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B Lymphocyte Stimulator Levels in Systemic Lupus Erythematosus: Higher Circulating Levels in African American Patients and Increased Production after Influenza Vaccination in Patients with Low Baseline Levels

Lauren L. Ritterhouse^{1,2}, Sherry R. Crowe, PhD¹, Timothy B. Niewold, MD³, Joan T. Merrill, MD¹, Virginia C. Roberts¹, Amy B. Dedek, MD¹, Barbara R. Neas, PhD², Linda F. Thompson, PhD¹, Joel M. Guthridge, PhD^{1,2}, and Judith A. James, MD, PhD^{1,2}

¹Oklahoma Medical Research Foundation, Oklahoma City, OK

²University of Oklahoma Health Sciences Center, Oklahoma City, OK

³Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, Chicago, IL

Abstract

Objective—Examine the relationship between circulating B lymphocyte stimulator (BLyS) levels and humoral responses to influenza vaccination in systemic lupus erythematosus (SLE) patients, as well as the effect of vaccination on BLyS levels. Clinical and serologic features of SLE that are associated with elevated BLyS levels will also be investigated.

Methods—Clinical history, disease activity measurements and blood specimens were collected from sixty SLE patients at baseline and after influenza vaccination. Sera were tested for BLyS levels, lupus-associated autoantibodies, serum IFN- α activity, 25-hydroxyvitamin D, and humoral responses to influenza vaccination.

Results—Thirty percent of SLE patients had elevated BLyS levels, with African American patients having higher BLyS levels than European American patients ($p=0.006$). Baseline BLyS levels in patients were not correlated with humoral responses to influenza vaccination ($p=0.863$), and BLyS levels increased post-vaccination only in the subset of patients in the lowest quartile of BLyS levels ($p=0.0003$). Elevated BLyS levels were associated with increased disease activity as measured by SLEDAI, PGA, and SLAM in European Americans ($p=0.035$, $p=0.016$, $p=0.018$, respectively), but not in African Americans. Elevated BLyS levels were also associated with anti-nRNP ($p=0.0003$) and decreased 25(OH)D ($p=0.018$). Serum IFN- α activity was a significant predictor of elevated BLyS in a multivariate analysis ($p=0.002$).

Conclusion—African American SLE patients have higher BLyS levels regardless of disease activity. Humoral response to influenza vaccination is not correlated with baseline BLyS levels in SLE patients and only those patients with low baseline BLyS levels demonstrate an increased BLyS response after vaccination.

Keywords

Systemic lupus erythematosus (SLE); Cytokines

Please direct correspondence to: Judith A. James, MD, PhD, Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104, Phone: (405) 271-4987, Fax: (405) 271-7063, Judith-James@omrf.org.

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B lymphocyte stimulator (BLyS, also known as TNFSF13B and BAFF) is a type II transmembrane protein and a member of the TNF superfamily (1, 2). BLyS is produced by several different cell types, including monocytes, activated neutrophils, T cells, and dendritic cells (3–5), and is expressed as a cell surface protein which can be furin-cleaved and released into the circulation. Although BLyS has been shown to be constitutively expressed, certain inflammatory cytokines such as IL-2, TNF- α , IFN- γ , and IFN- α can enhance its production and secretion (3, 4, 6, 7). BLyS binds to 3 receptors found primarily on B cells (8). Activation of these receptors leads to B cell proliferation, differentiation, survival, and IgG class switching (1, 4, 9). BLyS has been shown to play an important role in primary immune responses, as anti-BLyS treated mice show profoundly reduced numbers of naïve B cells with accompanying attenuated responses to both T-dependent and T-independent antigens (10).

Transgenic mice that overexpress BLyS display a myriad of autoimmune features, such as high levels of rheumatoid factor, anti-DNA, circulating immune complexes, and immunoglobulin deposition in the kidneys (9). Mice treated with exogenous BLyS develop an increase in B cell numbers, particularly those directed against chromatin (11), and autoreactive cells encountering transitional B cell checkpoints in the spleen require higher concentrations of BLyS to survive than do non-autoreactive B cells (8, 9, 12). Mouse models have also demonstrated that deletion of either BLyS or its receptor severely impairs B cell development beyond the transitional stage with a resulting decrease in peripheral B cell populations (9, 13–15).

