EFFECTS OF DIFFERENTIAL B CELL RECEPTOR SIGNALING ON MEMORY AND NAÏVE B CELL SUBSET FUNCTION AND IDENTIFICATION OF NOVEL IGM$^+$ MEMORY B CELL SUBSETS VIA SURFACE EXPRESSION OF IGD

by

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ABSTRACT: EFFECTS OF DIFFERENTIAL B CELL RECEPTOR SIGNALING ON MEMORY AND NAÏVE B CELL SUBSET FUNCTION AND IDENTIFICATION OF NOVEL IGM+ MEMORY B CELL SUBSETS VIA SURFACE EXPRESSION OF IGD

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The ability to remember antigens and respond quickly to repeat exposure is carried out by long-lived memory cells and is critical for preventing reoccurring infections. Of particular importance to the fields of vaccine development, immunotherapy, and autoimmunity is the study of humoral memory. Our lab has previously identified three subsets within the memory B cell (MBC) compartment delineated by B7-Family members CD80 and PD-L2. These subsets are $C80^+$ $PD\text{-}L2^+$ (Double Positive, DP), $C80^-$ $PD\text{-}L2^+$ (Single Positive, SP) and $C80^-$ $PD\text{-}L2^-$ (Double Negative, DN). B7-Family subsets are predictive of cell fate upon re-exposure to cognate antigen. DP cells quickly become antibody secreting cells (ASCs), while DN MBCs re-enter the germinal center reaction. SP cells possess the capacity to adopt either function. Despite the critical role humoral immunity plays in warding off disease, little is known about how these subsets adopt their respective roles in vivo.

The goal of this project was to further elucidate the differences between CD80/PD-L2 MBC subsets and to understand the significance of these differences in the context of of humoral memory. **Given these subsets share the splenic environment and therefore experience similar antigen exposure, we hypothesized that differential regulation of BCR signaling may explain the observed differences in cell differentiation and function.** We found that while B7-Family MBC subsets displayed similar proximal BCR signaling, the priming and activation of protein synthesis after BCR cross-linking was significantly different between subsets. Despite few differences in proximal BCR signaling cascades of B7 MBC subsets, we found surface expression of IgD divides the IgM^+ MBC compartment into further subsets based on pSyk signaling. IgD^+ MBCs have lower pSyk signaling upon re-stimulation with their cognate antigen than those IgM^+ cells that lack IgD on their surface. These differences may play a key role in MBC function, and may lead to the discovery of additional functional MBC subsets. Finally, we determined not only that MBCs signal much more strongly throughout the BCR signaling cascade than their naïve counterparts, but these differences are likely caused by a combination of cascade protein levels and decreased phosphatase activity in the MBC compartment.

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1.0 INTRODUCTION

1.1 MEMORY B CELL AND LONG-LIVED PLASMA BLAST RESPONSE

Immunological memory is a hallmark of the adaptive immune response and is critical for combating recurrent infections. The memory compartment consists of antigen-experienced effector cell that remain in the organism long after initial antigen exposure (often for the lifetime of the organism) and require no further interaction with antigen to be maintained [1, 2]. Upon re-exposure to their cognate antigen, memory cells offer a more rapid and robust response than naïve B cells (NBCs). In the case of humoral memory, memory B cells (MBCs) reconstitute the germinal center reaction (thus re-initiating somatic hypermutation) or form protective antibody secreting cells (ASCs) after secondary exposure. The generation of GCs and effective antibody responses are important goals in vaccine development, where the presence of these functions can lead to successful protection. Additionally, memory functions may play a role in B cell-mediated autoimmunity. Despite this, there is much we still do not understand about the generation and execution of humoral memory.

1.2 ROLE OF ANTIBODIES IN DISEASE STATES

Production of antigen-specific antibodies is the primary goal of humoral immune response. Antibodies are some of the most adaptable and potent weapons against invading pathogens, allowing for precise protection with relatively little collateral damage to healthy tissues. Effective generation of humoral immunity is critical to the containment and elimination of a wide variety of pathogens, including *C. difficile* [3, 4], HCMV [5] and Influenza [5, 6]. Additionally, vaccine technology (arguably one of humanity's greatest medical advances) relies heavily on the generation of humoral memory and long-lived antibody responses [7].

Antibodies are capable of dispatching threats through three methods. First is opsonization, where extracellular pathogens are coated with antibodies to enhance uptake and degradation by phagocytes, via Fc receptors [8]. Second, antibodies can neutralize pathogens by adhering to molecules necessary for disease progression. This includes blocking surface molecules on intracellular pathogens that mediate cellular entry on intracellular pathogens [9, 10], preventing adherence of extracellular bacteria [11, 12] and binding bacterial toxins (such as those produced by Tetanus or Diptheria) [3, 9]. Additionally, some antibody isotypes (such as IgM) are capable of effectively activating the complement system, a series of diverse proteins that disrupt plasma membranes and lyse invading cells [13].

The final strength of antibody-mediated protection is its ability to increase in affinity and specificity over the course of an immune response. This is achieved through directed mutation of immunoglobulin (Ig) genes in the germinal center reaction in a process called affinity maturation.

1.3 GERMINAL CENTER FORMATION AND AFFINITY MATURATION

The germinal center (GC) is a transient structure that forms in the B cell follicles of secondary lymphoid organs as a response to antigenic stimuli. Germinal centers are the site of rapid B cell turnover, with proliferation and cell death occurring at an accelerated rate [14, 15]. GCs begin when antigen is bound by the low-affinity BCR of NBCs, partially activating them. The antigen is internalized, degraded in the lysosome and presented on MHC Class II to $CD4^+$ T cells at the interface between B cell and T cell zones. Antigen specific T cells are activated by the presentation of antigen and co-stimulatory factors on the surface of the activated B cells. These T cells produce cytokines (such as IL-4 and IL-21) [16] that instruct the activated, antigen-specific B cells to begin rapid proliferation within the B cell follicle[17, 18].

The germinal center is responsible for two key aspects of humoral immunity. First is the rapid expansion of antigen-specific B cells upon exposure to an immunogen. This expansion allows the immune system to quickly build a repertoire of cells capable of recognizing the invading pathogen [14, 15, 19]. The second role of the germinal center is affinity maturation, a process by which the immune system adds to the already impressive utility of antigen-specific antibodies by honing their specificity for cognate antigen. This is achieved with the purposeful introduction of point mutations into the V region gene of the B cell receptor (BCR) in a stepwise, generational manner by the enzyme Activation-Induced Cytidine Deaminase (AID) through a process known as somatic hypermutation. Those cells with the highest affinity BCRs then preferentially survive to produce daughter cells [20], although the exact mechanism of selection is not well understood. The leading theory, known as the cyclic re-entry model, describes germinal center B cells migrating between the two main structures within the GC; the dark zone and the light zone. Proliferation and somatic hypermutation occur in the dark zone, after which the GC B cells migrate to the light zone. Once there, they receive antigen from follicular dendritic cells, internalize it and present it to T follicular helper cells (T_{fh}). It is believed that competition for antigen uptake and T_{fh} help is the driving factor behind affinity maturation. B cells that possess the highest affinity BCRs are better able to

acquire antigen and present it to T_{fh} and are thus preferentially given survival signals, while those with little to no affinity for the antigen do not receive these cues and perish [21, 22].

1.4 B CELL RECEPTOR SIGNALING CASCADE

B cell activation via the BCR is dependent upon the transduction of signals from the plasma membrane of the cell to transcription factors and eventually the nucleus of the cell and the genetic machinery [23]. This signal is propagated via the rapid phosphorylation of kinases and adaptor proteins, whose activation allows them to phosphorylate kinases further down the chain in turn.

