differences between various groups were evaluated by the Student's t-test, paired t-test, one-way ANOVA test, or spearman test, and were statistically significant at  $P < 0.05$ . **Results** **The frequencies of CD27<sup>+</sup>IgD<sup>+</sup>B cells are significantly decreased in SLE patients**

B cells could be divided into four subsets based on their expression of CD27 and IgD, including naïve B cells (CD27<sup>+</sup>IgD<sup>+</sup>), Innate-like B cells (CD27<sup>+</sup>IgD<sup>+</sup>), switched memory B cells (CD27<sup>+</sup>IgD<sup>+</sup>), and double negative B cells (CD27<sup>+</sup>IgD<sup>+</sup>) as demonstrated in Figure 1A. To reveal the role of CD27<sup>+</sup>IgD<sup>+</sup>B cells in SLE pathogenesis, we first compared their frequencies between SLE patients and healthy individuals. As shown in Figure 1B and C, compared to the 50 healthy volunteers, the frequencies of CD27<sup>+</sup>IgD<sup>+</sup>B cells were significantly decreased in the peripheral blood of 50 SLE patients.

### **CD27<sup>+</sup>IgD<sup>+</sup> B cells correlate with SLE patient clinical and immunological features**

The associations of the CD27<sup>+</sup>IgD<sup>+</sup> B cell frequencies with demographic and clinical characteristics were analyzed. As shown in Figures 2A–L and Table 3, WBC, Platelet count and serum C3 level positively correlated with CD27<sup>+</sup>IgD<sup>+</sup>B cells. We also found the frequencies of these CD27<sup>+</sup>IgD<sup>+</sup>B cells were negatively correlated with Serum Creatinine level, SLEDAI and Anti-dsDNA. However, no correlations were noted between the disease duration, ESR, serum C4 level and serum IgA, IgG, IgM level with CD27<sup>+</sup>IgD<sup>+</sup>B cells. These data demonstrated that CD27<sup>+</sup>IgD<sup>+</sup>B cells were numerically deficient under SLE circumstance.

### **CD27<sup>+</sup>IgD<sup>+</sup>B cells Demonstrate Dampened IgM-Producing competency in SLE**

To determine the functional changes of CD27<sup>+</sup>IgD<sup>+</sup>B cells, we then examined the production of IgM by CD27<sup>+</sup>IgD<sup>+</sup>B cells in SLE patients. CD27<sup>+</sup>IgD<sup>+</sup>B cells from active SLE patients (SLEDAI > 5) and healthy individuals were isolated by flow cytometry sorting and subjected to ELISPOT and QPCR analyses. ELISPOT analysis showed that under SLE circumstance, the IgM-producing capacities of these cells were dampened (Figure 3A). QPCR analyses further confirmed that the IgM transcripts of these CD27<sup>+</sup>IgD<sup>+</sup>B cells were significantly decreased in SLE (Figure 3B). Taken together, these results indicated that CD27<sup>+</sup>IgD<sup>+</sup>B cells were functionally impaired in producing IgM in SLE.

### **CD27<sup>+</sup>IgD<sup>+</sup>B cells are recovered in SLE patients with disease remission after therapy**

To ascertain the usefulness of CD27<sup>+</sup>IgD<sup>+</sup> B cells as a biomarker for disease activity, we further evaluated whether these cells would be recovered after effective therapy. 12 patients who were diagnosed as SLE were studied when they were in relapse and 4 weeks after the initiation of treatments. As shown in Table 2, after treatments reduced significantly disease activity as measured by SLEDAI. The anti-dsDNA and 24h urinary protein were decreased with the serum C3 and C4 normalized to some extent. The total number of leukocyte and platelet were increased after the treatments.

The frequency of CD27<sup>+</sup>IgD<sup>+</sup>B cells were examined before and after 4 weeks of initiation of treatments are shown in Figure 4A. As measured by SLEDAI, all patients showed a significant reduction in disease activity after treatment (Figure 4B) and the frequency of CD27<sup>+</sup>IgD<sup>+</sup>B cells was significantly increased (Figure 4C). All these results suggested that the impaired CD27<sup>+</sup>IgD<sup>+</sup> B cells in SLE patients could be recovered after effective therapy, indicating their deficiency might be correlated with the development of SLE.

