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THE BANK1 SLE-RISK VARIANTS ARE ASSOCIATED WITH ALTERATIONS IN PERIPHERAL B CELL SIGNALING AND DEVELOPMENT IN HUMANS

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Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the development of autoantibodies that drive disease pathogenesis. Genetic studies have associated nonsynonymous variants in the *BANK1* B cell scaffolding gene with susceptibility to SLE and autoantibodies in lupus. To determine how the *BANK1* SLE-risk variants contribute to the dysregulated B cell program in lupus, we performed genotype/phenotype studies in human B cells. Targeted phospho-proteomics were used to evaluate BCR/CD40 signaling in human B cell lines engineered to express the BANK1 risk or non-risk variant proteins. We found that phosphorylation of proximal BCR signaling molecules was reduced in B cells expressing the BANK1 risk protein compared to the non-risk protein. Similar to these findings, we observed decreased B cell signaling in primary B cells from genotyped healthy control subjects carrying the *BANK1* risk haplotype, including blunted BCR- and CD40-dependent AKT activation. Consistent with decreased AKT activation, we found that *BANK1* risk B cells expressed increased basal levels of FOXO1 protein and increased expression of FOXO1 target genes upon stimulation compared to non-risk B cells. Healthy subjects carrying the *BANK1* risk haplotype were also characterized by an expansion of memory B cells. Taken together, our results suggest that the SLE susceptibility variants in the *BANK1* gene may contribute to lupus by altering B cell signaling, increasing FOXO1 levels, and enhancing memory B cell development.

Graphical abstract

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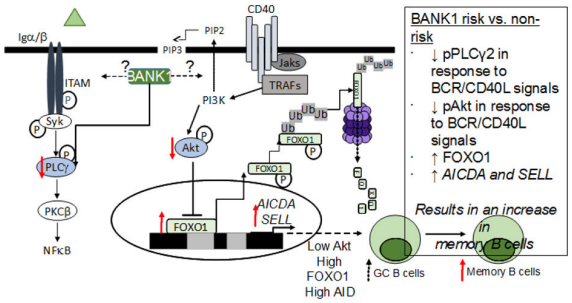

Figure 5. Model of the impact of BANK1 SNPs in B cell signaling and development. SLE pathogenesis is induced through environmental and genetic factors. Of these genetic factors, BANK1 has been identified as important in B cell signaling and development. We have demonstrated that in control subjects, risk compared to non-risk variants of BANK1 resulted in a decrease in B cell signaling through p-PLCγ and p-Akt. Further, we observe an enhancement in FOXO1 expression levels and in *AICDA* and *SELL* which are FOXO1 target genes. When we phenotyped these subjects we observed an increase in memory B cells which could be initiating SLE pathogenesis. Red arrows indicate findings described here.

1. INTRODUCTION

SLE is a complex autoimmune disorder with a strong genetic component. A cardinal feature of SLE is the development of autoantibodies specific for subcellular antigens. These self-reactive antibodies are essential for disease pathogenesis via tissue damaging immune complex deposition and parallel activation of innate immune cells [1].

Recent genome wide association studies have identified SLE susceptibility variants in numerous genes that function in B cells, implying that defects in B cell tolerance and the development of autoantibodies in SLE are due in part to genetic variants that confer disease risk [2-4]. Variants in the *BANK1* B cell scaffolding gene have been associated with SLE in European, Chinese, and African American populations [5-9] , and are also associated with susceptibility to rheumatoid arthritis and systemic sclerosis, suggesting *BANK1* may contribute to common mechanisms in autoimmunity [8, 10-13]. Three *BANK1* single nucleotide polymorphisms (SNPs) are associated with SLE susceptibility in Europeans including: a) two nonsynonymous substitutions in the inositol 1,4,5-triphosphaste receptor (IP3R) and ankyrin domains, rs10516487G>A in exon 2 encoding Arg61His and rs3733197G>A in exon 7 encoding Ala383Thr, respectively; and b) a noncoding SNP, rs17266594T>C, located in intron 1 of *BANK1* at a putative splice branch point for exon 2 (Figure S1) [5, 6].

The *BANK1* gene encodes a scaffolding protein that is expressed predominately in immature and mature B cells with functional BCRs [14]. Two isoforms are generated by alternative splicing, full-length and Δ2 that lacks exon 2 [5]. The BANK1 protein is comprised of three conserved domains: two ankyrin repeats, a coiled-coil domain, and a Dof/BANK1/BCAP or DBB motif which is conserved between the *Drosophila* Dof protein, the B cell-expressed adapter PIK3AP1 (BCAP) protein, and BANK1 (Figure S1) [15]. Additionally, BANK1

includes numerous tyrosine residues and several proline rich regions that may provide docking sites for SH2- and SH3-containing proteins.

The function of BANK1 has been studied primarily in model systems where BANK1 has been expressed ectopically or knocked out. These studies have pointed to a positive role in B cell signaling through interactions with the IP3R, the Src family kinases LYN and BLK, and phospholipase C, γ2 (PLCγ2) [14, 16, 17]. Upon BCR stimulation, BANK1 is phosphorylated and appears to promote the phosphorylation of the IP3R and PLCγ2 [14, 16]. Studies in mice using *Bank1* deficient B cells suggest that BANK1 inhibits AKT activation following CD40 stimulation and is required for TLR9 signaling via the p38-MNK1/2 pathway and TLR7 signaling [18, 19]. Further, *Bank1* also controls TLR7 induced type I IFN production in addition to regulating IgG production in the B6.*Sle1.yaa* mouse [20]. *In vivo*, *Bank1* deficiency results in increased germinal center (GC) formation and increased IgM primary immune responses to T-dependent antigens [18].

