

# The expansion of activated naive DNA autoreactive B Cells and its association with disease activity in Systemic Lupus Erythematosus patients

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# Abstract

## Background

Autoreactive B cells are well recognized as key participants in the pathogenesis of Systemic Lupus Erythematosus (SLE). However, elucidating the particular subset of B cells in producing anti-dsDNA antibodies is limited due to their B cell heterogeneity. This study aimed to identify B cell subpopulations that display autoreactivity to DNA and contribute to lupus pathogenesis.

## Methods

Flow cytometry was used to detect total B cell subsets (n = 20) and DNA autoreactive B cells (n = 15) in SLE patients' peripheral blood. Clinical disease activities were assessed in SLE patients using modified SLEDAI-2K and used for correlation analyses with expanded B cell subsets and DNA autoreactive B cells.

## Results

The increases of circulating double negative 2 (DN2) and activated naïve (aNAV) B cells were significantly observed in SLE patients. Expanded B cell subsets and DNA autoreactive B cells represented a high proportion of aNAV B cells with overexpression of CD69 and CD86. The frequencies of aNAV B cells in total B cell populations were significantly correlated with modified SLEDAI-2K scores. Further analysis showed that expansion of aNAV DNA autoreactive B cells was more related to disease activity and serum anti-dsDNA antibody levels than to total aNAV B cells.

## Conclusion

Our study demonstrated an expansion of aNAV B cells in SLE patients. The association between the frequency of aNAV B cells and disease activity patients suggested that these expanded B cells may play a role in SLE pathogenesis. Thus, aNAV B cells may provide a candidate biomarker for monitoring disease activity in these patients.

## Background

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by a loss of immunological tolerance. Recognition of self-antigens causes an abnormal autoreactive immune response resulting in B cell production of autoantibodies. The resulting immune complexes are deposited in multiple organs and cause severe inflammation, which is fatally damaged if delayed in treatment [1, 2]. The sensitive and specific biomarkers to the change of SLE disease activity will help predict the disease flares and be a useful tool for lupus care. Recently, several cytokines (IL-6, IL-8, and IL-18) have been shown a high sensitivity with SLE disease activity [3, 4]. However, the changes in cytokines could reflect

from the concomitant infection in lupus patients. Thus, the identification of a biomarker that is very specific to lupus activity is needed.

The anti-nuclear antibody (ANA) is a hallmark of SLE. Among these autoantibodies in SLE, anti-dsDNA antibodies are the most extensively studied. They are found in approximately 50% of SLE patients and are a specific diagnostic marker [5, 6]. However, the association between circulating anti-dsDNA antibody levels and lupus activity varies significantly across different studies [6–10]. The increases in these antibody levels are associated with SLE exacerbations, whereas reports of reductions associated with clinical benefit are limited [11–14]. The delayed clearance of autoantibody, which has a half-life of up to 30 days (depended on isotype), will not reflect the current SLE disease activity [15]. Although anti-dsDNA's specificity to SLE is better than cytokines, immunoglobulin's slow dynamic changes are not an excellent biomarker to reflect SLE disease activity. The anti-dsDNA producing cells may better reflect the disease activity and specific to SLE than serum anti-dsDNA or cytokines. Therefore, identifying the B cell population that produced anti-dsDNA is crucial, which may be a sensitive and specific biomarker to SLE disease activity.

In addition to pathogenic autoantibodies, B cells play other essential roles in SLE. Autoreactive B cells are phenotypically heterogeneous. Genetic background, hormonal milieu, and antigen exposure all contribute to this diversity [16–18]. Both naive and antigen-experienced B cell populations are correlated with SLE disease activity [19, 20]. Expansion of aNAV B cells (with highly expressed transcription factor T-bet and CD11c) was shown in patients with moderate to severe flares in disease activity [19, 21]. This B cell subset includes high clonality of the autoantibody secreting cells (auto-ASCs), indicating defective selection at the transitional stage and preferential differentiation contributing to SLE pathogenesis [21]. Also, antigen-experienced B cells [including atypical memory (AtMs), double negative 2 (DN2), or aged associated B cells (ABCs)] which share canonical phenotype and are identified as CD24<sup>-</sup>CD20<sup>hi</sup>, IgD<sup>-</sup>CD27<sup>-</sup> or CD19<sup>+</sup>CD21<sup>low</sup> B cells, are frequently detected in SLE patients [20, 22, 23]. The stimulation of these autoreactive B cells results in the production of anti-Ro, -Sm, and -RNP autoantibodies [20, 22, 23]. It remains controversial as to which subsets of B cells are autoreactive and secrete anti-dsDNA autoantibodies. Thus, more research regarding the autoreactive B cells contributing to SLE pathogenesis would help monitor disease activity and predict lupus flares.

In this study, we identified B cell subsets that were autoreactive against DNA. To assess these DNA autoreactive B cells' contribution in SLE pathogenesis, we determined their frequencies in SLE patients and analyzed the correlations with disease activity.

## Methods

### Subjects and study design

SLE patients were enrolled from the Department of Medicine, Ramathibodi Hospital, Bangkok, Thailand. Blood from 38 SLE patients was studied to detect the frequency of B cell subsets (n = 20) and their DNA

tetramer-binding B cells (n = 15) and to phenotype aNAV DNA tetramer-binding B cells (n = 3). Correlation analyses were done between total aNAV and aNAV DNA tetramer-binding B cells, and clinical parameters [modified SLEDAI-2K score, erythrocyte sedimentation rate (ESR), anti-dsDNA antibodies, complement 3 (C3), and complement 4 (C4) levels (Additional file 1: Tables S1 and Additional file 2: Tables S2).

Inclusion criteria for study subjects were: (1) a diagnosis of SLE based on Systemic Lupus International Collaborating Clinics (SLICC) criteria 2012 [24, 25]; (2) presence of SLE clinical manifestations and receipt of treatment with immunosuppressive drugs; (3) age above 18 years. Exclusion criteria were: (1) presence of a syndrome overlapping with SLE; (2) presence of infection; (3) a diagnosis of cancer. Disease activity was measured by the modified SLEDAI-2K. Laboratory parameters (ESR, anti-dsDNA antibodies, C3 and C4 levels) were determined and correlated with aNAV B cells' frequency. Characteristics of study subjects are presented in Additional file 1 and 2. Healthy donors (n = 24) were recruited as controls (HCs). The study was approved by the Ethical Committee of Mahidol University (approval number, MURA 2015/731).

