Increased Expression of B Cell Activation Factor Supports the Abnormal Expansion of Transitional B Cells in Systemic Lupus Erythematosus

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ABSTRACT. Objective. To examine the relationship between interferon-α (IFN-α) and dysregulation of B cell activation factor (BAFF) and specific B cell phenotypes in systemic lupus erythematosus (SLE).

Methods. Four-color flow cytometry was used to examine the peripheral B cell populations in patients with SLE. RNA was isolated from the peripheral blood of 87 patients and BAFF expression was determined by quantitative polymerase chain reaction (PCR) and normalized to GAPDH. The expression levels of 5 IFN-responsive genes (LY6E, OAS1, IFIT1, ISG15, and MX1) were determined by quantitative PCR and totaled to generate a global IFN score. The correlations were examined between peripheral B cell populations (including transitional, pregerminal, plasmablasts, and memory) and the expression of BAFF and the global IFN score.

Results. Examination of the peripheral B cell populations in SLE demonstrated a relative expansion of the transitional B cell and plasmablast compartment and a reduction in the memory B cell population. Expressions of BAFF and global IFN score were elevated in patients with SLE compared to healthy controls. A strong positive correlation was noted between BAFF expression and the relative proportion of late transitional (T2) B cells. The proportions of more mature B cell phenotypes did not correlate with BAFF expression. The global IFN score was strongly associated with the level of BAFF expression and moderately correlated with the proportion of late transitional B cells.

Conclusion. The findings suggest that elevated BAFF expression supports expansion of the T2 B cell compartment and contributes to a breach in tolerance in patients with SLE. (First Release Jan 15 2011; J Rheumatol 2011;38:642–51; doi:10.3899/jrheum.100214)

Key Indexing Terms:
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B CELL ACTIVATION FACTOR

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of high affinity autoantibodies directed against nuclear antigens, leading to formation of immune complexes. These deposit within target organs, resulting in organ dysfunction. B cell phenotypic and functional abnormalities are a prominent feature of the disease, including enhanced B cell responses to B cell receptor engagement, the presence of increased numbers of activated cells in diverse B cell subsets, and altered B cell homeostasis. Although the precise cellular and molecular mechanisms that lead to this immunologic derangement have not been fully characterized, both genetically determined intrinsic B cell functional alterations as well as extrinsic biochemical and cellular factors have been suspected of playing roles.

B cell activation factor (BAFF) is a member of the tumor necrosis factor family that is essential for mature B cell survival. Several lines of evidence suggest that BAFF plays an important role in systemic rheumatic autoimmune diseases: (1) elevated BAFF plasma levels have been documented in SLE, Sjögren’s syndrome, and rheumatoid arthritis; (2) mice with increased BAFF expression as a result of a B cell transgene develop an SLE-like phenotype characterized by increased levels of antinuclear antibodies and glomerulonephritis; and (3) BAFF blockade in lupus-prone mice, and more recently in humans with SLE, has been shown to ameliorate disease.

BAFF has multiple effects on immune function that could serve to enhance lupus. BAFF has been shown to promote germinal center B cell and plasmablast survival, as well as to augment T cell costimulation. Studies of human B cells confirm a graded effect of BAFF on B cell survival, with mature B cells > T2 cells > T1 cells, paralleling BAFF receptor expression.

The majority of patients with SLE have altered patterns of gene expression consistent with increased levels of type I interferons (IFN) and BAFF. We and others have found that these increases are associated with increased disease activity and production of autoantibodies. Among the multiple pleomorphic effects of type I IFN on immune function that serve to promote SLE are multiple effects on B cells. IFN-α induces BAFF secretion in vitro, and thus could affect B cell function indirectly through this mechanism. Alternatively, IFN-α also has direct effects on B cell function. In mice, IFN-α has been shown to lower the B cell activation threshold and enhance T-B cell collaboration.

Given the potential effect of BAFF and IFN-α on B cell function, we have sought to determine whether any of the B cell phenotypic abnormalities found in SLE are associated with elevated levels of these cytokines. Our previous work suggested that the increased proportion of B cells expressing recent activation markers in SLE did not correlate with the levels of IFN-α and BAFF. In this study we assessed the correlation between these cytokines and several B cell populations that, as outlined above, may be directly affected by their levels. We confirm that both BAFF and type I IFN levels are elevated in SLE and further demonstrate the association between elevated levels of these cytokines and expansion of the T2 B cell subset and autoantibody production in SLE. Our findings implicate these cytokines in the altered B cell function of patients with SLE and are consistent with the possibility that the elevated BAFF levels in these patients act to promote survival of autoantibiotic B cells, permitting their entry into the T2 compartment.