Elevated circulating BLyS levels have been found in patients with systemic autoimmune disorders such as SLE, rheumatoid arthritis (RA), and Sjören's syndrome (16–18). Early reports found increased serum BLyS levels in SLE patients compared to healthy individuals that correlated with anti-dsDNA titers, but not disease activity (16, 17, 19). BLyS levels did, however, decrease following high-dose corticosteroid treatment (19). Other studies found that peripheral blood leukocyte BLyS mRNA levels correlated with SLE disease activity (20, 21). While most of these initial studies, which were performed in mainly Hispanic SLE patients, failed to demonstrate a correlation between serum BLyS levels and disease activity, a later longitudinal study undertaken at 4 different clinical centers that contained ancestral diversity, found an association between increases in plasma BLyS levels from a previous visit and increases in SELENA-SLEDAI scores at the next visit (22). Additionally, another study involving Norwegian SLE patients, also found a correlation with serum BLyS levels and SLEDAI scores (23). A study examining plasma BLyS protein levels in pediatric SLE also demonstrated an association between elevated BLyS levels and increased disease activity (24).

IFN- α has proven to be a key cytokine in SLE pathogenesis, and has been shown to increase BLyS expression in antigen-presenting cells (4). Therefore, it is possible that IFN- α plays a role in driving BLyS production in SLE, although a direct correlation between circulating BLyS levels and serum IFN- α activity has yet to be demonstrated in SLE patients. Additionally, several environmental factors have been implicated in the pathogenesis of SLE (25), including vitamin D deficiency (26). As vitamin D has been shown to suppress the expression of the IFN signature in myeloid-derived dendritic cells, as well as suppress B cell activation and immunoglobulin production (27, 28), it is of interest to assess the relationship between vitamin D and BLyS levels.

Belimumab is a fully human monoclonal antibody that binds BLyS and inhibits its activity. Two recent large randomized, double-blind, placebo-controlled, multicenter phase 3 trials, BLISS-52 and BLISS-76, evaluated the efficacy of belimumab plus standard of care compared to placebo plus standard of care; positive results were obtained with both studies

reaching their primary efficacy endpoints of reductions in disease activity without ancillary organ flares (29, 30).

Taken together, these data suggest that excessive BLyS may be an important mechanism underlying SLE pathogenesis and that BLyS targeted therapies seem promising. However, normal B cell function requires some BLyS activity. To date no studies have evaluated whether specific levels of BLyS are required to mount appropriate vaccination responses or whether BLyS levels change after vaccination. The primary objective of this study is to investigate the relationship between circulating BLyS levels and humoral responses made to influenza vaccination, as well as the effect of influenza vaccination on BLyS production in SLE. The current study also examines select demographic, environmental, and clinical features of SLE for association with elevated BLyS levels in an effort to better understand the mechanisms of elevated BLyS in a heterogeneous SLE population.

MATERIALS AND METHODS

Study population

Experiments were performed in accordance with the Helsinki Declaration and approved by the Institutional Review Boards at the Oklahoma Medical Research Foundation and the University of Oklahoma Health Sciences Center. All of the study participants provided informed consent prior to enrollment. Sixty female SLE patients (defined as meeting at least 4 of the ACR classification criteria (31, 32)) who were enrolled in a longitudinal influenza study cohort at the OMRF were included in this study. Additionally, sixty healthy individuals were recruited to provide control samples and were matched to the patients based on age (± 5 years), race, and sex.

Data collection

Participants provided demographic information that included sex, age, and self reported race. Clinical information was extracted from the medical records using the Lupus Family Registry and Repository collection tool for ACR classification criteria (33), age at diagnosis, and medication use. Peripheral blood was collected from each participant before vaccination, and at 2, 6, and 12 weeks post vaccination. Post-vaccination time points were selected for determination of a variety of influenza vaccination immune responses, including predicted maximal T cell reactivity (2 weeks), humoral responses (6 weeks), as well as a more distant timepoint for potential autoantibody changes (12 weeks). Serum and plasma were isolated and stored at -20°C until further use. SLE patients were also evaluated by a board certified rheumatologist at the initial visit and at 6 and 12 weeks post-vaccination for disease activity by the SELENA modified SLE disease activity index (SELENA-SLEDAI), physician global assessment (PGA), and systemic lupus activity measure (SLAM) (34). The Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI flare composite score was computed for the 6 and 12 weeks visits (35, 36).

Plasma BLyS determination

Plasma BLyS levels were determined by an enzyme-linked immunosorbent assay (ELISA) from R&D Systems, Inc. (Minneapolis, MN, USA) according to manufacturer's instructions. Samples were analyzed in duplicate, and the mean coefficient of variation was 5.3%. Elevated plasma BLyS levels were defined as being greater than the 95th percentile of BLyS levels measured in healthy controls (> 1.285 ng/mL).

Serologic measurements

Plasma 25-hydroxyvitamin D (25(OH)D) levels were determined in duplicate using a commercial enzyme immunoassay (Immunodiagnostic Systems, Inc., Scottsdale, AZ)

according to manufacturer's instructions. SLE patient blood specimens were tested for complete blood counts, erythrocyte sedimentation rate (ESR), and creatinine measurements.