### **Tetramer generation**

The DWEYSVWLSN peptide can behave as a dsDNA mimotope [26]. Immunization of BALB/c mice with an octameric form of this peptide results in the production of anti-dsDNA antibodies, which are involved in lupus nephritis by deposition in renal glomeruli [27-29]. Here, a DWEYSVWLSN peptide was used to identify DNA tetramer-binding B cells in SLE patients. A biotinylated peptide was synthesized (GenScript, NJ, USA) and used in tetramerization by combining 5 µl of biotinylated peptide stepwise in 1/2 volumes to 3mM SA-R-phycoerythrin-PE at a molar ratio of 10:1 and incubating for 60 min at 4°C.

### **Tetramer enrichment**

To define tetramer-stained B cells, PBMCs from SLE patients (n = 15) were resuspended to 100 µl in FACS buffer (PBS + 0.1% BSA) and incubated with anti-CD32 Fc blocker for 2 min. Next, PE-conjugated peptide tetramer was added at a concentration of 0.001 M and incubated for 15 min at 4°C, then washed in 2.5 ml cold FACS buffer. Tetramer-stained B cells were resuspended to a volume of 100 µl FACS buffer per 10<sup>6</sup> cells and then mixed with 10 µl anti-PE-conjugated magnetic MicroBeads. These tetramer-stained B cells were enriched by positive selection with EasySep PE Positive Selection Kit (STEMCELL, Vancouver, Canada), incubated for 15 min at 4°C, and washed with 2.5 ml FACS buffer before tetramer surface staining.

### **B cell culture**

The purified tetramer-binding B cells (2 x 10<sup>2</sup>) were seeded into 96-well plates to demonstrate tetramer-binding B cells' capability that produces anti-dsDNA autoantibodies. These plates were pre-seeded overnight with CD154-expressing stromal cells (CD40L<sup>Low</sup> cell line, a gift from Garnett H. Kelsoe) [30]. B cells were cultured with R848 (1 µg/ml) and calf-thymus DNA (10 µg/ml) in R5 medium (RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM HEPES,

1 mM sodium pyruvate, and 1% MEM nonessential amino acids), supplemented with recombinant human IL-2 (50 ng/ml) and IL-21 (100 ng/ml) (PeproTech, NJ, USA) for 8 days. The supernatant from tetramer-binding B cell cultures was harvested and stored at -20°C for total IgG and anti-dsDNA antibody quantitation. DN2 B cells, representing pre-ASCs, were cultured as positive controls.

### **Antibody staining and flow cytometry**

To detect subpopulations and characterize phenotypes of B cells, PBMCs from SLE patients (n = 20) were separated and analyzed to determine the frequency of each B cell subset. Two x 10<sup>5</sup> cells were resuspended in 50 µl FACS buffer and incubated with surface antibodies for 15 min at 4°C. Antibodies used included FITC-CD19, APC-CD21, APC/Fire750-CD27, Alexa/fluor700-CD11c, PerCP/Cy5.5-CXCR5, PerCP/Cy5.5-IgG, PE/Cy7-IgD, APC-HLA-DR, PE/Cy5-CD69, and PE/Cy5-CD86 (Biolegend, San Diego, USA). After staining, cells were washed with 1 ml FACS buffer and resuspended in 300 µl FACS buffer for surface marker analyses.

The frequency and phenotype of DNA tetramer-binding B cells were detected by the tetramer staining technique, resuspended in 100 µl FACS buffer, and incubated with surface antibodies for 15 min at 4°C. The stained B cells were washed with 1 ml FACS buffer and resuspended in 500 µl FACS buffer for flow cytometric surface marker analysis. FACS data were acquired with a BD FACS Canto II (Becton-Dickinson Immunocytometry Systems, San Jose, USA) and analyzed with FlowJo software (v. 10.0; Tree Star Inc., CA, USA).

### **Enzyme-Linked Immunosorbent Assay (ELISA)**

To detect anti-dsDNA and anti-peptide IgG antibodies in plasma or B cell culture supernatant, 96-well plates were coated with calf thymus DNA (10 µg/ml) or synthesized DWEYSVWLSNK peptide (20 µg/ml), then held overnight at 4°C. After blocking with HBS containing 10% FBS for 2 h, plates were incubated with subject plasma (diluted 1:200) or culture supernatant (undiluted) for 1 h at RT. Then, plates were washed five times with HBS containing MnCl<sub>2</sub> and incubated with horseradish peroxidase (HRP)-conjugated, anti-human IgG antibodies (KPL, MA, USA) at a 1:1000 dilution for 1 h. Subsequently, plates were developed with tetramethylbenzidine (TMB) enzyme-substrate. After 15 min, the reaction was stopped by 1N HCl and absorbance measured at 450 nm using a microplate reader (Bio-Rad, CA, USA). To detect total IgG, plates were coated with 1 µg/ml of IgG anti-human immunoglobulin (Mabtech AB, Stockholm, Sweden), and undiluted culture supernatant was added, followed by the addition of HRP-conjugated, anti-human IgG antibodies. The optical density (OD) of the reaction was measured at 450 nm after adding TMB substrate.

### **Statistical analysis**

Data were analyzed using GraphPad Prism software version 8 (GraphPad Software Inc., San Jose, CA, USA). Group comparisons were performed using two-tailed, unpaired Mann-Whitney U tests [presented as group median (interquartile range)]. The significance of correlations were calculated using Spearman's

correlation coefficient ( $r$ ). Median  $\pm$  interquartile range (IQR) was used for all statistical tests. Group differences with  $p$  values less than 0.05 were considered statistically significant.

## Results

### Increased proportion of aNAV and DN2 B cells in lupus patients

Four B cell subpopulations including switched memory (SWM; IgD<sup>-</sup>CD27<sup>+</sup>), unswitched memory (USW; IgD<sup>+</sup>CD27<sup>+</sup>), double negative (DN; IgD<sup>-</sup>CD27<sup>-</sup>), and naïve (NAV; IgD<sup>+</sup>CD27<sup>-</sup>) B cells were detected in the peripheral blood of SLE patients (Fig. 1a). The percentage of DN B cells was greater in patients than controls [34.90% (23.95-50.00%) vs 20.70% (16.60-21.30%),  $p < 0.01$ ]. The frequencies of USW and naïve B cells were lower in SLE patients than controls ([1.57% (0.72-1.89%) vs 2.32% (2.01-3.85%),  $p < 0.01$ ] and [40.70% (24.75-66.55%) vs 65.30% (59.10-67.70%),  $p < 0.05$ ], respectively). The frequency of SWM B cells did not differ significantly in SLE patients and HCs (Fig. 1b).