MATERIALS AND METHODS

Subjects and data collection. Patients were recruited from the University of Toronto Lupus Clinic, a prospective longitudinal clinical cohort. A total of 87 patients, who satisfied 4 or more of the revised 1997 American College of Rheumatology classification criteria for SLE, participated in our study. Control blood samples (n = 26) were obtained from healthy donors who had no family history of SLE. All demographic, clinical, and treatment information for patients with SLE and controls is contained in Table 1. Blood and clinical data were obtained, enabling calculation of disease activity using the SLE Disease Activity Index (SLEDAI-2K). The Research Ethics Board of the University Health Network approved our study; all participants provided informed consent.

RNA isolation and real-time polymerase chain reaction (PCR). Total RNA

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Table 1. Demographic and clinical variables for patients with systemic lupus erythematosus (SLE) and control subjects.

<table>
<thead>
<tr>
<th>Demographic Variables</th>
<th>Patients with SLE, n = 87</th>
<th>Controls, n = 26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range, yrs</td>
<td>18.2–76.2</td>
<td>23.2–58.6</td>
</tr>
<tr>
<td>Mean ± SD (median)</td>
<td>39.4 ± 14.7</td>
<td>37.9 ± 10.7</td>
</tr>
<tr>
<td>Women, n (%)</td>
<td>77 (88.5)</td>
<td>24 (92.3)</td>
</tr>
<tr>
<td>Clinical features, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rash</td>
<td>17 (19.5)</td>
<td>NA</td>
</tr>
<tr>
<td>Mucocutaneous</td>
<td>8 (9.2)</td>
<td>NA</td>
</tr>
<tr>
<td>Alopecia</td>
<td>11 (12.6)</td>
<td>NA</td>
</tr>
<tr>
<td>Arthritis</td>
<td>13 (14.9)</td>
<td>NA</td>
</tr>
<tr>
<td>Serositis</td>
<td>2 (2.3)</td>
<td>NA</td>
</tr>
<tr>
<td>Nephritis (≥ 1 or more SLEDAI-2K criteria)</td>
<td>32 (36.8)</td>
<td>NA</td>
</tr>
<tr>
<td>Myositis</td>
<td>1 (1.2)</td>
<td>NA</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>1 (1.2)</td>
<td>NA</td>
</tr>
<tr>
<td>Cerebritis</td>
<td>6 (6.9)</td>
<td>NA</td>
</tr>
<tr>
<td>Medications*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prednisone, mean ± SD (median)</td>
<td>11.2 ± 15.2 (5.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Antimalarials, n (%)</td>
<td>60 (69.0)</td>
<td>NA</td>
</tr>
<tr>
<td>Immunosuppressives, n (%)</td>
<td>45 (51.7)</td>
<td>NA</td>
</tr>
<tr>
<td>Azathioprine, n (%)</td>
<td>17 (37.7)</td>
<td>NA</td>
</tr>
<tr>
<td>Mycophenolate mofetil, n (%)</td>
<td>20 (44.4)</td>
<td>NA</td>
</tr>
<tr>
<td>Methotrexate, n (%)</td>
<td>6 (13.3)</td>
<td>NA</td>
</tr>
<tr>
<td>Cyclosporine, n (%)</td>
<td>2 (4.4)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Clinical variables and treatment recorded at first clinical assessment.
† No patients received pulse steroids over the course of the study. NA: not applicable; SLEDAI-2K: SLE Disease Activity Index.
was isolated from whole blood archived in PAXgene tubes using the PAXgene RNA preparation kit (Qiagen, Basel, Switzerland) with a number of modifications to increase the quantity and quality of the RNA. A first-strand complementary DNA was produced using a High Capacity cDNA kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR (qRT-PCR) amplification was performed using a custom TaqMan Low Density Array, with GAPDH as the housekeeping gene. Genes including BAFF (assay identification Hs00198106_m1) were print- 
ed in duplicate with expression normalized to GAPDH. Normal BAFF expression levels were defined as the mean expression in healthy controls ± 2 SD. The expression of 5 (IFIT1, MX1, ISG15, LYN6e, and OAS1) IFN-responsive genes was determined as described and presented.

