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Expanded Population of Activated Antigen-Engaged Cells within the Naive B Cell Compartment of Patients with Systemic Lupus Erythematosus

Nan-Hua Chang,* Tamara McKenzie,* Gabriel Bonventi,* Carolina Landolt-Marticorena,* Paul R. Fortin,**†† Dafna Gladman,*** Murray Urowitz,***‡‡ and Joan E. Wither**††

Polyclonal B cell activation is a well-described feature of systemic lupus erythematosus (SLE), but the immune mechanisms leading to this activation are unclear. To gain insight into these processes, we extensively characterized the activated peripheral blood B cell populations in SLE. PBMC from lupus patients and healthy controls were stained with various combinations of conjugated Ab to identify distinct peripheral B cell subsets, and activation was assessed by measurement of forward scatter and CD80 or CD86 expression using flow cytometry. SLE patients had altered proportions of several B cell subsets, many of which demonstrated increased activation as assessed by forward scatter. This activation occurred at an early developmental stage, as B cells in the transitional (T2) stage were already significantly larger than those seen in controls. Increased proportions of CD80- or CD86-expressing cells were also seen in multiple B cell subsets, with the most striking differences observed in the naive CD27+/CD23hi population. Within the CD23hi subset, increased costimulatory molecule expression was most pronounced in an IgD+/IgMlo population, suggesting that activation follows Ag engagement. Although controls also had IgD+/IgMlo cells, they were reduced in number and not activated. Thus, there is an altered response to Ig receptor engagement with self-Ags in lupus. The Journal of Immunology, 2008, 180: 1276–1284.

The presence of multiple autoantibodies in systemic lupus erythematosus (SLE) reflects defective tolerance mechanisms leading to the activation of autoreactive B cells. However, the nature of the immune abnormalities resulting in these defects has proved elusive. Although disease manifestations in SLE are due predominantly to high avidity somatically mutated class-switched IgG autoantibodies, indicating that T cell-B cell collaboration is essential for the development of autoimmunity, increasing evidence suggests that intrinsically abnormal B cells may drive this process. Indeed, B cells from lupus patients are hyperresponsive to a variety of stimuli demonstrating enhanced proliferation to polyclonal activators (1, 2), increased anti-IgM and -IgD mediated intracellular Ca2+ concentration responses (3), increased anti-IgM-induced protein tyrosine phosphorylation (3), and decreased FcγRIIb1 inhibition of BCR signaling (4).

A hallmark of B cell activation is up-regulation of the costimulatory molecules CD80 and CD86. Previous work indicates that lupus patients have increased proportions of B cells expressing these activation markers (5, 6). Elevated expression of CD86 was seen in both small “resting” and large “activated” B cells and was also present in patients with inactive disease, suggesting that it arose from an intrinsic B cell defect. However, in these studies, it was unclear whether the elevation of these costimulatory molecules was due to an increased proportion of memory B cells, which are known to express higher levels of costimulatory molecules (7) or whether altered activation of other B cell subsets was present.

Recently, a number of cellular markers that enable classification of peripheral blood B cells into distinct B cell subsets have been identified. In this study, we have used these markers to perform a systematic analysis of activated B cell populations in lupus patients and examined their association with B cell-activating factor (BAFF) and IFN-α-induced gene expression. We show that increased activation is seen in multiple B cell subsets, including the naive compartment, and can already be seen at the transitional type 2 (T2) immature B cell stage. Within the mature naive B cell subset, up-regulation of costimulatory molecules was most pronounced on cells with features consistent with Ag engagement. Although normal controls also had B cells within this subset that appeared to have engaged Ags, these cells did not express costimulatory molecules. Thus, there is an altered response to Ig receptor engagement in lupus patients.

Materials and Methods

Subjects

Patients were recruited from the University of Toronto Lupus Clinic. A total of 87 patients, who satisfied four or more of the revised 1997 American College of Rheumatology classification criteria for SLE (8), participated in the study. All patients were between 18 and 44 years of age (mean...
Flow cytometry staining and analysis