The expansions of DN and NAV B cell populations were further analyzed in SLE patients. Double negative 1 (DN1) and double negative 2 (DN2) B cells were distinguished based on their expression of CXCR5, CD21 and CD11c (Fig. 2a). We detected a higher frequency of CXCR5<sup>-</sup>CD21<sup>-</sup>CD11c<sup>+</sup> DN2 B cells [22.95% (14.65-52.18%) vs 9.49% (4.88-11.20%),  $p < 0.001$ ] and a lower frequency of CXCR5<sup>+</sup>CD21<sup>+</sup>CD11c<sup>-</sup> DN1 B cells [26.55% (11.53-37.85%) vs 65.00% (57.40-68.50%),  $p < 0.0001$ ] when compared with HCs (Fig. 2b). Naïve B cells, resting naïve (rNAV) and activated naïve (aNAV) B cells were defined based on CXCR5, CD21 and CD11c expression. The frequency of aNAV B cells was markedly greater in SLE patients than controls [2.80% (1.10-14.20%) vs 0.84% (0.64-1.99%),  $p < 0.01$ ] (Fig. 2b). In contrast, the frequency of rNAV B cells was lower in patients than in HCs [53.75% (21.83-72.33%) vs 79.20% (73.50-83.60%),  $p < 0.001$ ] (Fig. 2b). Interestingly, patients' IgG expression levels within the cells of expanded DN2 B cells were higher than on the cell surfaces [intracellular: median = 50.25% (45.20-56.25%), surface: median = 20.00% (15.50-29.65%),  $p < 0.0001$ ] (Fig. 2c).

### Binding reactivity of anti-peptide IgG and anti-dsDNA antibody production of tetramer-binding B cells

To verify that the DWEYSVWLSN peptide can behave as a dsDNA mimetope [26], we first quantified the level of anti-dsDNA and anti-peptide IgG in serum SLE patients. Levels of anti-dsDNA IgG [0.248 (0.188-0.308) vs 0.098 (0.095-0.101),  $p < 0.05$ ] and anti-peptide IgG [0.189 (0.145-0.233) vs 0.0985 (0.0874-0.110),  $p < 0.05$ ] were significantly higher in subjects than controls (Fig. 3a, b). Furthermore, anti-dsDNA and anti-peptide IgG levels were significantly correlated ( $r = 0.6029$ ,  $p = 0.0004$ , Fig. 3c). Of 30 SLE patients, 24 exhibited an accordance between anti-peptide and anti-dsDNA reactivity (80%; 11 samples were seropositive, and 13 samples were seronegative), whereas 6 patients showed a discordance between these antibodies (Fig. 3d).

Next, we demonstrated the anti-dsDNA producing cells using the tetramer binding DWEYSVWLSNK peptide. Both tetramer-binding and non-tetramer-binding B cells secreted total IgG antibodies. Higher anti-

dsDNA antibodies were only detected in tetramer-binding B cell cultures (Fig 3e, f). As expected, DN2 (a pre-ASC subset) produced high levels of total IgG (Fig 3e, f).

### **Relation of aNAV B cells to tetramer-binding B cells**

The frequency of tetramer-binding B cells in SLE patients was gated on CD19<sup>+</sup> B cells (Fig. 4a). Tetramer-binding B cells were significantly more frequent in SLE patients than HCs [2.73% (2.28-3.14%) vs 1.00% (0.04-1.32%),  $p < 0.01$ ] (Fig. 4b). Tetramer-binding B cells were further characterized to determine whether the expanded DN and NAV B cells were the DNA autoreactive cells. We found that most bound DNA autoreactive B cells were naïve B cells (IgD<sup>+</sup>CD27<sup>-</sup>) [67.40% (60.00-86.70%)], whereas they were present in lower proportions in DN B cells (IgD<sup>-</sup>CD27<sup>-</sup>) [3.41% (1.88-4.67)] (Fig. 4c, d).

In addition, the phenotypic characterization of tetramer-binding B cells showed that aNAV B cells were more frequent in SLE patients than HCs [9.52% (3.29-30.80%) vs 6.42% (0.50-7.36%),  $p < 0.05$ ], whereas rNAV cells were less frequent [34.90% (13.60-66.30%) vs 78.30% (57.85-86.88%),  $p < 0.01$ ]. No significant difference was observed for DN B cells between DN B cell subsets of lupus patients and HCs (Fig. 4d). Of note, the activating profile, CD69, and CD86 were observed in an increased proportion of aNAV DNA tetramer-binding B cells, whereas no difference in the expression level of HLA-DR was observed between these B cells and non-tetramer-binding B cells (Additional file 4: Fig. S1).

### **Association of expanded aNAV B cells with clinical parameters**

Since aNAV B cells were found to be expanded in total CD19<sup>+</sup> B cells and DNA tetramer-binding B cells of SLE patients, we hypothesized that the frequency of this subset correlated with clinical parameters and that aNAV B cells could be biomarkers for monitoring disease activity. Therefore, correlations between cell frequencies and clinically measured parameters (modified SLEDAI-2K score, ESR, anti-dsDNA antibodies, C3 and C4 levels) were analyzed (Additional file 3: Table S3). The frequency of aNAV B cells was positively correlated with modified SLEDAI-2K score ( $r = 0.4991$ ,  $p = 0.0251$ ). Significantly higher modified SLEDAI-2K scores were observed in lupus patients with expanded aNAV B cells (Fig. 5a). In addition, the frequency of total aNAV B cells was positively correlated with ESR level ( $r = 0.4063$ ,  $p = 0.0358$ , Fig. 5b), whereas no correlation was found with anti-dsDNA antibody level (Fig. 5c). These aNAV B cells were inversely correlated with C3 ( $r = -0.4844$ ,  $p = 0.0304$ ) and C4 levels ( $r = -0.5753$ ,  $p = 0.008$ ) (Fig. 5d, e).

Correlation analysis of aNAV tetramer-binding B cell with clinical parameters, as described in Additional file 3 showed that the frequency of aNAV DNA tetramer-binding B cells was strongly (positively) correlated with modified SLEDAI-2K score ( $r = 0.7548$ ,  $p = 0.0017$ ). Higher frequencies of these cells were observed in patients with greater disease activity and active SLE [23.70% (7.58-33.30%) vs 1.96% (0.16-7.96%),  $p < 0.05$ ] (Fig. 6a). Moreover, the frequency of aNAV DNA tetramer-binding B cells was positively correlated with ESR ( $r = 0.8185$ ,  $p = 0.0006$ ) and anti-dsDNA antibody levels ( $r = 0.6302$ ,  $p = 0.0138$ ). Higher frequencies of these cells were observed in patients with high levels of ESR and anti-dsDNA antibody (Fig. 6b, c). However, no correlation was found between these B cells and C3, nor C4 levels in lupus patients (Fig. 6d, e).

