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Aakash Patel April 8, 2015

The Expanded IgD^{-} $CD27^{-}$ $CD11c^{+}$ B Cell Subpopulation of Systemic Lupus Erythematosus Patients Is Not Anergic, as Measured by Proximal B Cell Receptor–Associated Signaling

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Abstract

The Expanded IgD^- $CD27^-$ $CD11c^+$ B Cell Subpopulation of Systemic Lupus Erythematosus Patients Is Not Anergic, as Measured by Proximal B Cell Receptor–Associated Signaling

By Aakash Patel

Systemic Lupus Erythematosus (SLE) is a humoral and cell-mediated autoimmune disorder that leads to chronic inflammation of affected tissues such as the joints, skin, kidneys, lungs, heart, brain, and blood vessels. Over-activity of autoreactive B cells is believed to be central to the pathogenesis of SLE. Studies have elucidated an overrepresented subpopulation of potentially autoreactive IgD^- $CD27^-$ (double negative, DN) B cells in SLE patients. Further, unpublished studies have revealed that these B cells present a unique gene expression phenotype that would promote anergy; for example, they express large amounts of CD11c. Since previous studies have reported the presence of altered intracellular signaling in specific expanded B cell subpopulations from patients with autoimmune diseases such as SLE and RA, this study aims to examine the phosphorylation of B cell linker protein (BLNK), an upstream, activating proximal B cell receptor (BCR)-associated signaling protein specific to B cells, in the expanded DN $CD11c^+$ B cell subpopulation from SLE patients. Peripheral Blood Mononuclear Cells were isolated from 17 SLE patients and one healthy control donor and either stimulated with anti-human IgG or IgM or unstimulated by the addition of just PBS. Cells were paraformaldehyde fixed, methanol permeabilized, and stained with a panel of antibodies to measure phosphorylation of BLNK (Y84) in the various B cell subpopulations using phosphospecific flow cytometry. Our findings show that total DN IgA^- B cells induced phosphorylation of BLNK (Y84), although this was statistically lower than in IgD^-, $CD27^+$, IgA^- B cells. Further, there was no statistically significant difference in proximal BCR-associated signaling between the DN $CD11c^+$ and DN $CD11c^-$ B cell subpopulations. Taken together, my data suggest these potentially autoreactive, expanded DN B cells in SLE patients failed to become anergic despite their expression of proteins that would promote anergy; they still induced proximal BCR-associated signaling following stimulation. This failure of potentially autoreactive B cells to become anergic could be due to ineffective anergy-promoting mechanisms and is a characteristic of many autoimmune disorders. Consequently, B cell signaling pathways serve as a major target for future treatments.

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Introduction

Systemic lupus erythematosus (SLE) is a humoral and cell-mediated autoimmune disorder characterized by chronic inflammation caused by autoantibodies, such as anti-double stranded DNA antibodies, that lead to inappropriate immune complex deposition in the body's own tissues (1); affected tissues often include the joints, skin, kidneys, lungs, heart, brain, and blood vessels (2). As a result, the major symptoms of SLE are joint pain, malar rash, nephritis, pleuritis, pericarditis, photosensitivity, fatigue, and atherosclerosis. This multi-organ autoimmune disorder is more prevalent in females and African Americans (2). With effective immunosuppressant treatments such as prednisone and cyclophosphamide, patients can delay kidney failure, one of the most serious complications of SLE, and experience less severe symptoms. However, there is currently no cure for SLE, and current treatments that rely on immunosuppression are unsatisfactory due to their negative side effects.

The dysregulation, specifically over-activity, of B cells expressing autoantibodies is believed to be central to the pathogenesis of SLE (3). B cell specificity, activation, and differentiation are mediated through B cell receptors (BCRs) (4, 5). These are located on the surface of B cells and contain a membrane immunoglobulin (Ig) domain, which binds antigen, and an Ig-α and Ig-β heterodimer that facilitates signal transduction through its immunoreceptor tyrosine-based activation motifs (ITAM) (4, 5). Cross-linking of the BCR with its cognate antigen induces a signal transduction cascade that activates the B cell. This begins with the phosphorylation of ITAMs and is followed by successive phosphorylation of activating signaling proteins via tyrosine

kinases and generation of intracellular secondary messengers like calcium. Then, downstream transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFKB), become activated and up-regulate the expression of genes associated with differentiation and proliferation. B cells also contain inhibitory receptors with immunoreceptor tyrosine-based inhibitory (ITIM) motifs in the cytoplasmic domain; upon ligation of these receptors, ITIM motifs on these inhibitory receptors become phosphorylated, leading to activation of phosphatases which dephosphorylate activating proteins in the BCR signaling cascade (4, 5). When these inhibitory receptors are engaged, there is decreased activation of the B cell following cross-linking of the BCR with its cognate antigen.

Primary activation of B cells leads to class-switching, affinity maturation of the immunoglobulin variable domain, and clonal expansion, allowing for a more specific, rapid response in the future when its cognate antigen is introduced into the body (5). Following secondary activation, B cells undergo further affinity maturation and clonal expansion before differentiating into antibody-secreting plasma cells. This process of B cell activation is a highly regulated process with the B cell requiring a variety of signals in order for it to become activated (6). When B cells fail to receive all the appropriate signals, they continue to circulate in the periphery but fail to respond to BCR-associated stimuli because of inhibited signal transduction, a state called anergy. One of the primary reasons B cell become anergic is because they have autoreactive BCRs. Thus, anergy serves as a way to minimize autoimmune-related damage and inflammation (6). In non-autoimmune individuals and mouse models of anergy, anergic B cells have a shorter lifespan and undergo apoptosis, or programmed cell death, soon after becoming anergic (6).

During class-switching, B cells switch from IgD^+ and IgM^+ to either IgG^+ or IgA^+ (B cells rarely express surface IgE in healthy controls (5)). Cells that have encountered their cognate antigen also begin to express CD27, a classical marker of memory (7). Thus, human B cells can be categorized into four distinct subpopulations depending on their expression of IgD and CD27: naïve (IgD^+, $CD27^-$), unswitched memory, USM (IgD^+, $CD27^+$), switched memory, SM (IgD^-, $CD27^+$), and double negative, DN (IgD^-, $CD27^-$). However, these subpopulations are dysregulated in SLE patients compared to healthy controls (7). In SLE, the USM B cell population is greatly diminished, and the DN B cell population is markedly expanded compared to healthy controls. Approximately 50% of SLE patients have an increased proportion of DN cells, and in some SLE patients, DN B cells can comprise up to 80% of the total B cell population (7). These DN B cells present a unique phenotype because although they have already engaged their respective antigen, as evidenced by somatically hypermutated regions in the immunoglobulin variable domain (8) and class-switching, they do not express the memory B cell marker CD27.

Aberrant signal transduction in B cells is implicated in SLE (3). Several studies have identified specific signaling components that are altered in B cells from patients with SLE and other B cell-mediated autoimmune disorders. One study elucidated increased phosphorylation of spleen tyrosine kinase (SYK), an upstream, activating protein that is part of the BCR signaling pathway, in B cells from patients with rheumatoid arthritis (RA) compared to healthy controls (9). More recently, a study involving SLE patients discovered an increased population of $CD27^-$ B cells, most of which displayed greater expression of SYK. Furthermore, $CD27^-$ B cells with enhanced

expression of SYK increased induced phosphorylation of SYK following stimulation of the BCR compared to $CD27^-$ cells with lower expression of SYK (8). However, in contrast to the above studies which implicate an activated state in subpopulations of B cells, a separate study in RA patients identified an expanded population of unresponsive $CD21^-$ naïve B cells that failed to activate and generate calcium flux following cross-linking of the BCR with its cognate antigen (10). Interestingly, the lack of CD21 expression is a characteristic of DN B cells in SLE that will be discussed in further detail below. Taken together, these studies suggest the presence of altered intracellular signaling in specific expanded B cell subpopulations from patients with autoimmune diseases such as SLE and RA.

