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Interferon-α induces altered transitional B cell signaling and function in Systemic Lupus Erythematosus

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ABSTRACT

Previous studies suggest that the B cells of patients with Systemic Lupus Erythematosus (SLE) are hyper-responsive to BCR crosslinking; however, it has been unclear whether this is the result of altered B cell signaling or differences in various B cell subpopulations in SLE patients as compared to healthy controls. Here we have developed a novel Phosflow technique that permits examination of cell signaling in distinct B cell subpopulations stratified based upon developmental stage and cell surface IgM levels, which we use to show that the naïve B cells of SLE patients are hyper-responsive to IgM receptor crosslinking, resulting in increased SYK phosphorylation. We further demonstrate that this hyper-responsiveness is most marked in the transitional B cell subset and that it is associated with altered function, resulting in decreased apoptosis and increased proliferation of these cells. Examination of repeated samples from the same patients revealed that the hyper-responsiveness fluctuated over time, suggesting that it may be mediated by pro-inflammatory factors rather than genetic variations between patients. In support of this concept, incubation of healthy control B cells with IFN-α or SLE plasma induced the hyper-responsive phenotype, which was blocked by anti-IFN-α antibody. Furthermore, no obvious correlation was seen between genetic variants that are proposed to alter BCR signaling and the increased SYK phosphorylation. The findings suggest that pro-inflammatory factors, in particular Type I IFNs, modulate B cell function in SLE in a way that could contribute to the breach of tolerance in this condition.

1. Introduction

The presence of diverse auto-antibodies (Abs) in Systemic Lupus Erythematosus (SLE) suggests that the mechanisms that prevent activation of self-reactive B cells are defective. Studies in mice indicate that the strength of the B cell receptor (BCR) signal plays an important role in determining the fate of auto-reactive B cells, with genetic manipulations that enhance or impair BCR signaling, promoting lupus-like autoimmunity [1–8]. Recently, several lupus risk variants for genes that encode molecules downstream of the BCR have been described that could lead to altered B cell signaling in humans [9–14]. While the precise impact of some of these variants on B cell signaling remains to be determined, both increased (*CSK* risk variant) and decreased (*PTPN22* risk variant) B cell signaling has been reported [12,15]. However, these reports stand in contrast to older work suggesting that the B cells from SLE patients are hyper-responsive with increased anti-IgM- and -IgD-mediated $[Ca^{2+}]_i$ responses [16,17] and anti-IgM-induced protein tyrosine phosphorylation [17]. A potential resolution to this disparity lies in the observation that the B cells of SLE patients are subjected to high levels of pro-inflammatory cytokines, including Type I interferon (IFN) and BAFF, which could affect B cell signaling [18–21]. Thus, the impact of genetically determined differences in B cell signaling could be modulated in SLE patients as compared to those in healthy controls.

Adding a further level of complexity to examination of signaling in SLE patients are the alterations in B cell homeostasis that are

Abbreviations: Abs, antibodies; SLE, Systemic Lupus Erythematosus; BCR, B cell receptor; IFN, interferon; p-, phospho-.

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seen in this condition. SLE patients have altered proportions of plasma cells, pre-germinal center B cells, and memory B cells as compared to controls [22–29], which could affect the B cell activation threshold when the cell population is examined as a whole [29–31]. Additionally, SLE patients have a large proportion of B cells, even within the naïve B cell subset, that demonstrate changes consistent with prior activation *in-vivo* [23,28,29,32]. Thus, it is possible that the changes previously observed in SLE patients as compared to controls arise from these differences rather than from intrinsically altered B cell function.

In this study, we have used Phosflow to contrast BCR signaling in well-defined peripheral blood B cell subsets of SLE patients and healthy controls. We show that the naïve B cells of SLE patients are indeed hyper-responsive to IgM receptor crosslinking. This heightened responsiveness is most marked in the transitional B cell subset, does not correlate with lupus risk variants that are proposed to alter BCR signaling, and is at least in part IFN-α-induced. Furthermore, the serum levels of IFN-α seen in SLE patients appear to be sufficient to modulate transitional B cell function in a way that could contribute to the breach of tolerance observed in this condition.

2. Materials and methods

2.1. Subjects

SLE patients (n = 39) were recruited from the University of Toronto Lupus Clinic. All patients satisfied ≥4 of the revised 1997 American College of Rheumatology classification criteria for SLE [33], were between the ages of 18 and 44 years (mean 33.88 ± 8.16) and taking 20 mg or less of prednisone per day (mean 8.43 ± 5.75 mg). The mean duration from diagnosis of SLE was 13.40 ± 7.05 years (range 1.80–31.88). Thirty (76%) of the patients were taking anti-malarial drugs and 25 (64%) were on immunosuppressive medications, which included: azathioprine (n = 12), methotrexate (n = 5), and mycophenolate mofetil (n = 8). Disease activity was measured using the SLE Disease Activity Index (SLEDAI)-2K [34]. The mean SLEDAI-2K for the study patients was 3.97 ± 4.33, (range 0–20). Healthy controls (n = 27) were between the ages of 18 and 44 years (mean 29.94 ± 7.29) with no family history of autoimmune disease. The study was approved by the Research Ethics Board of the University Health Network and all subjects provided informed consent.