The signaling cascade begins with the cross-linking multiple BCRs by multivalent antigen, bringing associated Igα and Igβ proteins associated with the BCR into close proximity with one another. This recruits and activates the Src family kinase Lyn, which phosphorylates the ITAM regions of Igα and Igβ. Spleen tyrosine kinase (Syk) then binds the phosphorylated ITAMs via its SH2 domains and is activated. Syk is responsible for the phosphorylation of numerous downstream targets, including (but not limited to), Burkett's tyrosine kinase (Btk), 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2 (PLC-γ2), CD19 and BLNK [24].

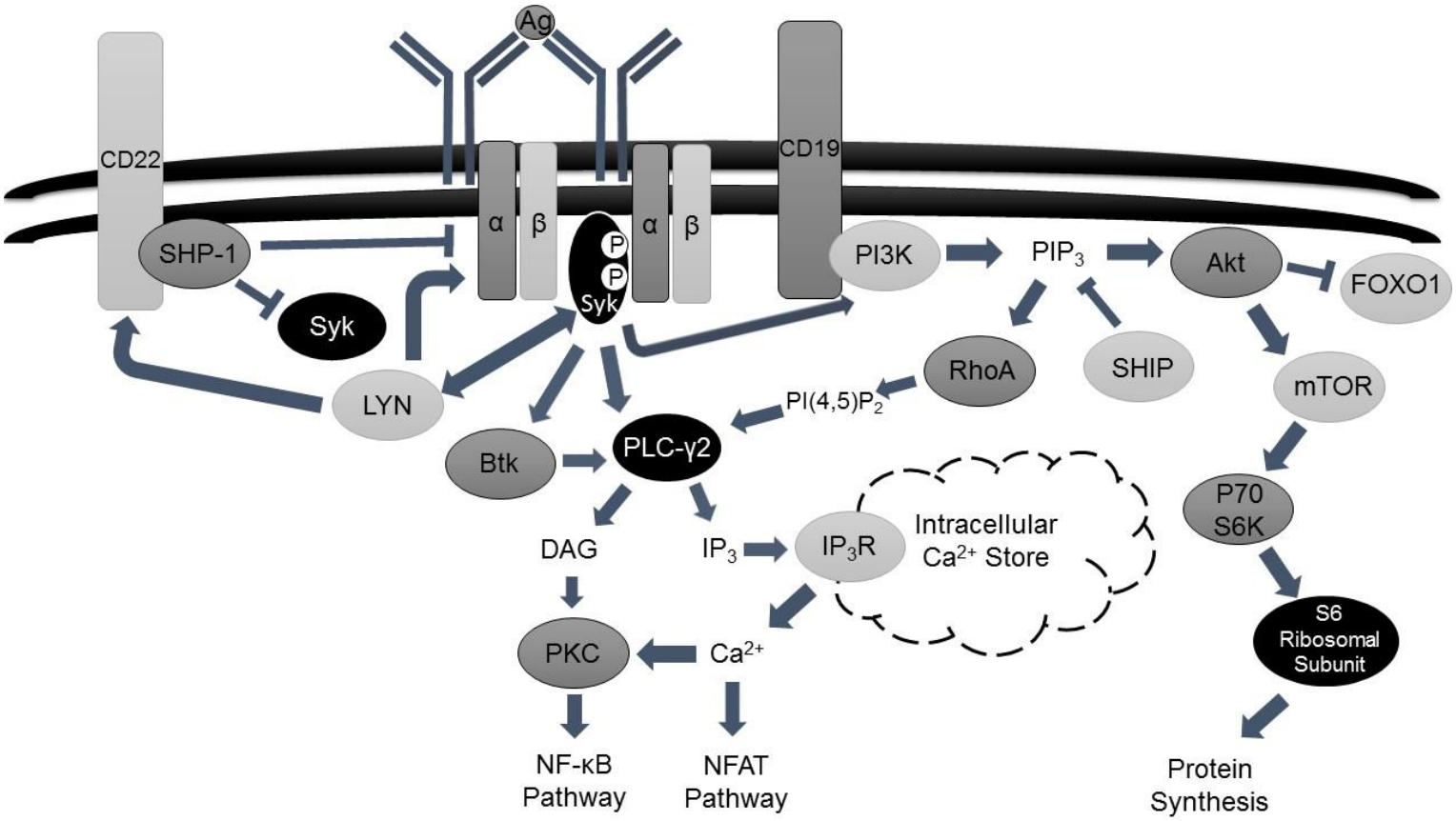

Figure 1: Focused Map of the BCR Signaling Pathway. *Cross-linking of two or more BCRs by their cognate antigen triggers a signaling cascade. For the purposes of our research we focused on the activation of three major pathways: NF-κB, NFAT and protein synthesis. The signaling cascades responsible for the activation of these pathways share several common elements. Syk, an extremely proximal determinant and regulator of BCR signaling, is a useful metric for the assessment of total BCR signaling. PLC-γ2 is one of a myriad of proteins activated by Syk and is responsible for the cleavage of PIP_2 into DAG and IP_3. IP_3 triggers the release of intracellular calcium, and the combination of DAG and Ca^{2+} together are responsible for the eventual activation of both NF-κB and NFAT pathways. While not directly involved in the cascade directly, S6 is a subunit of the ribosomal assembly and critical for the transcription of proteins. It is therefore these three proteins that served as the primary readouts for our research.*

After Syk activation, the BCR signal branches into several distinct pathways. PLC-γ2 (whose activation requires both Syk and Btk) cleaves PIP_2 into diacylglycerol (DAG) and IP_3. IP_3 levels lead to the release of intracellular Ca^{2+} from storage. This activates calcineurin and results in the translocation of transcription factor NFAT. Combined DAG and intracellular Ca^{2+} levels activate protein kinase C, which begins a cascade resulting in the degradation of IκB and the translocation of NFκB into the nucleus. Additionally, phosphorylation of CD19 recruits PI3K and leads to the generation of PIP_3. PIP_3 activates Akt, which in turn activates mTor and eventually protein synthesis pathways through the phosphorylation of ribosomal protein S6 (S6) (a component of the 40s-ribosomal subunit).

1.5 B7-FAMILY EXPRESSING MEMORY B CELL SUBSETS

The memory B cell compartment is far more heterogeneous than originally believed. Variations in isotype [25] and V region mutation content [26] have been reported. It has also been indicated that the classic functions of activated MBCs upon re-stimulation (reconstitution of the germinal center and rapid generation of antigen-specific plasma cells) are carried out by separate MBC subsets [27, 28], rather than being intrinsic properties of all MBCs.

Using a novel murine adoptive transfer system (see section 3.1), our lab has previously described three unique MBC subsets distinguished by their selective expression of B7-1 (CD80) and programmed death ligand-2 (PD-L2) [29]. As B7-Family members, CD80 and PD-L2 are both involved with B cell -T cell interactions. CD80 is one of two "signal 2" ligands responsible for stimulating T cell help for activated immune cells. CD80 is predominantly expressed on myeloid cells, and has thus far only been observed on memory cells in the B cell compartment. Interactions between PD-L2 and Programmed Death 1 (PD-1) are necessary for the formation of the T_{fh} compartment, germinal center formation and optimal ASC formation [30].

Three MBC subsets are delineated by surface expression of CD80 and PD-L2: $CD80^{-}PD\text{-}L2^{-}$ double negative (DN), $CD80^{-}PD\text{-}L2^{+}$ single positive (SP) and $CD80^{+}PD\text{-}L2^{+}$ double positive (DP) subsets. These subsets are genetically distinct [26, 31] and demonstrate differences in V region mutational content and isotype switching. The DN compartment predominantly expresses IgM BCRs with few germ line mutations, while DP MBCs are significantly mutated and have a high percentage of class-switched cells (although IgM^{+} DP cells do exist) [29, 31]. Furthermore, Weisel et. al [32] have shown that the generation of these subsets is temporally dispersed over the course of the early germinal center reaction.