Recent unpublished studies from my lab conducted flow cytometry and mRNA sequencing experiments to identify an expanded population of DN B cells in SLE patients with a unique phenotype that is not observed in any other B cell subpopulations in either SLE patients or healthy controls. These specific cells are predominantly IgG^+, but they express reduced levels of IgG compared to $CD27^+$ IgG^+ B cells. This has significant implications for receptor binding and signal transduction; the lack of immunoglobulin receptors hinders its extracellular cognate antigen from generating a response in the B cell. Thus, a B cell with this phenotype might not be as reactive to the presence of its cognate antigen. This expanded population of IgG^{low} DN B cells in SLE patients has several other unique features. It expresses increased levels of CD11c, a protein generally found on cells involved in the innate immune response, such as dendritic cells (11). On B cells, CD11c serves as an adhesion molecule by enabling the B cell to bind to components of the extracellular matrix such as fibrinogen, thereby mediating endothelial cell

adhesion (11). CD11c also functions to promote activation and proliferation upon ligation by endothelial ligands and/or inactivated complement component 3b (ic3b) (11). These DN $CD11c^+$ cells also exhibit increased expression of CD32b, CD22, and Fc receptor-like protein 5 (FCRL5). CD32b and CD22 are both inhibitory co-receptors that contain an ITIM in their cytoplasmic domain, dampening the BCR-associated signaling pathway; CD32b is ligated by the Fc domain of soluble IgGs and CD22 is ligated by *cis* or *trans* ligands (12-14). FCRL5 recruits protein tyrosine phosphatases that dephosphorylate ITAM-based tyrosines in the BCR complex, thereby attenuating the signal induced by cross-linking of the BCR with its cognate antigen (15).

Other unique features of this population are the lack of CXCR5, CD38, CD21, and CD24. CXCR5 is chemokine receptor that binds CXCR13 and is responsible for homing B cells to light zones in the germinal centers of secondary lymphoid tissues such as the spleen and Peyer's patches (16). CD38 is a cell surface receptor that facilitates BCR-dependent activation and proliferation of B cells (17). Furthermore, CD38 is expressed on plasma cells and B cells in the germinal center (18). CD21 induces BCR-associated signaling after binding to complement-coated antigens (19). CD24 signals through binding of a lectin-like ligand to activate the mitogen activated protein kinase (MAPK) pathway which modulates functions such as cell proliferation and apoptosis (20, 21). Naïve B cells exiting the bone marrow typically display the highest levels of CD24, while most B cells in the germinal centers of secondary lymphoid organs do not express any CD24 (22). Lastly, patients with this expanded subpopulation of B cells have increased serum interferon activity. The overrepresentation of these DN B cells with a unique phenotype in SLE

patients makes them an interesting target of research, especially as many of the above features of these cells influence signal transduction, a process implicated in the pathogenesis of SLE.

This study examines the phosphorylation of B cell linker protein (BLNK), an upstream, activating proximal BCR-associated signaling protein specific to B cells, in the IgD^-, $CD27^-$, $CD11c^+$ subpopulation of B cells from patients with SLE. CD11c was used as a marker to specifically investigate proximal BCR-associated signaling in the DN B cell subpopulation with the unique phenotype that is expanded in SLE patients. Because most of these unique features of this expanded population of DN B cells contribute to an anergic phenotype, I hypothesized that these cells would fail to induce activation following stimulation.

Materials and Methods

Acquisition of Patient Blood Samples

Approval from the Emory University Institutional Review Board was obtained prior to the start of this project. Furthermore, informed consent was obtained from all patients involved in this study. Blood samples from 17 SLE patients (15 females, 2 males; mean age of 43.0 years, range 20-71 years; 16 African American, 1 Caucasian; 16 non-Hispanic ethnicity, 1 unknown) were obtained by the clinical coordinators at patient clinics at Grady Memorial Hospital, Emory University Hospital Midtown, or the Emory Clinic. All classified SLE patients were diagnosed by a rheumatologist and met three or more of the criteria for SLE diagnosis outlined by the

American College of Rheumatology (23). The majority of SLE patients in this study were concurrently utilizing medications such as prednisone, hydrochloroquine, other immunosuppressants, and over-the-counter medications to manage their symptoms. SLE patients treated with B cell depletion therapy were excluded. Healthy controls did not have any autoimmune disease, diabetes, kidney disease, cancer, or history of cancer and were not taking any medications that can affect the immune system such as antibiotics or steroids.

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Stimulation of Lymphocytes

The following protocol for phosphospecific flow cytometry reflects that used by John Irish and colleagues (24). PBMCs were isolated from the patient blood samples using Ficoll-Paque Plus (GE Healthcare Life Sciences) density gradient separation. One to five million PBMCs were centrifuged and resuspended in 0.5% bovine serum albumin (BSA – Miltenyi Biotec) in RPMI 1640 medium without L-glutamine (Corning) and warmed to 37°C for five minutes. Cells were either stimulated by the addition of goat $F(ab')_2$ anti-human IgG or goat $F(ab')_2$ anti-human IgM (Southern Biotech) or unstimulated by the addition of just phosphate buffered saline (PBS – Corning), achieving a final concentration of 10 μg/mL. Immediately following addition of stimulants, cells were stimulated at 37°C for five minutes. The cells were fixed with paraformaldehyde (Electron Microscopy Services) to a final concentration of 1.4% for five minutes at room temperature. The PBMCs were permeabilized and resuspended in 100% methanol and stored in -20°C until being stained for flow cytometry.

Staining of Lymphocytes for Phosphospecific Flow Cytometry

Stimulated cells were washed with fluorescence-activated cell sorting (FACS) buffer (5% BSA in PBS) and subsequently stained with the following conjugated antibodies: mouse anti-human CD27 – brilliant violet 711 (BV 711, clone L128, BD Biosciences), mouse anti-human CD3 – peridinin-chlorophyll proteincyanin5.5 (PerCp cy5.5, clone SP34-2, BD Biosciences), mouse anti-human CD11c – phycoerythrin (PE, clone Bu15, BioLegend), goat anti-human IgD – biotin (BIO, Southern Biosciences), streptavidin – pacific orange (PacO, Life Technologies), mouse anti-human phosphorylated BLNK (Y84)– alexa 647 (A647, clone J117-1278, BD Biosciences), mouse anti-human CD20 – brilliant violet 421 (BV421, clone H1, BD Biosciences), and goat anti-human IgA – fluorescein isothiocyanate (FITC, Life Technologies). Manufacturer's suggestions were taken into consideration when choosing antibody clones. Anti-mouse IgG compensation beads (Bangs Laboratories, Inc.) were used for compensation controls for each fluorochrome.

Phosphospecific Flow Cytometry and Analysis

A BD Biosciences three-laser LSR2 instrument was used for biomarker detection of stained cells. FlowJo software from TreeStar was used to perform flow cytometric analysis. When cells stained with a fluorochrome pass through the appropriate laser, they will emit a fluorescence intensity that is proportional to the amount of the specific epitope that the fluorochrome-tagged antibody recognizes on the cell. Thus, mean fluorescence intensity of phosphorylated