PBMCs were isolated from heparinized whole blood with Ficoll density gradient centrifugation and treated with Gey’s solution to remove residual RBC. Half a million cells were stained with various combinations of conjugated mAbs followed by PerCP-Cy 5.5-conjugated streptavidin (BD Pharmingen) to reveal bixin-conjugated mAb staining. Stained cells were fixed with 2% formaldehyde and examined by flow cytometry using a dual-laser FACS Calibur instrument (BD Pharmingen). Results were analyzed using CellQuest software, with at least 50,000 lymphoid events acquired per sample. The following mAbs were purchased from BD Pharmingen: bixin-conjugated mouse IgG1 (MOPC-21) and anti-CD23 (M-LE23), IgD (IA-6-2), -CD38 (HIT2); PE-conjugated IgG2b (27–35), IgG2a (G155-178), and anti-IgD (IA6-2), -CD80 (B71), -CD86 (FUN-1), -CD27 (M-T271), -CD24 (ML5), and allophycocyanin-conjugated mouse IgG2b (27-35) and anti-CD20 (H1102); PE-conjugated IgG2b (27–35), IgG1, and anti-IgM (G20–127); FITC-conjugated IgM (MOPC-21), IgG2a (G155-178), and anti-IgD (IA6-2), -CD80 (B71), -CD86 (FUN-1), -CD27 (M-T271), -CD24 (ML5), and allophycocyanin-conjugated mouse IgG2b (27-35) and anti-CD20 (H1102). For analysis, the cells were first gated on the lymphocyte population based on forward and side scatter characteristics. This lymphoid gate contained all CD20-staining cells regardless of cell size. Positively staining populations were determined by comparison with isotype controls, gated on the relevant population. For measurement of the proportion of CD80- and CD86-expressing cells, the percent background staining with isotype controls was subtracted. Cell size was quantitated by measurement of forward scatter (FSC) for each population, the number of cells per microliter was calculated from the number of PBMC per microliter of blood, the percent of B cells in the total PBMC population, and the proportion of the total B cell population that the subset represented.

RNA isolation and real-time PCR

Total RNA was isolated from PBMC using an RNeasy Mini Kit (Qiagen), treated with DNase I, and a first-strand complementary DNA produced using a standard reverse transcription reaction. Quantitative real-time PCR (qRT-PCR) amplifications were performed with SYBR Green Master Mix on an ABI/PRISM 7900 HT sequence detector system (both from Applied Biosystems). PCR amplification of the housekeeping gene, GAPDH, was done for each sample as a control for sample loading. Normalization and quantification of the PCR signals was performed by comparing the cycle threshold value of the gene of interest, in duplicate, with the geometric average of the reference genes. 

Table I. Cell surface markers used to identify different B cell subsets

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Surface Markers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD27* subsets</td>
<td>CD23-CD38* /IgG1 IgD*</td>
<td>15–17</td>
</tr>
<tr>
<td>T1 transitional</td>
<td>CD38* /CD24hi</td>
<td>18, 20</td>
</tr>
<tr>
<td>T2 transitional</td>
<td>CD38* / /CD24mhi</td>
<td>18, 20</td>
</tr>
<tr>
<td>Early class-switched</td>
<td>CD23* CD38* /IgM IgD*</td>
<td>19</td>
</tr>
<tr>
<td>CD27* subsets</td>
<td>CD23* /IgM CD38* –/ +</td>
<td>13, 14</td>
</tr>
<tr>
<td>Pre-GC</td>
<td>CD23* /IgM CD38* –/ +</td>
<td>11</td>
</tr>
</tbody>
</table>

Measurement of serum BAFF levels

Serum BAFF was measured using a human BAFF ELISA detection kit (Antigenic America). All serum samples were pretreated with protein A-coupled Sepharose twice for 1 h at 4°C before performing the ELISA.

Statistical analysis

Data are expressed as the mean ± SD. The Mann-Whitney nonparametric test was used for comparisons between patients and controls, or between patient groups. For analysis of correlations between variables, a linear regression analysis was performed using Prism 3.0 software. Values of $p < 0.05$ were considered statistically significant.

Results

Altered activation and distribution of peripheral blood B cell subsets in lupus patients

The increased B cell activation in SLE could result from expansion of B cell subsets that are normally present within the peripheral blood of healthy individuals, such as pre-germinal center (GC) cells, or activation of B cell subsets not normally activated. To address this question, freshly isolated PBMC from lupus patients and controls were stained with anti-CD20, -CD27, -CD38, and -CD38 mAb to identify different B cell subsets and analyzed by flow cytometry (see Table I). As previously reported, lupus patients had reduced numbers of peripheral blood B cells and a reduced proportion of CD20* B cells in the PBMC population (Table II) (10–12). CD27 staining has been used to discriminate between CD27+ putatively naive B cells and CD27* B cells with somatically mutated Ig molecules (13, 14). SLE patients had an increased proportion of CD27+ and conversely decreased proportion of CD27- cells (Table II). Each of these populations can be further subdivided into six distinct B cell subsets based upon their staining pattern with anti-CD23 and -CD86 (Fig. 1) (15, 16).

In normal controls, the majority of CD27* B cells are contained within the CD23*CD38* and CD23*CD38- B cell subsets, which represent mature naive B cells (Fig. 1, Table I) (16, 17). Lupus patients have reduced proportions of these cells and a shift toward the CD23*CD38+ and CD23*CD38+ populations, which have been recently proposed to contain transitional B cells (18) but may also contain pre-GC B cells (16). The CD23*CD38- and CD23*CD38+ populations are also expanded in lupus. A significant proportion of these cells likely represent early class-switched memory B cells that have not yet acquired CD27 (19).

