

CD38, CD81 and BAFFR combined expression by transitional B cells distinguishes active from inactive systemic lupus erythematosus

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Abstract In view of its heterogeneous presentation and unpredictable course, clinical management of systemic lupus erythematosus (SLE) is difficult. There is a need for biomarkers and diagnostic aids to monitor SLE disease activity and severity prior to, during and after treatment. We undertook this study to search for unique phenotypic patterns in each peripheral blood (PB) B cell subset, capable of distinguishing SLE patients with inactive disease versus SLE patients with active disease versus controls by using an automated population separator (APS) visualization strategy. PB was collected from 41 SLE patients and 28 age- and gender-matched controls. We analyzed the cell

surface markers (in a tube CD20/CD27/CD19/CD45/CD38/CD81/BAFFR combination) expression on PB B cell subsets using principal component analysis, implemented in the APS software tool. Overall, our analysis indicates that active SLE can be distinguished from inactive SLE on the basis of a single tube analysis, focused on the decreased expression of CD38, CD81 and BAFFR in transitional B cells. The cluster analysis of immunophenotypic profiles of B cell subsets highlighted disease-specific abnormalities on transitional B cells that emerge as promising surrogate markers for disease activity. Further validation is needed with larger samples and prospective follow-up of patients.

Keywords Systemic lupus erythematosus · Transitional B cells · 3D automated population separator (APS) view · BAFFR · CD81 and CD38

Ana Henriques and Isabel Silva have contributed equally to this study.

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Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disorder, which presents a broad spectrum of symptoms and signs of disease, a diverse degree of severity, course and prognosis. Currently, the pathogenesis of SLE remains only partially understood and results of therapy are frequently unsatisfactory. Such disease complexity underlines the need for new biomarkers of disease activity, and it also highlights the need for further understanding the pathogenic mechanisms for the identification of new and more effective targeted therapies [1].

Studies conducted over the past 15 years indicate that B cells play a crucial role in SLE pathogenesis [2]. The presence of multiple autoantibodies reflects defective tolerance mechanisms leading to the activation of

autoreactive B cells and the production of autoantibodies often long before the first expression of the disease [3]. Although some intrinsic B cell abnormalities may be central to the disease process, the nature of the immune abnormalities resulting in these defects remains elusive. Notably, increased proportions of transitional B cells from peripheral blood have been described in patients with SLE, although bone marrow production and selection appeared to be normal. Since a significant proportion of the immature B cell repertoire is autoreactive, an assessment of the percentage of self- or poly-reactive B cells in early B cell populations has revealed two tolerance checkpoints: one at the immature to transitional junction and another one at the transitional to naïve junction [4]. Furthermore, several data suggest that B cell antigen receptor (BCR) ligation and B cell activation factor member of the tumor necrosis factor (TNF) family (BAFF) may play an essential role in the differentiation, survival and maturation of transitional B cells [5]. However, until now, few reports have evaluated the function of the interaction signaling through the colligation of the BCR and the complement (C3)-binding CD21/CD19/CD81 costimulatory complex which has been proposed as a co-receptor for CD38 in human B cells [6]. According to some studies in patients with autoimmune diseases, CD38 seems to participate in Ig class switching and plays a role in distinct pathological situations [7], although little is known about its role in lupus development. In addition, current knowledge has demonstrated the potential of BAFF to break immune tolerance when over-expressed [8]. Most importantly, some data indicate that elevated BLYS levels may correlate with SLE disease activity [9].

Taking into account the above considerations, we decided to investigate the potential utility of these combined markers (CD19, CD38, CD81 and BAFFR) on each PB B cell subset to distinguish disease activity levels in SLE patients by using the automatic population separator (APS) graphical representation of the Infinicyt software.

Patients and methods

Patients and controls

Sixty-nine adults were enrolled in the study, including 41 SLE patients and 28 age- and gender-matched healthy individuals (NC: 90 % female; 30 ± 6 years) recruited among healthy blood donors and research staff.