Given the critical importance of CD80 and PD-L2 in coordinating B cell activation and function, it should come as little surprise that Zuccarino Catania et al. [31] demonstrated vastly different functions among the three described MBC subsets. Double negative cells readily proliferate and re-enter the germinal center reaction

(complete with somatic hypermutation). Conversely, double positive cells undergo a short proliferative burst before differentiating into plasma cells. Single positive cells adopted a middling approach, demonstrating the capacity to either regenerate GCs or differentiate. These data extend previous work by Dogan et al. [27], who proposed that isotype was indicative of MBC function, mirroring our findings. However, it should be noted that, while DP MBCs have a higher incidence of IgG than DN, the IgM^+ contingent of $CD80^+$ $PD\text{-}L2^+$ cells react to antigen stimulation in the same manner as their isotype switched counterparts [31].

Despite the critical therapeutic importance of distinct functional MBC subsets, very little is understood about what allows these subsets to carry out their individual duties. Given these subsets share the splenic environment and therefore experience similar antigen exposure, we hypothesized that differential regulation of BCR signaling may explain the observed differences in cell differentiation and function.

2.0 MATERIALS AND METHODS

2.1 ADOPTIVE TRANSFER OF ANTIGEN-SPECIFIC B CELLS AND GENERATION OF HUMORAL MEMORY IN A TRANSGENIC MURINE MODEL

mVh186.2 (B1-8) knock-in BALB/c mice were generated as previously described [33] and maintained as $J\kappa^{-/-}$ to enrich the frequency of λ^{+} NP-specific B cells for use in the transfer system to generate MBCs. AM14 transgenic (Tg) $V\kappa8R^{+/-}$ BALB/c mice were generated as previously described [34] and used as recipients for NP^{+} B cells due to their inability to generate endogenous NP-specific B cells. $B18^{+/-}$ $J\kappa^{+/+}$ mice were used as naïve controls.

$B18^{+/-}$ $J\kappa^{-/-}$ splenocytes were harvested and enriched for B cells via complement-mediated depletion of T cells (anti-CD4 (clone GK1.5, grown in house) and anti-CD8 (clone TIB05, grown in house) along with rabbit complement). Frequency of NP^{+} B cells was determined by flow cytometry and $2x10^{5}$- $3x10^{5}$ NP^{+} B cells (appx. 10^{6} total cells) were transferred intravenously to each AM14 Tg $V\kappa8R^{+/-}$ recipient. After 24 hours, the recipient mice were immunized with 50µg NP_{33}-chicken γ-globulin (4-hydroxy-3-nitrophenyl) acetyl chicken γ-globulin (NP-CGG) precipitated in alum intraperitoneally. Splenocytes were harvested from recipients (now designated memory mice) only 8 weeks post immunization.

All mice were bred and maintained in accordance with University of Pittsburgh Department of Laboratory Animal Resources guidelines and all animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2 IN VITRO B CELL STIMULATION

Spleens of individual mice were harvested, homogenized, depleted of erythrocytes and kept at room temperature until approximately 20 minutes before stimulation, at which time they were warmed to 37°C. Single-cell suspensions of approximately $5x10^6$ RBC-depleted splenocytes were stimulated with 5µg NP_{33}-Fluroscein-AECM-Ficoll (Biosearch Technologies), 20µg LPS- free anti-IgM (µ-chain specific) (goat polyclonal, Jackson Immunoresearch, LPS removed in house) or 1-5mM hydrogen peroxide at 37°C. Stimulation was halted with the addition of 2x cytofix/cytoperm solution (final concentration 4% PFA, Perm/Wash™ buffer, BD).

2.3 ANALYSIS OF BCR SIGNALING EVENTS VIA FLOW CYTOMETRY

After stimulation and fixation cells were washed twice and stained in 1x BD perm/wash buffer. Cells were incubated with Anti-CD80 and fixable viability stain (FVS) before stimulation and fixation, all other antibodies were stained post-fixation/permeabilization.

Antibodies used were: Anti-CD45R (clone RA3-6B2, eBiosciences), anti-CD80 (clone 16-10A1, BD Horizon), anti-FITC (clone 5D6.2, EMD Millipore), anti- IgD (clone 11-26c.2a, BD Horizon), anti-IgM (µ-chain specific) (goat polyclonal, Southern Biotech), anti- PD-L2 (clone TY25, Biolegend), anti- PLCγ2 (Y759) (clone K86-659.37, BD), anti-S6 Ribosomal Protein (Ser235/236) (clone D57.2.2E, Cell Signaling), anti-SHP-1 (rabbit polyclonal, Santa Cruz), anti-Syk (clone 5F5, Biolegend) and anti-ZAP70(pY319)/Syk(pY352) (clone 17A/P-ZAP70, BD).

Detection reagents used were: Ghost Dye™ Violet 510 fixable viability stain (Tonbo Biosciences) and NIP-APC ((4-hydroxy-5-iodo-3nitrophenyl) acetyl, Prozyme, conjugated in-house).

3.0 RESULTS AND ANALYSIS

3.1 ADOPTIVE TRANSFER SYSTEM FOR THE GENERATION AND DETECTION OF ANTIGEN-SPECIFIC HUMORAL MEMORY

As previously stated, the murine system has no unique marker that designates the MBC compartment. Many researches have used IgG as a proxy marker for memory, as class-switched B cells must be antigen-experienced [35]. However, the field now recognizes that IgM^+ B cells make up a large portion of the memory compartment, and that excluding them when considering humoral memory gives us an incomplete picture of MBC biology [27]. To address this issue, we use an adoptive transfer system that allows for the identification of MBCs based on antigen binding rather than BCR isotype.

mVh186.2 (B1-8) is a BCR heavy chain which, when paired to the λ1 light chain, forms a BCR with specificity for the hapten nitrophenol (NP). B1-8 knock in mice on the kappa-chain knock out background (B1-$8^{+/-}$ $J\kappa^{-/-}$) produce a B cell compartment that is approximately 60% NP-reactive [22]. These NP-reactive cells are adoptively transferred into mice with a transgenic AM14 BCR heavy chain and Vκ8R light chain. AM14-Vκ8R BCRs are specific to IgG (i.e. have rheumatoid factor specificity), and the recipient mice are therefore unable to generate endogenous NP-reactive B cells [34]. Once immunized with NP, the host mice will generate an immune response deriving from the B1-$8^{+/-}$ $J\kappa^{-/-}$ cells they received. Once the primary response has run its course, the NP-reactive B cells that remain in the animal are MBCs and can be identified by staining with fluorophore-conjugated NP (Figure 2). In this way, we can assess the entire MBC compartment, regardless of isotype.