Lupus-associated autoantibodies

Anti-nuclear antibodies (ANA) were detected using a HEp-2 indirect immunofluorescent assay (INOVA Diagnostics, Inc., San Diego, CA). Detection of antinuclear antibodies at a dilution of 1:120 or greater was considered a positive result. Double stranded DNA (dsDNA) antibodies were detected using a *Crithidia luciliae* indirect immunofluorescent assay (INOVA Diagnostics, Inc., San Diego, CA). Detection of anti-dsDNA at a dilution of 1:30 or greater was considered a positive result. Enzyme-linked immunosorbent assays were used to evaluate sera for antibodies to Sm, nuclear ribonucleoprotein (nRNP), Ro, La, ribosomal P, and cardiolipin as described (37, 38). Samples were run in duplicate and normalized to a known positive control. Western blot analysis using HeLa cell extracts was also performed to determine autoantibody specificities (39). Briefly, electrophoresis was performed on HeLa cell extracts using 12.5% polyacrylamide gels under denaturing conditions and transferred to nitrocellulose. Autoantigens were determined by identifying distinct bands that corresponded to the appropriate molecular weight and to the band from the known positive control serum used in the assay.

Serum IFN- α activity

The reporter cell assay for serum IFN- α activity has previously been described in detail (40). Briefly, reporter cells were used to measure the ability of patient sera to upregulate IFN-induced gene expression. The reporter cells (Wistar Institute, Susan Hayflick (WISH) cells, ATCC no. CCL-25, American Type Culture Collection, Manassas, VA) were cultured with 50% patient sera for 6 hours and then lysed. mRNA was purified from cell lysates, and cDNA was made from total cellular mRNA. cDNA was then quantified using real-time PCR. Forward and reverse primers for the genes MX1, PKR and IFIT, which are known to be highly and specifically induced by IFN- α , were used in the reaction (41). Background gene expression was controlled by amplifying GAPDH in the same samples.

Trivalent Influenza Vaccination

This study extended over four influenza vaccination seasons and included individuals vaccinated with the 2005–2006, 2006–2007, 2007–2008, and 2008–2009 vaccines. All patients received the currently licensed influenza vaccine approved for use in the United States. Hemagglutinin assays were performed at 4°C, using human red blood. The highest dilution of serum to prevent hemagglutination was designated the HAI titer.

Anti-influenza vaccine humoral response

Three measures of vaccine responsiveness – $B_{\max}$ (relative amounts of native/denatured anti-influenza antibodies), K_a (antibody affinity, the inverse of the dissociation constant K_d), and hemagglutination inhibition (HAI) – were performed as previously described cells (42). A sandwich ELISA was used to quantify antibodies to native glycoproteins, and data were subjected to a nonlinear regression model to calculate $B_{\max}$ and K_a (42). Hemagglutinin assays were performed at 4°C using human red blood cells (42). The sum of the ranks of the three measures was used to determine an individual's combined antibody score within each vaccination year.

Statistical analysis

Categorical variables were analyzed using Fisher's exact test. Normally distributed continuous variables were analyzed using an unpaired t-test, and Welch's correction was used in instances of unequal variance. The Mann-Whitney test was used in instances of non-

normality. The false discovery rate (FDR) was used to correct for multiple comparisons. Spearman correlation was used to assess the relationship between serum IFN- α activity, combined antibody scores for vaccination responses, and BLYS levels. A Wilcoxon matched-pairs signed rank test was performed to assess changes in BLYS levels after vaccination. Multivariate logistic regression was performed after candidate predictors were identified and a model with all candidates was fitted and reduced using likelihood ratio tests with data transformation performed as necessary. Potential two-way interactions and confounders were assessed in the multivariate model. Analyses were performed using GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego, CA, USA), SAS 9.2 (SAS Institute, Raleigh, NC, USA), and NCSS (NCSS, LLC, Kaysville, UT, USA).

RESULTS

African American SLE patients have higher plasma BLYS levels than European Americans

Plasma BLYS levels were determined at baseline for all SLE patients and controls. BLYS levels in both groups were non-normally distributed. SLE patients had a higher median (interquartile range) BLYS level, 1.00 (0.65–1.51) ng/mL, than did healthy controls 0.73 (0.64–0.84) ng/mL ($p=0.0003$, Mann Whitney test) (Figure 1A). African American SLE patients ($n=26$) had a higher median plasma BLYS level 1.22 (0.82–2.70) ng/mL than European American SLE patients ($n=33$) 0.85 (0.57–1.21) ng/mL ($p=0.006$, Mann Whitney test) (Figure 1B). No difference was noted in the median plasma BLYS level between African American controls 0.77 (0.61–0.89) ng/mL and European American controls 0.72 (0.69–0.79) ng/mL ($p=0.440$, Mann Whitney test).