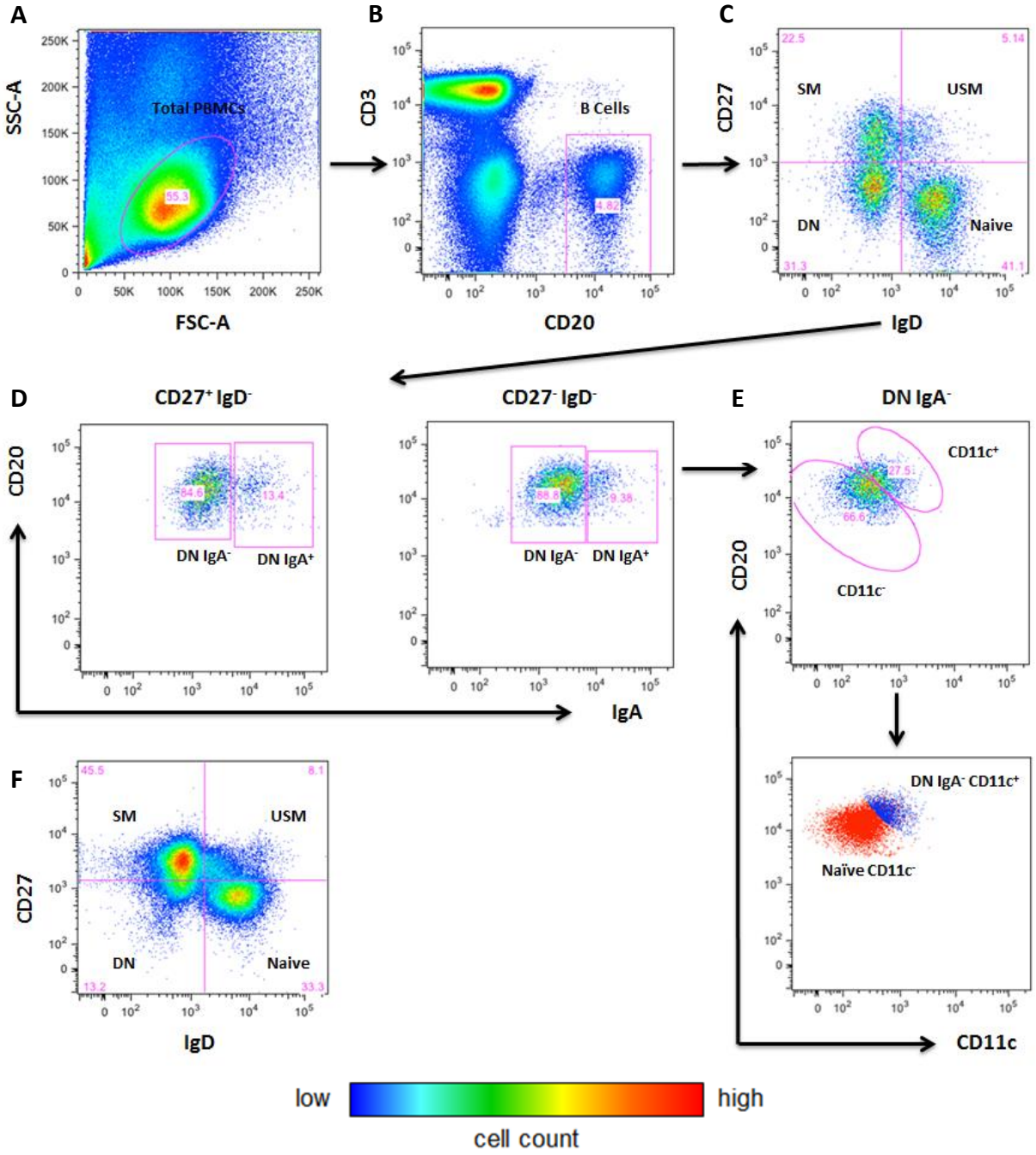
BLNK (Y84) was obtained and compared between B cell subpopulations using a two-tailed paired t-test with an alpha level of 0.05 to determine significance.

Results

Optimization of Phosphospecific Flow Cytometry Requires Use of Antibody Clones which Bind Cell Surface Epitopes that Can Withstand Fixation and Permeabilization

Since fixation and permeabilization of cells alter cell surface proteins (24), several different antibodies were tested for their ability to effectively separate B cell populations. Figure 1 (A-E) shows the final staining protocol and gating strategy through which flow cytometric analysis was used to separate B cells from SLE patients into the various subpopulations, including the expanded DN IgA^- $CD11c^+$ subpopulation. Levels of phosphorylated BLNK (Y84) were measured within the subpopulations of B cells exposed to the different stimulants. The quadrant gating method displayed in Figure 1C demonstrates the expanded DN B cell subpopulation and the diminished USM B cell subpopulation in SLE patients, compared to the healthy control donors in which the USM subpopulation is relatively expanded while the DN subpopulation is diminished (Figure 1F). A similar staining protocol and gating strategy was used to identify the B cell subpopulations in the healthy control donor. However, B cells from the healthy control were not separated on the basis of CD11c expression since this protein is expressed only on a small subset of B cells in healthy control donors (11).

Figure 1



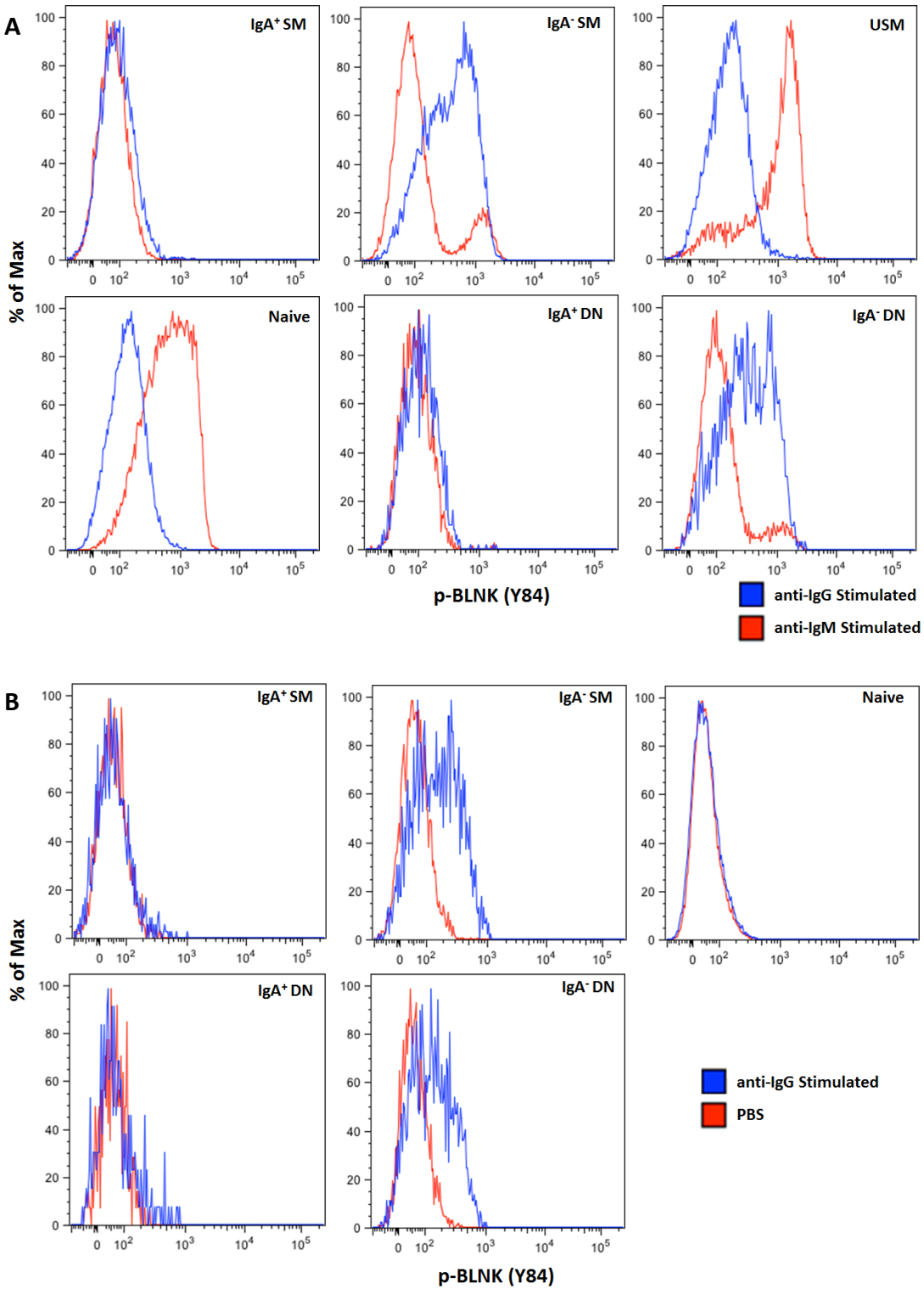
Representative dot plots for gating strategy to identify B cell subpopulations. Phosphospecific flow cytometry was used to identify the DN IgA$^-$, DN IgA$^+$, DN IgA$^-$ CD11c$^+$, DN IgA$^-$ CD11c$^-$, SM IgA$^+$, SM IgA$^-$, and naïve B cell subpopulations in SLE patients by staining with the following conjugated antibodies: mouse anti-human CD27, mouse anti-human CD3, mouse anti-human CD11c, goat anti-human IgD– biotin, streptavidin, mouse anti-human CD20, and goat anti-human IgA. Purple polygons and circles indicate gated cell subsets. **(A)** Side scatter-area (SSC-A), a measure of cellular complexity and forward scatter-area (FSC-A), a measure of cell size, were used to identify PBMCs. **(B)** Next, the CD3$^-$ and CD20$^+$ B cell population was selected. **(C)**