In contrast, the functional and biochemical impact of the *BANK1* SLE risk variants in human peripheral B cells is not completely understood. Previously, Kozyrev et al. observed different quantities of full-length and Δ2 isoforms in PBMC from healthy subjects in relation to their risk status for *BANK1* [5]. Specifically, they found increased quantities of the full-length transcript compared to the Δ2 transcript in *BANK1* risk subjects and similar quantities of the full-length and Δ2 transcripts in *BANK1* non-risk subjects, suggesting that the full-length BANK1 protein is associated with higher risk for autoimmune disease development [5]. In these studies there was no difference in total BANK1 levels in subjects with the risk genotype versus the non-risk genotype [5]. Further studies revealed that the rs10516487 R61 risk variant correlates with decreased splicing of exon 2 of *BANK1* due to loss of a splice enhancer site, leading to the reduction of *BANK1* Δ2 transcript levels relative to full-length transcripts [21]. Exon 2 encodes the region of BANK1 that is reported to interact with the IP3R, but the consequences of decreased levels of the Δ2 isoform in peripheral B cells are unclear. Additionally, the rs10516487 R61H risk variant is associated with altered subcellular distribution of BANK1 protein in non-B cells [21]. The impact of the rs3733197 A383T risk variant has not been studied; however, this amino acid residue is conserved evolutionarily, suggesting a functional role. Interestingly, the *BANK1* risk variants have been associated with the presence of anti-DNA and anti-RNP autoantibodies in SLE patients, consistent with a role for BANK1 in antibody responses *in vivo* [6, 22].

The purpose of this study was to examine the impact of the *BANK1* SLE-risk variants on BCR/CD40 signaling and the development and function of human peripheral B cells to understand the contribution of the risk alleles to dysregulated B cell function in SLE. We found that the *BANK1* risk haplotype was associated with decreased BCR and CD40-induced AKT activation, increased FOXO1 levels and an expansion of memory B cells. Our results suggest the SLE susceptibility variants in the *BANK1* gene may contribute to lupus by altering B cell signaling, increasing FOXO1 levels, and ultimately enhancing memory B cell development.

2. MATERIALS AND METHODS

2.1 Subjects

Local healthy control subjects were recruited through the Immune Mediated Disease Registry at the Benaroya Research Institute (BRI). Our control cohort subjects are in good health with no history of autoimmune disease, the majority also have no first degree relatives with a history of autoimmunity (screened controls). All subjects signed informed consent and the study was approved by the BRI Institutional Review Board. Peripheral blood samples were processed and PBMC banked by the BRI Clinical Core laboratory using standardized protocols to minimize sample variability. Genomic DNA from control subjects was genotyped for the *BANK1* SNPs rs17266594, rs10516487, and rs3733197, as well as *PTPN22* rs2476601, *BLK* rs13277113, and *CSK* rs34933034 using minor groove binding Eclipse genotyping probes (ELITech Group). All genotyping assays were validated using HapMap DNAs of known genotype. *BANK1* risk and non-risk three SNP haplotypes at rs17266594, rs10516487, and rs3733197 were defined based on Kozyrev et al. [5] where TGG=risk, CAA=protection, TGA=neutral, and CAG=protection. For the studies reported here, *BANK1* non-risk subjects were primarily CAA/CAA, and CAA/CAG, and *BANK1* risk subjects were TGG/TGG and TGG/TGA. Subjects were also held constant for the SLE risk alleles at *PTPN22, BLK* and *CSK*. PBMC from an additional seven *BANK1* non-risk (CAA/CAA) healthy control subjects were obtained from the Genotype and Phenotype Registry at the Feinstein Institute for Medical Research and the North Shore-Long Island Jewish Tissue Donation Program. Table S1 lists the subjects included in the experiments reported here. Experiments performed on a daily basis consisted of control subjects carrying the *BANK1* non-risk and risk haplotypes to eliminate batch effects.

2.2 Ramos B cell lines

BANK1 non-risk and risk cDNAs were expressed in human Ramos B cells using lentiviral transduction. A full-length *BANK1* cDNA encoding the main full-length isoform that utilizes exon 1a (Origene Technologies, Inc), was modified by PCR to encode the non-risk (nucleotide/amino acid A/His, A/Thr) or risk variants (G/Arg, G/Ala) at position 61 and 383, with an HA epitope tag at the N-terminus. BANK1 cDNAs were cloned into the pRRL-MND- MCS-T2A-eGFP lentiviral vector in frame with a T2A cleavage site and eGFP coding sequence to enable transduced cells to co-express BANK1 and GFP [23]. The sequence-confirmed BANK1 lentiviral plasmids, along with an empty plasmid control, were packaged and lentiviral supernatants were used to transduce Ramos B cells. After 72-96 h, GFP positive Ramos cells were sorted by flow cytometry and stable GFP expressing cell lines were established. Ramos lines were cultured in RPM1 supplemented with 10% FBS, 100U/ml penicillin, 100ug/ml streptomycin, and 1mM sodium pyruvate.

2.3 Phospho-peptide mass spectrometry

Ramos B cells stably expressing GFP, the BANK1-non-risk, or the BANK1-risk proteins (10^8 cells/line) were cultured with light amino acids (Sigma-Aldrich) and in parallel, untransduced Ramos cells were cultured with labeled heavy amino acids (U-13C6 L- Lysine: 2HCL and U-13C6; U-15N4 L-Arginine:HCL, Cambridge Isotope Laboratories) for 2 weeks at 37°C. Following labeling, all four cell populations were harvested, serum starved for 1 h

at 37°C, and then were stimulated with CD40L 2.5μg/ml (Peprotech) plus F(ab′)2 fragment goat anti-human IgM 10μg/ml (Jackson ImmunoResearch) and F(ab′)2 fragment goat anti-human IgD 10μg/ml (Southern Biotech) for 5 min at 37°C. Cells were lysed in 3mL of 8M urea supplemented with 1mM sodium orthovanadate and cell lysates from each experimental light sample were combined at a 1:1 protein ratio with a fraction of the stimulated heavy sample. Each lysate mix was then independently reduced, alkylated, trypsinized and desalted [24]. Phosphopeptide enrichment was performed as detailed previously [25].

Peptides were loaded onto a 3cm self-packed C18 capillary pre-column (Reprosil 5μM, Dr. Maisch). After a 10 min rinse (0.1% Formic Acid), the pre-column was connected to a 25cm self-packed C18 (Reliasil 3μM, Orochem) analytical capillary column (inner diameter, 50μm; outer diameter, 360μm) with an integrated electrospray tip (~1μm orifice). Online peptide separation followed by mass spectrometric analyses was performed on a 2D-nanoLC system (nanoAcquity UPLC system, Waters Corp.). Peptides were eluted using a 90-min gradient with solvent A (H_2O/Formic Acid, 99.9:1 (v/v)) and B (Acetonitrile/Formic Acid, 99.9:1 (v/v)): 10 min from 0% to 10% B, 55 min from 10% to 40% B, 5 min from 40% to 80% B, and 20 minutes with 100% A. Eluted peptides were directly electrosprayed into a Orbitrap QExactive mass spectrometer (Thermo Fisher Scientific) equipped with a high energy collision cell (HCD).