## Discussion

Autoreactive B cells, found among the expansion of various B cell subsets in SLE patients, are considered as pathogenic since they recognize self-antigens and produce autoantibodies [20, 22, 23]. These cells contribute to the development of SLE and the activity of disease [19, 31, 32]. Such autoreactive B cells in SLE may provide a candidate biomarker for B cell therapeutic interventions. We found the expansions of DN2 and aNAV B cells in the blood from SLE patients. Within these expanded B cell subsets, aNAV B cells were mainly tetramer-binding B cells, and their antibodies mostly recognized DNA, suggesting their role in producing autoantibodies in SLE patients. Notably, the frequencies of aNAV B cells in both total and DNA autoreactive B cells were positively correlated with SLE disease activity. Altogether, our data suggested that the expansion of these B cells plays a pathogenic role in SLE by the active autoantibody production.

We demonstrated that SLE patients had high frequencies of DN2 and aNAV B cells. The aNAV B cells strongly correlated with modified SLEDAI-2K score and ESR level, indicating that aNAV B cells may exhibit pathogenic functions and are associated explicitly with SLE progression. In assessing the pathogenic effect of DN2 and aNAV B cells, it was demonstrated that they shared a canonical phenotype characterized by  $CXCR5^-CD21^-CD11c^+$  expression. This suggests that DN2 and aNAV B cells originate and develop from resting naive B cells [20]. Recently, both DN2 and aNAV B cells were demonstrated to have the capacity to differentiate into ASCs and produce autoantibodies [20, 21]. Upon stimulation of naive B cells with TLR-7, IFN- $\gamma$  and IL-21, these aNAV B cells can directly differentiate into DN2 B cells and plasma cells at days 3 and 5, respectively, indicating that DN2 B cells exhibit a pre-ASC stage of pathogenic relevance to autoantibody production [20, 33]. In support of previous studies, we present more evidence that DN2 B cells may be pre-ASCs by showing their high amounts of intracellular IgG. The simultaneous expansion of both aNAV and DN2 B cells in active SLE patients suggested that these B cell subsets cooperate to produce the autoantibodies that play such a crucial role in SLE pathogenesis. Further investigations are required to clarify their contributions to SLE pathogenesis and the exact mechanisms of activation and differentiation that produce auto-ASCs and whether localized in germinal centers or extra-follicular reactions.

Our study demonstrated that circulating aNAV B cells correlated with increased modified SLEDAI-2K score, suggesting that these cells' expansion plays a crucial role in SLE pathogenesis. The aNAV DNA autoreactive B cells, using a tetramer tool, were strongly related to disease activity and anti-dsDNA antibody levels compared with the aNAV B cells within the total B cell population. Using this methodology will be informative in tracking the frequency of aNAV DNA autoreactive B cells in individual patients who develop clinical disease flares.

Reports that heterogeneous autoreactive B cells produce anti-dsDNA antibodies and contribute to SLE pathogenesis have long been a matter of debate. Which specific B cell subset (s) includes DNA autoreactive B cells is still unclear. Using a peptide surrogate (DWEYSVWLSN) for dsDNA and the tetramer tool, we were able to identify and enrich DNA autoreactive B cells that produced anti-dsDNA antibodies. They were mostly accounted for by aNAV B cells, which expressed high surface densities of

CD69 and CD86. Interestingly, the expansions of aNAV DNA autoreactive B cells were strongly and positively correlated with modified SLEDAI-2K scores and anti-dsDNA antibody levels and found in active SLE patients. The explanation is likely that more significant impairment of central tolerance eliminating self-reactive B cells led to the survival of aNAV DNA autoreactive B cells, which then expanded under autoimmune conditions.

Consequently, these expanded aNAV B cells were likely precursors of auto-ASCs and produced the anti-dsDNA autoantibodies central to SLE pathogenesis. The aNAV B cells may be the key players associated with SLE development. However, the study of pathogenic functions of aNAV B cells, such as autoantibody production, pro-inflammatory cytokine secretion, and T cell induction, in the pathogenesis of SLE is required in future studies.

## Conclusion

Our data demonstrated that aNAV B cells could engage with self-antigens expanded under SLE's autoimmune conditions, and the number of these aNAV B cells was strongly correlated with disease activity. Therefore, aNAV B cells might be used as a biomarker for monitoring SLE patients.

## Abbreviations

aNAV: activated naïve; Anti-dsDNA: Anti-double stranded DNA; ASCs: Antibody-secreting cells; AtMs; Atypical memory B cells; ABCs: Aged-associated B cells; APC: Allophycocyanin; C3: Complement 3; C4: Complement 4; DN1: Double negative 1; DN2: Double negative 2; ESR: Erythrocyte sedimentation rate; FITC: Fluorescein isothiocyanate; FBS: Fetal bovine serum; HBS: HEPES buffer saline; HCs: Healthy controls; Hb: Hemoglobin; IFN-g: Interferon-g; IL: Interleukin; IgG: Immunoglobulin G; MS: Master of science; PBMCs: Peripheral blood mononuclear cells; PBS: Phosphate buffer saline; PerCP/Cy5.5: Peridinchlorophyll protein/ Cyanine 5.5; PE: Phycoerythrin; R: correlation coefficient; R848: Resiquimod; SLE: Systemic lupus erythematosus; modified SLEDAI-2K: modified SLE disease activity index-2K; SLICC: Systemic lupus erythematosus international collaborating clinics; SWM: Switched memory B cells; TLR-7: Toll-like receptor 7; UPCR: Urine Protein to Creatinine; USW: Unswitched memory B cells; WBC: White Blood Cell count.

## Declarations

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## **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## **Ethics approval and consent to participate**

The study was approved by the Faculty of Medicine Ramathibodi Hospital ethics committee (approval number is MURA 2015/731, protocol number ID 12-58-12) and conducted according to the guidelines of the Declaration of Helsinki. Each participant gave written informed consent before enrollment.

## **Competing interests**

The authors declared that they have no competing interests.

## **Consent for publication**

Not applicable.

## **Authors' Contributions**

KW designed and planned the study, performed the experiments, statistical analysis, data interpretations, and wrote the manuscript. CT analyzed clinical data. TK performed flow cytometric analyses. CL analyzed flow cytometric data. TS collected the samples and laboratory analyses. PN analyzed clinical data. LK helped interpreting data. PK analyzed statistic data. CS performed anti-dsDNA antibody detection and analyses. PP designed and planned the study, analyzed clinical data, and wrote the manuscript. PC designed and planned the study, reviewed the data, analyses, data interpretations, and the final draft. All authors have read and approved the final manuscript.