## **Discussion**

In this study, we observed that the rate of CD27<sup>+</sup>IgD<sup>+</sup>B cells were decreased and functionally impaired in producing natural antibody-like IgM in patients with SLE when compared with healthy controls. The number of CD27<sup>+</sup>IgD<sup>+</sup>B cells is correlated with the clinical characteristics of SLE patients. After effective therapy, these CD27<sup>+</sup>IgD<sup>+</sup>B cells could get recovered.

In SLE, the number of apoptotic cells increases while the phagocytosis function is impaired (14, 15). The contemporaneous occurrence of enhanced apoptosis and impaired phagocytosis is considered to be a key process in the pathogenesis of SLE and can lead to the cumulative exposure to the autoantigens which resulted in autoantibody production and autoimmunity (16). Apoptotic cells are normally phagocytosed

by professional phagocytes such as macrophages, but the clearance of apoptotic cells depends not only on functioning phagocytes but also on soluble proteins that act as opsonins and/or bridging molecules. Phagocytosis of apoptotic cells can be enhanced by C-reactive protein (CRP), serum amyloid P component (SAP), C1q, IgM, MBL and other proteins, forming a redundant backup mechanism. Previous studies have found that phagocytic activity of macrophages is reduced three-fold to four-fold in the absence of IgM (17). Decreased natural IgM level in SLE patients may lead to inefficient clearance of apoptotic cells, leading to the accumulation of dead cells in peripheral blood(18). SLE patients were reported to demonstrate lower levels of anti-PC natural IgM, reductions of which correlated with duration of the disease. Lower levels of anti-PC natural IgM were also reported to be associated with more frequent cardiovascular events in patients with SLE (19). However, till now little is known about the causes of natural IgM defects in SLE patients.

In our previous study, we proved that CD27<sup>+</sup>IgD<sup>+</sup>B cells can spontaneously secrete

IgM that were polyreactive and low affinitive. We termed these CD27<sup>+</sup>IgD<sup>+</sup>B cells-derived IgM as natural antibody-like IgM. In this study, we found that CD27<sup>+</sup>IgD<sup>+</sup>B cells in SLE patients were significantly reduced and negatively correlated with SLEDAI and anti-dsDNA autoantibodies. These data are consistent with previous reports (20, 21) and, taken together, indicate that the B cell-subsets are disordered in SLE patients. Since different B cell subsets have different functional characteristics, the imbalance of their proportions will lead to the imbalance of immune homeostasis and promote the development of the disease to some extent. Cytokines such as IFN-gamma, BAFF, TNF-alpha, il-6, il-21 in the serum of SLE patients will affect the B cell signaling pathway and thus increase its activation and differentiation(22-24). Therefore, we speculated that the inflammatory environment in SLE patients is one of the reasons for the reduction of CD27<sup>+</sup>IgD<sup>+</sup>B cells, but this speculation need to be further studied.

In addition, QPCR and ELISPOT analyses showed that the ability of CD27<sup>+</sup>IgD<sup>+</sup>B cells to secrete IgM in SLE patients was significantly lower than that in healthy control, indicating that in SLE patients, CD27<sup>+</sup>IgD<sup>+</sup>B cells has defects not only in quantity, but also in function. Therefore, this is also a reason for the accumulation of apoptotic cells and the generation of autoimmunity in SLE patients. The decreased TCR or BCR diversity has been observed in cancers and autoimmune diseases, which may contribute to the development of the diseases(25). Our previous research has shown that the BCR repertoire of CD27<sup>+</sup>IgD<sup>+</sup>B cells was altered in RA. Compared with healthy controls, the variable region of the CD27<sup>+</sup>IgD<sup>+</sup>B cells-derived IgM  $\mu$  chain showed much narrower spectrum. Therefore, we speculated that the BCR repertoire of CD27<sup>+</sup>IgD<sup>+</sup>B cells was also changed in SLE, and the following study will be conducted to score the BCR repertoire of CD27<sup>+</sup>IgD<sup>+</sup>B cells in SLE patients.

In summary, we demonstrated that CD27<sup>+</sup>IgD<sup>+</sup>B cells were numerically and functionally impaired in SLE. CD27<sup>+</sup>IgD<sup>+</sup>B cells are a reliable biomarker for active SLE and that the role of these cells in the pathogenesis of SLE deserve to further investigation.