To further explore the cellular origin of the increased B cell activation within the CD27*CD38+ populations, we examined the mean FSC following staining with anti-CD24. CD24 can be used to discriminate between pre-GC cells that are CD24hi, mature B cells that are CD24lo, and immature transitional B cells that are CD24hi in this population (18, 20). As previously reported, the vast majority of cells within the CD27*CD38* B cell subset of both patients and controls were CD24hi transitional cells, suggesting that the increased activation of the CD27*CD38* subset in lupus patients is due to abnormal activation of their transitional B cells (Fig. 2). Transitional B cells down-regulate CD24 and CD38 as they mature, permitting discrimination between the less...
mature peripheral B cell subset, termed T1, and a more mature T2 or intermediate cell population (18, 20). As shown in Fig. 2B, the increased activation of B cells in lupus patients appears to coincide with acquisition of a T2 phenotype. Although there was a trend to an increased proportion of T2 cells within the CD20+ population of lupus patients, this did not achieve statistical significance.

**FIGURE 1.** B cell subsets in lupus patients and controls separated according to surface expression of CD27, CD23, and CD38. Freshly isolated PBMC were analyzed by four-colour flow cytometry following staining with various combinations of conjugated mAbs. A, Representative dot plots from a control and lupus patient showing how each B cell subset was gated. B, Scatter plots showing the percentage of cells within the CD20+ population and the mean FSC for the indicated subsets, gated as shown in the dot plots. Each circle represents the determination for an individual with open circles indicating healthy controls (n = 28) and filled circles indicating SLE patients (n = 53). Horizontal lines show the mean for each population examined. Values of p were calculated for the difference between controls and lupus patients using the Mann-Whitney U test. Statistically significant p values, <0.05 (*), <0.005 (**), or <0.0005 (** *) are indicated.

### Table II. Comparative analysis of peripheral blood B cell subsets and activation markers in controls and lupus patients

<table>
<thead>
<tr>
<th>B Cell Subpopulation</th>
<th>Control (Median)</th>
<th>SLE (Median)</th>
<th>p&lt;sup&gt;ab&lt;/sup&gt;</th>
<th>No. Cells per Microliter Mean ± SD (Median)</th>
<th>p&lt;sup&gt;ab&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>na</td>
<td>na</td>
<td></td>
<td>2077 ± 790.4 (2000)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD20+/PBMC</td>
<td>10.41 ± 3.68 (10.33)</td>
<td>8.78 ± 5.15 (8.13)</td>
<td>0.0169</td>
<td>216.8 ± 103.7 (212.2)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD27+/CD20+</td>
<td>61.67 ± 13.41 (61.74)</td>
<td>67.25 ± 19.49 (72.34)</td>
<td>0.0258</td>
<td>134.6 ± 75.28 (123.0)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD27+/CD20+</td>
<td>38.24 ± 13.44 (38.17)</td>
<td>32.76 ± 19.33 (27.54)</td>
<td>0.0323</td>
<td>81.94 ± 54.81 (70.66)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD80+/CD20+</td>
<td>10.30 ± 5.93 (10.97)</td>
<td>15.69 ± 13.02 (13.25)</td>
<td>0.1228</td>
<td>22.97 ± 17.26 (17.61)</td>
<td>0.0021</td>
</tr>
<tr>
<td>CD86+/CD20+</td>
<td>5.50 ± 4.81 (4.09)</td>
<td>11.31 ± 9.58 (8.54)</td>
<td>0.0006</td>
<td>11.89 ± 13.21 (8.71)</td>
<td>0.6610</td>
</tr>
<tr>
<td>CD80+/CD27+/CD20+</td>
<td>0.92 ± 1.09 (0.61)</td>
<td>8.04 ± 10.49 (5.08)</td>
<td>0.0001</td>
<td>1.22 ± 1.66 (0.40)</td>
<td>0.0281</td>
</tr>
<tr>
<td>CD86+/CD27+/CD20+</td>
<td>2.81 ± 3.12 (1.85)</td>
<td>10.25 ± 10.56 (7.46)</td>
<td>0.0001</td>
<td>3.44 ± 4.69 (1.87)</td>
<td>0.0366</td>
</tr>
<tr>
<td>CD80+/CD27+/CD20+</td>
<td>18.34 ± 10.87 (18.35)</td>
<td>27.20 ± 15.61 (27.51)</td>
<td>0.0057</td>
<td>14.96 ± 12.88 (10.63)</td>
<td>0.0010</td>
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<tr>
<td>CD86+/CD27+/CD20+</td>
<td>5.94 ± 5.09 (4.63)</td>
<td>13.47 ± 9.90 (11.67)</td>
<td>&lt;0.0001</td>
<td>4.58 ± 4.91 (2.98)</td>
<td>0.4901</td>
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<tr>
<td>CD80+/IgM+/CD23+/CD20+</td>
<td>1.00 ± 2.08 (0.00)</td>
<td>9.11 ± 12.52 (5.25)</td>
<td>0.0002</td>
<td>0.24 ± 0.45 (0.00)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CD86+/IgM+/CD23+/CD20+</td>
<td>2.00 ± 2.64 (1.08)</td>
<td>7.47 ± 6.30 (5.46)</td>
<td>0.0009</td>
<td>1.03 ± 1.49 (0.63)</td>
<td>0.0009</td>
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<tr>
<td>IgM+/IgG+/CD23+/CD20+</td>
<td>27.90 ± 9.43 (30.23)</td>
<td>41.00 ± 13.47 (44.07)</td>
<td>0.0048</td>
<td>23.88 ± 16.15 (24.18)</td>
<td>0.1543</td>
</tr>
<tr>
<td>CD80+/IgM+/CD23+/CD20+</td>
<td>1.02 ± 2.33 (0.00)</td>
<td>3.04 ± 4.17 (0.98)</td>
<td>0.0234</td>
<td>0.12 ± 0.23 (0.00)</td>
<td>0.0385</td>
</tr>
<tr>
<td>CD86+/IgM+/CD23+/CD20+</td>
<td>2.24 ± 3.44 (0.84)</td>
<td>5.27 ± 5.69 (3.56)</td>
<td>0.0134</td>
<td>0.63 ± 1.07 (0.48)</td>
<td>0.2946</td>
</tr>
<tr>
<td>CD80+/IgM++/CD23+/CD20+</td>
<td>2.18 ± 2.77 (1.26)</td>
<td>13.82 ± 12.82 (10.18)</td>
<td>0.0001</td>
<td>0.35 ± 0.51 (0.21)</td>
<td>0.0023</td>
</tr>
<tr>
<td>CD86+/IgM++/CD23+/CD20+</td>
<td>2.69 ± 2.80 (1.40)</td>
<td>9.98 ± 7.99 (8.19)</td>
<td>0.0004</td>
<td>0.52 ± 0.73 (0.22)</td>
<td>0.0237</td>
</tr>
</tbody>
</table>