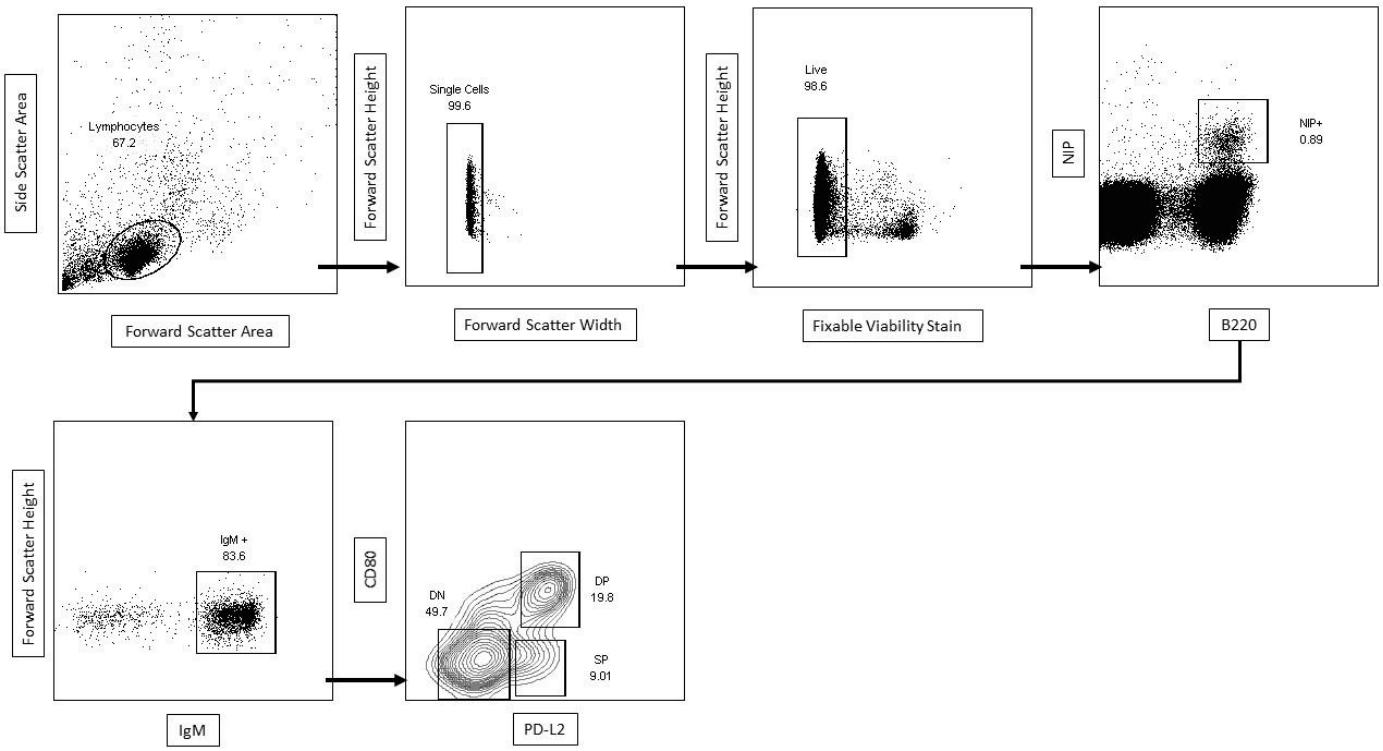

Figure 2: Gating Strategy for Identification of IgM+ B7-Family MBC subsets. *A complex gating strategy was employed to identify B7-Family MBC in whole splenocytes harvested from the B18-Jκ KO into AM14-Vκ8R$^{+/-}$ adoptive transfer system (section 3.1). Lymphocytes were selected via forward and side scatter. Lymphocytes tend to be small (low forward scatter) and have low granularity (low side scatter). Cell doublets (two cells attached to one another) were excluded by height and width to prevent false readings where two cells are read as one. Next, dead cells were excluded with the use of a fixable viability stain. Pre-fixation, Cells are stained with a reagent that is easily pumped out by live cells but which stays present in dead or dying cells. In the live cell population, B cells were identified by the surface expression of B220. Of the B220+ population, MBCs were identified by their ability to bind NIP conjugated to a fluorophore. IgM$^+$ B cells were selected for to ensure our assessment of BCR signaling was not influenced by comparing IgM and IgG-expressing cells (as IgG has previously been show to activate the BCR signaling cascade more strongly than IgM [36, 37]). Finally, the IgM$^+$ MBCs were divided into three subsets defined by surface expression of CD80 and PD-L2 (DN, SP and DP respectively).*

3.2 DP MBCS ARE PRIMED FOR ENHANCED PROTEIN SYNTHESIS

Zuccarnio-Catania et. al (2014) previously demonstrated that MBC subsets defined by CD80 and PD-L2 perform vastly different functions upon re-exposure to their cognate antigen. Given that these differences occur despite identical BCR stimulation in each subset, we hypothesized that divergent BCR signal processing might be responsible. Thus, we investigated proximal BCR signaling as a potential mechanism for differential MBC function.

We examined three signaling molecules for this purpose. Syk, an extremely proximal effector molecule in the BCR pathway, is responsible for activating important downstream effectors such as PI3K, Btk, and PLCγ2. The second, 1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2 (PLCγ2), cleaves diacylglycerols into DAG and IP_3, important signaling molecules involved in the activation of the NF-κB pathway (among others) and calcium flux respectively. The third molecule, ribosomal protein S6 (S6) is a component of the ribosome complex and an indicator of Akt pathway signaling.

Single-cell suspensions of splenocytes harvested from transfer recipient mice (hitherto referred to as "memory mice") were stimulated with cognate antigen (NP-Ficoll, Figure 3 A, C, D) or BCR cross-linking antibody (μ-chain-specific anti-IgM, Figure 3 B, E). Each B7-Family MBC subset was then assessed for the phosphorylation of the signaling molecules described above at specific activation sites (Syk pY352, S6 pSer235/236, PLCγ2 pY759) (Figure 3).

There were no statistically significant differences among B7-Family subsets in either Syk or PLCγ2 phosphorylation. This suggests very proximal signaling in these subsets is relatively similar and unlikely to play a significant role in the diversification of MBC function. However, BCR crosslinking did induce significantly higher levels of pS6 in DP MBCs when compared to the other two subsets. While SP MBCs did not signal as strongly as their DP counterparts, S6 signaling in this compartment was still significantly higher than that in DN

MBCs. This indicates tiered levels of protein synthesis subsets (as S6 is part of the 40S subunit) that correspond with functional B7-Family MBC subsets.