Flow cytometry staining and analysis. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by Ficoll density gradient centrifugation and treated with Gey’s solution to remove contaminating erythrocytes. Half a million cells were stained with various combinations of conjugated monoclonal antibodies followed by PerCP-conjugated streptavidin (BD PharMingen, Mississauga, ON, Canada) to reveal biotin-conjugated monoclonal antibody staining. Stained cells were fixed with 2% formaldehyde and examined by flow cytometry using a dual-laser FACScanlibur instrument (BD PharMingen). Results were analyzed using Cell Quest software, with at least 50,000 lymphoid events acquired per sample. The following monoclonal antibodies were purchased from BD PharMingen: biotin-conjugated mouse IgG1 k (MOPC-21) and anti-CD38 (HIT2); phycoerythrin-conjugated IgG2b (27-35) and IgG1 (MOPC-21), and anti-CD27 (M-T271), and -CD38 (HIT2); FITC-conjugated IgG1 (MOPC-21) and IgG2a (G155-178), and anti-CD27 (M-T271), and -CD24 (ML5); and allophycocyanin-conjugated mouse IgG2b (27-35) and anti-CD20 (HIT2). To quantify the various B cell populations, PBMC were stained with anti-CD20, –CD27, –CD38, and ± –CD24, and 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. For determination of the proportion of plasmablast/plasma cells, all cellular events were gated, with these populations being defined as CD27++, CD38++. Positively staining populations were determined by comparison with isotype controls.

Serologic and circulating BAFF testing. Anti-dsDNA was measured by Farr assay and anti-Ro, –La, –Sm, and –RNP antibodies were measured by ELISA at the hospital laboratory. Serum BAFF levels were measured by a commercial ELISA (Antigenix America, Huntington Station, NY, USA). All serum samples were pretreated with protein A-coupled Sepharose twice for 1 h at 4°C before the ELISA.

Statistical analysis. The Mann-Whitney U 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 4.0 software. P values < 0.05 were considered statistically significant.

RESULTS

Altered distribution of B cell subsets in patients with SLE. Alterations in the proportions of specific peripheral B cell subsets have been described in patients with SLE compared to healthy controls, including a relative increase in the plasma cell21 and pregerminal center B cell compartments22. A trend toward an increased proportion of T2 cells has also been noted. To determine whether these findings were repeated in our patient population, PBMC were stained with anti-CD20, –CD27, –CD38, and –CD24, and the proportion of various cell populations determined by flow cytometry (Figure 1A). As reported, transitional B cells are found in the naive (CD20+CD27−) B cell subset and can be further gated as T1 and T2 cells, based upon their levels of CD24 and CD38 within the CD24++ CD38++ population.23 Pregeminal and memory B cells were gated within the CD20+CD27+ B cell compartment as CD38++ and CD38±, respectively.25 The proportion of CD27+++CD38+++ plasmablast/plasma cells was quantified in the total cell population rather than the lymphoid gate, because we have noted that these cells are not restricted to the lymphoid gate. As shown in Figure 1A, these cells were predominantly in the CD20− cell population, as reported.23 Consistent with our previous results, examination of the transitional cell compartment revealed that patients with SLE had an increased frequency of T2 B cells compared to healthy controls (Figure 1C; p = 0.03), while no statistically significant differences were noted in the T1 cell compartment. Linear regression analysis showed a moderate positive correlation between the proportions of T2 compared to T1 B cells (r = 0.69, p < 0.0001) in patients with SLE (data not shown). Also, in accordance with our previous findings2, an increased frequency of pregerminal B cells (Figure 1D; p = 0.02) and plasmablast/plasma cells (Figure 1F; p = 0.007) and a relative reduction in the proportion of memory cells (Figure 1E; p = 0.02) was noted in patients with SLE. With the exception of a reduced number of memory cells in patients with SLE, no differences were found between controls and patients with SLE in the absolute number of cells/ml for any of the peripheral B cell subsets examined (data not shown).

Changes in the absolute number of CD27high plasma cells have been shown to correlate with disease activity.21 To examine the relationship between disease activity and peripheral B cell populations, patients were segregated into high (SLEDAI-2K ≥ 7) and low/moderate (SLEDAI-2K ≤ 7) activity groups (Figure 2). Increased disease activity was associated with a statistically significant increase in the absolute number of plasmablast/plasma cells (Figure 2D; p = 0.02) and a reduction in the absolute number of pregerminal center (Figure 2B; p = 0.003) and memory B cells (Figure 2C; p = 0.0004). Linear regression analysis (data not shown) between the SLEDAI-2K and plasmablast/plasma cells noted a moderate positive correlation (r = 0.3, p = 0.03), while a moderate negative correlation was found between memory cells and disease activity (r = -0.34, p = 0.008). These findings suggest that cells are recruited out of the pregerminal center and memory B cell compartments into the plasmablast/plasma cell compartment in active disease. In contrast, no association between the absolute number of T2 B cells and disease activity was noted.