<sup>a</sup>Freshly isolated PBMC were analyzed by four-colour flow cytometry following staining with various combinations of conjugated mAb.

<sup>b</sup>The combination of stains used to gate the various cell populations are shown in the left column of the table. For each population, the percentages given are for the proportion of cells with the staining pattern indicated by the numerator within the cell population shown in the denominator. Cell numbers indicate the total number of cells with the staining pattern shown in the left column per microliter of blood.

n = 39 controls and 64 lupus patients were examined for anti-CD20, or anti-CD20- and -CD23-stained populations, and n = 15 controls and 25 lupus patients were examined for anti-IgM-stained populations.

**Bold numbers indicate statistically significant p values <0.05.**
In contrast to the findings in the CD27− compartment, the mean FSC for all CD27+ populations examined was comparable in lupus patients and controls (Fig. 1). However, different proportions of the various CD27+ B cell subsets likely also contribute to the increased B cell activation in lupus. Lupus patients had significantly decreased proportions of CD23−CD38+ and CD23+CD38− B cells, which represent memory B cells (11), but increased proportions of CD23−CD38++ and CD23+CD38++ B cells, which have previously been identified as pre-GC B cells and are significantly larger than memory cells (11).

Taken together, the findings indicate that both expansion of B cell subsets that are normally activated, such as pre-GC cells, and abnormal activation of B cell subsets that are not normally activated, such as naive and transitional B cells, contribute to the increased proportion of activated B cells seen in lupus patients.

FIGURE 2. Increased B cell activation within the CD27− B cell subsets of SLE patients. Freshly isolated PBMC were analyzed with four-color flow cytometry following staining with anti-CD20, -CD27, -CD3, and anti-CD23 or -CD24. A. Dot plots gated on CD20+ CD27− cells from a representative control and patient (bottom panels). Top panel, The stains and regions used to identify the B cell subsets. B. Scatter plots showing the percentage of cells within the CD20+ CD27− population and the mean FSC for the indicated subsets, gated as shown in the dot plots. Each circle represents the determination for an individual with open circles indicating healthy controls (n = 14) and filled circles indicating SLE patients (n = 21 for CD38++ populations, 19 for T1/T2 populations). Horizontal lines show the mean for each population examined. Values of p were calculated for the difference between controls and lupus patients using the Mann-Whitney U test. Statistically significant p values, <0.05 (*), <0.005 (**), or <0.0005 (***) are indicated. Note that the FSC values cannot be directly compared with those in Fig. 1, because the two experiments were done at different times and the flow cytometer was recalibrated during the intervening period.