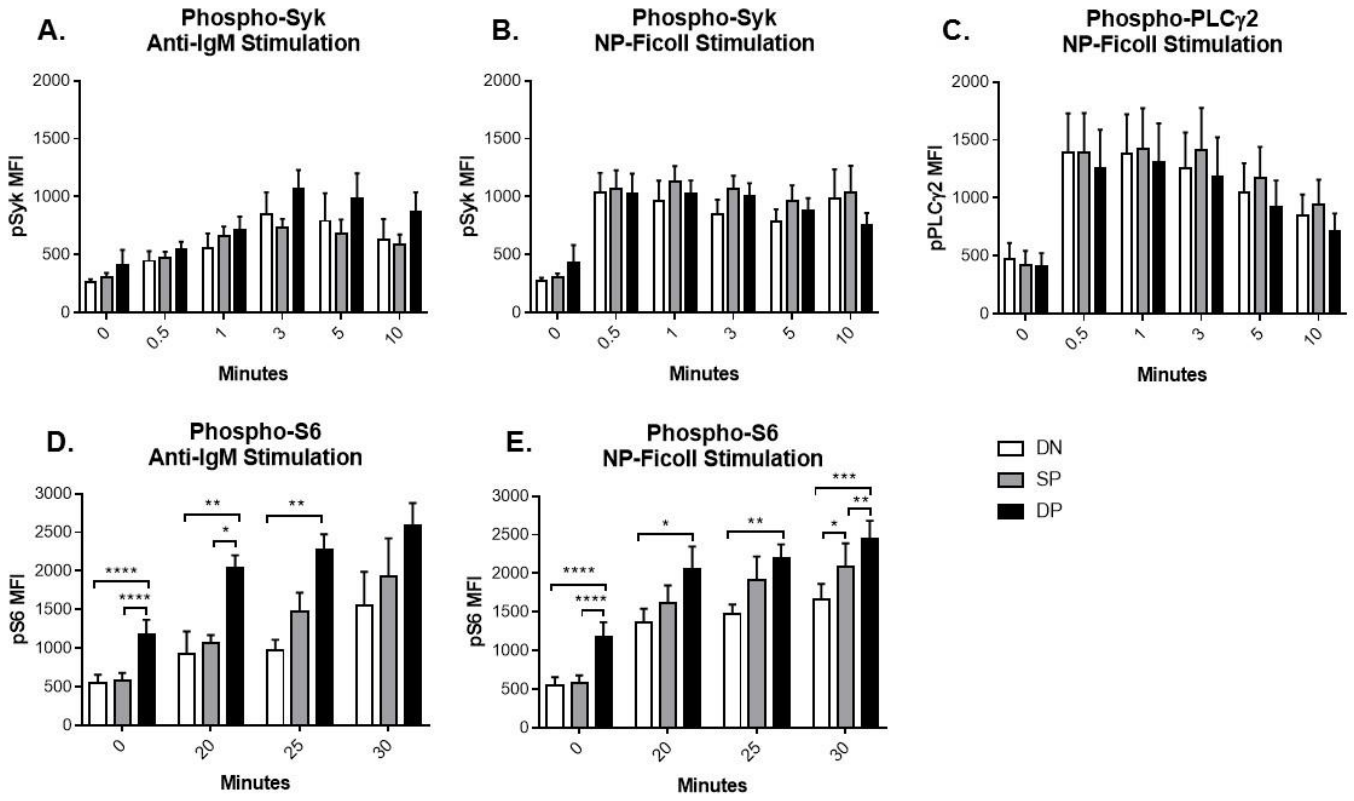

Figure 3: DP MBCs are Primed for Enhanced Protein Synthesis. Whole splenocytes were harvested from adoptively transferred AM14-Vκ8R$^{+/-}$ mice and stimulated, depleted of erythrocytes and stimulated with 20μg/ml Anti-IgM (A, D) or 5μg/ml NP-Ficoll (B, C, E). Cells were then fixed to halt BCR signaling, permeabilized and assessed for phosphorylation of BCR pathway molecules Syk (A, B), PLC- γ2 (C) or S6 (D, E). While no statistical differences emerged in early events of BCR signaling (Syk, PLC- γ2), the B7-Family MBC subsets differed significantly in terms of S6 phosphorylation. DP, SP and DN MBCs formed a gradient of pS6 that mirrored previously established functions; DP MBCs displayed the highest levels of pS6 after stimulation, DN MBCs displayed the least, and the SP compartment contained moderate levels of pS6. Bars represent mean and SEM. (A) n=7-8 (4 experiments), (B) n=6 (3 experiments), (C) n=4-5 (3 experiments), (D) n=6 (3 experiments), (E) n=6 (3 experiments). * = $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$, ****= $P \leq 0.0001$.

3.3 STRENGTH OF PROXIMAL BCR SIGNALING CORRELATES WITH IGD/IGM EXPRESSION IN BOTH MEMORY AND NAÏVE B CELLS

While no statistically significant difference among B7-Family subsets was observed in either Syk or PLCγ2 phosphorylation, within each MBC population we observed bimodal signals in the phosphorylation of all three signaling molecules (Syk, PLCγ2 and S6) (Figure 4 A). Further investigation revealed that the extent of pSyk generated corresponds with the expression of surface IgD and IgM. MBCs with high expression of IgD (IgD^{Hi}) had low levels of Syk phosphorylation after BCR cross linking, while those MBCs with low expression of IgD (IgD^{Lo}) had significantly higher levels of pSyk (Figure 4 B). This pattern of correlation between BCR isotypes and pSyk generation was found within each of the B7-family MBC subsets (Figure 4 C, D).

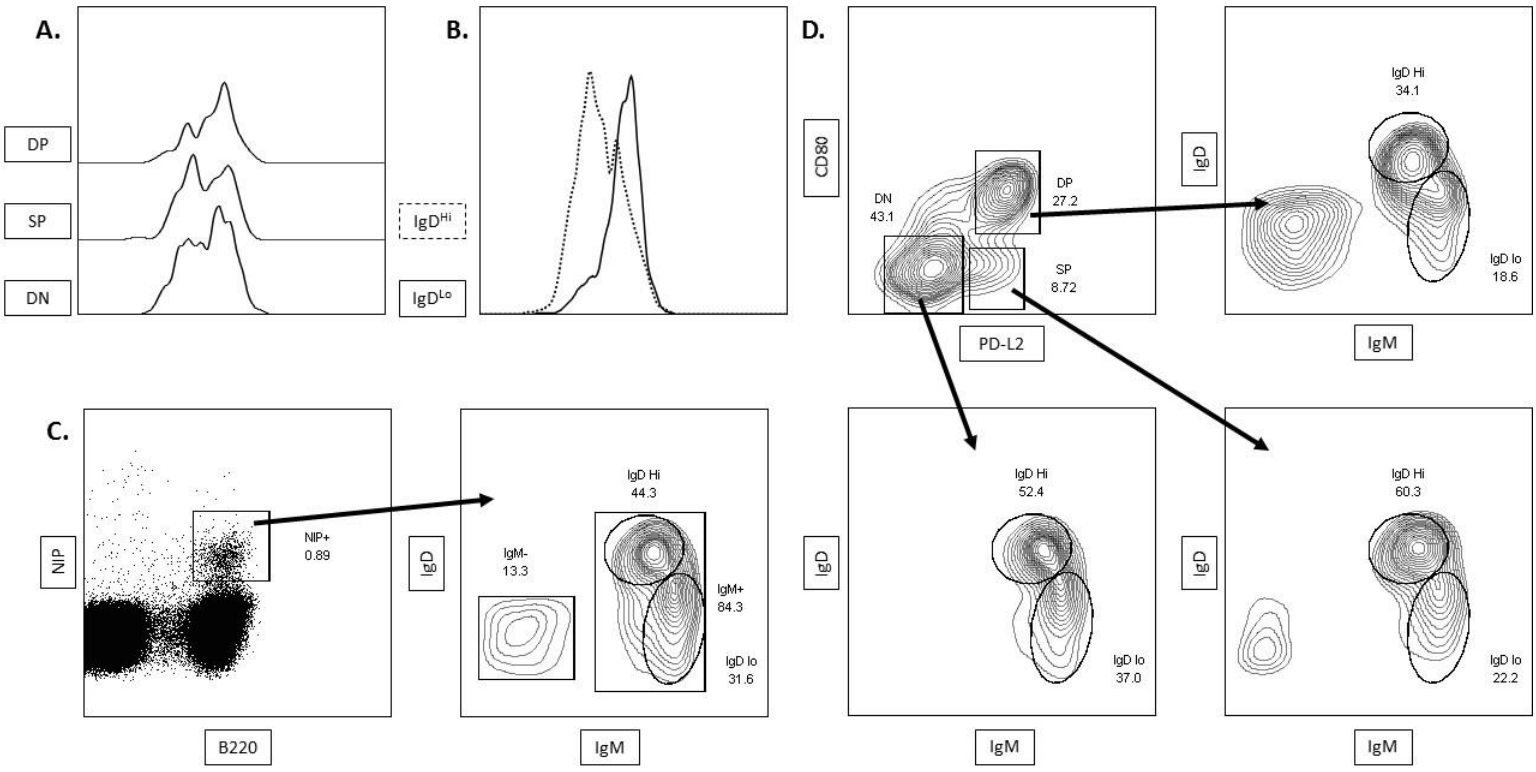

Figure 4: Bimodal pSyk Signals Exist within B7-Family MBC Subsets. *While no statistical significance was found when comparing pSyk levels between B7family MBC subsets, analysis of individual subsets revealed bimodal pSyk signals (A). These signals correlated heavily with the expression of surface IgD, with IgD^{Lo} MBCs demonstrating higher levels of pSyk than IgD^{Hi} cells (B). These IgD-defined subsets exist within all three B7-Family MBC subsets (C, D).*

Given that Syk phosphorylation correlated well with Ig expression (Figure 5A), we assayed MBCs and NBCs (which also possess IgD^{Hi} and IgD^{Lo} populations) for PLCγ2 and S6 phosphorylation in the context of BCR isotype expression in response to BCR crosslinking. We found that, as with Syk, increased PLCγ2 phosphorylation correlated with IgD expression (Figure 5 C), while S6 phosphorylation levels did not (Figure 5 D, E). Additionally, we found that all MBC populations demonstrated significantly higher phosphorylation of all three molecules when compared to NBCs. For molecules involved in proximal signaling (Syk and PLCγ2), cells demonstrated significantly more phosphorylation after stimulation, while, for the distal indicator S6, MBCs had significantly higher basal levels of phosphorylation in addition to stronger pS6 responses after activation. This led us to three conclusions. First, differential expression of IgM and IgD correlates with capacity for proximal BCR signaling. Second, MBCs demonstrate a more vigorous response to BCR cross-linking than their NBC counterparts. Finally, in terms of protein synthesis (as indicated by basal pS6), MBCs may be more primed than NBCs for activation.