Each sample was run three times and acquired in the mass spectrometer using a data-independent mode of acquisition (DIA) over independent scan ranges (400-600 m/z, 600-800 m/z, and 800-1000 m/z). For each analysis, we iteratively repeated one full scan acquired in the Orbitrap analyzer (resolution = 70,000) followed by DIA (resolution = 17,500; normalized collision energy 25.0) using overlapping 10 m/z windows to flank the entire 200 m/z window. The data files were imported into the Skyline software package [26] whereupon the retention time for each peptide was determined based upon co-elution of at least three product ions in the DIA analysis. For each peptide that we analyzed in Figure 1, the relative intensity of the co-eluting isotopes of the heavy and light precursor and product ions (at least three per peptide) were quantified using the identified retention times. Following calculation of the mean fold-change and a p-value using the student's t-test between replicate results for stimulated BANK1 non-risk over BANK1 risk, we applied the Benjamini-Hochberg method to correct for our testing of multiple hypotheses (false discovery rate (FDR) = 0.15). The raw mass spectrometry proteomics data will be deposited to the ProteomeXchange Consortium (www.proteomexchange.org; [27]) via the PRIDE partner repository.

2.4 Immunoblot analysis

Whole-cell protein extracts were prepared from Ramos B cell lines by cell lysis with buffer containing 50mM Tris (pH 7.4), 150mM sodium chloride, 1mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 0.25% sodium deoxycholate, and protease inhibitors. Cell lysates were cleared by centrifugation and were separated by SDS-PAGE under reducing conditions. Following electrophoretic transfer, nitrocellulose membranes were blocked, probed with primary Ab, followed by IRDye-labeled secondary Abs (LI-COR Biosciences). Antibody binding was detected and quantified using the Odyssey infrared imaging system

software (LI-COR Biosciences). Abs used for immunoblot analysis included anti-pPLCγ2 (Y1217, Cell Signaling Technology (CST)), anti-PLCγ2 (CST), anti-pS6 (S235/236, CST), anti-S6 (CST), anti-tubulin (Sigma), anti-pY100 (pan pY, CST), and anti-HA (Covance).

2.5 FACS analysis and sorting

B cell population studies were performed using previously frozen PBMC samples that were thawed and stained using the following Abs: PE Cy7-anti-CD19 (Biolegend, clone HIB19); V450-CD27 (BD Biosciences, clone M-T271) or V500-CD27 (BD Biosciences, clone M-T271); APC-CD10 (eBioscience, clone eBioCB-CALLA); FITC-IgM (BD Biosciences, clone G20-127); PE-CD24 (Biolegend, clone ML5); PerCP Cy5.5-CD38 (BD Biosciences, clone HIT2); Alexa Fluor700-IgD (BD Biosciences, clone 1A6-2); APC-CD62L (Biolegend, clone DREG-56) and V450-CD40 (BD Biosciences, clone 5C3). Flow cytometry was performed using FACS Canto or LSR II flow cytometers (BD Biosciences), and analysis was performed with FlowJo software (Tree Star). B cell developmental populations within the peripheral blood $CD19^+$ B cell gate were defined based on CD24 and CD38 surface expression: transitional ($CD19^+$ $CD38^{hi}$ $CD24^{hi}$ $CD10^+$), naïve ($CD19^+$ $CD38^{mid}$ $CD24^{lo/mid}$ $CD10^{lo/neg}$), memory ($CD19^+$ $CD38^{lo}$ $CD24^{+/hi}$) and plasmablasts ($CD19^+$ $CD38^{hi}$ $CD24^{lo}$). Memory B cells were further separated into switched (IgM^{neg} IgD^{neg}) and unswitched (IgM^+ IgD negative and positive). To assess FOXO1 levels, we performed transcription factor staining (clone C29H4, CST) according to the manufacturer's protocol (BD Biosciences). To measure BCL2 and BIM levels, we utilized the cytofix/cytoperm buffer kit (BD Biosciences) to perform intracellular staining for BCL2 (clone Bcl-2/100, BD Biosciences) and BIM (clone 2819, CST).

2.6 B cell activation and signaling

To assess BCR signaling in primary B cells, total B cells were enriched from previously frozen PBMC by negative selection using the Human B cell isolation kit II (Miltenyi Biotec), rested for 1h at 37°C in RPM1 supplemented with 1% human serum (Gemini Bio-Products), then stimulated with F(ab′)2 fragment goat anti-human IgM 10 μg/ml (Jackson ImmunoResearch) and F(ab′)2 goat anti-human IgD 10μg/ml (Southern Biotech) for 5 min. Immediately thereafter, cells were fixed with BD Fix Buffer I, permeabilized with BD Perm Buffer III (BD Biosciences), and stained with APC-anti-CD27 (clone L128), Alexa Fluor 488-anti-CD20 (clone H1), and PE-anti-PLC-γ2 (Y759) (clone K86-689.37, BD Biosciences), according to the manufacturer's instructions for intracellular phosphoprotein staining.

To quantify pAKT (Ser473) in B cell subsets, previously frozen PBMC were thawed and pre-stained with BV421-anti-CD24 (Biolegend, clone ML5) and BV510-anti-IgD (Biolegend, clone IA6-2) in RPMI 1% HS, rested for 1 hour in RPMI 1% HS, then co-stimulated with goat anti-human IgM $F(ab')_2$ 10 μg/ml (Jackson ImmunoResearch) and goat anti-human IgG $(F(ab')_2$ 10 μg/ml (Jackson ImmunoResearch), with or without anti-CD40L (2.5 μg/ml, Peprotech) for 20 min. Cells were fixed with BD Cytofix/Cytoperm and permeabilized with BD Perm Buffer III. Following a block step with Human TruStain FcX™ (Biolegend) in 1X BD PermWash buffer, cells were stained in 1X BD PermWash with the following antibody conjugates: Alexafluor488-CD20 (BD Biosciences, clone H1);