Then, CD27 and IgD were used to characterize B cells into the four major subpopulations: double negative (DN, IgD^- $CD27^-$), switched memory (SM, IgD^- $CD27^+$), naïve (IgD^+ $CD27^-$), and unswitched memory (IgD^+ $CD27^+$). **(D)** Class-switched SM and DN B cells were separated on the basis of IgA expression. **(E)** Lastly, CD11c was used to separate the DN IgA^- B cells. Due to non-optimal CD11c separation, the naïve $CD11c^-$ B cell subpopulation was used as an aid to identify the $CD11c^+$ cells in the DN IgA^- B cell subpopulation. **(F)** A similar gating strategy was used for the healthy control donor. However, B cells were not separated on the basis of CD11c expression. The gating quadrant for CD27 and IgD is presented. Values on the x and y-axis correspond to fluorescence intensity on a linear scale (A) or fluorescence intensity on a logarithmic scale (B-F).

Anti-IgG induces phosphorylation of BLNK (Y84) only in the DN IgA^- and SM IgA^- B cell subpopulations and anti-IgM induces phosphorylation of BLNK (Y84) only in the Naïve and USM B cell subpopulations

SM and DN B cells that are IgA^- are IgG^+ because B cells express negligible surface IgE. Thus, SM and DN IgA^- B cells incubated with goat $F(ab')_2$ anti-human IgG represent the stimulated, or experimental condition, while those incubated with either goat $F(ab')_2$ anti-human IgM or PBS represent the unstimulated, or control condition. Likewise, naïve and USM B cells, which are IgM^+, incubated with goat $F(ab')_2$ anti-human IgM represent the experimental condition, while those incubated with either goat $F(ab')_2$ anti-human IgG or PBS represent the control condition. Thus, SM IgA^-, total DN IgA^-, and naïve B cells from the healthy control donor induced phosphorylation of BLNK (Y84) following incubation with their respective stimulant (Figure 2A). This was also observed in B cells from SLE patients (Figure 2B). The USM subpopulation in SLE patients was not examined because it is greatly diminished in most SLE patients.

Figure 2

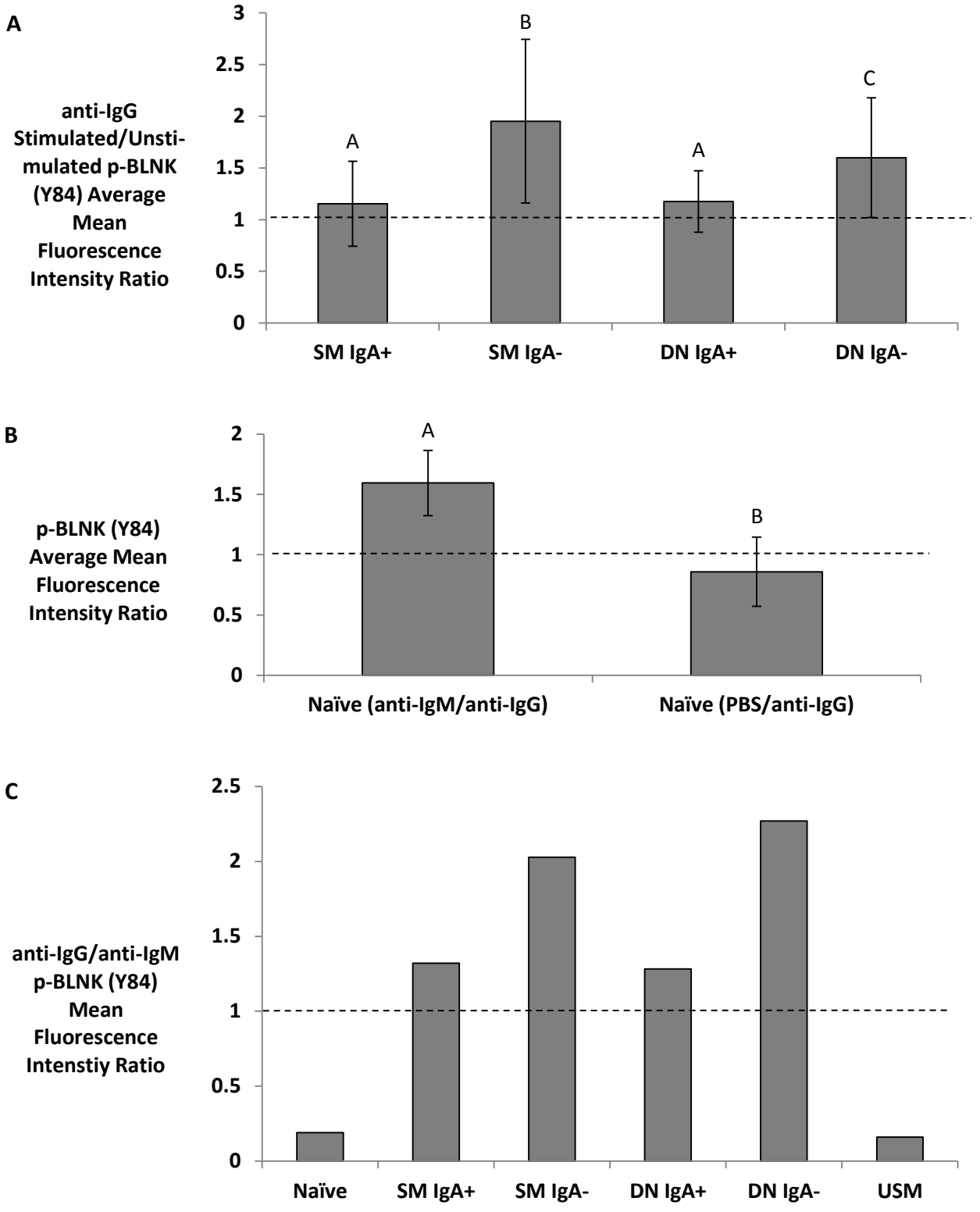

A
IgA+ SM
IgA- SM
USM
Naive
IgA+ DN
IgA- DN
% of Max
p-BLNK (Y84)
anti-IgG Stimulated
anti-IgM Stimulated
B
IgA+ SM
IgA- SM
Naive
IgA+ DN
IgA- DN
% of Max
p-BLNK (Y84)
anti-IgG Stimulated
PBS

Phosphorylation of BLNK (Y84) in B cell subpopulations from a healthy control donor and SLE patient following incubation with the appropriate stimulant. PBMCs were isolated from patient blood samples and either stimulated by the addition of goat F(ab')$_2$ anti-human IgG or goat F(ab')$_2$ anti-human IgM or unstimulated by the addition of just PBS. Cells were then fixed and permeabilized. The staining protocol and gating strategy described in Figure 1 was used to identify the B cell subpopulations, also described in Figure 1. Further, in order to measure proximal BCR-associated signaling, cells were also stained with mouse anti-human phosphorylated BLNK (Y84). **(A)** Histograms depicting mean fluorescence intensity (MFI) of phosphorylated BLNK (Y84) in the different B cell subpopulations (excluding the DN IgA$^-$ CD11c$^+$ and DN IgA$^-$ CD11c$^-$ populations) from a healthy control donor stimulated with goat F(ab')$_2$ anti-human IgG and goat F(ab')$_2$ anti-human IgM. **(B)** Histograms presenting MFI of phosphorylated BLNK (Y84) in the different B cell subpopulations (excluding the USM and DN IgA$^-$ CD11c$^+$ and DN IgA$^-$ CD11c$^-$ populations populations) from an SLE patient stimulated with goat F(ab')$_2$ anti-human IgG and unstimulated with PBS.