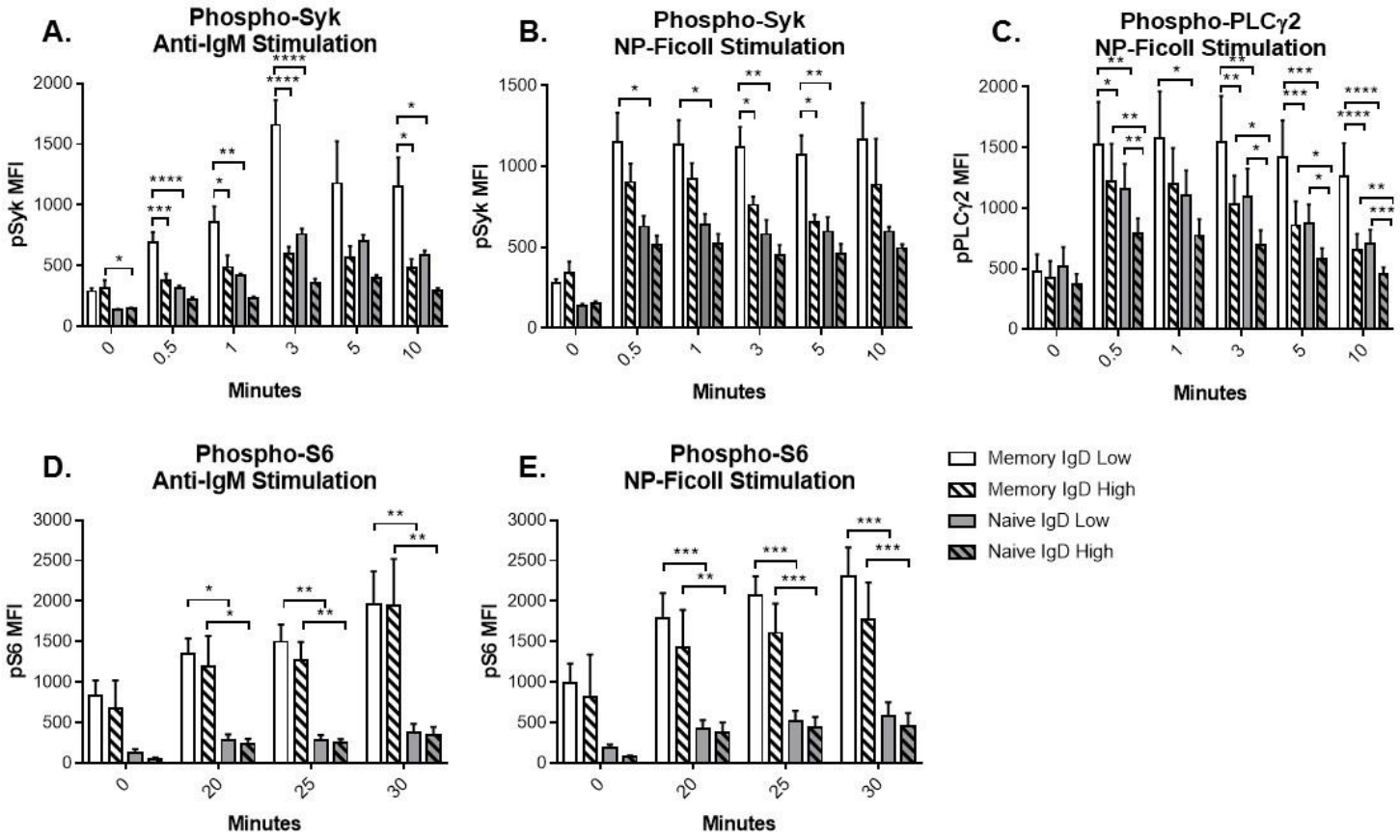

Figure 5: Strength of Proximal, but not Downstream Signaling Correlates with IgD/IgM Expression in Both Memory and Naïve B cells.
*Whole splenocytes were harvested from adoptively transferred AM14-Vκ8R$^{+/-}$ mice and stimulated, depleted of erythrocytes and stimulated with 20μg/ml Anti-IgM (A, D) or 5μg/ml NP-Ficoll (B, C, E). Cells were then fixed to halt BCR signaling, permeabilized and assessed for phosphorylation of BCR pathway molecules Syk (A, B), PLC- γ2 (C) or S6 (D, E). Syk and PLC- γ2 phosphorylation was significantly higher in IgD^{Lo} MBCs when compared to IgD^{Hi} cells (A, B, C). However, bimodality between IgD subsets did not reach statistical significance when comparing pS6 levels (D, E). Additionally, MBCs were significantly enriched in pSyk, PLC-γ2 and pS6 compared to NBCs with similar IgD expression (A, B, C, D, E). Bars represent mean and SEM. (A) n=7-8 (4 experiments), (B) n=6 (3 experiments), (C) n=4-5 (3 experiments), (D) n=6 (3 experiments), (E) n=4 (2 experiments). * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.*

3.4 ENHANCED SYK SIGNALING CAPACITY IN MBCS IS ASSOCIATED WITH INCREASED LEVELS OF SYK PROTEIN AND DECREASED LEVELS OF PHOSPHATASE ACTIVITY

We next wanted to further elucidate mechanisms responsible for enhanced proximal BCR signal in MBCs. Among MBC subsets there was little difference total Syk content (Figure 6A). However, MBCs possess significantly higher levels of Syk protein compared to NBC (Figure 6B). This suggests that the lower Syk signaling observed in NBCs comes in part due to lower maximum signaling capacity as reflected by the total amount of Syk.

Hydrogen peroxide (H_2O_2) has previously been shown to initiate intracellular signaling by deactivating phosphatase activity and allowing basal kinase activity to drive phosphorylation [38, 39]. Both maximal phosphorylation of signaling molecules and the amount of phosphatase activity that must be overcome to reach that maximum can be inferred via titration of H_2O_2 stimulation. To investigate this, MBCs and NBCs were stimulated with increasing concentrations of H_2O_2 and Syk phosphorylation within individual MBC and NBC subsets was compared. As with BCR cross-linking, H_2O_2 stimulation resulted in no significant differences in maximum pSyk signaling among MBC B7-Family subsets (Figure 6C). There was also no statistically significant difference between memory or naïve B cell subpopulations defined as IgD^{Hi} or IgD^{Lo} (Figure 6D).

Despite no significant differences among MBC or NBC subsets, there was a striking difference between maximum pSyk signal in MBCs compared to NBCs. Upon stimulation with H_2O_2, MBCs had between three and four times the Syk phosphorylation levels of their naïve counterparts (Figure 5D). Furthermore, NBCs required twice the concentration of H_2O_2 to achieve maximum pSyk signal when compared to MBCs, suggesting that NBCs must overcome significantly more phosphatase activity to achieve their more-limited signaling potential. Taken together, it appears that the BCR pathway in MBCs of all subsets has been re-wired at several levels to be more responsive to BCR stimulation.