2.2. B cell Phosflow

PBMCs were isolated over a Ficoll (GE Healthcare) gradient, treated to remove residual RBCs, and washed twice at room temperature. The cells were resuspended in 5% FBS/RPMI (plus additives), rested at 37 °C for 1 h, and then stimulated for 2 min with media alone, or 2 and 10 min with media containing 20 μg/ml of $F(ab')_2$ goat anti-human IgM (Jackson ImmunoResearch). Following stimulation, the cells were fixed in 1% paraformaldehyde for 10 min at 37 °C and then frozen at −80 °C. After thawing, the cells were washed with PBS, and stained with various directly-conjugated Abs followed by Streptavidin-Pacific Blue™ (Invitrogen). Abs used for surface staining included: anti-IgD-FITC (IA6-2), -CD27-allophycocyanin (L128), -CD19-allophycocyanin-H7 (HIB19), and -IgM-Biotin (G20-127) from BD Biosciences; and anti-CD38-PE-Cy7 (HIT2) from eBioscience. The cells were then permeabilized by incubation in 70% methanol on ice for 30 min. Following washing in PBS, the cells were resuspended in Perm/Wash™ buffer (BD Biosciences) and stained with the following Ab: anti-phospho (p)-SYK-PE (pY348; 1120-722), -p-ERK1/2-PE (pT202/Y204; 20A), or -p-PLCγ2-PE (pY759; K86-689.37), all from BD Biosciences. Approximately 1 million lymphoid events were acquired per sample, using an LSRII instrument (BD Biosciences) and were analyzed using Flow Jo software (TreeStar).

2.3. Measurement of Ca^{2+} mobilization

B cells were enriched from PBMCs using RosetteSep (StemCell Technologies), serum-deprived for 1 h in Tyrode's buffer, and then labeled with 5 μM Indo-1 AM (Molecular Probes) and 0.03% pluronic F-127 (Molecular Probes) for 30 min at 37 °C. After washing, the cells were stained with anti-CD20 (2H7), -CD27, -CD3 (HIT3a), -IgG (G18-145), -CD24 (ML5), and -CD38 Abs (all mAb BD Biosciences except CD38 eBioscience) and rested at 37 °C for 10 min. Events were acquired for 1 min before addition of $(Fab')_2$ goat anti-human IgM (20 μg/ml).

2.4. B cell co-culture with plasma

Healthy control PBMCs (2×10^6 cells/ml) or purified B cells (0.4×10^6 cells/ml, isolated using a Human B cell Enrichment Kit, StemCell Technologies), were incubated for 1 h in 5% FBS/RPMI (plus additives) and various concentrations of IFN-α (PBL Interferon Source) or 50% plasma from healthy controls or SLE patients. For blocking studies, 1 μg/ml of neutralizing anti-human IFN-α (MMHA-2, PBL Interferon Source, sufficient to block 50 units IFN-α) or purified isotype-matched mouse IgG1 (MOPC-31C, BD Biosciences) was added. In some experiments, plasma was treated with RNase (20 μg/ml, Thermo Scientific) at 37 °C for one hour or depleted of IgG by incubation with Protein G Sepharose gel (GE Healthcare) for one hour at 4 °C.

2.5. B cell functional assays

Naïve B cells were enriched from PBMCs using immunomagnetic beads (Naïve B cell isolation kit, Miltenyi Biotec). For measurement of B cell apoptosis, 1×10^6 PBMCs (for healthy control cells co-cultured with IFN-α) or 1×10^5 enriched naïve B cells (for SLE patients) were cultured overnight in medium supplemented with avidin alone (20 μg/ml, Sigma) together with biotinylated anti-IgM $F(ab')_2$ (10 μg/ml, Jackson ImmunoResearch). For B cell proliferation experiments, cells were labeled with CFSE (Molecular Probes) and cultured for 3 days in medium alone or supplemented with anti-IgM $F(ab')_2$ (10 μg/ml). Following culture, cells were stained with anti-CD19, -IgD, -CD27, -CD24, and -CD38 Abs to enable gating of specific B cell subsets. The proportion of apoptotic cells was determined by Annexin V-FITC (BD Biosciences) staining and the proportion of proliferating cells was assessed by quantifying the $\%CFSE^{lo}$ cells above background without stimulation.

2.6. Measurement of SYK protein expression

B cells were isolated using RosetteSep, lysed in sample buffer, separated on a 10% SDS-PAGE gel, and transferred to a polyvinylidene difluoride membrane. Following blocking with 5% nonfat milk for 1 h, SYK was detected using a polyclonal rabbit anti-SYK Ab (1:2000, Cell Signaling) followed by HRP-conjugated anti-rabbit IgG Ab (1:1000, Cell Signaling). ACTIN levels were detected using a rabbit anti-ACTIN Ab (1:5000; AC-40, Sigma). Bound antibodies were detected using ECL Western Blotting Detection Reagent (Amersham), with the resultant bands being imaged with Kodak film and quantified using a Luminescent Image Analyzer (LAS-3000; FUJIFILM).