## Acknowledgements

This work was supported by the National Nature Science Foundation of China (81860295), the Inner Mongolia Autonomous Region Science and Technology Plan Project (201802089, 2019GG052), the Nature Science Foundation of Inner Mongolia(2017MS0809).

## Disclosures

The authors have no financial conflict of interest.

## Author's contributions

Conceived and designed the experiments: W. Z., Y. W., K.L.; Performed the experiments: W. Z., T.W.; Analyzed the data: W. Z., Y. W., F. H.; Contributed reagents, materials and analysis tools: L.F., F. H., Y. W.; Wrote the manuscript: W. Z.; Reviewed and edited the manuscript: F. H., K.L.

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Table 1 Clinical characteristics of SLE patients

Characteristics	SLE (N = 50)
Age, mean(range), years	45.6(17-67)
Female/male	46/4
Duration of diagnosis, mean(range), years	6.8(1-30)
SLEDAI score, mean(range)	3.8(0-12)
Anti-dsDNA, IU/mL, mean(range)	244.5(100-659)
IgG, g/L, mean(range)	14.35(7.4-29.2)
Serum C3, g/L, mean(range)	0.94(0.26-1.51)
Serum C4, g/L, mean(range)	0.19(0.03-0.40)
Serum Creatinine, $\mu$ mol/L, mean(range)	87.99(56-189)
WBC, 109/L (mean $\pm$ SD)	3.75 $\pm$ 1.04
Platelet, 1012/L (mean $\pm$ SD)	171.76 $\pm$ 70.76
Urinary proteins, g/24h, mean(range)	0.4(0-3.4)

Note: SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index; WBC, White blood cell

Table 2 Demographic and clinical characteristics of

SLE patients experiencing remission before and after treatment.

Characteristics	Before treatment (N=12)	After treatment (N=12)
Age, years	50 $\pm$ 12	50 $\pm$ 12
Female/male	10/2	10/2
SLEDAI score	8 $\pm$ 2	2 $\pm$ 2***
Anti-dsDNA, IU/mL	177.8 $\pm$ 51.32	98.92 $\pm$ 28.55**
Serum C3, g/L	0.72 $\pm$ 0.34	1.25 $\pm$ 0.46*
Serum C4, g/L	0.09 $\pm$ 0.07	0.21 $\pm$ 0.10*

Characteristics	Before treatment (N=12)	After treatment (N=12)
WBC, 10 <sup>9</sup> /L	2.98±1.57	3.53±0.63
Platelet, 10 <sup>12</sup> /L	124.0±65.9	157.08±69.94
Serum Creatinine, µmol/L	102.58±31.16	87.83±20.39
Urinary proteins, g/24h	1.73±1.58	0.20±0.19**

Note: All data are expressed as mean ± SD. \*p<0.05, \*\*p < 0.01,\*\*\*p <0.001 vs. the values before treatment