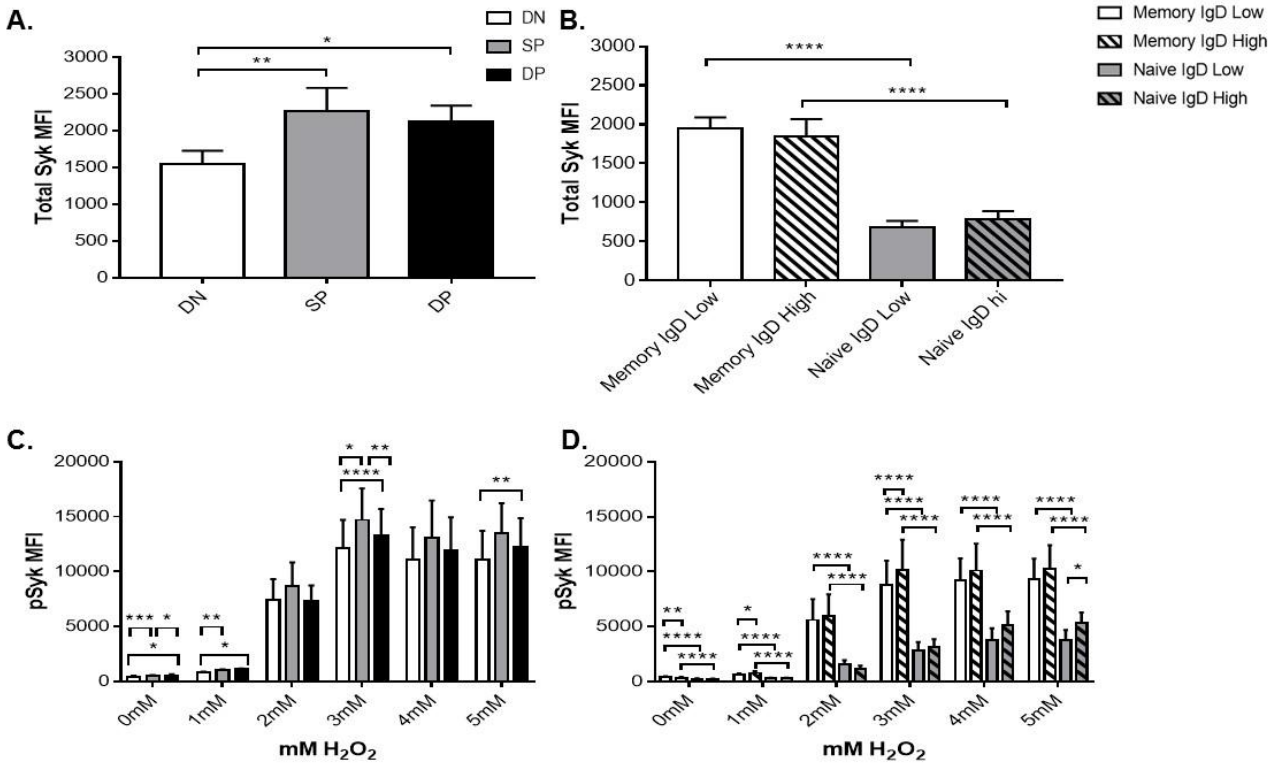

Figure 6: Enhanced Syk Signaling Capacity in MBCs is Associated with Increased levels of Syk Protein and Decreased Levels of Phosphatase Activity. *To elucidate the mechanisms responsible for increased Syk phosphorylation observed in IgD^{Lo} B cell subsets and MBCs, total Syk protein was analyzed via flow cytometry (A, B). As with Syk phosphorylation, no significant difference was found between B7-Family subsets (A). However, no statistically significant differences in total Syk protein were observed when comparing IgD^{Hi} and IgD^{Lo} subsets within the MBC or NBC compartments (B). Where total Syk was found to be different was between MBCs and NBCs, with MBCs having significantly more Syk protein than their naïve counterparts (B). These trends were mirrored in our analysis of total phosphatase activity among B cell subsets (C, D). MBCs and NBCs were stimulated with a titrated concentration of hydrogen peroxide and assayed for Syk phosphorylation as a measure of phosphatase activity. As with total Syk, no difference in phosphatase activity was detected among B7 MBC subsets or IgD subsets (C, D). However, as with total Syk, there was a profound difference in both the intensity of Syk phosphorylation and the and concentration need ed to elicit Syk phosphorylation in MBCs as compared to NBCs. MBCs generated significantly more pSyk upon stimulation with H_2O_2 when compared to NBCs (C). Additionally, while it did not reach significance, MBC activation began at 2mM and peaked at 3mM, while NBCs hit their highest levels of Syk phosphorylation in the given range at 5mM. Bars represent mean and SEM. (A) n=6 (3 experiments), (B) n=6 (3 experiments), (C) n=6 (3 experiments), (D) n=7-9 (4 experiments). * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$.*

4.0 DISCUSSION

4.1 DP MBCS ARE PRIMED FOR RAPID PROTEIN SYNTHESIS

Zuccarnio-Catania et. al [31] have previously shown DP MBCs rapidly differentiate into ASCs upon re-exposure to cognate antigen, while DN cells undergo significant proliferation and re-enter the germinal center reaction. This gradient of function in mirrored by the activation of S6 in these subsets (Figure 2 C, D). DN cells display the least pS6 after stimulation, while DP cells activate S6 the most. As in function, SP MBCs fall in between the other MBC subsets with intermediate amounts of pS6 expression.

Given their propensity to form ASCs, it would make sense that DP MBCs have a heightened capacity to activate S6 upon BCR stimulation. The primary role of ASCs is to produce large quantities of antigen-specific antibodies in response to external threats, a process that would require the upregulation of protein synthesis machinery to facilitate. It should also be noted that DP MBCs have higher levels of pS6 than other MBCs even before stimulation, indicating that these cells may be primed for rapid protein synthesis even in in their resting state.

The presumed higher capacity of DP MBCs to initiate and carry out protein synthesis offers a plausible mechanism to support their bias towards ASC differentiation. We suggest that the biochemical infrastructure already in place in the DP compartment allows them to carry out protein synthesis more easily than other MBC subsets, making the conversion of these cells into ASCs a simpler process. This then may bias the cells towards differentiation and away from re-entry into the GC. Better understanding this process may in turn help us to generate treatments that boost or hinder this differentiation, as antibody production is the most critical function of B cells in disease.

Another implication of higher S6 activation in the DP MBC compartment is the potential for increased AKT signaling in the subset as well. In addition to being a potent regulator of protein synthesis (via mTOR and S6), AKT is a critical regulator of the cell cycle via its interaction with the transcription factor FOXO1 [40-42] (Figure1). By phosphorylating FOXO1, AKT prevents the transcription factor from entering the nucleus and flags it for degradation. This in turn arrests the progression of cell cycle. This feature would be critical for cells wishing to differentiate into long-lived determinate cells (such as DP MBCs differentiating into plasma cells) and would be detrimental to cells needing to undergo rapid proliferation (such as DN MBCs re-entering the GC). Thus, higher AKT signaling (as indicated by increased phosphorylation of S6 protein) may also be responsible for driving the observed functions of B7-Family MBCs in vivo.

4.2 BIMODALITY IN PROXIMAL BCR SIGNALING CORRELATES WITH BCR ISOTYPE EXPRESSION IN MBCS

It is only recently that the B cell memory community has more widely acknowledged the existence of IgM^+ MBCs due to the difficulty inherent in identifying them amongst naïve populations. IgD^+CD27^+ and IgD^-CD27^+ B cells have been previously identified in humans [43-45]. These cells are confirmed to contain somatic V region mutations, demonstrating that they are in fact MBCs [44]. We speculate that our IgD^{hi} and IgD^{lo} MBC populations may correlate with MBC subsets identified in humans.