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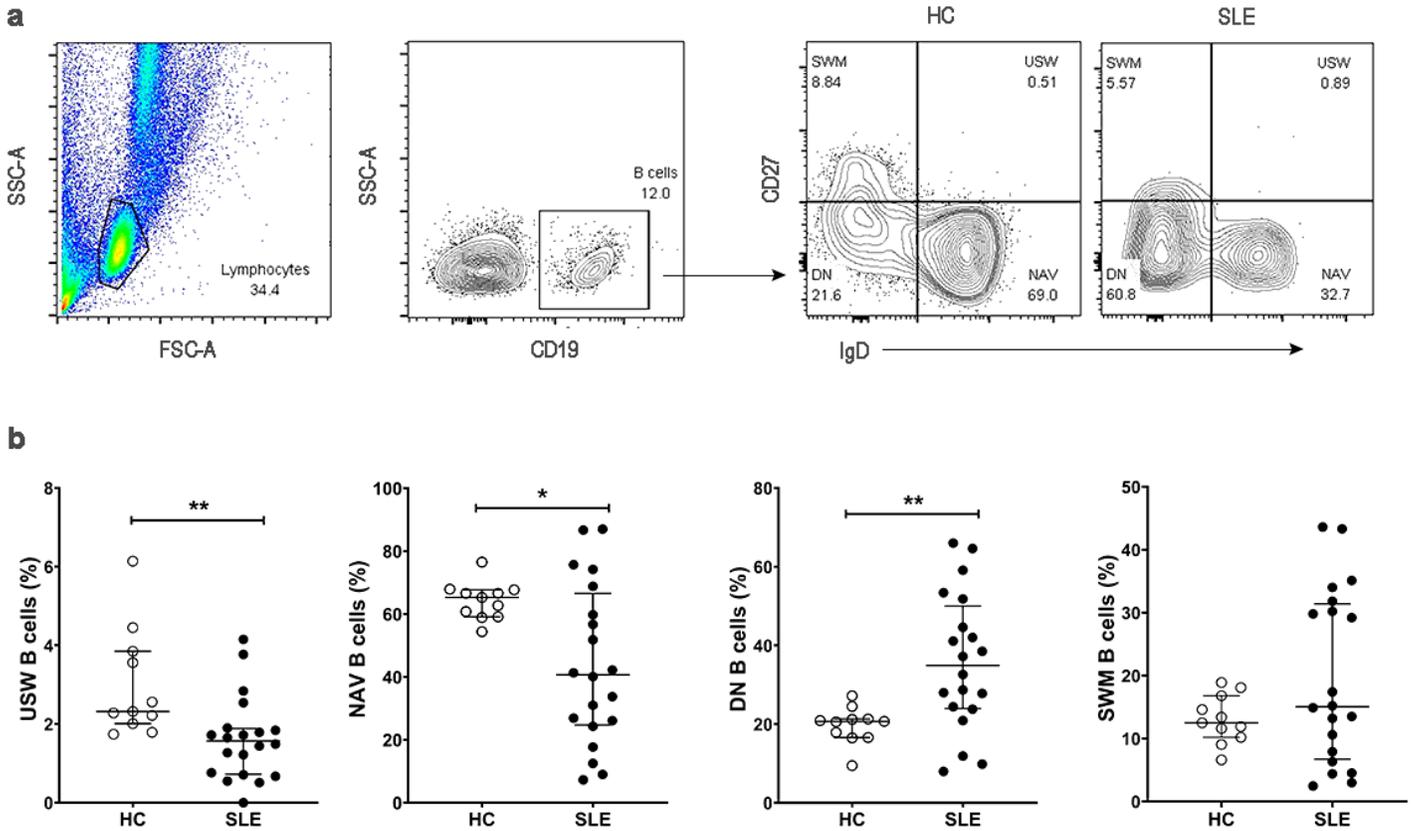
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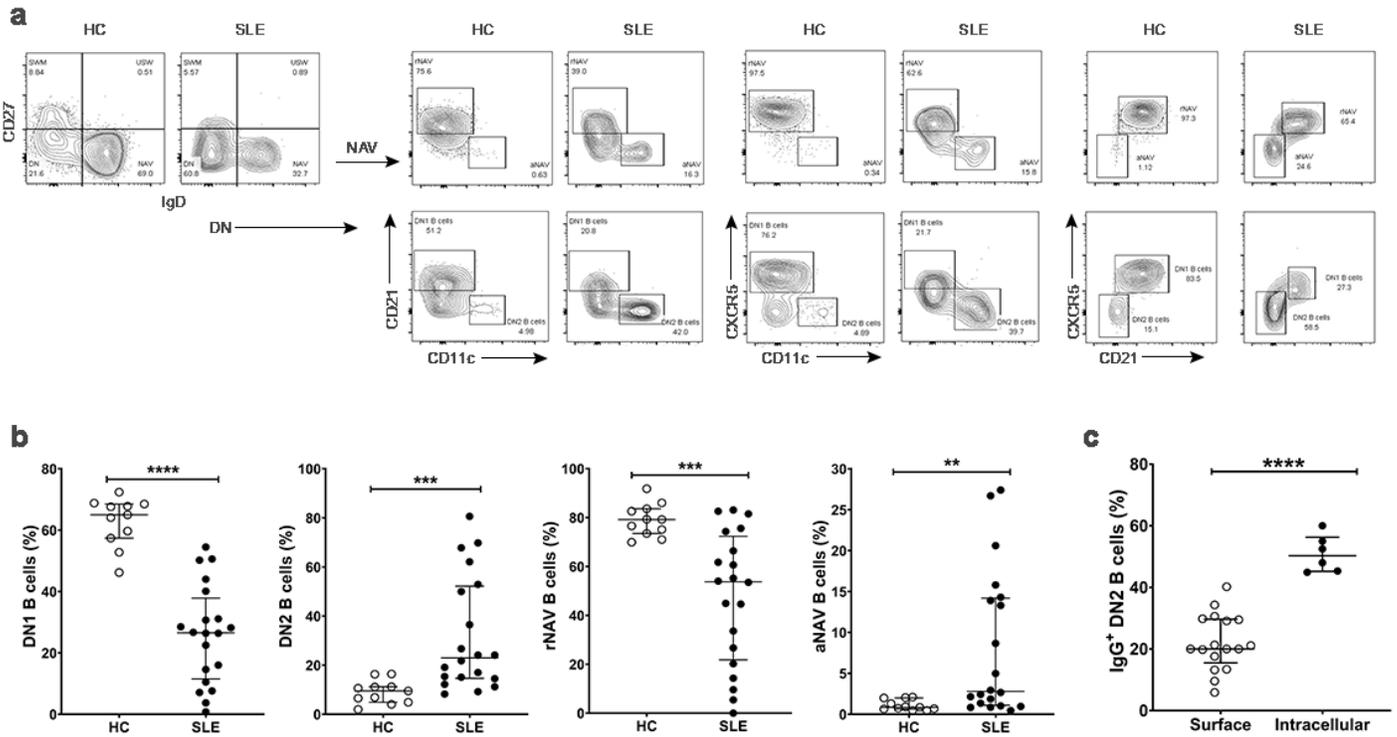
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## Figures