A convenience sample of consecutive patients fulfilling the 1997 American College of Rheumatology (ACR) classification criteria for SLE [10], with either active or inactive disease according to the criteria below, was

recruited from a University Hospital-based Lupus Clinic. Patients were evaluated according to the SLE Disease Activity Index 2000 (SLEDAI 2k) [10, 11] and classified as having active (SLEDAI 2k ≥ 5 ; 100 % female, 33 ± 11 years) or inactive disease (SLEDAI 2k < 5 ; 84 % female, 33 ± 10 years). Table 1 summarizes the clinical and therapeutic data of all patients.

The number of subsequent flares and the SLEDAI score were measured during the study period. Follow-up was defined as, at least, two visits 2–6 months apart during 12-month period. Flare episode was defined as an increase in SLEDAI-2K score of ≥ 4 from the previous visit.

Disease features cumulatively observed from the beginning of the disease until the time of the study were registered. Exclusion criteria: known or suspected ongoing infections and, for NC, any history of autoimmune disease or immunosuppressive therapy.

All participants were asked to provide a morning sample of peripheral blood which was processed fresh.

Informed consent was obtained from all individual participants included in the study.

Flow cytometric analysis

Distribution of different B cell compartments was performed on erythrocyte-lysed and washed PB samples according to procedures which have been previously described in detail [12]. Briefly, all samples were stained and lysed using a direct immunofluorescence technique. PB white blood cells (WBC) were stained with the following monoclonal antibody (mAb) combinations: anti-CD20-PB (*Pacific Blue*; clone 2H7; BioLegend, San Diego, California, USA), anti-CD27-PC5 (phycoerythrin–cyanine 5; clone 1A4LDG5; Beckman Coulter; USA), anti-CD19-PC7 (phycoerythrin–cyanine 7; clone J3-119; Beckman Coulter, France), anti-CD45-KO (Krome Orange; clone J.33; Beckman Coulter), anti-CD81-APC-H7 (clone JS-81; BD Pharmingen), anti-BAFFR-PE (phycoerythrin; clone 11C1; BD Pharmingen, San Diego, California, USA) and anti-CD38-FITC (fluorescein isothiocyanate; clone HIT2, BD Pharmingen).

Cells were acquired on a FACS Canto™ II (BD) using FACSDiva software (BD), and 100,000 events were analyzed using Infinicyt 1.7 software (Cytognos, Salamanca, Spain).

B cell subsets were identified on the basis of the following immunophenotypic features: immature transitional ($CD19^+/CD20^+/CD27^-/CD38^+$), naïve ($CD19^+/CD20^+/CD27^-/CD38^-$), memory ($CD19^+/CD20^+/CD27^+/CD38^-$) and plasmablasts ($CD19^+/CD20^{-/+low}/CD27^{++}/CD38^{++}$). In each subset, the expression of CD19, CD20, CD38, CD81 and BAFFR was evaluated. Results represent the mean fluorescence intensity (MFI) for each marker

Table 1 Clinical features and active medication of SLE patients

	ISLE (<i>n</i> = 24)	ASLE (<i>n</i> = 17)
Mean SLEDAI scores	1.6 ± 0.9	9.7 ± 3.2 [#]
Mean time since diagnosis	9.0 ± 6.0	7.6 ± 7.4
Lupus nephritis ^a	61.3 %	44.4 %
Neurolupus ^a	19.4 %	0 % [#]
Lupus arthritis ^a	58.1 %	66.7 %
Hematological involvement ^a	87.1 %	100 %
Lupus skin disease ^a	74.2 %	77.8 %
Severe lupus ^a	71 %	44.4 % [#]
Anti-dsDNA antibodies ^b		
Negative (<4.2 IU/ml)	42.3 %	11.1 % [#]
Low positive (<20 IU/ml)	32.3 %	11.1 % [#]
Moderately positive (20–50 IU/ml)	22.6 %	22.2 %
High positive (>50 IU/ml)	6.5 %	55.6 % [#]
Treatment ^b		
Hydroxychloroquine	87.1 %	94.4 %
Immunosuppressants ^c	32.3 %	66.7 % [#]
Steroids	12.9 %	83.4 % [#]
Low dose (up to 10 mg/day)	100 %	38.9 %
Moderate dose (10–30 mg/day)	0 %	27.8 %
High dose (more than 30 mg/day)	0 %	16.7 %

[#] Statistically significant differences were considered when $P < 0.05$ for Mann–Whitney U and Chi-squared (χ^2) test