Notably, on linear regression analysis, neither the proportion of B cells in the various subsets, nor their size showed any strong correlations with disease activity, as measured by the SLEDAI-2K (all p > 0.05, except p = 0.032 for a weak inverse relationship with FSC in the CD27−CD23−CD38− population). Furthermore, prednisone dose was not positively correlated with the expanded or activated populations in lupus patients, with the exception of the CD27−CD23+CD38− population (p = 0.018). Indeed, a weak inverse correlation was seen between prednisone dose and the proportion of CD27−CD23−CD38++ cells (p = 0.012) and FSC in the CD27−CD23−CD38− population (p = 0.012). Although there was no association between treatment with anti-malarials and the proportion of, or FSC in, the B cell subsets (all p > 0.05, Mann-Whitney U test), immunosuppressive use showed a weak positive association with the proportion of CD27−CD23−CD38− cells (p = 0.012) and FSC in the CD27−CD23+CD38− and CD27−CD23−CD38+ compartments (p = 0.048 and 0.015, respectively). Given the weak and inconsistent associations observed, the abnormal activation of B cells in lupus does not appear to reflect disease activity, nor does it arise from drug treatment effects.

Increased expression of B cell activation markers, CD80 and CD86, on several distinct peripheral B cell subsets in lupus patients

To further examine the origin of increased B cell activation in lupus patients, expression of CD80 and CD86, which are increased following B cell activation, was examined in the various B cell subsets. Consistent with previous reports (5, 6), lupus patients had increased proportions of CD20+ B cells expressing CD80 or CD86 as compared with healthy controls, which achieved statistical significance for CD86 (Table II). Significant increases were seen for both costimulatory molecules when the CD27− and CD27+ B cell subsets were analyzed separately (see Fig. 3A and Table II) and for the CD27− B cell subset they remained significant even when corrected for the reduced number of CD20+ cells in the blood (Table II). In contrast, the number of CD86+CD27+ B cells was similar in lupus patients and controls, whereas the number of CD80+CD27+ B cells was significantly decreased in lupus patients. It is likely that this reflects the marked reduction of CD27+ B cells in lupus patients, together with the more moderate increases in costimulatory molecule expression within the CD27+ B cell compartment. Comparison of CD80 and CD86 expression with FSC revealed that only a subset of the FSChigh population expressed these molecules, indicating that the increased expression of these molecules was not simply due to increased cell size.

To investigate the unexpected finding of activated B cells within the putatively naive CD27− B cell subset of lupus patients, we used CD23 staining to subdivide the CD27− B cell subset into two compartments. In lupus patients, there was a significant increase in the percentage of CD80+ and CD86+ cells in both of these compartments (Fig. 3B). The CD23− subset has been reported to contain mature naive B cells; however, early class-switched B cells (19, 21) and transitional B cells (Ref. 18 and Fig. 2A) can also be found in this compartment. Consistent with the presence of early switched cells in this population, a significant proportion of the CD23+ cells in our lupus patients were IgM− and/or IgM+, and the proportion of these cells was increased as compared with controls (percent IgM−; controls 3.78 ± 1.74, patients = 15.57 ± 13.54, p = 0.032; percent IgM+; controls = 7.00 ± 5.32, patients = 19.88 ± 17.27, p = 0.034). Thus, it is likely that the increased expression of CD80 and CD86 in the CD27−CD23− cell subset of lupus patients arises, at least in part, from contamination with these cells. In contrast, staining of the CD27−CD23+ compartment with
anti-IgD revealed that the vast majority (96%–99%) of these cells in controls and lupus patients were IgD\(^+\), confirming that naive B cells are abnormally activated in lupus patients.

In general, there was a strong positive correlation, on linear regression analysis, between the proportion of CD80- and CD86-positive cells in each of the B cell compartments (all \(p < 0.007\)). There was also a strong correlation for each costimulatory molecule between the proportion of positive cells in the CD27\(^-\) and CD27\(^+\) compartments (all but one \(p < 0.05\)). Notably, the proportion of costimulatory molecule expressing cells within the two (CD23\(^-\) or CD23\(^+\)) subsets of the CD27\(^-\) compartment was positively correlated with the proportion of mature naive CD38\(^-\) cells and negatively correlated with the proportion of transitional CD38\(^++\) cells in the relevant (CD23\(^-\) or CD23\(^+\)) B cell subset (all \(p < 0.036\)).