Total DN IgA$^-$ B cells from SLE patients induce phosphorylation of BLNK (Y84) following stimulation with anti-IgG

In the larger cohort consisting of 16 SLE patients, anti-IgG stimulation induced phosphorylation of BLNK (Y84) in the SM IgA$^-$ and total DN IgA$^-$ B cell subpopulations while anti-IgM stimulation induced phosphorylation of BLNK (Y84) in naïve B cells (Figure 3A, B). When specifically investigating the DN B cell subpopulation, the anti-IgG stimulated/unstimulated average mean fluorescence intensity (MFI) ratio for phosphorylated BLNK (Y84) was 1.18±0.30 for the DN IgA$^+$ subpopulation, compared to 1.60±0.58 for the DN IgA$^-$ subpopulation (p=0.0016). However, DN IgA$^-$ B cells did not induce phosphorylation of BLNK (Y84) as strongly as SM IgA$^-$ B cells; the anti-IgG stimulated/unstimulated average MFI ratio for phosphorylated BLNK (Y84) was 1.95±0.79 for the SM IgA$^-$ subpopulation, compared to 1.60±0.58 for the DN IgA$^-$ subpopulation (p=0.0144). Thus, total DN IgA$^-$ B cells from SLE patients induced phosphorylation of BLNK (Y84) above baseline but not as strongly as SM IgA$^-$ B cells. Data for the healthy control donor is also presented for reference (Figure 3C). The B cell subpopulations from this healthy control donor presented similar levels of proximal BCR-associated signaling compared to those from SLE patients, except that the DN IgA$^-$ subpopulation induced greater phosphorylation of BLNK (Y84) compared to the SM IgA$^-$ B cell subpopulation (anti-IgG stimulated/anti-IgM unstimulated MFI ratio = 2.27 and 2.03, respectively).

Figure 3


A
anti-IgG Stimulated/Unsti-mulated p-BLNK (Y84) Average Mean Fluorescence Intensity Ratio
3
2.5
2
1.5
1
0.5
0
A
B
A
C
SM IgA+
SM IgA-
DN IgA+
DN IgA-
B
p-BLNK (Y84) Average Mean Fluorescence Intensity Ratio
2
1.5
1
0.5
0
A
B
Naïve (anti-IgM/anti-IgG)
Naïve (PBS/anti-IgG)
C
anti-IgG/anti-IgM p-BLNK (Y84) Mean Fluorescence Intenstiy Ratio
2.5
2
1.5
1
0.5
0
Naïve
SM IgA+
SM IgA-
DN IgA+
DN IgA-
USM

Levels of phosphorylated BLNK (Y84) in B cell subpopulations from 16 SLE patients and a healthy control donor. PBMCs were isolated from patient blood samples and either stimulated by the addition of goat $F(ab')_2$ anti-human IgG or goat $F(ab')_2$ anti-human IgM or unstimulated by the addition of just PBS. Cells were then fixed and permeabilized. The staining protocol and gating strategy described in Figure 1 was used to identify the B cell subpopulations, also described in Figure 1. Further, in order to measure proximal BCR-associated signaling, cells were also stained with mouse anti-human phosphorylated BLNK (Y84). **(A)** The average ratio of anti-IgG/anti-IgM or PBS MFI of phosphorylated BLNK (Y84) for each B cell subpopulation (excluding the naïve, USM, DN IgA^- $CD11c^+$, and DN IgA^- $CD11c^-$ subpopulations) from 16 SLE patients. Statistically different means ($P < 0.05$), on the basis of a two-tailed paired t-test, have different letters. **(B)** The average ratio of anti-IgM/anti-IgG or PBS/anti-IgG MFI of phosphorylated BLNK (Y84) for the naive B cell subpopulation from 16 SLE patients. Statistically different means ($P < 0.05$), on the basis of a two-tailed paired t-test, have different letters. **(C)** The ratio of anti-IgG/anti-IgM MFI of phosphorylated BLNK (Y84) for the different B cell subpopulations (excluding the DN IgA^- $CD11c^+$ and DN IgA^- $CD11c^-$ subpopulations) of the healthy control donor stimulated with goat $F(ab')_2$ anti-human IgG and goat $F(ab')_2$ anti-human IgM.

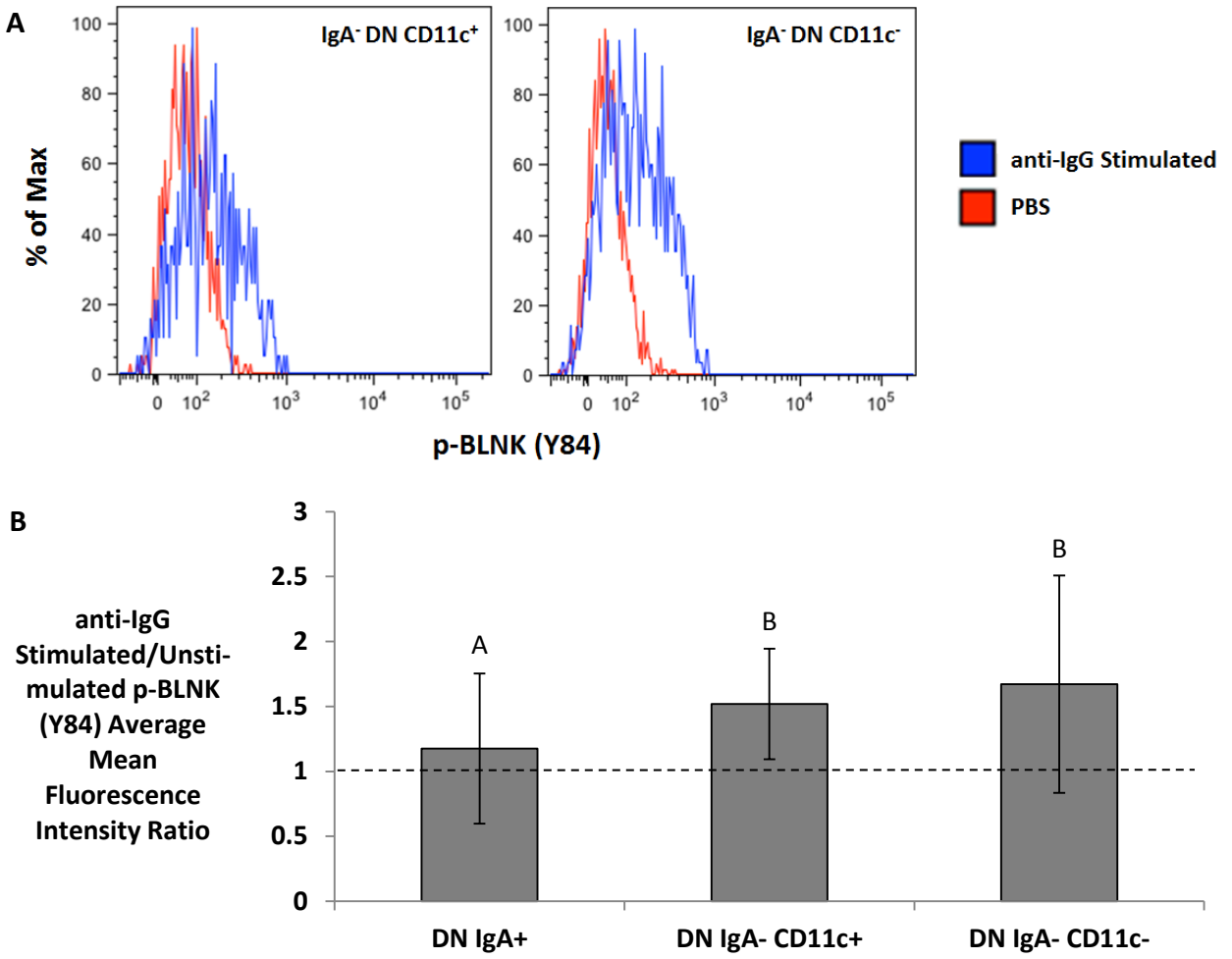
The expanded subpopulation of IgA$^-$, CD11c$^+$ DN B cells from SLE patients displays a similar level of induced phosphorylation of BLNK (Y84) compared to the IgA$^-$, CD11c$^-$ DN B cells