PerCP-Cy5.5-CD38 (clone HIT2); APC-IgM (clone G20-127); BV605-CD27 (Biolegend, clone L128); and PE-pAKT (Ser473) (BD Biosciences, clone M89-61). Flow cytometry was performed using a BD FACS Canto flow cytometer and analysis was performed with FlowJo software. A slightly different flow panel was used to quantify pAKT (Ser473) in naïve and memory B cell subsets from a replicate group of samples. Previously frozen PBMC were thawed, rested, and stimulated as described above. Immediately thereafter, cells were fixed with BD Fix Buffer I, permeabilized with BD Perm Buffer III and blocked with Human TruStain FcX™ in FACS buffer prior to staining with APC-anti-CD27 (clone L128), Alexa Fluor 488-anti-CD20 (clone H1), PerCP-Cy5.5-anti-CD38 (clone HIT2) and PE-anti-pAKT(Ser473) (clone M89-61) in FACS buffer, and samples were analyzed using a BD FACS Calibur flow cytometer.

Signaling was quantified as the fold change in mean fluorescence intensity (MFI) of the stimulated sample divided by the baseline media alone except for signaling through p-PLCγ2 in memory B cells where there was a bi-modal shift in p-PLCγ2 levels. In this case, the positive peak was gated and the mean identified from the positive population. The positive peak MFI was divided by the baseline media alone to obtain the fold change.

For FOXO1 target induction, total B cells were isolated by negative magnetic bead separation (Miltenyi) from thawed PBMC and plated at $0.5\text{-}1\times10^6$ cells/well in a 96 round bottom plate in RPM1 supplemented with 10% human serum, 100U/ml Penicillin, 100μg/ml streptomycin, 2mM L-glutamine, and 1mM sodium pyruvate. B cells were stimulated with CD40L 2.5ug/ml together with F(ab′)2 fragment goat anti-human IgM 10 μg/ml and F(ab′)2 goat anti-human IgG 10μg/ml for 72h.

2.7 Quantitative PCR (qPCR)

To assess expression of the FOXO1 target genes *AICDA* and *SELL*, RNA was isolated from purified B cells −/+ stimulation for 72h and cDNA was subjected to qPCR using gene specific Taqman assays (Applied Biosystems) and a *B2M* Taqman assay that was used as a housekeeping control. *AICDA* and *SELL* transcript levels were normalized to expression of *B2M* and expressed relative to a control sample included on each plate. Levels of BANK1 full-length RNA were detected using a commercial Taqman assay (Hs01009382_m1) and Δ2 transcript levels were detected using a custom Taqman assay spanning the exon 1-exon 3 boundary (forward primer 5'- CTTGGGAGCCCGGACC-3', reverse primer 5'-TCTGTTGGAATGTTGACCTCAAAGT-3', probe 5'- CCCCAGATTCTGAAGACT-3').

2.8 Statistical analysis

Statistical analysis of the phospho-proteomic analysis is described above. All other analyses were performed using Prism GraphPad version 6.02 (GraphPad Software, Inc.) and are described in the figure legends.

3. RESULTS

3.1 Decreased B cell signaling in Ramos B cells expressing the BANK1 risk protein

BANK1 is reported to interact with BCR signaling proteins and impact signaling in cell lines and *Bank1* deficient cells [14, 16-19]. To test whether the SLE-variants in BANK1 alter B cell signaling, we generated Ramos B cell lines expressing the risk or non-risk BANK1 protein and assessed the response to BCR+CD40 co-stimulation. Ramos B cells are a Burkett's lymphoma B cell line; we found this cell line was homozygous for the *BANK1* risk haplotype, but expressed nearly undetectable levels of endogenous *BANK1* RNA and protein (data not shown). Full-length cDNAs encoding BANK1 non-risk (61H, 383T) and risk (61R, 383A) proteins were introduced into Ramos B cells by lentiviral infection and stable cell lines were established that expressed the risk or non-risk BANK1 proteins at similar levels (Figure S1).

We found BANK1 expression did not alter surface expression of IgM, IgD, or CD40 on Ramos cells (Figure S1). Thus we investigated B cell signaling in the Ramos cell lines following stimulation with anti-IgM/IgD+CD40L or media for 5 min. We used spiked-in SILAC coupled with phosphopeptide enrichment and high-resolution quantitative mass spectrometry to quantify 28 phospho-peptides that we have previously found to be responsive to BCR stimulation. As shown in Figure 1A, we detected an increase in abundance of several tyrosine phosphorylated peptides derived from a subset of these proximal BCR pathway proteins in stimulated Ramos cells expressing the non-risk BANK1 protein compared to unstimulated cells. In contrast, the abundance of these peptides was significantly decreased in Ramos cells expressing the BANK1 risk protein compared with those expressing the non-risk protein (Benjamini-Hochberg FDR=0.15).

We also examined signaling for selected B cell molecules by western blot, including phosphorylation of PLCγ2 and S6 as well as total tyrosine phosphorylation. We chose to monitor S6 phosphorylation rather than AKT because we have previously observed that S6 phosphorylation is more faithfully responsive to BCR stimulation than AKT in Ramos cells (data not shown). Upon stimulation with anti-IgM/IgD+CD40L, we detected decreased total tyrosine phosphorylation and decreased levels of phospho-PLCγ2, and a significant decrease in phospho-S6 in Ramos cells expressing the BANK1 risk protein compared to non-risk BANK1 and the vector control transduced cells (Figure 1B, C). These effects were apparent at both early and later times following BCR+CD40 costimulation and are consistent with the mass spectrometry findings. Taken together, these results suggest that BCR signaling is diminished in cells expressing the BANK1 risk protein, consistent with an inhibition of function.

3.2 B cell signaling is decreased in BANK1 risk peripheral B cells

To study the impact of the *BANK1* risk variants in primary B cells, we genotyped healthy control subjects for the *BANK1* SNPs associated with SLE: rs17266594 T/C, rs10516487 G/A, and rs3733197 G/A. Our prior studies have shown that phenotypes associated with genetic risk variants are more apparent in healthy control subjects where disease related factors do not obscure their effects [28-32]. We selected subjects based on their 3-SNP

haplotype as defined in the methods. In addition, we held genotype constant for the non-risk alleles at the *PTPN22, BLK,* and *CSK* genes, which are associated with SLE and have been correlated with alterations in B cell function in human peripheral B cells [28, 30, 32-35].