The increased expression of costimulatory molecules in lupus patients was not simply a function of disease activity. Only a weak positive correlation between costimulatory molecule expression in some, but not all, of the B cell subsets and the SLEDAI-2K was noted (Fig. 3D) (percent of CD80\(^-\)CD27\(^-\)CD23\(^+\) \(p = 0.039\), percent of CD80\(^-\)CD27\(^-\)CD23\(^+\) \(p = 0.034\), percent of CD86\(^-\)CD27\(^-\)CD23\(^+\) \(p = 0.011\), percent of CD86\(^-\)CD27\(^-\)CD23\(^+\) \(p = 0.041\)). Furthermore, increased levels of costimulatory molecules could still be seen in patients with a SLEDAI-2K of 0 (Fig. 3D and data not shown). There was no association between costimulatory molecule expression and specific disease manifestations such as hypocomplementemia, anti-dsDNA Abs as measured by the Farr assay, or renal disease (as measured by renal indices of the SLEDAI-2K).

Overall, drug treatment, including the dose of prednisone, and anti-malarial or immunosuppressive use, also had no impact on the levels of B cell costimulatory molecule expression. Specifically, there was no correlation between prednisone dose (all \(p > 0.09\) on linear regression analysis) nor was there an association between anti-malarial use (all \(p > 0.33\), Mann-Whitney \(U\) test) and costimulatory molecule expression in any of the subsets tested. Although, in general, there was no association between use of immunosuppressives and costimulatory molecule expression, a weak positive association was found between the levels of costimulatory molecules in the CD27\(^-\)CD23\(^-\) B cell subset (\(p = 0.017\) for CD80 and \(p = 0.024\) for CD86) and immunosuppressive use.

**The abnormal up-regulation of costimulatory molecules occurs predominantly on Ag-engaged naive B cells in lupus patients**

Ag engagement of B cells down-regulates cell surface IgM, while leaving IgD expression relatively unaffected (22–25). Therefore, to determine whether Ag engagement contributes to the activation of B cells in SLE, we examined the association between cell surface
IgM levels and up-regulation of costimulatory molecules. As almost all of the CD23−CD20+ B cells that express IgM are IgD+ in both lupus patients and controls, indicating a naive phenotype (see Fig. 4A), staining with anti-CD20 and -CD23, together with anti-IgM and -IgD or anti-IgM and -CD80, A, Dot plots gated on CD23−CD20+ B cells from a representative control and two lupus patients. Note that IgM−CD80+ B cells in lupus patients were IgD+ and that two patterns of staining (SLE P1 and SLE P2) were observed. B, Scatter plot of the percentage of CD80+ B cells in the IgM−CD23−CD20+, IgMhighCD23−CD20+, or IgMlowCD23+CD20+ populations. Each circle represents the determination for an individual with open circles indicating healthy controls and filled circles indicating SLE patients. Horizontal lines show the mean for each population examined. Values of p were calculated for the differences between 15 controls and 26 SLE patients using the Mann-Whitney U test. Statistically significant p values, <0.05 (●), <0.005 (**), or <0.0005 (***) are indicated.

FIGURE 4. Increased expression of CD80 is seen on IgM+IgD− naive B cells in lupus patients. Freshly isolated PBMC were analyzed by four-color flow cytometry following staining with anti-CD20 and -CD23, together with anti-IgM and -IgD or anti-IgM and -CD80. A, Dot plots gated on CD23−CD20+ B cells from a representative control and two lupus patients. Note that IgM−CD80+ B cells in lupus patients were IgD+ and that two patterns of staining (SLE P1 and SLE P2) were observed. B, Scatter plot of the percentage of CD80+ B cells in the IgM−CD23−CD20+, IgMhighCD23−CD20+, or IgMlowCD23+CD20+ populations. Each circle represents the determination for an individual with open circles indicating healthy controls and filled circles indicating SLE patients. Horizontal lines show the mean for each population examined. Values of p were calculated for the differences between 15 controls and 26 SLE patients using the Mann-Whitney U test. Statistically significant p values, <0.05 (●), <0.005 (**), or <0.0005 (***) are indicated.

Table II), whereas in the lupus patients there was a progressive increase in the proportion of CD80+ cells as IgM cell surface expression decreased. Indeed, in the majority of patients (15 of 26) increased expression of CD80 was seen only in the IgMlow B cell population (as exemplified by SLE P1 in Fig. 4A). Similar findings were observed for CD86 expression (Table II and data not shown). Thus, the abnormal up-regulation of CD80 and CD86 in lupus patients appears to be driven, at least in part, by engagement with Ag.