Elevated BLYS levels were those determined to be greater than the 95th percentile of plasma BLYS levels in healthy controls (> 1.285 ng/mL). Eighteen SLE patients (30%) and three controls (5%) fell above this cut-off and were classified as having “elevated” BLYS levels. No difference in age between the SLE patients with elevated BLYS levels (mean 40.5 years) and with normal BLYS levels (mean 43.7 years) ($p=0.345$, Fisher’s exact test) (Table 1) was noted. Twelve of the patients with elevated BLYS levels were African American (67%), while the remaining six were European American (33%) [OR 4.0 (95% CI 1.2–12.9), $p=0.024$ Fisher’s exact test, FDR $q=0.144$] (Table 1).

Humoral immune response to influenza vaccination is not affected by baseline plasma BLYS levels in SLE patients

Baseline BLYS levels were not correlated with anti-influenza humoral responses as measured by a combined antibody score ($B_{\max}$, K_a , HAI) following influenza vaccination in SLE patients ($r^2=0.001$, $p=0.863$, Spearman correlation) (Figure 2A). There was also no significant correlation between baseline BLYS levels and anti-influenza humoral responses in controls ($r^2=0.045$, $p=0.105$, Spearman correlation). Also of interest was the effect of vaccination on circulating BLYS levels. In addition to plasma BLYS levels that were obtained at baseline before vaccination, BLYS levels were determined from samples that were taken two weeks after vaccination. There was no significant change in plasma BLYS levels in SLE patients between baseline and two weeks after influenza vaccination (Figure 2B). SLE patients had a median (IQR) BLYS level of 1.0 ng/mL (0.6–1.5) at baseline and a median BLYS level of 1.0 ng/mL (0.7–1.4) 2 weeks after vaccination ($p=0.337$, Wilcoxon matched-pairs signed rank test). However, when examining the subset of patients in the lowest quartile of BLYS levels at baseline, there was a significant increase in BLYS levels 2 weeks post-vaccination ($p=0.0003$, paired t-test) (Figure 2C). This subset of patients had a mean (SD) BLYS level of 0.53 ng/mL (0.07) before vaccination and 0.86 ng/mL (0.29) post-vaccination.

Elevated BLyS levels are associated with increased disease activity in European Americans but not in African Americans

European American SLE patients with elevated BLyS levels had higher SLEDAI, PGA, and SLAM scores than did those with normal BLyS levels (Figure 3A, 3B). European American patients with elevated BLyS levels had a median (IQR) SLEDAI of 8 (5–12), PGA of 60 (39–75), and SLAM of 11 (9–15), while European American patients with normal BLyS levels had a median SLEDAI of 2 (0–6), PGA of 23 (9–39), and SLAM of 7 (5–10) ($p=0.035$, $p=0.016$, $p=0.018$, respectively, Mann Whitney test). African American patients with elevated BLyS levels, however, did not have increased disease activity scores compared to those with normal levels (Figure 3C, 3D). African American patients with elevated BLyS levels had a median (IQR) SLEDAI of 4 (2–8), PGA of 47 (19–53), and SLAM of 11 (6–15), while African American patients with normal BLyS levels had a median SLEDAI of 5 (2–8), PGA of 43 (12–59), and SLAM of 9 (6–11) ($p=1.000$, $p=0.837$, $p=0.225$, respectively, Mann Whitney test).

Elevated BLyS levels were associated with discoid rash, renal disease, serositis, and lymphopenia but did not predict subsequent disease flare

Historical cumulative ACR classification criteria in SLE patients with elevated BLyS levels were compared to criteria in SLE patients with normal BLyS levels (31, 32). The following historical ACR criteria were present in this study population: malar rash (57%), discoid rash (20%), photosensitivity (65%), oral ulcers (63%), arthritis (83%), serositis (57%), renal disease (32%), CNS disease (8%), hemolytic anemia (5%), leucopenia (28%), lymphopenia (33%), thrombocytopenia (13%), and immunologic criteria (62%). SLE patients with elevated BLyS levels were more likely to have the following ACR criteria: discoid rash [OR 7.6 (95% CI 1.9–30.5), $p=0.004$, FDR $q=0.056$], renal disease [OR 6.7 (95% CI 2.0–22.7), $p=0.002$, FDR $q=0.042$], serositis [OR 3.9 (95% CI 1.1–13.7), $p=0.046$, FDR $q=0.176$], and lymphopenia [OR 4.0 (95% CI 1.2–12.9), $p=0.034$, FDR $q=0.159$] (Fisher's exact test) (Table 1) from medical record review. Active disease features as collected as part of the SLEDAI evaluation did not show any differences between patients with elevated and normal BLyS, although the number of individuals with each active criteria was low.

The Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI flare composite score was calculated 6 and 12 weeks after baseline plasma BLyS levels were measured (35, 36) (Table 1). Twenty-two individuals experienced a mild/moderate flare at either 6 or 12 weeks after baseline, and 3 individuals experienced a severe flare. Fifty percent of SLE patients with elevated BLyS levels experienced a flare during the 12-week follow-up period, while 38% of SLE patients with normal BLyS levels at baseline experienced a flare during this time [OR 1.6 (95% CI 0.5–5.0), $p=0.409$, Fisher's exact test]. The median (IQR) BLyS level of SLE patients at their visit prior to a flare was 1.14 ng/mL (0.76 – 1.50), while the median (IQR) BLyS level of SLE patients at the time of flare was 1.00 ng/mL (0.79 – 1.55) ($p=0.687$, Wilcoxon matched-pairs signed rank test).

Medication use was also assessed in both groups of SLE patients (Table 1). The median (IQR) prednisone dosage in patients with normal BLyS levels was 0 (0–5) mg/day, compared to a median of 5 (0–10) mg/day in patients with elevated BLyS levels ($p=0.064$, Mann Whitney test). There was no significant difference between use of azathioprine, hydroxychloroquine, mycophenolate mofetil, or methotrexate between those with elevated BLyS levels and those with normal levels. Only one patient was taking cyclophosphamide. Additionally, one patient with elevated BLyS levels had received rituximab approximately 10 months before the baseline study date, and another patient with normal BLyS levels was currently receiving infliximab treatment.

Elevated BLYS levels in SLE are associated with anti-nRNP

SLE patients with elevated BLYS levels had a higher median (IQR) ANA titer 1:1080 (1:360–1:3240) than patients with normal BLYS levels 1:120 (1:40–1:1080) ($p=0.006$, Mann Whitney test). Patients with elevated BLYS levels also had higher anti-dsDNA titers than patients with normal BLYS levels, although the difference did not reach statistical significance (median titer 10 vs. 0, respectively, $p=0.061$, Mann Whitney test). However, there was a significant correlation between anti-dsDNA titers and BLYS levels in SLE patients (Spearman $r=0.39$, $p=0.002$).

Additional lupus-associated autoantibody specificities were tested in all SLE patients. Twenty-five patients were positive for anti-Ro (42%), 22 patients for anti-nRNP (37%), 20 patients for anti-dsDNA (33%), 12 patients for anti-La (20%), 11 patients for anti-Sm (18%), 9 patients for anti-cardiolipin (15%), and 8 patients for anti-ribosomal P protein (13%). When the presence of each autoantibody specificity was assessed categorically, anti-nRNP was significantly associated with elevated BLYS levels, present in 72% of these patients vs. only 21% of patients with normal BLYS levels [OR 9.5 (95% CI 2.7–33.9), $p=0.0003$, Fisher's exact test, FDR $q=0.013$] (Table 2). The total number of lupus-associated autoantibody specificities was determined for each patient, and those with elevated BLYS levels had a median of 2 autoantibodies (range 0–5), while those with normal BLYS levels had a median of 1 autoantibody (range 0–4) ($p=0.137$, Mann Whitney test).

HeLa cell extract Western blots were used to identify the specificities of the nRNP autoantibody production in those patients with elevated and normal BLYS levels. Interestingly, anti-nRNP positive SLE patients with elevated BLYS levels more frequently had autoantibody responses directed against nRNP 70K (67%) than those patients with normal BLYS levels (25%), although this was not statistically significant ($p=0.170$, Fisher's exact test). There were no significant associations between the presence of autoantibodies against dsDNA, Ro, La, Sm, ribo P, or phospholipids and elevated BLYS levels.

Serum IFN-alpha activity is positively correlated with BLYS levels in SLE

Serum IFN- α (IFN- α) activity was measured by a reporter cell assay and strongly correlated with BLYS levels in SLE patients ($r^2=0.40$, $p<0.0001$, Spearman correlation) (Figure 4A). Additionally, SLE patients with elevated BLYS levels ($n=18$) had a significantly higher median (IQR) serum IFN- α activity 6.7 (0.5–12.4), than did patients with normal levels ($n=42$) 0.0 (0.0–0.9) ($p<0.0001$, Mann Whitney test) (Figure 4B). African American patients ($n=26$) had higher median serum IFN- α activity 1.0 (0.1–9.2), than did European American patients ($n=33$), 0.0 (0.0–1.2) ($p=0.001$, Mann Whitney test).