DN IgA$^-$ B cells were separated on the basis of CD11c expression to identify the expanded DN IgA$^-$ CD11c$^+$ subpopulation in SLE patients. Both CD11c$^+$ and CD11c$^-$ DN IgA$^-$ B cells were capable of inducing phosphorylation of BLNK (Y84) upon stimulation with IgG (Figure 4A). However, there was no statistically significant difference in the anti-IgG stimulated/unstimulated average MFI ratio for phosphorylated BLNK (Y84) between the DN IgA$^-$ CD11c$^+$ (1.52±0.43) and DN IgA$^-$ CD11c$^-$ (1.67±0.84) B cell subpopulations from SLE patients ($p=0.4435$, Figure 4B). Thus, proximal BCR-associated signaling operated at a similar level within both these subpopulations of DN IgA$^-$ B cells from SLE patients.

Further, since B cells from patients with an expanded DN subpopulation may differ in their responsiveness to the stimulant, we compared B cell subpopulations from SLE patients with a high and low proportion of total DN B cells in their level of induced phosphorylation of BLNK (Y84) following stimulation. When SLE patients were divided into high and low proportion of DN B cell groups (Figure 5A), there was no statistically significant difference in the anti-IgG stimulated/unstimulated average MFI ratio for phosphorylated BLNK (Y84) between the two cohorts in any of the B cell subpopulations, including the total DN IgA$^-$ (high DN: 1.62±0.49, low DN: 1.58±0.68), DN IgA$^-$ CD11c$^+$ (high DN: 1.50±0.36, low DN: 1.53±0.50), and DN IgA$^-$ CD11c$^-$ (high DN: 1.70±0.78, low DN: 1.65±0.91) subpopulations (Figure 5B). This suggests that DN B

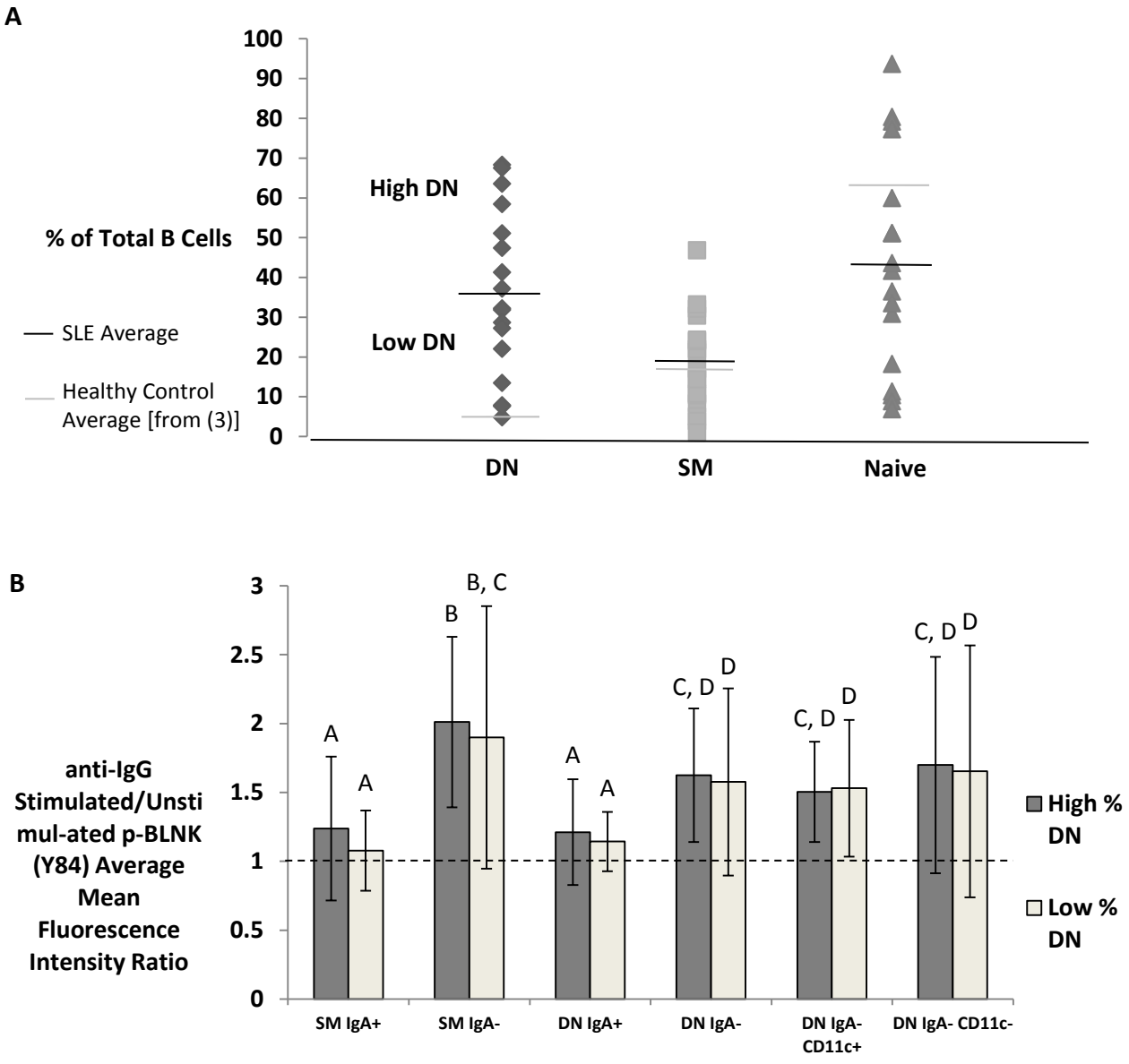
cells have similar proximal BCR-associated signaling phenotypes in patients with both high and low proportions of DN B cells.

Figure 4



Levels of phosphorylated BLNK (Y84) in the DN IgA$^-$ B cell subpopulations from 16 SLE patients. PBMCs were isolated from patient blood samples and either stimulated by the addition of goat F(ab')$_2$ anti-human IgG or goat F(ab')$_2$ anti-human IgM or unstimulated by the addition of just PBS. Cells were then fixed and permeabilized. The staining protocol and gating strategy described in Figure 1 was used to identify the B cell subpopulations, also described in Figure 1. Further, in order to measure proximal BCR-associated signaling, cells were also stained with mouse anti-human phosphorylated BLNK (Y84). **(A)** Histograms presenting MFI of phosphorylated BLNK (Y84) for the DN IgA$^-$, DN IgA$^-$ CD11c$^+$, and DN IgA$^-$ CD11c$^-$ B cell subpopulations from an SLE patient stimulated with goat F(ab')$_2$ anti-human IgG and unstimulated with PBS. **(B)** The average ratio of anti-IgG/anti-IgM or PBS MFI of phosphorylated BLNK (Y84) for the DN IgA$^+$, DN IgA$^-$ CD11c$^+$, and DN IgA$^-$ CD11c$^-$ B cell subpopulations from 16 SLE patients. Statistically different means ($P < 0.05$), on the basis of a two-tailed paired t-test, have different letters.