Activation of the various B cell subsets did not correlate with IFN-α-induced gene expression or BAFF

In lupus, there is increased expression of IFN-inducible genes, reflecting increased generation of IFN-α (26–30). Because IFNs enhance B cell activation (31–33), we examined whether the increased cell size or altered expression of costimulatory molecules in lupus patients was associated with increased expression of four IFN-α-inducible genes, ifit4, ly6e, mxi1, and oasl, in PBMC (30). As shown in Fig. 5, expression of all four genes was significantly increased in SLE patients as compared with controls, with the majority of patients demonstrating values above those seen for controls. Expression of these genes was significantly associated with each other, reduced levels of the serum complement component C4 (mxi1 p = 0.014, oasl p = 0.028, ly6e p = 0.047), and increased levels of anti-dsDNA Abs as detected by the Farr assay (mxi1 p = 0.030). However, there was no correlation with disease activity or B cell activation.

BAFF is also elevated in SLE (34–36). Elevated levels of BAFF have been shown to increase survival of autoreactive B cells, permitting their recruitment into autoantibody-producing responses
patients for B cell activation. Cell surface IgM levels and up-regulation of costimulatory molecule engagement (22–25), we examined the association between patients, reflects increased activation of naive cells.

By analyzing FSC in the CD23\(^{+/+}\) population, in contrast to original reports defining this as subset, making CD23 a particularly good marker for defining the naive B cell population. Thus, the increased activation observed by analysis of FSC in the CD23\(^{+/+}\) and CD23\(^{++}\) populations, together with the increased costimulatory molecule expression in the CD27\(^{-}\)CD23\(^{+}\) population of lupus patients, reflects increased activation of naive cells.

Because naive B cells down-regulate IgM in response to Ig receptor engagement (22–25), we examined the association between cell surface IgM levels and up-regulation of costimulatory molecules in the CD23\(^{+}\) cell subset. In all lupus patients, the proportion of costimulatory molecule expressing B cells increased as cell surface IgM levels decreased, implicating BCR engagement in the activation of their B cells. Indeed, in the majority of patients, up-regulation of costimulatory molecules was seen only on the IgM\(^{low}\) population, suggesting that BCR engagement is required for B cell activation.

In contrast to the findings in lupus patients, the IgM\(^{low}\)CD23\(^{+}\) cells in healthy controls demonstrated minimal expression of costimulatory molecules. Thus, in the absence of a specific exogenous antigenic exposure such as infection, low levels of cell surface IgM on the CD23\(^{+}\) cell population are generally not associated with B cell activation. Studies of nonautoimmune wild-type mice (39) indicate that the IgM\(^{low}\)CD23\(^{+}\) subset contains both chronically Ag-engaged anergic B cells and functionally competent B cells. Although similar studies have not been performed in humans, it is likely that a significant proportion of the IgM\(^{low}\)CD23\(^{+}\) population in humans is also chronically Ag-engaged and anergic. Approximately 5–10% of naive human B cells express the innate autoantibody V\(^{\gamma}_{4}\)–34 H chain that recognizes carbohydrate epitopes displayed at high density on the surface of RBCs (24). In normal individuals these cells are IgM\(^{low}\)IgD\(^{+}\), functionally anergic, and located within the mature naïve compartment. Thus, in normal individuals the lack of costimulatory molecule expression on the IgM\(^{low}\)CD23\(^{+}\) B cell subset likely reflects the lack of a sufficient BCR signal to induce costimulatory molecule expression in the functionally competent cells together with the impaired ability of chronically engaged B cells to up-regulate costimulatory molecules in response to BCR engagement (40). With this in mind, there are several potential explanations for the increased expression of costimulatory molecules on the IgM\(^{low}\)CD23\(^{+}\) B cells of lupus patients. First, the increased costimulatory molecule expression could result from the B cell hyperresponsiveness to BCR stimuli reported in lupus patients (1–4). This could lead to a decreased threshold for the activation of naive self-reactive B cells that have escaped anergy induction, and would not normally be activated by virtue of their low BCR affinity or poor availability of self-Ag, or could overcome the relative block in costimulatory molecule induction in anergic self-reactive B cells. In mice, genetic manipulations that decrease expression and/or impair function of inhibitory molecules such as lyn, CD22, or CD45 (41–44), or increase expression of stimulatory molecules such as CD19 (45, 46), lead to lupus-like autoimmunity and/or aberrant activation of anergic B cells, presumably by reducing the B cell activation threshold. The observation that there are lower levels of lyn in the B cells of lupus patients suggests that similar signaling abnormalities may be implicated in human lupus (47, 48).

Alternatively, the increased costimulatory molecule expression could result from a defect in the induction of anergy in the self-reactive B cells of individuals with lupus. Finally, additional stimuli may be present in lupus patients that can activate B cells that have engaged self-Ags, such as those delivered by T cells, cytokines, and TLR ligands. In contrast to BCR engagement, anergic B cells retain the capacity to up-regulate costimulatory molecules in response to CD40L- and T cell-derived cytokines, such as IL-4 (40).