^a Clinical parameters were evaluated from the beginning of the disease until the time of the study. Severe lupus defined as cumulative major organ involvement

^b Parameters were evaluated at the time of blood sample collection

^c Azathioprine, mycophenolate mofetil, cyclosporine, tacrolimus, methotrexate, cyclophosphamide or rituximab; n = sample size. For categorical variables, frequencies are reported. For continuous variables, data are presented as means and standard deviation

within each cell compartment, as graphically displayed with the 3D automated population separator (APS) view—principal component 1 (PC1) versus PC2 versus PC3—of the Infinicyt™ software. As previously described in detail [13], in this APS view, each axis of a plot is represented by a different PC as a linear combination of parameters with distinct statistical weights. Selected clusters were compared with each other to find significantly different bins in a search for cellular subsets that would serve as hallmarks of the cluster.

Statistical analysis

Multiple group comparisons were first performed by means of Kruskal–Wallis nonparametric test to determine whether any group differed from the others. Individual pairings were analyzed using a Mann–Whitney U test and Chi-squared (χ^2) test to determine significance. P values lower than 0.05 were considered statistically significant. SPSS version 21.0 software (SPSS Inc., Chicago, USA) was used to perform the statistical analyses.

Results

B cell subsets in healthy individuals and SLE patients

Lower absolute numbers of total B lymphocytes were found in active SLE patients when compared with healthy controls and inactive SLE patients ($P < 0.05$). This was particularly reflected in the marked reduction in naïve and memory B cells, whereas both transitional B cells and plasmablasts were apparently not affected. Moreover, a higher frequency of plasmablasts, among B cells, was detected in active disease (3 %) as compared with inactive disease (0.9 %) and controls (1.3 %). Regarding the pattern of B cell distribution in inactive SLE, a significant increase in the numbers of transitional B cells followed by a decrease in memory cells and plasmablasts was observed—additional data are given in Online Resource 1.

To clarify the mechanisms underlying all these previous alterations, we further analyzed cell surface signal transduction molecules (CD19, CD20, CD38, CD81 and

BAFFR) on B cell subsets from both SLE patients and healthy individuals—additional data are given in Online Resource 2.

Differential expression of CD19, CD20, CD38, CD81 and BAFFR on B cell compartments from SLE patients versus normal controls

The mathematical tools used in the present study allowed the calculation of the complete immunophenotypic information derived from each PB B cell subset among SLE patients and NC. Herein, we demonstrated that with the analysis of transitional B cells, we could identify two major groups, as shown in Fig. 1a. In contrast, no clusters could be distinguished in the remaining B cells subsets (naïve, memory and plasmablasts)—additional data are given in Online Resource 3.

Regarding transitional B cells, Cluster 1 represented a total overlap between controls and the majority of SLE patients with inactive disease (79 %), reflecting the intrinsic biological similarity of transitional B cells in these cases. Only 18 % (three cases) of the SLE patients with active disease were included in Cluster 1 (Fig. 1a, b). Of note, the three patients with active disease assigned to Cluster 1 showed a relatively low SLEDAI (SLEDAI = 8 ± 0) compared with the remaining ASLE patients (mean SLEDAI = 10 ± 3.7) and all became inactive upon the 12-month follow-up period (Mean SLEDAI: 3 ± 1.1).

On the other hand, Cluster 2 integrated 82 % of all SLE patients with active disease and only 21 % (5 cases) with inactive disease, these ones showing a SLEDAI = 2 and a medical history of inactive nephritis and severe lupus. In addition, a flare episode during a follow-up of 12 months