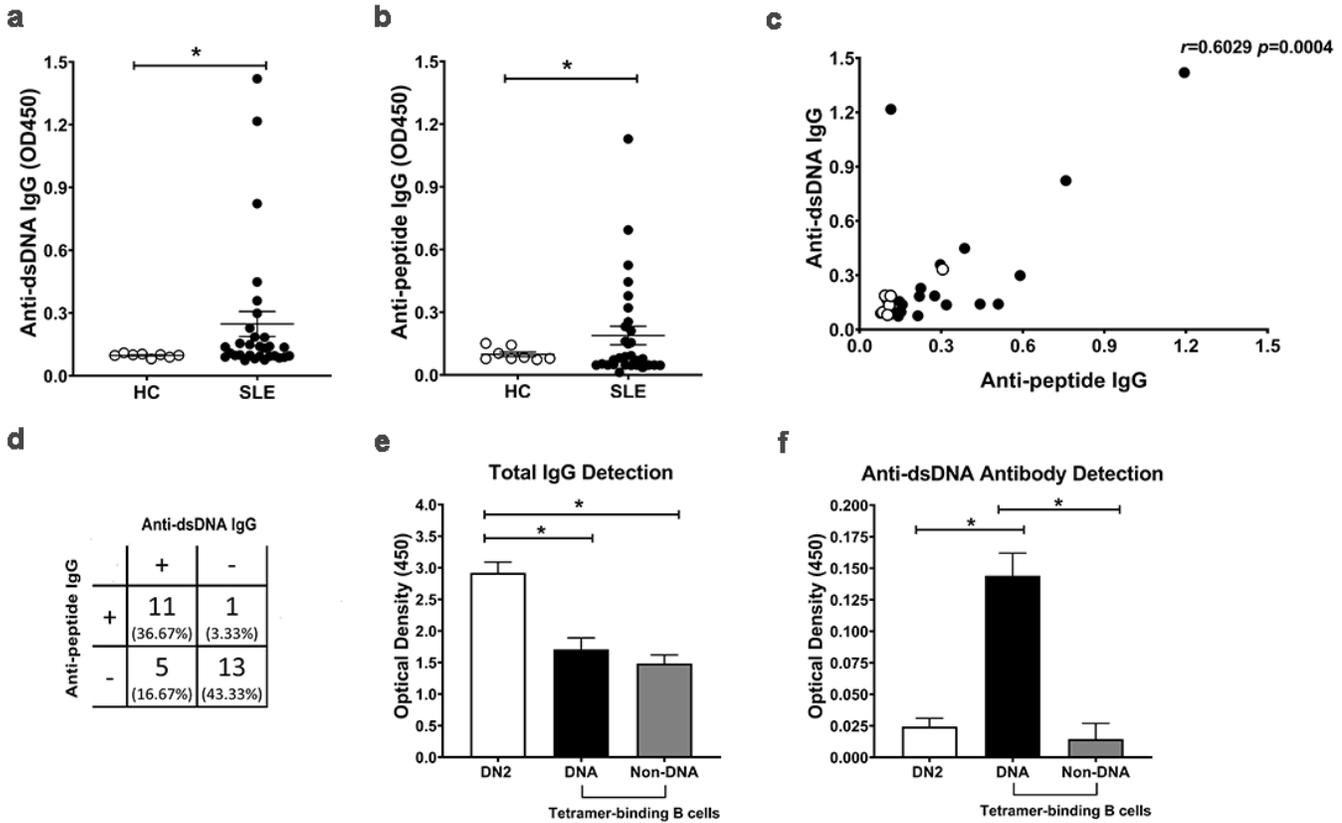
**Figure 1**

Phenotype and frequency of B cell subsets in SLE patients. (a) Representative gating strategy of CD19+ B cells from one SLE patient and one HC. The four independent subpopulations were gated: unswitched memory B cells (USW, IgD+CD27+); naïve B cells (NAV, IgD+CD27-); double negative B cells (DN, IgD-CD27-); and switched memory B cells (SWM, IgD-CD27+). (b) The frequency of USW, NAV, DN and SWM B cells in SLE patients (n = 20) compared to HCs (n = 11). Bars represent median with interquartile range. p values were determined by the Mann-Whitney U-test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.



