Increased CD21⁺CD27⁺ atypical memory B cells as a biomarker of clinical disease activity in patients with systemic lupus erythematosus

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Research Article

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Abstract

Background Determining disease activity in systemic lupus erythematosus (SLE) patients is challenging and limited by the lack of reliable biomarkers. Analysis of the altered distribution of circulating B cells has shown promise for assessment disease activity. Yet studies are limited by variable classifications of B subsets. Therefore, we studied peripheral B cells using high-dimensional tools to identify a valid biomarker of disease activity.

Methods We studied B cells in two separate cohorts of patients included in the Swiss SLE Cohort Study. In discovery cohort A, we analyzed cryopreserved PBMCs from 30 SLE, and 30 age-, sex- and ethnicity matched healthy controls (HC) by mass cytometry. In validation cohort B, fresh blood from 63 SLE, 14 Sjögren syndrome (pSS), 14 Sarcoidosis (Sarc), and 39 age-matched HC were analyzed by flow cytometry.

Results In cohort A, using unsupervised clustering analysis, we identified 7 metaclusters within B cells. Two metaclusters were increased in SLE and exhibited a phenotype of atypical memory B cells (aMBC): CD21⁻, CD27⁻, CD11c⁺ and CXCR5⁻. Based on cohort A results, we confirmed in cohort B the increase in CD21⁻CD27⁻ aMBC in SLE, compared to healthy and disease controls. In both cohorts, aMBC were associated with the severity of clinical manifestations. Compared to classical biomarkers, aMBC showed a significant correlation with clinical signs of disease activity.

Conclusion aMBC were expanded in SLE, and the increase correlated with clinical disease activity. According to our data, aMBC represents a robust and easily accessible biomarker to assess disease activity in patients with SLE.

Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by periods of remission and exacerbation. Determining disease activity in patients is crucial to fine-tune therapy. Given the heterogeneity of SLE and the lack of consistent biomarkers, assessing disease activity is challenging(1). Identifying reliable, widely available, and non-invasive biomarkers remains an unmet need.

Examination of the altered distribution of peripheral blood B cells has been the subject of extensive research in the pursuit of biomarkers(2). Despite discrepancies in the definition of B subpopulations, studies have repeatedly reported an imbalance of memory B cell (MBC) subsets that increase with SLE activity. Previous studies on MBC in SLE have shown promising results but their use as biomarker of disease activity has been limited due to the lack of consensual markers.

This study aimed to explore peripheral B cells using high dimensional analysis mass cytometry and flow cytometry in two cohorts of SLE patients included in the Swiss SLE Cohort Study (SSCS). We identified that an atypical MBC (aMBC) population accumulates in SLE periphery. This population lacked the

expression of classical memory surface markers - CD21 and CD27 - but showed signs of activation. aMBC increase correlated with clinical manifestations of disease activity independently from classical serological markers.

Materials And Methods

Participant's characteristics. We studied 93 adults included in the SSCS and diagnosed with SLE according to the 1997 revised ACR criteria and/or the 2012 SLICC criteria. Patients who received rituximab were excluded. Samples from two cohorts of SLE patients, separated in time, were analyzed by mass cytometry (cohort A) or flow cytometry (cohort B). In cohort A (discovery), we compared 30 SLE patients to 30 age-, sex- and ethnicity-matched healthy controls (HC; Supplementary table 1A). Cohort B (validation) included 63 SLE patients and 39 age-matched HC as well as 14 patients with Sjogren's syndrome (pSS), meeting the 2002 American-European Classification Criteria and 14 patients with biopsy-proven sarcoidosis (Sarc; Supplementary table 1B). Detailed characteristics of SLE patients included are available in Table 1. Disease activity was assessed using the Safety of Estrogens in Lupus Erythematosus National Assessment modification (SELENA)-SLEDAI and the Physician's Global Assessment score (PGA). We categorized SLE patients into three groups of disease activity: inactive (SELENA-SLEDAI 0-3), moderate (SELENA-SLEDAI 4-10) and active (SELENA-SLEDAI >10).⁽⁵⁾ Clinical SLEDAI was used to exclude complement and anti-dsDNA from the score. All patients and controls were recruited at the Service of Immunology and Allergy of the Lausanne University Hospital and gave written informed consent. The study was approved by the Institutional Review Board (SwissEthics 2017-01434).

Sample processing

For cohort A, peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation (FICOLL 400, Merck, Switzerland) and then cryopreserved in liquid nitrogen. For cohort B, we analyzed fresh blood by flow cytometry within a maximum delay of 24 hours following sampling.