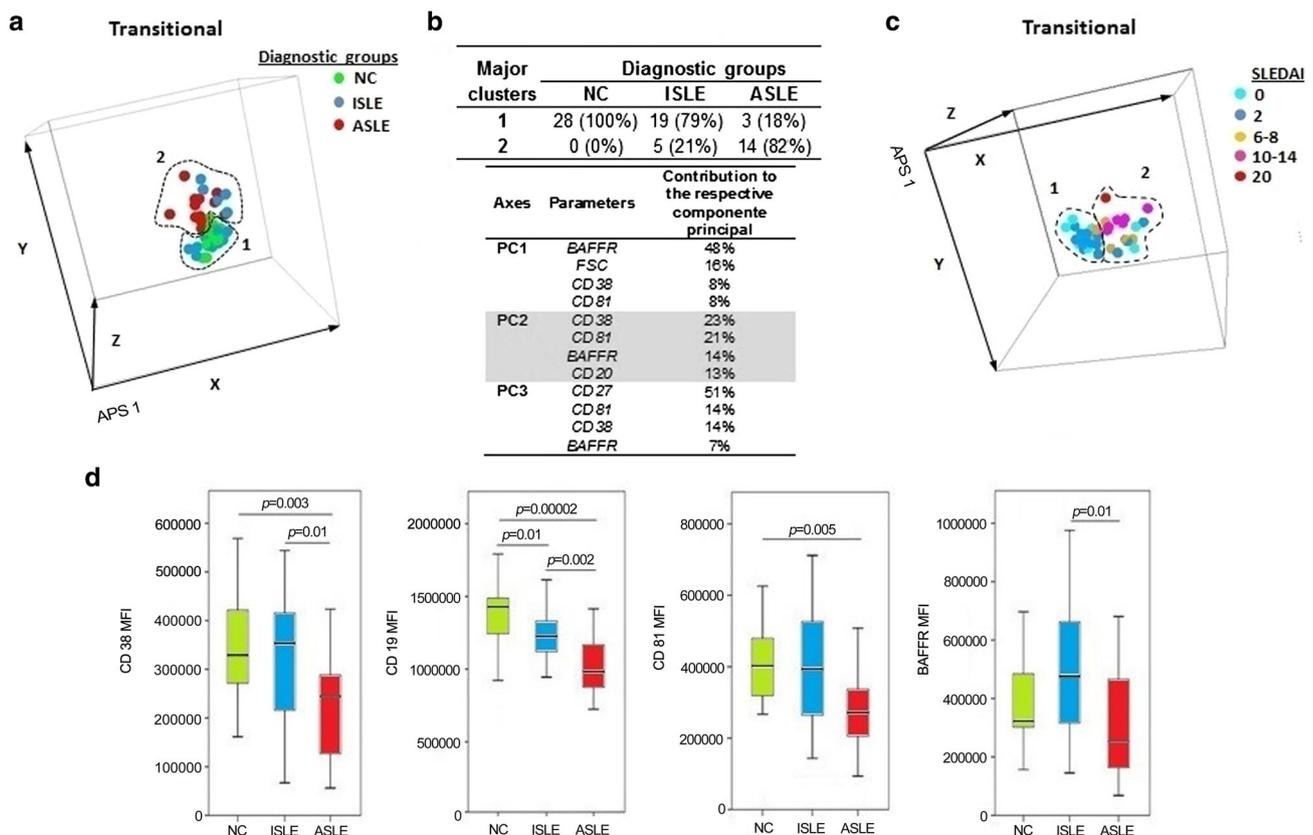


Fig. 1 Principal component analysis (three-dimensional X–Y–Z axis view of PC1 vs. PC2 vs. PC3, respectively) for comparison of SLE patients with inactive disease (ISLE), SLE patients with active disease (ASLE) and normal controls (NC) according to the expression of CD19, CD20, CD27, CD38, CD45, CD81, BAFFR as well as FSC and SSC parameters using the Infinicyt™ software. Each circle represents the overall median position of an individual SLE patient and NC in the PC1 versus PC2 versus PC3 representation of the whole immunophenotypic profile of transitional B cell subset; overall, NC,

ISLE and ASLE cases are distinguished by different colors (a). The most informative parameters contributing to the best discrimination between the two clusters are displayed in a decreasing order of percentage contribution to each of the principal component (b); the distribution of the SLEDAI among SLE patients is colored differently (c). Individual expression of CD19, CD38, CD81 and BAFFR on transitional B cells from SLE patients and NC (d); PC principal component

and an increased SLEDAI score (SLEDAI = 6) was exclusively observed in one of these misclassified patients. Moreover, a reduction in SLEDAI score, although only six patients become inactive, was found 12 months after the study (mean SLEDAI = 6 ± 2.7) in active SLE patients included in this cluster—see additional data are given in Online Resource 4.