Treatment of lupus patients with anti-CD40L mAb has demonstrated that signals mediated by CD40-CD40L interactions lead to the generation of the CD38\(^{++}\)GC cell population (49), an important intermediate in the pathway to Ig-secreting cell development (11, 50–52). Thus, raising the possibility that CD40-CD40L interactions contribute to some other aspects of the B cell activation phenotype. However, the proportion of costimulatory molecule expressing B cells in our patients did not correlate positively with either the proportion of CD38\(^{++}\)GC cells or plasma cells (data not shown), suggesting that it is unlikely that this is the sole mechanism leading to the activation of B cells in lupus patients. Experiments in lupus-prone mouse models also suggest that provision of T cell stimuli, while necessary, may not be sufficient to produce the B cell activation phenotype in lupus patients. We have previously shown that B6 mice with a New Zealand Black chromosome 1 interval produce high-titer IgG anti-chromatin Abs associated with spontaneous activation of histone-reactive T cells that have been proposed to provide support for pathogenic autoantibody production in human lupus. Production of mice with a mixture of bone marrow-derived from normal and chromosome 1-congenic mice revealed that the autoimmune phenotype in these mice resulted from intrinsic T and B cell defects (53). Importantly, despite provision of T cell help to both normal and congenic B cells, only congenic B cells up-regulated costimulatory molecules and differentiated into Ig-secreting cells. In addition, congenic B cells were
preferentially recruited into germinal centers. Thus, it is likely that additional defects affecting B cell function, such as those outlined previously, are required to permit B cells in human lupus to up-regulate costimulatory molecules and enter the pre-GC and plasmablast compartments in response to T cell signals.

Engagement of TLR by nuclear Ags has also been shown to lead to induction of costimulatory molecules on nuclear Ag-specific self-reactive B cells (54, 55). However, it is unlikely that this is the predominant mechanism leading to induction of costimulatory molecule expression on the B cells of lupus patients, particularly in the naive and transitional B cell subset. One of the consequences of TLR engagement is induction of TLR expression (56, 57). Thus, if the increased B cell activation in the various B cell subsets results from TLR engagement, one would have expected to see increased TLR expression in these subsets. However, expression of TLR-9 and -3 are reported to be normal in the CD27– B cell subset of lupus patients and up-regulation of TLR-9 in the CD27+ B cell subset was only seen in active patients (58), whereas increased B cell activation in our study was seen in active and inactive patients. Furthermore, treatment with anti-malarials, which has been shown to decrease TLR activation (59), had no impact on the B cell activation phenotype in lupus patients.

In addition to an increased proportion of IgMlowCD23+ B cells that express costimulatory molecules, lupus patients also had an increased proportion of IgMlow cells within their CD27+ naive B cell compartment. As the extent of IgM down-regulation following Ag engagement depends not only on the concentration of Ag and affinity of the BCR, but also on the BCR-signaling apparatus (60, 61), it is possible that this increased proportion of IgMlow cells reflects B cell hyperresponsiveness to BCR stimuli in lupus patients. Alternatively, the increased proportion of IgMlow B cells in lupus patients could indicate an expanded pool of self-reactive B cells within the CD23+ population. This possibility is compatible with the results of Yurasov et al. (62, 63), who found increased proportions of self-reactive B cells in the mature naive B cell compartment of active and, to a lesser extent, inactive lupus patients as compared with normal controls (62, 63). Notably, many anergic autoreactive B cells are removed from the peripheral B cell repertoire at the T1 to T2 transition, never advancing into the mature B cell subset. BAFF has been shown to play a critical role in this process, with high serum levels of BAFF enhancing survival of self-reactive immature B cells and promoting their entry into the mature B cell pool (37). However, there was no correlation between serum BAFF or BAFF mRNA expression and expansion of the IgMlow population in our lupus patients. Nevertheless, it is possible that neither of these measures of BAFF accurately reflects the tissue levels of BAFF that are “seen” by the B cells and an association with BAFF was missed.

In this study, we also demonstrate for the first time that increased activation of naive B cells can already be seen at the T2 phase of B cell development. Significantly, it is at the T2 stage that B cells first acquire the capacity to proliferate in response to BCR stimuli (18, 64). Thus, the increased activation of this subset in lupus likely reflects acquisition of this functional competence together with the B cell hyperresponsiveness and/or expansion of the self-reactive B cell pool outlined above. Indeed, it has been proposed that immune mechanisms that lead to removal of self-reactive B cells before the T2 stage play an important role in preventing the potential for activation of autoreactive B cells (37). In this study, we provide evidence that patients with SLE have a disturbance of tolerance resulting in an increased proportion of the naive B cell population becoming activated as a consequence of self-Ag engagement. Further characterization of the precise immune mechanisms leading to the generation of this phenotype will be an important step in our understanding of this condition.

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