Mass Cytometry

Cryopreserved PBMC from SLE and matched HC were processed and analyzed as previously published⁽⁵⁾. Lists of mass cytometry antibodies are provided in the Supplementary Table 3 and were previously described⁽⁵⁾. To avoid batch effect, PBMCs of SLE and matched HC were barcoded, processed, and examined at the same time. Manual gating of FCS files was performed using FlowJoTM Software version 10.7.1 (Becton, Dickinson and Company; 2019). Data analysis was performed by exploiting the following FlowJoTM plugins: DownSample v3.3 and FlowSOM⁽⁶⁾ v2.9. Dimensionality reduction was realized using the Barnes-Hut implementation of t-stochastic neighboring embedding (t-SNE) algorithm. Unsupervised clustering analysis was performed using self-organizing map in combination with consensus clustering (FlowSOM) using 100 clusters and 7 metaclusters.

Flow Cytometry

Fresh blood from SLE and controls were labeled, washed after red cell lysis (BulkLysis, Cytognos) and added to bead containing TruCount tubes (BD Biosciences). Lists of mass and flow cytometry antibodies are provided in the Supplementary Table 3 Cells were acquired on BD FACS Canto II (BD Biosciences).

Statistics

Statistical analysis were performed using GraphPad Prism (version 8). We used parametric tests to compare the B cell populations in different groups of individuals as they were normally distributed after log-transformation. We used two-sided unpaired student t-tests to compare the cell frequencies or counts in two groups or two-way ANOVA to compare cells in more than two groups. For multiple comparison, we applied Bonferroni's method for pairwise comparison of the means between groups. We used Pearson's test for correlation between two log-transformed B cell subsets. As continuous clinical variable, such as SELENA-SLEDAI, remained skewed after log-transformation, we used Spearman's rho test for correlation between clinical variable and aMBC. P value < 0.05 was considered significant.

Results

Unsupervised clustering of B cells in SLE patients reveals increased subsets of B cells characterized by a lack of expression of CD21 and CD27

In accordance with previous reports(7), the frequency of B cells within lymphocytes were not significantly different between SLE and HC in both cohorts (Supplementary Figure 1). We first characterized the alteration of the SLE B cell compartment in discovery cohort A. To visualize B cells distribution according to their phenotype, we used a high-dimensionality reduction method (t-SNE) incorporating a total of 240,000 B cells (50% from SLE and 50% from HC) (Supplementary Figure 2A-C). Unsupervised clustering analysis using FlowSOM identified seven B cell metaclusters (Figure 1A). We found a significant increase in the abundance of metaclusters 4 and 5 and decrease in metacluster 3 in SLE compared to HC (Figure 1B). While metaclusters 4 and 5 differed in their expression of IgD, the two populations were characterized by lack of expression of CD21, CD27, and CXCR5, low CD38, and expression of CD11c and CXCR3 (Figure 1C). The frequencies of metaclusters 4 and 5 were significantly correlated ($r = 0.47$, $p = 0.0021$). Based on our results and previous reports(4), we defined metaclusters 4 and 5 as aMBC.

In contrast, the frequency of metacluster 3, which corresponds to unswitched memory B cells (USM), was reduced in SLE compared to HC (Figure 1B-C). Other metaclusters, corresponding to naive (metacluster 1), switched memory (SM, metacluster 2), early plasmablast-like (metacluster 6) and plasmablasts (PB, metacluster 7)(2) showed no alteration in their percentage between SLE and HC.

aMBC defined as CD21⁻CD27⁻ B cells are increased in SLE patients compared to other autoimmune diseases

Next, we performed manual gating of B cells in patients included in cohort A (Supplementary Figure 3A). We defined manually aMBC as CD21⁻CD27⁻ based on our initial results. Frequencies of manually gated

aMBC and metaclusters 4+5 significantly correlated ($r = 0.43$, $p = 0.0027$). In addition, aMBC identified manually exhibited a cell surface phenotype ($CD11c^+CXCR3^+CXCR5^-CD38^-IgD^{+/-}$) similar to that observed with unsupervised clustering (Figure 2A, Supplementary Figure 4).

Using manual gating, we confirmed the increase in aMBC and the decrease USM frequency in SLE patients compared to HC (Figure 2B). In contrast to our initial results, we also observed a significant increase in total PB in SLE patients, as previously reported(8).