We examined the expression of the *BANK1* gene in human peripheral B cells to ensure that BANK1 was expressed in these cells. We found that *BANK1* RNA was expressed in memory and naïve peripheral blood B cells at equivalent levels (data not shown).

To determine whether changes in B cell signaling were observed in primary B cells with the *BANK1* risk haplotype, we investigated AKT activation since *Bank1*$^{-/-}$ B cells were reported to have altered AKT signaling upon CD40 stimulation [18]. PBMC samples from *BANK1* risk or non-risk subjects were stimulated with CD40L, BCR+CD40L, or media alone for 20 minutes and AKT phosphorylation was assessed by phospho-flow cytometry. Representative histograms are shown in Figure S2, top. We detected significantly reduced AKT activation in naïve and memory B cells from control subjects carrying the *BANK1* risk haplotype compared with non-risk subjects (Figure 2A). AKT signaling was decreased in response to both CD40 stimulation alone, as well as co-stimulation. Decreased signaling was not due to reduced surface expression of BCR or CD40 in BANK1 risk subjects (Figure S3). Within the memory subset, we found that AKT activation was significantly reduced in IgM memory B cells with the *BANK1* risk haplotype and we observed a trend towards decreased AKT signaling in switched memory B cells (data not shown). The decrease in AKT signaling was confirmed in a replicate set of samples, demonstrating the reproducibility of the assay (Figure S4). These results are consistent with decreased S6 phosphorylation observed in Ramos B cells following BCR/CD40 stimulation (Figure 1C).

We also examined phosphorylation of the proximal B cell signaling molecule PLCγ2 since BANK1 is reported to interact with PLCγ2 [16]. For these experiments, purified total B cells were stimulated with anti-IgM/IgD or media for 5 min and PLCγ2 phosphorylation was detected by phospho-flow cytometry. Similar to a trend observed in Ramos cells, we found that PLCγ2 phosphorylation was significantly decreased in naïve B cells carrying the *BANK1* risk haplotype compared with non-risk B cells (Figure 2B, representative data in Figure S2, bottom). We did not detect a significant decrease in PLCγ2 phosphorylation in total memory B cells. Taken together, these results demonstrate a clear reduction in B cell signaling in response to BCR and CD40 stimulation in the presence of the *BANK1* risk variants.

3.3 FOXO1 levels and activity are increased in BANK1 risk B cells

An important target of the PI3K/AKT pathway is the FOXO1 transcription factor. AKT-mediated phosphorylation of FOXO1 elicits export of FOXO1 from the nucleus and subsequent proteasomal degradation, with the result that levels of AKT activation and FOXO1 are inversely related [36, 37]. Given the blunted AKT activation detected in *BANK1* risk B cells, we quantitated intracellular FOXO1 protein levels in peripheral B cells by flow cytometry. We found that basal FOXO1 protein levels were significantly increased in B cells from subjects with the *BANK1* risk haplotype compared to non-risk subjects (Figure 3A).

To further investigate FOXO1 levels in *BANK1* risk B cells, we measured induction of FOXO1 transcriptional targets following B cell stimulation. Total B cells were purified from *BANK1* risk and non-risk control subjects and stimulated with anti-IgM/IgG+CD40L for 0-72h. As shown in Figure 3B, we found that steady-state RNA levels for the FOXO1 target genes *AICDA* (AID) and *SELL* (CD62L) were significantly induced in *BANK1* risk B cells compared to non-risk B cells at 72h, suggesting that FOXO1 transcriptional activity is increased in cells carrying the *BANK1* risk haplotype. This was evident at the protein level as well; we measured CD62L and FOXO1 protein levels by flow cytometry at day 0 and at 48 hrs after stimulation with anti-IgM/IgG+CD40L. We determined that at baseline, FOXO1 and CD62L protein were significantly negatively correlated (Figure 3C, left) but that after 48 hrs of stimulation FOXO1 and CD62L induction resulted in a significant positive correlation (Figure 3C, right).

We also quantified BANK1 levels to determine whether BANK1 expression correlated with *AICDA* and *SELL* induction. RNA levels for the full-length and Δ2 isoforms of *BANK1* were measured in the same samples used to determine *AICDA* and *SELL*. While we observed no significant differences in RNA levels for the Δ2 transcript of *BANK1* in non-risk or risk subjects after 72 hours of anti-IgM/IgG plus CD40L stimulation (data not shown), we did observe a significant increase in full-length *BANK1* RNA in the *BANK1* risk subjects (Figure 3B) which was significantly correlated with *AICDA* and *SELL* RNA levels at 72 hours (Figure 3D).

To rule out the possibility that decreased AKT activation altered cell survival differentially in B cells expressing the *BANK1* risk variants, we quantified levels of the anti-apoptotic BCL2 protein and the pro-apoptotic BIM protein by flow cytometry. We found that levels of BCL2, BIM, and the ratio of BCL2/BIM were not significantly different in B cells from subjects carrying the *BANK1* risk haplotype versus the non-risk haplotype (Figure S5). Thus, the *BANK1* risk variants do not appear to alter BCL2 family protein expression levels.

3.4 Memory B cells are increased in BANK1 risk subjects

FOXO1 regulates B cell development in both the bone marrow and periphery [38-41]. To determine whether the *BANK1* risk haplotype alters B cell development, we assessed the peripheral B cell compartment in genotyped control subjects. B cell subsets were analyzed in PBMC samples by flow cytometry as shown in Figure S6A. No significant difference was detected in the frequency of total B cells, transitional B cells, or plasmablasts in subjects carrying the risk versus non-risk *BANK1* haplotype (data not shown). However, we found that the *BANK1* risk haplotype was associated with a significant increase in the frequency of memory B cells in the $CD19^+$ B cell compartment compared to the non-risk haplotype, and a compensatory decrease in naïve B cell frequency (Figure 4A). When the frequency of naïve and memory B cells was calculated as a percentage of live lymphocytes, the increase in memory B cells remained significant, while the decrease in naïve B cells was no longer detected (Figure S6B).