Figure 5



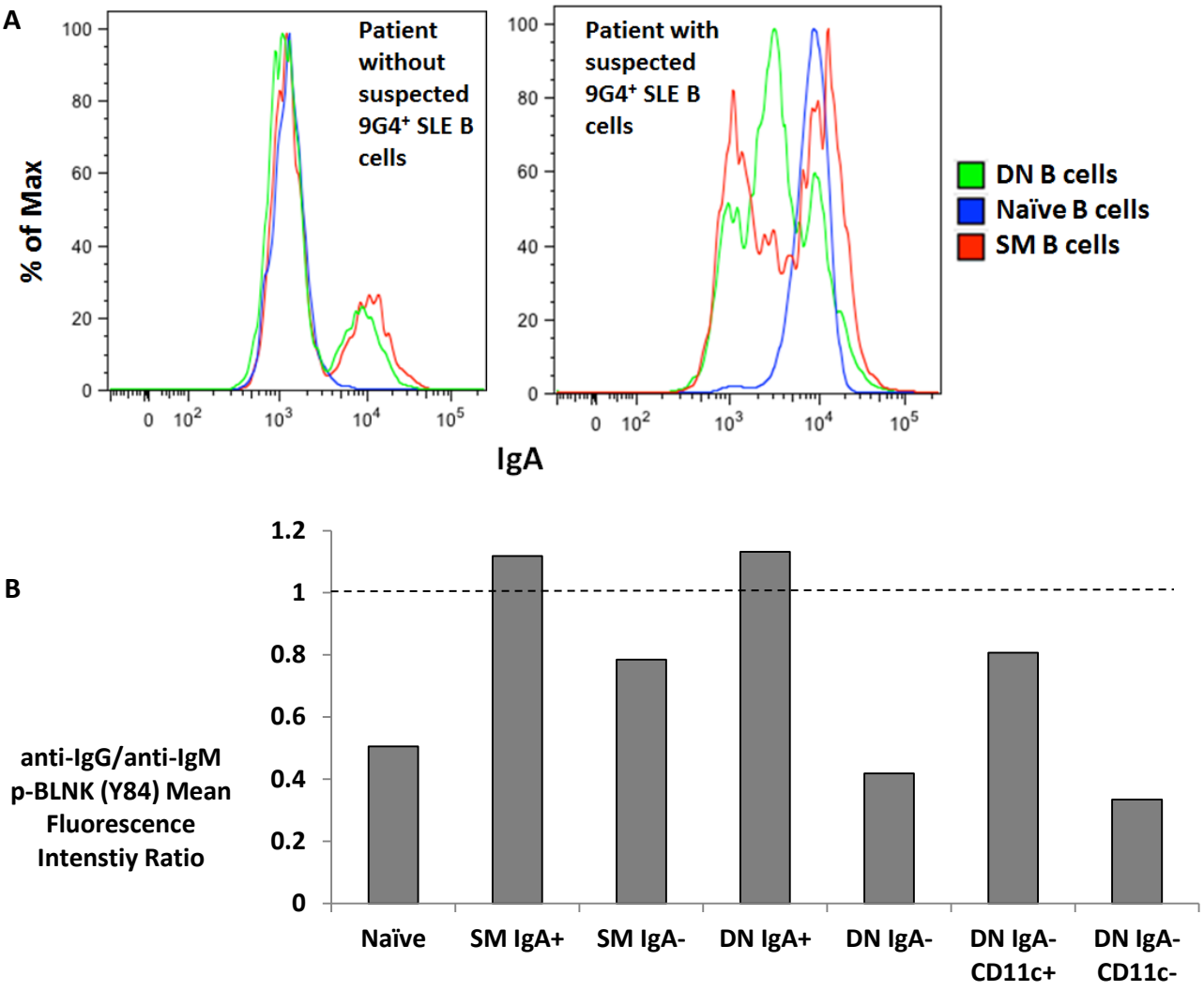
A comparison of the levels of phosphorylated BLNK (Y84) in B cell subpopulations from 16 SLE patients with a high and low proportion of IgD$^-$ CD27$^-$ DN B cells. PBMCs were isolated from patient blood samples and either stimulated by the addition of goat F(ab')$_2$ anti-human IgG or goat F(ab')$_2$ anti-human IgM or unstimulated by the addition of just PBS. Cells were then fixed and permeabilized. The staining protocol and gating strategy described in Figure 1 was used to identify the B cell subpopulations, (excluding the naïve and USM populations), also described in Figure 1. Further, in order to measure proximal BCR-associated signaling, cells were also stained with mouse anti-human phosphorylated BLNK (Y84). **(A)** Displays the average proportion of DN, SM, and naïve B cells out of total B cells in the 16 SLE patients compared to values from healthy

controls obtained from (3). The SLE average obtained from this study served as the cutoff for grouping patients into high and low proportion of DN B cells groups. **(B)** A comparison of the average ratio of anti-IgG/anti-IgM or PBS MFI for phosphorylated BLNK (Y84) in each B cell subpopulation from 16 SLE patients with a high and low proportion of DN B cells. Statistically different means ($P < 0.05$), on the basis of a two-tailed paired t-test, have different letters.

In an SLE patient with suspected surface 9G4$^+$ anti-B cell lymphocyte antibodies, only naive B cells induce phosphorylation following stimulation

One SLE patient was eliminated from the analyses described above due to suspected surface 9G4$^+$ anti-B cell lymphocyte antibodies. 9G4 is a rat anti-human idiotype antibody that recognizes VH4-34-encoded autoreactive antibodies; these could potentially confound proximal BCR-associated signaling studies since it has been suggested that they can dampen signal transduction by promoting phosphatase activity (25, 26). This SLE patient displayed poor IgA separation that suggests the presence of surface 9G4$^+$ anti-B cell lymphocyte antibodies (Figure 6A). Interestingly, with the exception of the naïve B cell subpopulation, the other B cell subpopulations showed reduced phosphorylation of BLNK (Y84) upon incubation with the anti-IgG stimulant, compared to those that were unstimulated (Figure 6B). The anti-IgG stimulated/anti-IgM unstimulated MFI ratio for phosphorylated BLNK (Y84) was 0.79 for the SM IgA$^-$ subpopulation (1.12 for the SM IgA$^+$ subpopulation), 0.42 for the DN IgA$^-$ subpopulation (1.13 for the DN IgA$^+$ subpopulation), 0.81 for the DN IgA$^-$ CD11c$^+$ subpopulation, and 0.33 for the DN IgA$^-$ CD11c$^-$ subpopulation. Thus, the SM and DN IgA$^+$ B cell subpopulations surprisingly displayed greater phosphorylation of BLNK (Y84) than the SM and DN IgA$^-$ B cell subpopulations following stimulation. Given that these results from this SLE patient are not consistent with those from the rest of the SLE cohort, it is possible that surface 9G4$^+$ anti-B cell lymphocyte antibodies can lead to altered proximal BCR-associated signaling in SLE patients.

Figure 6



Levels of phosphorylated BLNK in B cell subpopulations from an SLE patient with Suspected Surface 9G4$^+$ anti-B Cell Lymphocyte Antibodies. PBMCs were isolated from the patient's blood sample and stimulated by the addition of goat F(ab')$_2$ anti-human IgG and goat F(ab')$_2$ anti-human IgM. Cells were then fixed and permeabilized. The staining protocol and gating strategy described in Figure 1 was used to identify the B cell subpopulations (excluding the USM population), also described in Figure 1. Further, in order to measure proximal BCR-associated signaling, cells were also stained with mouse anti-human phosphorylated BLNK (Y84). **(A)** Displays the MFI of IgA for the DN, naïve, and SM B cell subpopulations from a patient with and without suspected surface 9G4$^+$ anti-B cell lymphocyte antibodies. **(B)** Presents the ratio of anti-IgG/anti-IgM MFI for phosphorylated BLNK (Y84) in each of the B cell subpopulations in this SLE patient with suspected surface 9G4$^+$ anti-B cell lymphocyte antibodies.