Next, we performed a similar analysis in validation cohort B (Supplementary Figure 3B). As expected, SLE patients had increased frequencies and absolute counts of aMBC compared to HC (Figure 2C-D). Interestingly, SLE patients also had significantly more aMBC than patients with pSS or Sarc (Figure 2C and D).

aMBC correlate with clinical SLE manifestations

By evaluating the relationship between aMBC and SLE disease activity, we observed a significant association with both aMBC frequencies and absolute counts (Figure 3A-C, Supplementary figure 5). We also observed a significant correlation between aMBC and clinical SLEDAI. Similar correlation was not observed when classical serum biomarkers (elevated anti-dsDNA antibodies and decreased complement C3, C4) were compared to clinical SLEDAI (Figure 3E). Additionally, aMBC frequencies were not increased in serologically positive patients who showed no signs of clinical activity compared to patients with a SELENA-SLEDAI of 0 points (Figure 3F). Of note, there were no correlation between aMBC frequency and age nor disease duration (data not shown).

Next, as various definitions were used to define aMBC, we examined the importance of additional markers (CD11c, CD38, CXCR5, or IgD) for $CD21^-CD27^-$ B cells. Compared to aMBC defined as $CD21^-CD27^-$ B cells, the addition of these surface receptors did not help predict the disease activity (Supplementary Figure 6).

These results show that the frequency of aMBC is strongly associated with the clinical manifestations of SLE, independently from classical serological markers. In addition, aMBC frequency is more accurate than anti-dsDNA antibodies elevation and complement components C3 and C4 decrease to predict SLE severity.

Discussion

Here, we demonstrated an enrichment in $CD19^+CD21^-CD27^-$ in the peripheral blood in a discovery cohort of SLE patients using initially unsupervised analysis followed by manual gating on mass cytometry data. Additionally, we confirmed our results in a validation cohort using flow cytometry. This population of $CD21^-CD27^-$ B cells exhibited increased levels of CD11c and CD20 compared to other subsets in addition to $CD38^{low}$, $CXCR5^-$, and $CXCR3^+$, resembling previously reported aMBC(3, 4, 9, 10). We have also shown that their increase is associated with the activity and severity of SLE disease. The association between aMBC and disease activity was correlated with clinical manifestations of SLE independently from dsDNA

autoantibodies. Since classical biomarkers only inconsistently correlate with disease activity and exhibit disparities due to variable methods of detection (11-14), determination of the frequency of aMBC represents a highly valuable and easily accessible tool to identify clinical activity in SLE patients. This test can be easily performed by flow cytometry and requires only a limited number of cell surface markers (CD19, CD21 and CD27).

Recent studies suggested that aMBC, expressing similar markers than those found in our study, expand during (sub-)acute stimulation such as COVID-19 or following vaccinations(9) and rise during acute systemic inflammation (3, 4, 15). Furthermore, these cells were reported to have the capacity to differentiate into antibody secreting cells (3, 4). Initial studies describing increased aMBC in the peripheral blood of active SLE patients defined these cells as CD19^{hi}CD21^{low}(7). Further work pointed out that CD11c and Tbet expression was observed in a subset of B cells displaying overlapping features with previously reported aMBC(3, 4, 16) and aMBC reported in our study. These previously described subsets - CD27⁺IgD⁻CD11c⁺, CD11c⁺⁺Tbet⁺, CD11c⁺ - were positively associated with SLE disease activity, autoantibody production and renal disease(3, 4, 16). Recently, the frequency of CD11c⁺ B cells was shown to be enriched within MBC subsets lacking the expression of CD21 and CD27(10, 16). In our study, using additional surface markers such as CD11c did not improve disease activity association. Since the expression of these additional phenotype markers may be influenced by the duration of exposure to the antigen (10) or may be less accessible in daily routine lab tests (such as Tbet, the detection of which requires cell permeabilization), assessment of aMBC defined as CD21⁻CD27⁻ appears powerful enough to capture SLE disease activity despite patient heterogeneity.

Our study showed that the critical expansion of aMBC is specific to SLE compared to pSS and Sarc controls. Although pSS is also associated with B cell dysfunction, we did not identify any increase in aMBC. These data are consistent with those reported by Jenks et al., showing that expansion of aMBC is mostly observed in SLE compared to other connective tissue diseases (3). Interestingly, in pSS, only patients who have pSS associated-lymphoproliferation show a significant increase in aMBC(17). Overall, our data supports a critical role of systemic B cells activation in SLE patients compared to other connective tissue diseases and sarcoidosis.

Our study has limitations. One limitation is its cross-sectional design. Despite good correlation of aMBC with disease activity, the evolution of their frequency over the time remains unknown. Yet our study shows that aMBC frequency was associated with disease activity but not with disease duration. Thus, we hypothesize that patients in whom SLE disease activity improves will have reduced peripheral aMBC levels. Nevertheless, a longitudinal study is needed to determine the evolution of aMBC over the time in order to confirm this hypothesis.