Elevated BLYS levels are associated with lower 25-hydroxyvitamin D and increased ESR in SLE

Laboratory tests obtained from SLE patients included 25-hydroxyvitamin D (25(OH)D), erythrocyte sedimentation rate (ESR), complete blood counts (CBC), and serum creatinine levels (Table 2). SLE patients with elevated BLYS levels had a lower mean (SD) 25(OH)D level than patients with normal BLYS levels, 16.3 ng/mL (4.8) vs. 20.6 ng/mL (8.7), respectively ($p=0.018$, unpaired t-test, FDR $q=0.144$). Seventy-two percent of patients with elevated BLYS levels were considered to be vitamin D deficient (25(OH)D < 20 ng/mL) and 57% of patients with normal BLYS levels were considered deficient ($p=0.387$, Fisher's exact test). There was no significant difference in mean (SD) 25(OH)D levels between European American and African American patients in this study, 19.2 ng/mL (7.6) vs. 19.3 ng/mL (8.8), respectively ($p=0.967$, unpaired t-test).

Patients with elevated BLYS levels also had a greater mean (SD) ESR of 35.7 (22.1) mm/hr compared to patients with normal BLYS levels, which had a mean ESR of 21.1 (13.1) mm/hr ($p=0.034$, unpaired t-test with Welch's correction, FDR $q=0.159$). No significant differences were found between the two groups regarding percent hematocrit, hemoglobin levels, lymphocyte counts, or platelet counts. Both groups of patients had the same mean serum creatinine level, 0.9 mg/dL.

Increased serum IFN- α activity is associated with elevated BLYS levels in a multivariate analysis

A multivariate logistic regression analysis was performed to determine the relationship between African American ancestry, nRNP autoantibodies, disease activity, serum IFN- α activity, 25(OH)D, and BLYS levels (Table 1). A model with all candidates was fitted and reduced using likelihood ratio tests. The only significant variable in the final model was serum IFN- α activity [OR 1.71 (95% CI 1.22 – 2.39), $p=0.002$]. Two-way interactions between all variables were assessed and none were found to be significant. African American ancestry and 25(OH)D levels were considered as potential confounders. Inclusion of either variable did not change the point estimate by greater than 10% and therefore neither ancestry nor 25(OH)D levels were considered to be confounding.

DISCUSSION

Several studies have documented elevated circulating BLYS in SLE patients (16, 17, 19, 22) but have suggested inconsistencies in the relationship between increased BLYS and disease activity. This is possibly accounted for by differences in the populations studied or the timing or type of disease activity measured. Results of the belimumab trials underscore the likely significance of the BLYS pathway in SLE, however they also confirm that many patients remain unresponsive to BLYS pathway intervention. The current report explored subsets of SLE patients who may be more likely to have BLYS elevation underlying their disease and whether circulating BLYS levels affected the humoral response to vaccination.

Baseline BLYS levels at vaccination did not correlate with influenza-specific antibody responses in SLE patients. This is an encouraging finding, since BLYS inhibition may be a promising new treatment for SLE, and BLYS has previously been shown to play a role in primary immune responses (10). However, subnormal BLYS levels might be induced in some patients receiving agents antagonistic to the BLYS pathway and in such cases poor response to this or other immunizations cannot be excluded. Additionally, vaccination only induced an increase in BLYS production in those patients with low levels at baseline. This phenomenon suggests that patients with high levels at baseline are already producing BLYS at a maximal level and may be restricted from producing additional BLYS by the number of cellular sources or other limiting factors. It is also possible that a certain threshold of BLYS production exists which is sufficient for the formation of primary humoral immune responses, and increases in BLYS production occur following vaccination only in those individuals that fall below this threshold.

We found elevated BLYS levels in 30% of SLE patients, which is a similar frequency as has been reported in other studies looking at a single time point (16, 17). In our study population, we found significantly higher BLYS levels in African American SLE patients. However, in multivariate analyses, African American race was not selected in the final model. This suggests that factors which are more common in African American SLE patients, such as nRNP autoantibodies or high IFN- α activity (43, 44), may be important BLYS related variables. Interestingly, the association between elevated BLYS levels and increased disease activity was only seen in European American patients and not in the African American patients, and suggests the BLYS-mediated pathogenesis and response to

BLyS directed therapies may differ in patients depending on their ancestral background. The presence of elevated IFN- α activity and BLyS levels in African American patients may contribute to the poor response to belimumab that was seen in this ancestral subset of patients in phase III clinical trials, and are factors that should be considered in the upcoming clinical trial that will focus on the efficacy of the drug in African American SLE patients (45).