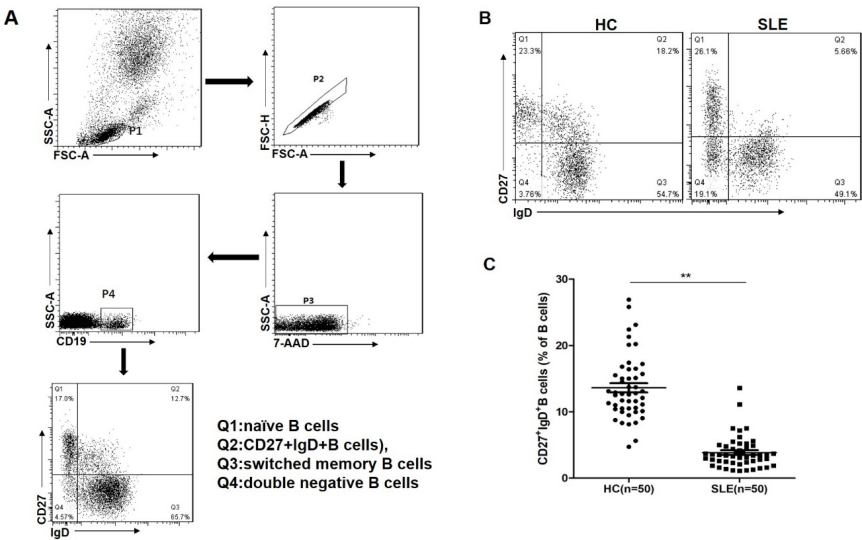


Fig. 1. CD27<sup>+</sup>IgD<sup>+</sup>B cells are decreased in the blood of patients with SLE compared to healthy controls. (A) Gating strategy to identify naïve B cells (CD27<sup>+</sup>IgD<sup>+</sup>), Innate-like B cells (CD27<sup>+</sup>IgD<sup>+</sup>), switched memory B cells (CD27<sup>+</sup>IgD<sup>-</sup>), and double negative B cells (CD27<sup>-</sup>IgD<sup>-</sup>) in human blood. (B) CD27<sup>+</sup>IgD<sup>+</sup>B cells from a representative healthy control (left panel) and an SLE patient (right panel). (C) Percentage of CD27<sup>+</sup>IgD<sup>+</sup>B cells in patients with SLE(n=50)and healthy controls(n=50).

Data are represented as mean ± SD or median ± interquartile range. Data points represent individual subjects. NS = not significant, \*\*p<0.01, \*\*\*p<0.001

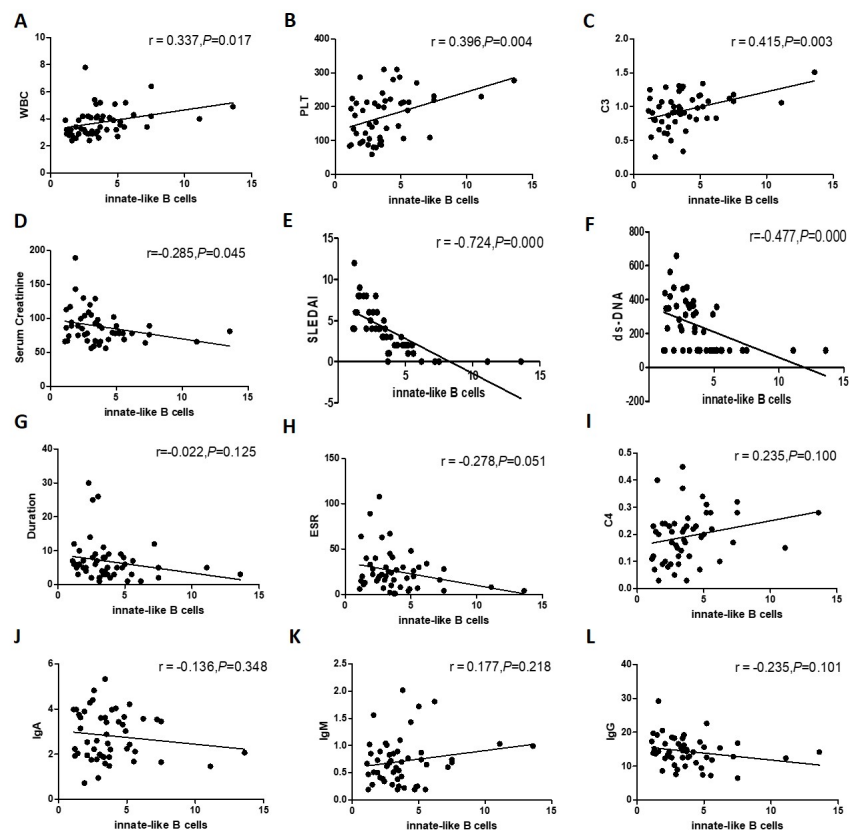


Fig.2 Correlation of CD27<sup>+</sup>IgD<sup>+</sup> B cells with SLE patient disease manifestations, including the WBC count(A), Platelet count(B), serum C3 (C), Serum Creatinine (D), SLEDAI(E), Anti-dsDNA (F), disease duration(G), ESR(H), serum C4(I),IgA(J), IgG(K),and the IgM(L) was analyzed by the Spearman test (\*P < 0.05, \*\*P < 0.01).