The dispersion observed within this latter group was proportional to the wide range of SLEDAI typically found in active disease. Moreover, even within Cluster 2, cases with higher SLEDAI (SLEDAI 10–14) appeared to cluster together, between those with lower SLEDAI (SLEDAI ≤ 8) and an isolated case with SLEDAI = 20 (Fig. 1c).

The most informative markers in this comparative analysis were: (1) CD38, CD81 and BAFFR for the identification of active SLE patients (Fig. 1b). Cluster 2 was mainly characterized by SLE patients with active disease showing a marked decrease in CD19, CD38, CD81 and BAFFR expression on transitional B cells ($P < 0.05$; Fig. 1d).

Discussion

Our findings describe a novel visualization strategy that combines expression of BAFFR, CD81 and CD38 on transitional B cells as a useful tool in the assessment of disease activity in SLE patients.

B cells from SLE patients display signaling defects that may underlie disease pathogenesis activity. Reportedly, complement receptor (CR) type 1 and type 2 are decreased on the surface of SLE B cells and their function appears to maintain B cell immune tolerance to self-antigens [14].

Using a mathematical procedure for the immunophenotypic analysis of PB B cell populations, we evaluated the potential of B cell surface receptors in differentiating between SLE and healthy subjects.

Our visualization strategy revealed that the combined expression of BAFFR, CD81 and CD38 on transitional B cells had a greater weight in the discrimination of active SLE patients, clearly clustering together a major fraction of SLE patients with active disease (82 %, 14 out of 17 active SLE patients), whereas most SLE patients with inactive disease were considered to be clearly different and closely related to controls (79 %, 19 out of 24 inactive SLE patients). Of note, although eight cases were misclassified and could be viewed as “failures” of the proposed procedure, it more likely reflected the need for additional markers to be included in this monoclonal antibody combination. Alternatively, it is also conceivable that misclassified patients are in progress toward a modification of their clinical disease activity status. In this sense, we also assessed the relationship between immunophenotypic

profiles on well-defined subsets of peripheral blood B cells and the risk of further SLE flare-ups during 12-month follow-up. Notably, the only patient with inactive disease who had followed a flare episode was identified as a “misclassified” case within Cluster 2. Further long-term longitudinal studies on larger series of patients will be crucial to definitely clarify these hypotheses and define the precise value of this new tool in SLE. Noteworthy, the potential of this strategy is highlighted by successes obtained in the immunophenotypic screening and classification of individual patients into pre-established and well-defined WHO diagnostic entities [15]. To the best of our knowledge, this is the first time that such a procedure, based on information derived from phenotypic profiling of individual cells, is proposed in the disease stratification of SLE.

Overall, the results obtained showed that B cell surface receptors BAFFR, CD81 and CD38 are significantly affected in patients with active SLE, pointing to their possible involvement in the etiopathogenesis of the disease. Support for this concept comes from previous observations of decreased expression of CD19 and CD21 (CR2), a C3d receptor, as a result of subsequent interaction with circulating immune complexes (CICs) bearing C3 fragments [16] in SLE patients. Interestingly, the decreased expression of CD81, a negative regulator of B cell activation, and CD38 observed on transitional B cells from active SLE patients may suggest an enhanced early B cell activation or a more mature phenotype of transitional B cells. Although CD38 has been used extensively to classify various subpopulations of lymphocytes, in recent years, several publications also have linked CD38 with different pathologies, including autoimmune diseases. Indeed, apoptotic effect mediated by CD38 in immature cells is well described and absence of CD38 in *lpr* mice has been associated with an accelerated development of a lupus-like disease [17]. Furthermore, an increased acquisition of CD38 expression on memory B cells along with the expansion of plasmablasts observed in active SLE patients further supports the hypothesis of a stronger stimulation on SLE B cells and the subsequent generation of plasmablasts in active disease. Finally, it is well established that BAFF, through BAFFR, plays a key role in B cell activation and survival. It has been demonstrated that the chronically elevated overproduction of BAFF in SLE patients with active disease down-regulates BAFFR expression on transitional B cells [18].

In conclusion, the present study demonstrates that the combined expression of BAFFR, CD81 and CD38 in transitional B cells may be used as a practical tool to disease classification of SLE. Further studies evaluating its efficiency in larger series of patients, where an extended follow-up monitoring is also included, are required to confirm these results.

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Conflict of interest None.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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