Most previous studies have shown consistent correlations between BLyS protein and mRNA levels with anti-dsDNA titers (16, 17, 19, 22). A significant correlation between BLyS levels and anti-dsDNA titers was also observed in this study, although categorical analysis did not find an increased likelihood of anti-dsDNA positivity in patients with elevated BLyS levels. Additionally, patients with anti-nRNP responses were more than 9 times as likely to have elevated BLyS levels. These findings highlight the need for additional mechanistic studies examining the impact of BLyS on B cell subsets including whether there is preferential selection for autoreactive cells that are located in the naïve compartment, antigen exposed B cells present in germinal centers, or both.

A significant correlation between BLyS levels and serum IFN- α activity was found, and serum IFN- α activity was the only significant variable in the multivariate analysis. A similar association has been previously reported between BLyS mRNA expression and a global IFN score in SLE patients (46). Another study examining Sjögren's Syndrome patients after treatment with etanercept demonstrated that serum BLyS levels varied with IFN- α activity following therapy (47). Additionally, serum IFN- α activity has been shown to strongly correlate with autoantibody production in SLE, and specifically with antibodies against dsDNA and nRNP (44). *In vitro*, the addition of IFN- α to dendritic cells and monocytes results in increased BLyS expression (4). It is therefore reasonable to hypothesize that increased serum IFN- α in SLE patients helps to drive BLyS production and that an overlapping spectrum of patients might respond to treatments inhibiting these two pathways. Also of interest, was the association between elevated BLyS levels and decreased plasma 25(OH)D levels that was independent of racial differences in vitamin D levels. As vitamin D deficiency is present in approximately two-thirds of SLE patients (48), and has been shown to modulate autoantibody production and the IFN signature (27, 28), it is plausible that vitamin D is capable of either directly or indirectly regulating BLyS levels in SLE.

In conclusion, African American SLE patients have increased BLyS levels regardless of their disease activity, and BLyS levels are strongly associated with serum IFN- α activity in SLE patients. Elevated BLyS levels were also associated with anti-nRNP and decreased 25(OH)D levels. Baseline BLyS levels are not correlated with humoral responses to influenza vaccination in these patients, and only those patients with low BLyS levels at baseline demonstrate an increase in BLyS production after vaccination. These findings support the idea that BLyS is important in SLE disease pathogenesis, although increased levels of BLyS are not necessarily associated with improved humoral responses to vaccination.

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Figure 1. African American SLE patients have increased plasma BLyS levels

A, BLyS levels (ng/mL) in SLE patients and matched controls. Each symbol represents one individual and the median and interquartile range (IQR) is displayed. Mann Whitney test was performed. **B**, BLyS levels (ng/mL) in African American and European American patients. Boxes represent median and IQR. Mann Whitney tests were performed.

Figure 2. No association between baseline BLyS levels and influenza vaccination responses in SLE patients

A, Baseline BLyS levels and correlation with influenza vaccination response as measured by a combined antibody score (B_{max} , K_a , HAI) in SLE patients ($r^2=0.001$, $p=0.863$). **B**, BLyS levels at baseline and 2 weeks after vaccination in all 60 SLE patients ($p=0.337$). **C**, BLyS levels at baseline and 2 weeks after vaccination in the subset of SLE patients in the lowest quartile ($n=15$) of baseline BLyS levels. Each symbol represents one individual.

Figure 3. Elevated BLyS levels are associated with increased disease activity in European American SLE patients but not in African American SLE patients
 SLEDAI (Panel A) and PGA (Panel B) scores in European American patients. SLEDAI (Panel C) and PGA (Panel D) scores in African American patients ($p=1.000$ and $p=0.837$, respectively). Elevated BLyS levels were defined as being greater than the 95th percentile of healthy matched controls. Median and IQR displayed. Each symbol represents one individual.

Figure 4. Serum IFN- α activity is positively correlated with circulating BLyS levels in SLE patients

A, Correlation between IFN- α activity and BLyS levels. Black circles and gray squares represent individual African American and European American patients, respectively. Spearman correlation was performed. **B**, Serum IFN- α activity in patients with normal and elevated BLyS levels. Elevated BLyS levels were defined as being greater than the 95th percentile of healthy matched controls. Bars represent median and IQR.