The expansion of memory B cells was apparent in both the IgM and switched memory subsets, but only reached statistical significance in the IgM memory subset (Figure S6C). Consistent with a mechanistic role for FOXO1 in memory B cell differentiation, we found

that basal FOXO1 levels and the frequency of memory B cells were significantly correlated in a separate cohort of healthy subjects (Figure 4B). Thus, our findings demonstrate that the *BANK1* variants are associated with an expansion of memory B cells, and suggest FOXO1 levels may impact memory B cell development (Figure 5).

4. DISCUSSION

Genome wide association studies have identified a large number of genetic variants that confer risk for SLE [2-4, 42]. These findings have the potential to enhance our understanding of the pathogenesis and treatment of lupus. However, a crucial challenge moving forward is to develop a mechanistic understanding of how a candidate risk allele promotes loss of immune tolerance and/or influences disease progression.

In this study we investigated the SLE-risk variants in the *BANK1* B cell scaffolding gene to elucidate how the risk alleles contribute to a dysregulation of the B cell program in SLE. Using peripheral blood B cells from genotyped healthy control subjects in conjunction with human B cell lines engineered to express the BANK1 risk and non-risk proteins, we found that the *BANK1* risk variants are associated with decreased BCR and CD40 signaling. BANK1 is reported to interact with the B cell Src kinases LYN and BLK, as well as PLCγ2 [14, 16, 17], which may explain the decreased PLCγ2 phosphorylation we observed with the *BANK1* risk haplotype. Further, we detected blunted AKT activation in *BANK1* risk B cells that was associated with increased levels of the FOXO1 transcription factor and increased expression of FOXO1 target genes upon stimulation, consistent with regulation of FOXO1 levels by AKT. Control subjects carrying the *BANK1* risk haplotype were also characterized by an expansion of the memory B cell compartment which correlated with FOXO1 levels. Thus, our combined findings define alterations in AKT activation and FOXO1 levels that may impact memory B cell development (Figure 5).

Our results in Ramos B cell lines expressing the BANK1 variant proteins suggest that the risk protein inhibits B cell signaling relative to what is observed with the non-risk protein. These observations imply that the risk variants impart altered function on the BANK1 protein. In mice, the data regarding *Bank1* and B cell signaling is contradictory. On one hand, decreased p38- MNK1/2 signaling is detected following CpG stimulation in *Bank1* deficient B cells and in TLR7 signaling during type I IFN induction [14, 19, 20]. In contrast, Aiba *et al.* observed an increase in AKT signaling in *Bank1* deficient B cells upon CD40 stimulation, but no alterations in antigen receptor signaling [18]. These possible inconsistencies between the murine data and our results likely represent a difference between the complete absence of Bank1 protein in $Bank1^{-/-}$ mice, versus altered BANK1 function with the nonsynonymous SLE variants. It may also reflect a mouse strain-dependent effect as experiments with $Bank1^{-/-}$ mice backcrossed to C57BL/6 did not reveal a strong difference in AKT signaling in B cells [19]. We are actively investigating the exact biochemical alterations associated with the risk variants individually and in combination.

The mechanism whereby the *BANK1* risk variants enhance memory B cell differentiation may be due in part to the AKT/FOXO1 pathway [43]. We observed decreased AKT activation in naïve and memory peripheral B cells and decreased S6 phosphorylation in

Ramos B cells expressing the BANK1 risk protein. AKT has a well-documented role in B cell development and homeostasis, mediated in large part through the regulation of FOXO1 levels by AKT [36-41, 43]. Consistent with blunted AKT activation, we found that *BANK1* risk B cells expressed higher levels of FOXO1 protein and increased expression of FOXO1 target genes upon stimulation, including *AICDA* which mediates class switch recombination and *SELL* (CD62L) which is required for homing of B cells to lymph nodes [38, 39, 44]. Notably, we observed a significant correlation between basal FOXO1 levels and the corresponding frequency of memory B cells, highlighting the importance of FOXO1 in memory B cell development (Figure 4). In murine models, preferential reduction of FOXO1 levels in mature B cells results in altered GC formation, reduced class switch recombination, and reduced affinity maturation following immunization, with a concomitant increase in marginal zone B cells [38-41, 45, 46]. Thus, the increase in memory B cells observed here may reflect altered GC differentiation, although we are unable to assess GC B cells in peripheral blood to directly address this question.

Interestingly, we observed that *BANK1* risk B cells stimulated with BCR/CD40L for 72h expressed increased levels of RNA for the full-length *BANK1* transcript, but not the Δ2 isoform, compared to B cells from non-risk subjects. Further, full-length *BANK1* transcript levels were positively correlated with RNA levels of the FOXO1 target genes *AICDA* and *SELL* at 72h. Previous studies have suggested that the full-length isoform of BANK1 is pathogenic and the Δ2 isoform is protective in the development of SLE based on increased quantities of RNA for full-length *BANK1* compared to the Δ2 isoform in *BANK1* risk subjects [5]. The preferential induction of full-length RNA levels in *BANK1* risk B cells versus non-risk B cells following stimulation may support these observations.

Together, our data suggest that BANK1 contributes to the development of SLE by inducing the proliferation and/or differentiation of memory B cells, which may result in the expansion of autoreactive memory B cells. In healthy individuals, *BANK1* variants in isolation induce memory B cell accumulation which is analogous to what occurs in SLE [47]. Mechanistically, our data suggests that the BANK1 risk protein acts through an inhibition of function resulting in a decrease in BCR/CD40L signaling, enhanced FOXO1 expression and subsequent memory B cell expansion as depicted in Figure 5. A recent study has shown that 7% of healthy control individuals are ANA^+ and that these subjects demonstrate an expansion of memory B cells, indicating that this is an early event in B cell dysregulation [48]. As B cells start to accumulate in at-risk subjects, other genetic and environmental factors could act as additional triggers resulting in a shift towards BCR hyper-responsiveness, due in part to loss of negative regulation of BCR signaling via decreased FCγRIIB and Lyn expression [49, 50]. While hyper-responsive BCR signaling can promote SLE by increasing the degree of positive selection in the GC [51], there are examples where genetic alterations that limit GC B cell signaling lead to increased autoreactive B cell differentiation [52]. Decreases in B cell signaling could promote autoreactive populations by limiting negative selection of self-reactive antigens in the periphery and it is possible that the *BANK1* risk haplotype might impact this process through decreased BCR, CD40 and TLR signaling [19, 20], in addition to its role in memory B cell expansion. Future experiments will address these possibilities.