Conclusion

In conclusion, the measurement of CD21⁻CD27⁻ aMBC in the peripheral blood is a relevant, reliable and valid tool to assess disease activity in patients with SLE. Using a limited panel of flow cytometry

antibodies, these cells can be easily identified in blood samples from patients with SLE during follow-up visits.

Abbreviations

SLE : systemic lupus erythematosus

HC : healthy controls

pSS : Sjögren's syndrome

Sarc : sarcoidosis

aMBC : atypical memory B cells

MBC : memory B cells

SSCS : Swiss SLE Cohort Study

SELENA - SLEDAI : Safety of Estrogens in Lupus Erythematosus National Assessment modification of the Systemic Lupus Erythematosus Disease Activity Index

PGA : Physician's Global Assessment score

PBMC : peripheral blood mononuclear cells

t-SNE : t-stochastic neighboring embedding

FlowSOM : self-organizing map in combination with consensus clustering

USM : unswitched memory B cells

SM : switched memory B cells

PB : plasmablasts

Declarations

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

All patients and controls were recruited at the Service of Immunology and Allergy of the Lausanne University Hospital and gave their written informed consent. The study was approved by the Institutional Review Board (SwissEthics 2017-01434).

CONSENT FOR PUBLICATION

Not applicable. No identifiable details on patients are provided in this manuscript.

DATA AVAILABILITY

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

COMPETING INTERESTS

The authors have declared no conflict of interest.

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AUTHORS' CONTRIBUTIONS

AH, CR, DC: study design and data analysis. MH, FB, NF: conducting experiments, data acquisition and analysis. AH, CR, DC: recruitment of SLE patients and healthy controls. CF: responsible of CyTOF facility. AH, DC: writing and editing of manuscript. All authors contributed to the article and approved the submitted version.

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Tables

Table 1 is not available with this version

Figures

Figure 1

Analysis of B cells phenotype by mass cytometry in systemic lupus erythematosus (SLE) in comparison to healthy controls (HC) using unsupervised clustering analysis. (A) Merged t-SNE plots incorporating 120k B cells from 30 SLE and 120k B cells from 30 HC and showing B cell clusters identified by FlowSOM unsupervised clustering analysis (each color represents a different cluster). (B) Frequency of each B cell clusters in 30 SLE patients compared to 30 HC. Scatter bar plots represents mean $\pm$ SEM. Statistical analysis were performed on log-transformed data to obtain normal distributions ***p value < 0.001. (C) Heatmap showing B cell clusters and their mean level of expression of each surface markers used for FlowSOM analysis.

Figure 2

Analysis of B cell subsets using manual gating in systemic lupus erythematosus (SLE) compared to healthy and disease controls. (A) Heatmap showing mean levels of expression of surface markers in manually gated B cell subsets: naive (N), switched memory (SM), unswitched memory (USM), atypical memory B cells (aMBC) and plasmablast (PB). B cells were analyzed by mass cytometry in 30 HC and 30 SLE (cohort A). Data was normalized by rows. (B) Frequency of B cell subsets defined by manual gating using mass cytometry data in 30 SLE compared to 30 HC (cohort A). (C-D) Frequency and absolute counts of aMBC, defined by manual gating of flow cytometry data, in 64 SLE patients compared to 14 primary Sjögren's syndrome, 14 sarcoidosis and 39 HC (cohort B). Scatter bar plots represents mean $\pm$ SEM. Statistical analysis were performed on log-transformed data to obtain normal distributions, *p value < 0.05 , **p value < 0.01 , ***p value < 0.001 .

Figure 3

Atypical memory B cells (aMBC) frequencies in systemic lupus erythematosus (SLE) patients according to disease activity parameters evaluated in two cohorts. B cells are analyzed by mass cytometry in 30 SLE and 30 HC in cohort A and by flow cytometry in 64 SLE and 39 HC in cohort B. (A-B) Frequencies of aMBC in SLE patients according to disease activity (cohort A (A): 17 inactive, 8 moderate, 5 active; cohort B (B): 28 inactive, 25 moderate, 10 active). (C-D) Correlation between disease activity scores (PGA and

SELENA-SLEDAI) and the frequency of aMBC in SLE patients in cohort A (C) and cohort B (D). (E) Correlation between clinical SLEDAI (SELENA-SLEDAI excluding anti-dsDNA and complement scores) and aMBC frequency, anti-dsDNA value and complement (C3 and C4) values in cohort B. (F) Frequencies of aMBC in SLE patients according to their clinical disease activity. Patients with disease activity limited to positive serology were classified as clinically inactive, serologically active. Scatter bar plots in (A), (B), (F) represents mean $\pm$ SEM and statistical analysis were performed on log-transformed data to obtain normal distributions. *p value < 0.05, **p value < 0.01, ***p value < 0.001.

Supplementary Files

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