Univariate analysis of demographic, ACR criteria, disease activity, and medication use of SLE patients with normal and elevated BLyS levels

Table 1

	Normal BLyS levels n=42	Elevated BLyS levels* n=18	p-value [†]	FDR q-value ^{††}	Odds Ratio (95% CI)
Race					
European American	64%	33%	0.046	0.176	0.3 (0.1–0.9)
African American	33%	67%	0.024	0.144	4.0 (1.2–12.9)
Age	43.7	40.5	0.345		
Cumulative ACR criteria					
Malar rash	50%	72%	0.157		2.6 (0.8–8.6)
Discoid rash	10%	44%	0.004	0.056	7.6 (1.9–30.5)
Photosensitivity	67%	61%	0.771		0.8 (0.3–2.5)
Oral ulcers	64%	61%	1.000		0.9 (0.3–2.7)
Arthritis	88%	72%	0.149		0.4 (0.1–1.4)
Serositis	48%	78%	0.046	0.176	3.9 (1.1–13.7)
Renal disease	19%	61%	0.002	0.042	6.7 (2.0–22.7)
CNS disease	12%	0%	0.310		0.2 (0.0–3.5)
Hemolytic anemia	5%	11%	0.576		2.5 (0.3–19.3)
Leukopenia	26%	44%	0.227		2.3 (0.7–7.2)
Lymphopenia	24%	56%	0.034	0.159	4.0 (1.2–12.9)
Thrombocytopenia	12%	28%	0.149		2.8 (0.7–11.5)
Immunologic	45%	67%	0.164		2.4 (0.8–7.7)
Disease activity measures					
SLEDAI, median (IQR)	2 (0–6)	6 (2–9)	0.085		
SLAM, median (IQR)	8 (5–10)	11 (8–14)	0.006	0.063	
PGA, median (IQR)	23 (9–49)	49 (36–64)	0.021	0.144	
SLICC, median (IQR)	1 (1–3)	2 (1–4)	0.158		
Flare (over 12 weeks)	38%	50%	0.409		1.6 (0.5–5.0)
Medication					
Prednisone§	0 (0–5)	5 (0–10)	0.064		
Azathioprine	17%	22%	0.719		1.4 (0.4–5.7)

	Normal BLyS levels <i>n</i> =42	Elevated BLyS levels* <i>n</i> =18	p- value [†]	FDR q- value ^{††}	Odds Ratio (95% CI)
Hydroxychloroquine	74%	72%	1.000		0.9 (0.3–3.2)
Mycophenolate mofetil	21%	39%	0.207		2.3 (0.7–7.8)
Methotrexate	14%	11%	1.00		0.8 (0.1–4.1)
Cyclophosphamide	2%	0%	1.00		0.9 (0.0–24.2)

* Elevated BLyS levels were defined as being greater than the 95th percentile of healthy controls.

[†] Differences in age were assessed using an unpaired t-test. Categorical variables were assessed using Fisher's exact test. Differences in number of ACR criteria, disease activity scores, and prednisone dosages were assessed using the Mann-Whitney test. IQR, interquartile range.

^{††} False discovery rate (FDR) q-value

[§] Prednisone dosage, median and IQR (mg/day)

Table 2
Univariate analysis of serologic studies in SLE patients with normal and elevated BLyS levels.

	Normal BLyS levels n=42	Elevated BLyS levels* n=18	p-value [†]	FDR q-value ^{††}	Odds Ratio (95% CI)
Autoantibodies					
anti-dsDNA	29%	44%	0.249		2.0 (0.6–6.3)
anti-Ro	45%	33%	0.569		0.6 (0.2–1.9)
anti-La	21%	17%	1.000		0.7 (0.2–3.1)
anti-nRNP	21%	72%	0.0003	0.013	9.5 (2.7–33.9)
anti-Sm	14%	28%	0.279		2.3 (0.6–8.9)
anti-ribo P	14%	11%	1.000		0.8 (0.1–4.1)
anti-cardiolipin	12%	22%	0.431		2.1 (0.5–9.0)
Serologic studies					
25(OH)D (ng/mL)	20.6	16.3	0.018	0.144	
ESR (mm/hr)	21.1	35.7	0.034	0.159	
Hematocrit (%)	37.2	37.3	0.531		
Hemoglobin (g/dL)	13.2	17.9	0.616		
Leukocyte count (10 ⁶ cells/mL)	6.6	7.0	0.933		
Lymphocyte count (10 ⁶ cells/mL)	2.87	2.28	0.058		
Platelet count (10 ⁶ cells/mL)	263	283	0.219		
Creatinine (mg/dL)	0.9	0.9	0.390		

* Elevated BLyS levels were defined as being greater than the 95th percentile of healthy matched controls.

[†] Categorical variables were analyzed by Fisher's exact test. Continuous variables were analyzed by an unpaired t-test. Welch's correction was used in instances of unequal variance. Ln-transformation was done to correct for non-normality; Mann-Whitney test was used when Ln-transformation failed to correct the normality. 25(OH)D, 25-hydroxyvitamin D.

^{††} False discovery rate (FDR) q-value