Table 3 Correlation analysis of CD27+IgD+ B cells with SLE patient clinical manifestations

clinical manifestation	<i>r</i>	<i>P</i>
WBC	<b>0.337</b>	<b>0.017*</b>
PLT	<b>0.396</b>	<b>0.004*</b>
C3	<b>0.415</b>	<b>0.003*</b>
Serum Creatinine	<b>0.285</b>	<b>0.045*</b>
SLEDAI	<b>-0.724</b>	<b>0.000**</b>
ds-DNA	<b>-0.477</b>	<b>0.000**</b>
Duration	-0.022	0.125
ESR	-0.278	0.051
C4	0.235	0.100
IgA	-0.136	0.348
IgM	0.177	0.218
IgG	-0.235	0.101

Bold font indicates having statistical significance. \*P < 0.05, \*\*P < 0.01.

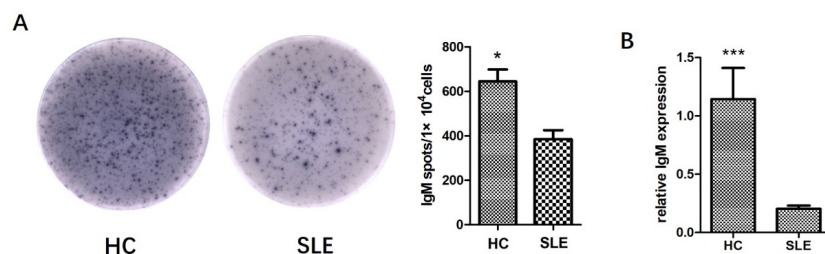


Figure 3 Difference of dampened IgM-producing capacities of  $CD27^+IgD^+$  B cells between SLE and normal control. Flow cytometry-sorted  $CD27^+IgD^+$  B cells from four healthy donors and four active SLE patients (SLEDAI>5) were subjected to ELISPOT (A) Real-time PCR (B) analyses of IgM. The representative charts as well as the statistical results were shown (t-test, \* $P < 0.05$ , \*\*\* $P < 0.001$ ).

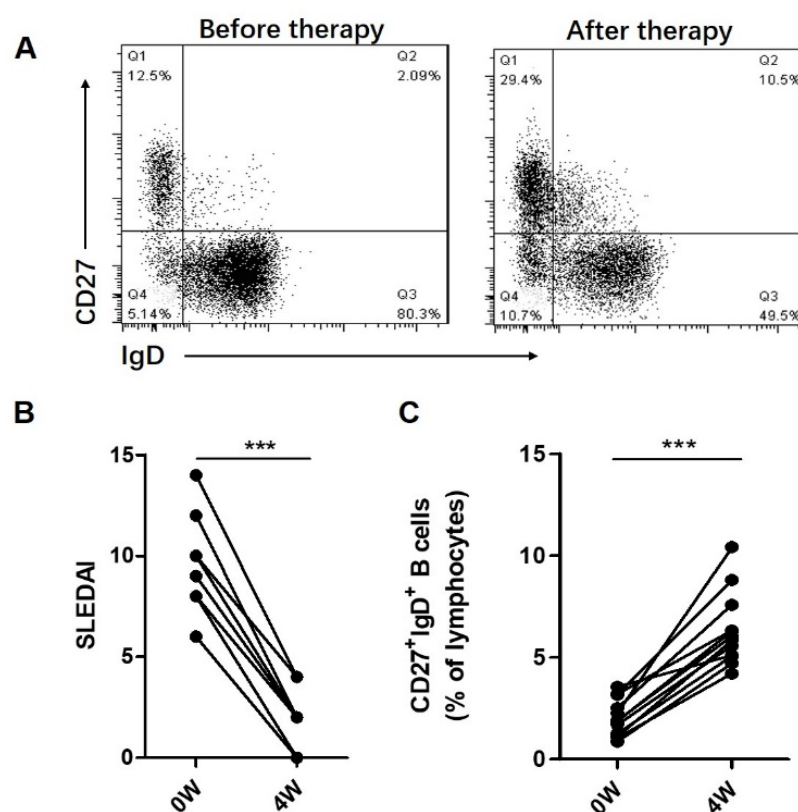


Fig. 4.  $CD27^+IgD^+$  B cells were increased after treatment in SLE patients. (A) Flow cytometric analysis of the frequencies of  $CD27^+IgD^+$  B cells before and after 4 weeks of initiation of treatments. (B) SLEDAI before and after treatment in SLE patients ( $n = 12$ ). (C) The frequencies of  $CD27^+IgD^+$  B cells in SLE patients before and after treatment ( $n = 12$ ). (\* $P < 0.05$ , \*\* $P < 0.01$ ).