Discussion

Hyperactivity of autoreactive B cells is believed to be central to the pathogenesis of SLE (3). Recent unpublished studies from my lab have identified a phenotypically distinct subpopulation of expanded DN IgA^- $CD11c^+$ B cells with features suggestive of an anergic state in SLE patients. The current study involving SLE patients addressed the nature of proximal BCR-associated signaling in this subpopulation and compared it to that of other B cell subpopulations. Total DN IgA^- B cells induced phosphorylation of BLNK (Y84) but not as strongly as SM IgA^- B cells, suggesting that these cells are not anergic. Further, to my surprise, within the DN IgA^- B cell subpopulation, the DN IgA^- $CD11c^+$ B cells induced phosphorylation of BLNK (Y84) to a similar extent as the DN IgA^- $CD11c^-$ B cells upon stimulation. Thus, the expanded DN IgA^- $CD11c^+$ B cell subpopulation did not display any difference in proximal BCR-associated signaling compared to other DN IgA^- B cells despite having a distinct gene expression phenotype including increased expression of inhibitory receptors. Lastly, total DN IgA^-, DN IgA^- $CD11c^+$, and DN IgA^- $CD11c^-$ B cell subpopulations from SLE patients with a high proportion of DN B cells displayed similar proximal BCR-associated signaling as those from SLE patients with a low proportion of DN B cells. As a result, DN B cells from SLE patients have similar signaling properties that are unaffected by variations in the makeup of the B cell compartment across patients.

Taken together, these data fail to support my hypothesis that this expanded population of DN IgA^- $CD11c^+$ B cells would be anergic despite the presence of features on these cells that would promote anergy. A justification for my hypothesis was grounded in increasing evidence that

suggests DN B cells possess autoreactive immunoglobulins due to their clinical correlations with the presence of certain autoantibodies, such as anti-double stranded DNA, and B cells expressing the 9G4 idiotype (7, 27, 28). This could lead to anergy, described as the inability of the B cell to induce activation and signal transduction in response to cross-linking of the BCR with its cognate antigen. B cell activation and proliferation in the germinal center during immune responses require two signals, one from the antigen and a second one from the cognate helper T cell in the form of CD40L (29). However, it is possible that the cognate helper T cell which recognizes the same autoantigen as the B cell was eliminated in the thymus during the negative selection process of central tolerance. As a result, the autoreactive B cell would receive signal one from the antigen but fail to receive signal two from its cognate helper T cell. Since both signals are required to avoid anergy (29), these B cells would become anergic and excluded from the germinal centers of secondary lymphoid follicles where proliferation and differentiation take place. Further, chronic stimulation of the BCR by antigen is needed to maintain an anergic state (30, 31). My hypothesis was grounded in the belief that these DN autoreactive B cells are chronically stimulated by their cognate autoantigen; they consequently maintain an anergic phenotype by up-regulating inhibitory molecules and down-regulating stimulatory molecules (CD27 (28)). In addition, my hypothesis was consistent with the features of this expanded DN IgA^- $CD11c^+$ population outlined above that should promote anergy and follicular exclusion: IgG^{low}, $CD32b^+$, $CD22^+$, $FCRL5^+$, $CXCR5^-$, $CD38^-$, $CD21^-$, and $CD24^-$.

However, peripheral tolerance mechanisms such as anergy are often impaired in patients with autoimmune disease as well as in mouse models of autoimmunity (32), and my results could be

a reflection of this phenomenon. Several processes could contribute to this failure to induce B cell anergy. First, elevated levels of B cell activation factor belonging to the tumor necrosis factor family (BAFF) rescue autoreactive anergic B cells from elimination and facilitate a breakdown of tolerance by enhancing production of autoantibodies, thereby driving autoimmunity (33, 34). In fact, elevated BAFF levels leading to overproduction of anti-dsDNA antibodies have been observed in SLE patients (34). Second, in regards to the two signal theory for B cell activation and proliferation in the germinal center, studies have provided evidence that signals from $CD11c^+$ dendritic cells and toll-like receptor (TLR) ligands can serve as the second signal and allow for extra-follicular B cell activation, class switching, and somatic hypermutation in a CD40-independent manner (7, 31, 35-39). This is consistent with findings that CD27 expression begins during the germinal center reaction involving the activated B cell and its cognate helper T cell (7, 40) and supports the idea that these $CD27^-$ DN B cells could potentially engage in extra-follicular activation. Further, signaling through TLRs on these B cells may modulate proximal BCR-associated signaling by interfering with various inhibitory receptors, such as CD32b and CD22, in order to induce activation of the B cell. Lastly, the lack of anergy in this expanded population of B cells could be attributed to dysfunctional inhibitory receptors. For example, numerous polymorphisms have been identified in the gene encoding CD32b in SLE patients (41). Most of these polymorphisms affect expression of the gene, but there remains the possibility that some unidentified polymorphisms can affect the functioning of the CD32b protein.

Investigating proximal BCR-associated signaling is difficult due to all these processes that can modulate signaling. The overrepresentation of disease specific autoantibodies, such as

those encoded by the VH4-34 heavy chain gene (recognized by the rat anti-human idiotype antibody 9G4), further complicates this. For example, analysis of the single SLE patient with suspected surface $9G4^+$ anti-B cell lymphocyte antibodies yielded contradictory results compared to those found in the rest of the SLE cohort. VH4-34 autoreactive antibodies likely attenuate signaling by altering phosphatase activity (25, 26). Interestingly, previous studies have demonstrated that naïve B cells from these patients displayed substantially reduced anti-IgM induced phosphorylation (25, 26). However, this is not consistent with what is observed in my analysis of the one patient. Additional studies need to be conducted on SLE patients with suspected surface $9G4^+$ anti-B cell lymphocyte antibodies in order to confirm a unique signaling phenotype in B cell subpopulations from these patients. Future research in this field needs to also investigate the contributions of each of the various signaling pathways associated with signaling stimuli, such as the inhibitory receptors and TLRs, towards inducing or failing to induce B cell activation. In addition, time course and kinetic-based experiments, as developed by John Irish and colleagues (24, 42), would also be highly useful in understanding the signaling dynamics in this expanded population of DN IgA^- $CD11c^+$ B cells.

This is an important area of research because of the inherent therapeutic potential in appropriately attenuating activation of autoreactive B cells. Current medications targeting B cells in autoimmune disorders either eliminate B cells completely, as in the case of Rituximab, or restrict clonal expansion of B cells, as in the case of many anti-proliferative drugs and glucocorticoids, often with substantial side effects. More recent therapeutic treatments for SLE have begun to exploit key pathways in lymphocyte signaling (43), so a greater understanding of

B cell signaling properties in SLE would enable the production of more effective treatments with lesser off-target effects.

Conclusion

Potentially autoreactive IgD^-, $CD27^-$ B cells are expanded in SLE patients. Further unpublished studies from my lab have demonstrated that these cells present a unique gene expression phenotype that would promote anergy. Despite this, they still induce proximal BCR-associated signaling following stimulation. This failure of potentially autoreactive B cells to become anergic could be due to multiple different faulty anergy-promoting mechanisms and is a characteristic of many autoimmune disorders. Thus, B cell signaling pathways serve as a major target for future treatments.

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Abbreviations used in this article:

BAFF B cell activation factor belonging to the tumor necrosis factor family
BCR B cell receptor
BLNK B cell linker protein
BSA bovine serum albumin
DN double negative
FACS fluorescence-activated cell sorting
FCRL5 Fc receptor-like protein 5

FSC-A forward scatter - area
ic3b inactivated complement component 3b
ITAM immunoreceptor tyrosine-based activation motifs
ITIM immunoreceptor tyrosine-based inhibitory
MAPK mitogen activated protein kinase
MFI mean fluorescence intensity
NFKB nuclear factor kappa-light-chain-enhancer of activated B cells
p-BLNK (Y84) phosphorylated B cell linker protein at tyrosine 84
PBMC peripheral blood mononuclear cells
PBS phosphate buffered saline
RA rheumatoid arthritis
SLE systemic lupus erythematosus
SM switched memory
SSC-A side scatter-area
SYK spleen tyrosine kinase
TLR toll-like receptor
USM unswitched memory

Disclosures

I have no financial conflicts of interest.

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