5. CONCLUSIONS

Our results highlight the importance of the *BANK1* SLE risk variants in the expansion of memory B cells of healthy subjects. In the setting of SLE, we believe that the *BANK1* variants act in combination with other factors, including genetic variants in *PTPN22,* to alter peripheral tolerance checkpoints in naïve B cells [35, 53, 54]. In combination with the expansion of memory B cells imparted by the *BANK1* risk variants, this results in the expansion of autoreactive B cells in SLE. These alterations drive a dysregulated B cell program and development of autoantibodies, consistent with the association of *BANK1* with the presence of anti-DNA and anti-RNP antibodies in lupus [6, 22]. The alterations in B cell signaling and development with the *BANK1* risk variants described here provide new insight into possible mechanisms through which this may occur.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FDR	false discovery rate
GC	germinal center
IP3R	inositol 1,4,5-triphosphaste receptor
PLCγ2	phospholipase C, γ2
qPCR	quantitative PCR
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism

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Highlights

- *BANK1* SLE-risk variants are associated with reduced BCR and CD40 through AKT
- *BANK1* risk variants are associated with increased levels of FOXO1
- Enhanced expression of FOXO1 target genes *AICDA* and *SELL* in *BANK1* risk subjects
- Expansion of memory B cells correlates with FOXO1 in *BANK1* risk subjects

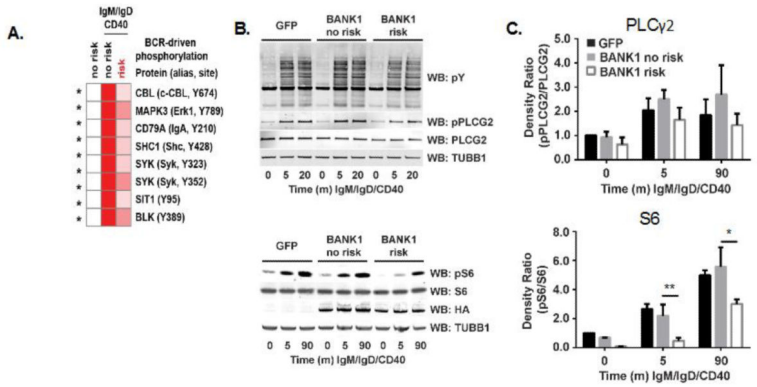

Figure 1. Reduced B cell signaling is detected in Ramos B cells expressing the BANK1 risk protein

(A) Ramos B cell lines expressing the BANK1 risk or non-risk protein were stimulated with 2.5μg/ml CD40L plus 10μg/ml each F(ab′)2 fragment anti-human IgM and IgD for 5 min. The degree of phosphorylation of 27 peptides known to be responsive to BCR stimulation in Ramos B cell lines was quantified using spiked-in SILAC coupled with phosphopeptide enrichment and high-resolution quantitative mass spectrometry. The indicated peptides exhibited significantly different phosphorylation in the BANK1 risk and non-risk cell lines (Benjamini-Hochberg FDR=0.15). (B) Immunoblot analysis of whole cell extracts from Ramos B cell lines stimulated as described in (A) for the indicated times. Blots were probed with Abs specific for HA, tubulin (TUBB1), total phospho-tyrosine (pY), PLCγ2 (PLCG2), S6 and their corresponding phospho-specific Abs. (C) Quantitation of the phosphorylation of PLCγ2 and S6 was determined by densitometry and normalized for total protein levels. Graphs depict the mean and SD of three independent immunoblot experiments. Significance was assessed with one-way anova and p-values were generated with the SIDAK post-hoc test.

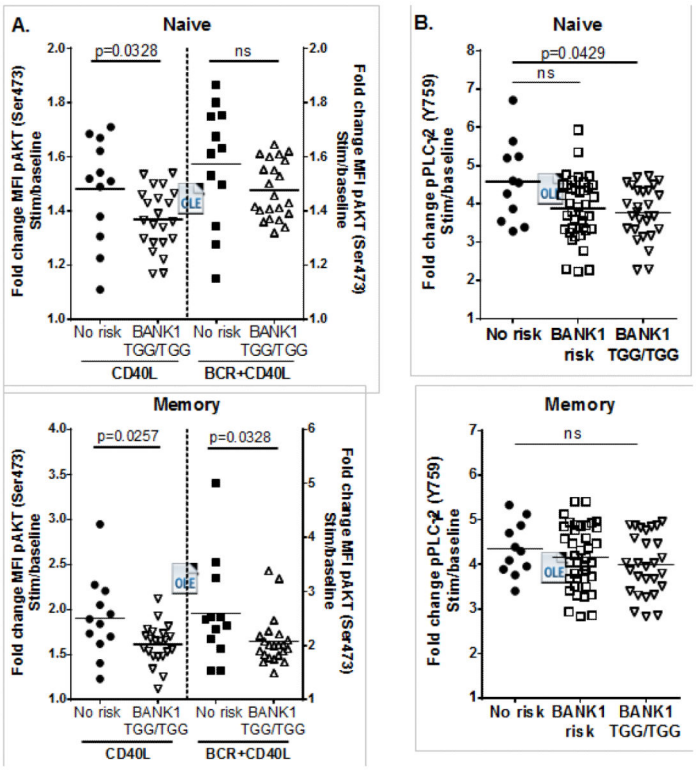

Figure 2. B cell signaling is decreased in *BANK1* risk primary B cells

(A) Phospho-AKT (pAKT Ser473) signaling in naïve (upper panel) and memory (lower panel) B cells from *BANK1* non-risk (No risk, n = 12) and *BANK1* risk (BANK1 TGG/TGG, n = 22) control subjects was assessed by flow cytometry following stimulation of PBMC with CD40L 2.5μg/ml or CD40L plus anti-IgM/IgG F(ab')$_2$ 10μg/ml each (BCR +CD40L) for 20 min. The fold change in pAKT MFI was calculated in stimulated cells compared to baseline (media alone). (B) Phospho-PLCγ2 (pPLCγ2 Tyr759) signaling in gated naïve (upper panel) and memory (lower panel) B cells from *BANK1* non-risk (No risk, n = 11), *BANK1* TGG/TGA and TGG/TGG subjects (BANK1 risk, n = 39), and *BANK1* TGG/TGG (n = 28) control subjects was assessed by flow cytometry following stimulation of purified total B cells with 10μg/ml each anti-IgM/IgD F(ab')$_2$ for 5 min. The fold change in pPLCγ2 MFI was calculated in stimulated cells compared to baseline (media alone). Significance was assessed using a Mann Whitney U test.