Elevated BAFF expression in SLE is correlated with the proportion of T2 B cells. Although both peripheral blood BAFF RNA expression and serum BAFF concentrations have been reported to be elevated in patients with SLE, studies have demonstrated that RNA expression correlates more closely with disease activity and serum immunoglobulin levels than
Figure 1. Peripheral B cell phenotypes in patients with SLE versus healthy controls. B cell subsets were analyzed on the basis of cell surface expression. Freshly isolated PBMC were stained with various combinations of fluorescently labeled antibodies and analyzed by 4-color flow cytometry (A). Top set of dot plots has been gated on CD20+CD27− lymphoid cells, with early transitional (T1) and late transitional (T2) cells differentiated within the CD24++CD38++ subset, as indicated by the labeled circles. The middle set of dot plots has been gated on CD20+CD27+ lymphoid cells, with the memory and pregerminal center (Pre-GC) populations gated as shown. The boxes in the bottom set of dot plots show how CD27+++CD38+++ plasmablast (PB)/plasma cells (PC) were gated on the total cell population. Panels B-E compare the percentage of CD20 cells that are T1 cells (B); T2 cells (C); pregerminal center cells (D); and memory cells (E). Panel F shows the percentage of total cells that are plasmablast/plasma cells. Each dot indicates an individual determination, with the mean indicated by a solid line. Significance determined by Mann-Whitney U testing. Statistically significant p values (<0.05) are shown.
The circulating BAFF concentration was measured in the study. As noted, patients with SLE had a statistically significant increase in peripheral BAFF expression (p = 0.0002) compared to normal controls (Figure 3A). In contrast, circulating BAFF levels were not elevated in patients with SLE and no correlation was noted between circulating BAFF levels and BAFF expression (data not shown).

Linear regression analysis was performed to examine the relationship between individual peripheral B cell populations and BAFF expression. This analysis demonstrated a moderate positive correlation (r = 0.61, p < 0.0001) between BAFF expression and the relative proportion of T2 cells in the lymphoid compartment (Figure 4B). No association was found between BAFF expression and the relative proportion of B cells in the T1, pregerminal center, memory, or plasmablast/plasma cell compartments (Figure 4). On further analysis, no statistically significant association between BAFF expression and the absolute number of any B cell subset was noted (data not shown). Elevated BAFF expression has also been reported to be associated with the presence of autoantibodies.

The current dataset includes published results in which the peripheral gene expression of 5 IFN-responsive elements was determined and totaled to generate a global IFN signature (IFN5). As noted, the global IFN signature was significantly elevated in patients with SLE compared to normal controls, with no significant difference in the IFN5 score noted in patients with high (SLEDAI-2K ≥ 7) versus low/moderate (SLEDAI-2K < 7) disease activity. Segregation of patients on the basis of autoantibody positivity demonstrated that an increased global IFN signature was associated with the presence of both anti-RNA binding protein and anti-dsDNA antibodies. The relationship between the presence of anti-RBP antibodies and an elevated IFN signature was confirmed by linear regression analysis.

Given the association between autoantibody positivity and an elevated IFN5 score, as well as reports noting that IFN-α augments the antibody response, we hypothesized that the global IFN score may correlate with specific B cell phenotypes. Linear regression analysis was performed between the IFN5 score and the relative proportion of each peripheral B cell population (Figure 5). A moderate positive correlation was found between the proportion of T2 B cells and the global IFN score (Figure 5B; r = 0.4, p = 0.01). A weak negative correlation was noted between the relative proportion of memory B cells and the global IFN score (Figure 5D) but this did not reach statistical significance.
Figure 3. Peripheral B cell activation factor (BAFF) expression correlates with the global interferon (IFN5) signature and the presence of autoantibodies in patients with SLE. The relative quantity of BAFF was calculated with expression normalized to GAPDH. (A) BAFF expression in PBMC of controls (n = 11) and patients with SLE (n = 87); (B) BAFF expression in patients with low/moderate SLEDAI-2K (< 7) and high SLEDAI-2K (≥ 7). Graphs are segregated based on BAFF expression, with normal levels defined as within 2 SD of the mean for healthy individuals. (C) Total number of different anti-RNA binding protein antibodies; (D) levels of anti-dsDNA antibodies; (E) global IFN score in patients with normal and high BAFF levels. (F) Linear regression analysis of the global IFN score (IFN5) versus BAFF expression.
correlation was found between IFN5 and the absolute number of any peripheral B cell subset examined (data not shown). A strong positive correlation ($r = 0.69$, $p < 0.0001$) was noted between the expression of IFN-responsive genes and BAFF (Figure 3F). The strength of this association suggests that IFN-α promotes BAFF production in patients with SLE. Thus the association between the global IFN score and T2 B cells may be mediated by BAFF, because activation of the IFN-α pathway augments BAFF production\textsuperscript{29,30}.