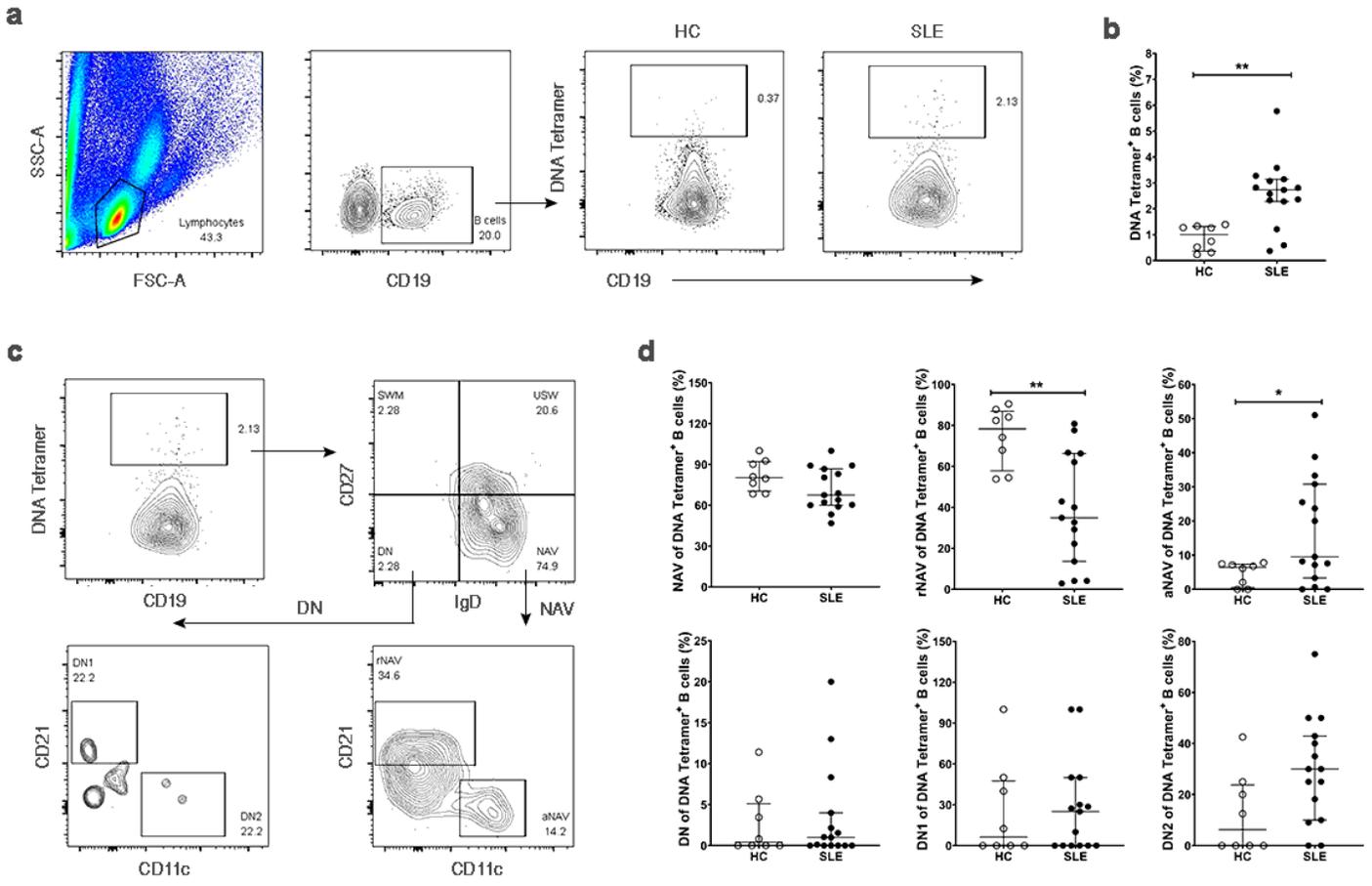
**Figure 2**

Subsets of double negative and naïve B cells. (a) Representative flow cytometry from SLE patient and HC. The NAV and DN B cells were classified and grouped as rNAV (CD21+, CD11c-, CXCR5+), aNAV (CD21-, CD11c+, CXCR5-), DN1 (CD21+, CD11c-, CXCR5+) and DN2 B cells (CD21-, CD11c+, CXCR5-). (b) The frequencies of DN and NAV B cell subsets in HCs (n = 11) compared to SLE patients (n = 20). (c) The frequency of intracellular IgG<sup>+</sup> DN2 B cells (n = 6) as compared to surface IgG<sup>+</sup> DN2 B cells (n = 17) in SLE patients. Bars represent median with interquartile range. p values were determined by the Mann-Whitney U-test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.



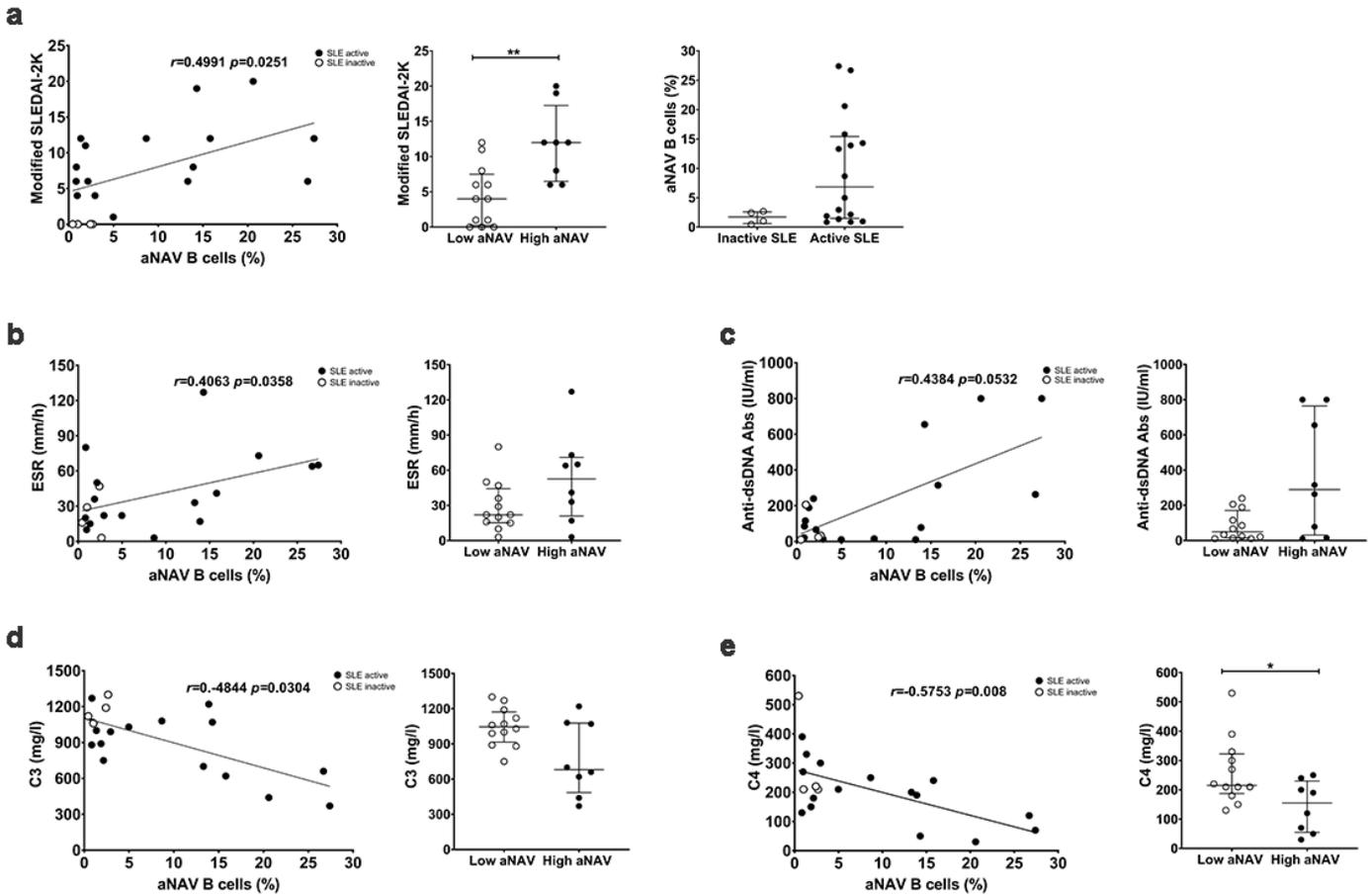
**Figure 3**

Characterization of anti-dsDNA and anti-peptide IgG reactivity. A significant difference exists between (a) anti-dsDNA IgG (b) anti-peptide IgG antibody levels in lupus patients ( $n = 30$ ) compared with HCs ( $n = 8$ ). (c) A significant correlation between anti-peptide IgG and anti-dsDNA IgG antibody levels in all individual patients. Healthy controls were shown in white ( $n = 8$ ), lupus patients in black ( $n = 30$ ). (d) The correlation analysis between anti-peptide and anti-dsDNA IgG levels in 30 SLE patients. Secretion levels of (e) total IgG (f) anti-dsDNA antibodies by DN2, DNA and non-DNA tetramer-binding B cells detected after 8 days of in vitro culture ( $n = 3$ ). Bars represent median with interquartile range. p values were determined by the Mann-Whitney U-test: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .



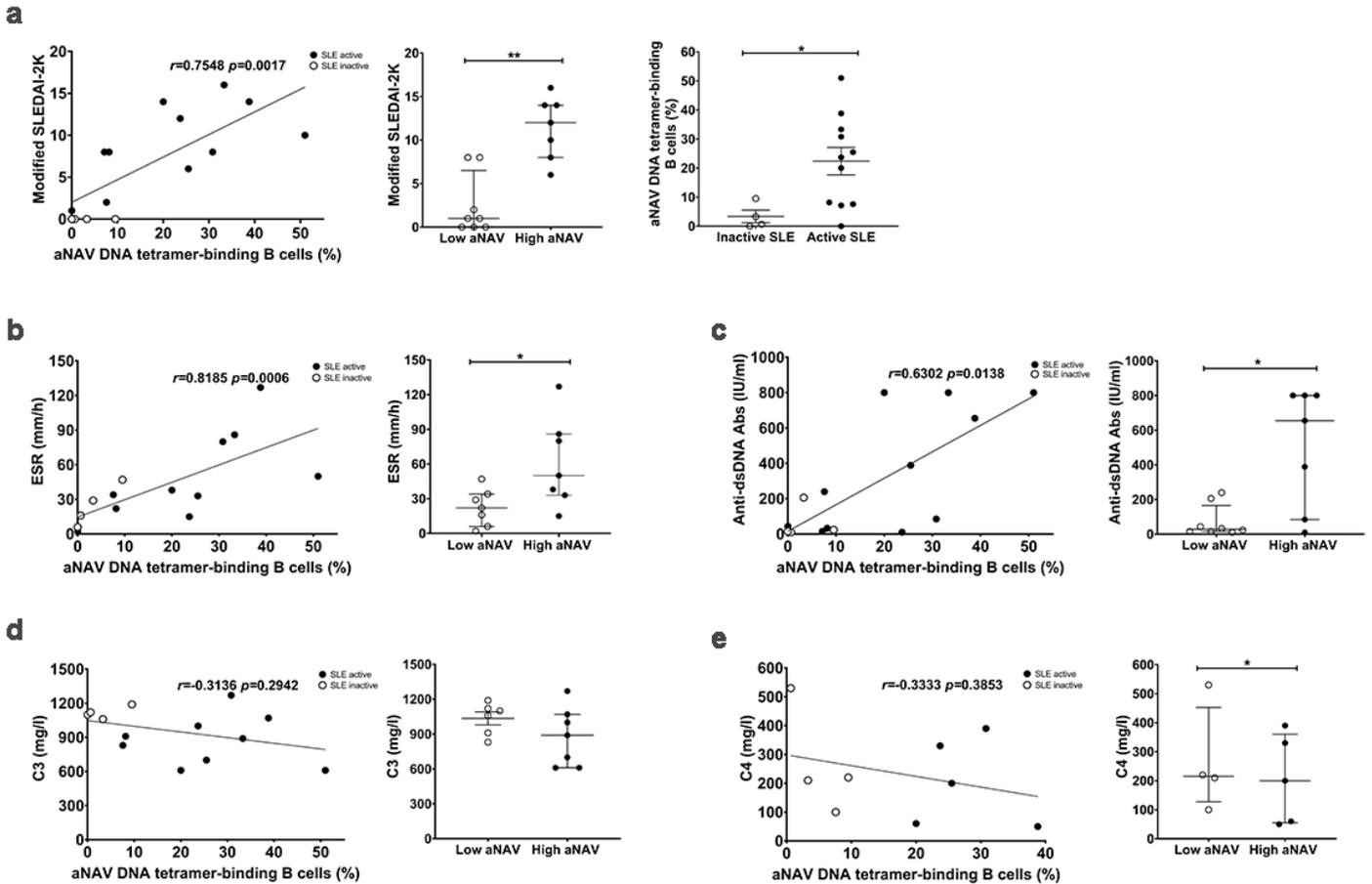
**Figure 4**

Identification and characterization of tetramer-binding B cells. (a) Gating strategy used to identify DNA tetramer-binding B cells. CD19<sup>+</sup> B cells were gated, and the frequency of DNA tetramer-binding B cells was determined as shown in HCs and SLE patient. (b) The frequency of DNA tetramer-binding B cells in HCs (n = 8) compared to SLE patients (n = 15). (c) DNA tetramer-binding B cells were defined as double negative and naïve B cells; the NAV B cells were further classified into resting (rNAV) and activated (aNAV) naïve B cells; double negative B cells were classified into double negative 1 (DN1) and double negative 2 (DN2) B cells. (d) The comparative frequencies of naïve and double negative B cell subsets in HCs (n = 8) and SLE patients (n = 15). Bars represent median with interquartile range. p values were determined by the Mann-Whitney U-test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.



**Figure 5**

Correlation of aNAV B cells with clinical parameters. Correlation between the frequencies of aNAV B cells and clinical parameters in SLE patients (n = 20). (a) modified SLEDAI-2K score (left panel), modified SLEDAI-2K score in patients with an expansion of aNAV B cells (middle panel) and frequencies of aNAV B cells in inactive and active SLE patients (right panel), (b) ESR, (c) Anti-dsDNA autoantibodies, (d) C3 and (e) C4 levels. Spearman's correlation coefficient (r) was used as metric to calculate correlations between numerical data. Bars represent median with interquartile range. p values were determined by the Mann-Whitney U-test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.



**Figure 6**

Correlation of activated naïve tetramer-binding B cells with clinical parameters. Correlation between the frequencies of aNAV DNA tetramer-binding B cells and clinical parameters in SLE patients. (a) modified SLEDAI-2K score (left panel), modified SLEDAI-2K score in patients with an expansion of aNAV DNA tetramer-binding B cells (middle panel) and frequencies of aNAV DNA tetramer-binding B cells in inactive and active SLE patients (right panel) (n = 15), (b) ESR (n = 15), (c) Anti-dsDNA autoantibodies (n = 15), (d) C3 (n = 13) and (e) C4 (n = 9) levels. Spearman's correlation coefficient (r) was used as metric to calculate correlations between numerical data. Bars represent median with interquartile range. p values were determined by the Mann-Whitney U-test: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

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