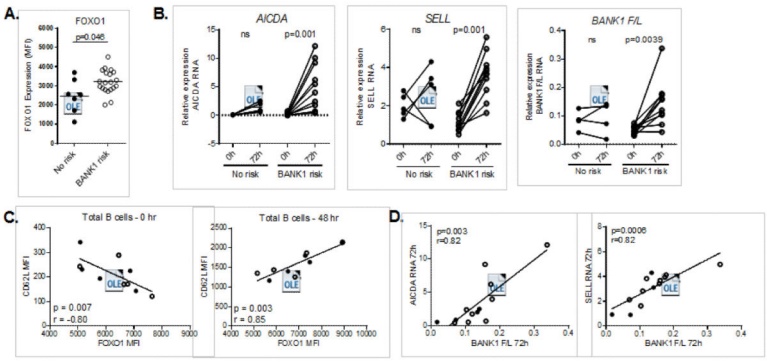

Figure 3. FOXO1 levels and transcriptional activity are increased in B cells carrying the *BANK1* risk variants

(A) Baseline FOXO1 levels were quantified in CD19+ total B cells from *BANK1* non-risk (No risk, n = 7) and risk (BANK1 risk, n = 20) control subjects by flow cytometry. (B) Transcript levels of the FOXO1 target genes *AICDA* (AID) and *SELL* (CD62L) were quantified by qPCR in purified total B cells from *BANK1* non-risk (No risk, n = 5) and risk (BANK1 risk, n = 11) control subjects before and after stimulation with 2.5μg/ml CD40L plus 10μg/ml anti-IgM/IgG F(ab')2 for 72h. Transcript levels of the *BANK1* full-length (F/L) transcript were quantified by qPCR in same samples (no-risk n = 4, risk n = 10). (C) CD62L and FOXO1 protein expression levels were quantified at baseline (left panel) and at 48 hours after stimulation with 2.5μg/ml CD40L plus 10μg/ml anti-IgM/IgG F(ab')2 (right panel). Correlation was determined between FOXO1 and CD62L in BANK1 risk and non-risk subjects (n = 10). (D) *AICDA* and *SELL* transcript levels at 72 hrs were correlated with *BANK1* full-length transcript levels from (B) (n = 14). Significance was determined using a Mann Whitney U test in (A), a Wilcoxon matched–pairs signed rank test in (B, D) and correlation was determined using Spearman correlation.

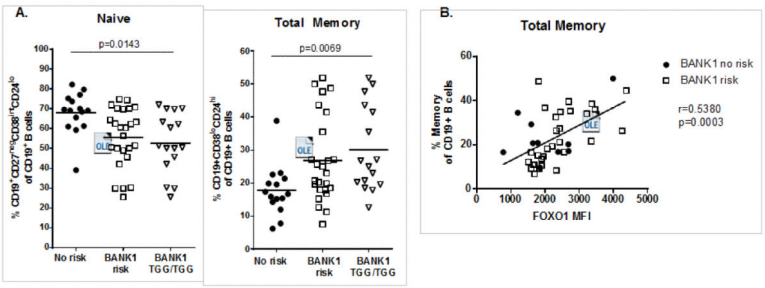

Figure 4. The *BANK1* risk haplotype is associated with an expansion of memory B cells

(A) Banked PBMC samples were stained with Abs to the indicated surface proteins and analyzed by flow cytometry. Naïve and memory B cell populations within the peripheral blood CD19^{+} B cell gate were defined based on CD24 and CD38 surface expression: naïve (CD19^{+} CD38^{mid} CD24$^{lo/mid}$ CD10$^{lo/neg}$); memory (CD19^{+} CD38^{lo} CD24$^{+/hi}$). The frequency of naïve and memory B cells in the CD19^{+} gate is shown for *BANK1* non-risk (No risk, n = 15), *BANK1* TGG/TGA and TGG/TGG subjects (BANK1 risk, n = 25), and *BANK1* TGG/TGG (n = 16) control subjects.

(B) orrelation between the frequency of memory B cells and basal FOXO1 MFI in memory B cells of the *BANK1* risk and non-risk subjects (n = 40) depicted in Figure 3A. Significance was determined by a Kruskal-Wallis test and the correlation was determined using Spearman correlation.

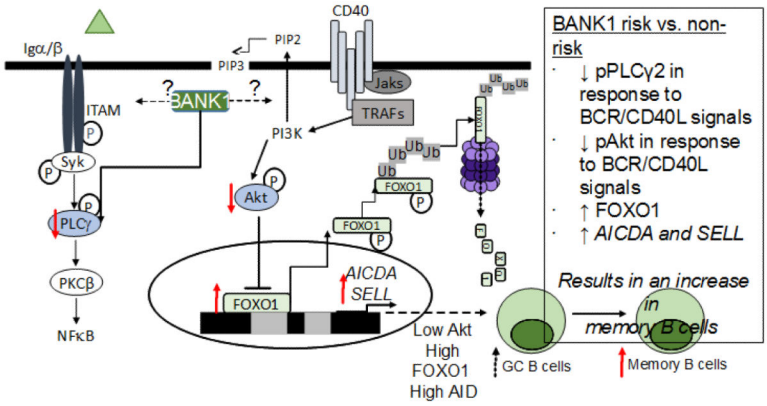

Figure 5. Model of the impact of *BANK1* SNPs in B cell signaling and development

SLE pathogenesis is induced through environmental and genetic factors. Of these genetic factors, *BANK1* has been identified as important in B cell signaling and development. We have demonstrated that in control subjects, risk compared to non-risk variants of *BANK1* resulted in a decrease in B cell signaling through p-PLCγ and p-Akt. Further, we observe an enhancement in FOXO1 expression levels and in *AICDA* and *SELL* which are FOXO1 target genes. When we phenotyped these subjects we observed an increase in memory B cells which correlated with FOXO1 levels. These alterations may represent an early event in B cell dysregulation contributing to SLE. Red arrows indicate findings described here.