**DISCUSSION**

A number of peripheral B cell abnormalities were found in patients with SLE compared to healthy controls. Notably, patients with SLE had an increased proportion of T2 B cells, pregerminal center B cells, and plasmablast/plasma cells, and a reduction in the relative proportion of memory cells. These observations are in agreement with previous publications\textsuperscript{2,21} and demonstrate that alterations in peripheral B cell phenotypes are a consistent feature of SLE. Despite the reproducibility of these findings, the factors promoting B cell derangements in SLE remain unknown.

We have explored the relationship between peripheral BAFF expression and specific B cell phenotypes. A moderate positive correlation was noted between the proportion of T2 B cells and BAFF expression. This observation is in keeping with a wealth of evidence supporting the central role of BAFF in the survival of T2 B cells. Murine models have demonstrated that in the absence of BAFF or its receptor, B cell maturation is arrested at an early transitional stage, highlighting the reliance of T2 cells on BAFF for survival\textsuperscript{31}. Moreover, BAFF blockade in mice reduces T2 B cells without affecting T1 cells\textsuperscript{32}. Human studies also illustrate the reliance of T2 cells on BAFF. In vitro studies have demonstrated that T2 B cells have enhanced survival in the presence of BAFF in line with the higher expression of BAFF receptors at this stage of B cell development\textsuperscript{13}. No correlation was noted between the absolute number of T2 cells and the level of BAFF expression. This observation is...
not surprising because the absolute number of transitional cells is restricted by the number of T1 B cells exiting the bone marrow. This process, as well as survival of T1 B cells, is a BAFF-independent process and would limit the number of precursor cells available for BAFF rescue.

The level of BAFF expression did not correlate with more mature B cell phenotypes including pregerminal center, plasmablast/plasma cells, nor memory B cells. These findings are in accord with previous murine studies demonstrating a decreased reliance on BAFF for both the memory B cell and plasmablast cellular compartments. As well, our results are in keeping with a recent analysis of the long-term effects of BAFF blockade on specific B cell populations in patients with SLE, which demonstrates that BAFF depletion results in a preferential decrease in transitional B cells with little effect on memory B cells and plasmablasts.

Given the reliance of T2 cells on BAFF, it is likely that this late stage of B cell development is when BAFF makes its most significant contribution to disease pathogenesis. Animal studies have demonstrated that the stringency of B cell selection at the late transitional stage is highly dependent on BAFF levels, with increased BAFF allowing for the maturation of autoreactive B cells normally lost at this transition.

In our study, BAFF expression was not associated with disease activity (Figure 3B), suggesting that disease flares are not driven by fluctuations in BAFF. This conclusion is supported by the observation that BAFF expression is closely linked to the global IFN score, which has been shown not to fluctuate with disease activity. Given that disease flares are associated with a marked reduction in pregerminal and memory B cells and a coupled increase in plasmablast/plasma cells, it is likely that additional factors, such as T cell help, are involved in supporting the B cell abnormalities seen in SLE and in promoting disease flares.

The observation that about a third of patients with SLE did not have elevated BAFF expression suggests that intrinsic B cell defects also promote SLE activity in these patients.
patients. In some patients these defects may lead to a decreased threshold for BAFF responsiveness that could result in enhanced rescue of autoreactive transitional B cells in the setting of normal BAFF expression. Although elevated BAFF expression levels were associated with increased production of autoantibodies, including anti-dsDNA and anti-RNP antibodies, normal levels of BAFF expression did not preclude the production of autoantibodies, suggesting that the available BAFF was sufficient to breach tolerance in the face of as yet unidentified intrinsic B cell abnormalities.

Increased IFN activity is an accepted feature of SLE and is associated with increased disease activity. Murine studies have demonstrated that IFN-α priming lowers the B cell activation threshold and augments the antibody response through enhancement of B cell and T cell interaction. The global IFN signature has a positive correlation with autoantibodies, supporting the contention that activation of the IFN pathway may correlate with distinct B cell phenotypes in SLE. In our current analysis no association between mature B cell phenotypes and the global IFN signature was noted. This observation does not exclude the possibility that IFN participates in the expansion of plasmablast/plasma cells in SLE, but rather suggests that additional factors, such as intrinsic B cell defects or T cell help, also contribute to this expansion.

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