We chose to study in greater detail IgD subsets among MBCs due observations of bimodality in Syk phosphorylation after Ig cross linking in B7-Family MBC subsets (Figure 3). This bimodality was found to correlate with the expression of surface IgD, with IgD^{hi} MBCs generating comparatively lower pSyk after activation than those cells that expressed little IgD (IgD^{lo}) (Figure 4 A, B). This bimodality is also propagated down the signaling pathway, where it is reflected in PLCγ2 activation (Figure 4 C).

We propose that the presence of surface IgD on cells is directly responsible for the observed differences in signaling, as opposed to merely being a correlate. IgD class B cell receptors have previously been found to have lower signaling capacity than that of IgM [46]. This is primarily due to the long hinge region of the BCR which, when grafted to an IgM heavy chain, significantly lowers the ability of said IgM to activate proximal and downstream signaling [47]. It has been suggested that IgD's role is to dampen extreme IgM signaling by interfering with minimum IgM clustering, thereby preventing apoptosis in the absence of T cell help [48]. Given this structural and functional dampening of BCR signaling, it is highly likely that high surface expression of IgD is responsible for the weaker activation of Syk and PLCγ2 observed in IgD^{hi} MBCs and NBCs.

IgD^{lo} IgM^{hi} B cells in the naïve compartment are typically located in the marginal zone (MZ), while IgD^{hi} IgM^{mid} B cells are found in the B cell follicles [49]. MZ B cells have been previously shown to generate effector cells more quickly and efficiently than follicular (FO) B cells [50-53]. It has been demonstrated that this effect is due to quicker, stronger phosphorylation of Syk and PLC-γ2 in MZ cells and the transmission of these signals downstream [54]. MZ B cells are also heavily implicated in T-cell independent (TI) humoral responses [55]. It is well documented that TI B cell responses are capable of generating MBCs [55-57], and it is possible that these MZ/FO NBC subsets are the precursors for the MBC IgD subsets we observed, respectively. If this is correct and some of the functions of their naïve precursors are carried into the MBCs, there may be functional differences tied to the distinct signaling patterns observed between MBC IgD subsets.

The rapid, strong BCR signaling observed in IgD^{lo} MBCs may be significant enough to induce rapid ASC formation without T-cell support. While it has been shown that T-cell help is critical for many secondary humoral responses [58, 59], TI activation of MBCs has been reported as well [60, 61]. This indicates to us the possibility of MBC subsets generated for the express purpose of rapid, TI responses upon re-exposure to cognate antigen. Cumulatively, these results suggest the existence of previously unknown functional subsets within the MBC compartment and may have implications in the generation and activation of MBCs.

4.3 BCR SIGNALING IN MBCS IS "RE-WIRED" FOR RAPID, MORE ROBUST SIGNAL PROPAGATION AND PROTEIN SYNTHESIS

For decades, the dogma surrounding immunological memory has been clear: memory cells react far faster and with greater potency than their naïve counterparts. B cell memory is no exception to this [7, 62]. However, due to the difficulty present in identifying and isolating MBCs, little progress has been made towards understanding the biochemical mechanisms responsible for this rapid activation. We found that MBCs signal much more strongly than NBCs in both proximal and distal BCR signaling. The comparative strength of these early signals may partially explain the ability of MBCs to respond to pathogens or other stimuli more quickly than do NBCs. This heightened signaling capacity is due to increased levels of the signaling molecules themselves (specifically Syk), suggesting that the BCR pathway is "re-wired" to prepare MBCs for rapid activation. The additional phosphatase activity that must be overcome by naïve cells to reach their more limited maximal signaling capacity also speaks to this re-wiring.

While all MBCs are not created equal in terms of S6 activation (see Section 4.1), MBCs display significantly higher basal pS6 than NBCs. This suggests that MBCs are not only more adept at propagating BCR signals via Syk and PLCγ2, but are primed for increased protein synthesis. This priming may explain in part the ability of MBCs to re-enter the germinal center reaction and differentiate into ASCs more rapidly than antigen-naïve cells.

In a recent paper, Moens et. al [63] examined similar phospho-proteins involved in BCR activation in human NBC and MBC (the latter as defined by expression of CD27). They found, as we did, that S6 activation was significantly higher in MBCs than in NBCs. However, in their experiments, Syk and PLCγ2 phosphorylation were found not to have significant differences between MBCs and NBCs. It is possible that their data did not find significance between early signaling in MBCs versus NBCs because the IgD subsets we describe were not separated. This would result in the IgD^{hi} population dragging down the average MFI of the total MBC compartment (as its pSyk and pPLCγ2 MFI is only marginally higher than that of naïve IgD^{hi} cells). Already their

data showed trends towards higher proximal signaling in MBCs, and we speculate that this trend would be intensified if there were distinction of IgD subsets. Additionally, we found that IgD subsets had no correlation with S6 phosphorylation, which may explain why Moens et. al found significant differences between MBCs and NBCs despite not singling out these subsets. Finally, in a somewhat contradictory finding (given that they failed to observe increased signaling through this pathway), Moens et. al also found PLCγ2 protein levels to be elevated in MBCs. This suggests a possible mechanism for the heightened PLCγ2 signaling we have observed in MBCs, and provides further evidence that MBCs are primed for more robust signaling than NBCs.

5.0 FUTURE DIRECTIONS

Our research has shown significant differences in BCR-mediated signaling between MBCs and NBCs and suggests the existence of additional MBC subsets defined by the expression of surface IgD. We also provided evidence that MBCs produce much stronger proximal BCR signaling than NBCs due to a combination of increased total Syk and lower global phosphatase activity. Finally, we demonstrated MBC B7 subset function may be tied to their ability to activate protein synthesis machinery after stimulation with cognate antigen. The future of this research revolves around further expanding our understanding of the mechanisms responsible for these observations and assaying the function of these new subsets.

Our research revealed significant differences in the activation of protein-synthesis machinery (specifically S6 phosphorylation) among MBC B7-Family subsets and between MBCs and NBCs. In the case of MBCs vs NBCs, we also observed significantly higher pSyk signaling. However, how this signal is transmitted from BCR to ribosomal complex (or, in the case of B7-Family subset MBCs, where this differential signaling originates) is still unknown. To elucidate this "black box", it is important to analyze the AKT signaling pathway. AKT is an important mediator of cell growth, proliferation and protein synthesis, and is regarded as a critical component of this arm of BCR signaling (Figure 1). Thus, AKT association analysis may be a powerful tool to identify players involved in this phenomenon. This will involve sorting subsets in both MBCs and NBCs, stimulation with anti-IgM or cognate antigen and lysis. Proteins with phosphorylated AKT consensus sequences will then be isolated via immunoprecipitation. These proteins can be identified via mass spectroscopy, allowing insight into which proteins are associating with- or being modified by- AKT.

While our research has revealed significant differences in Syk signaling between MBCs and NBCs and tied this phenomenon in part to variations in overall phosphatase activity, it is still unknown which specific

phosphatases are responsible for these effects. To determine this, we propose stimulating cells in the presence of increasing concentrations of phosphatase inhibitors for critical BCR pathway phosphatases, such as SHP-1 (inhibitor NSC-87877, ED Millipore) and SHIP (inhibitor 3AC, ED Millipore). This may allow us to determine how active these phosphatases are and what role they play inhibiting BCR signaling in MBCs.

In section 4.2 we proposed that IgD^{Lo} MBCs may be derived from- or act functionally similar to- MZ NBCs, while IgD^{Hi} MBCs may be linked to FO NBCs. Genetic screening of these populations may therefore reveal similarities. It has been established that MBCs are genetically distinct from NBCs and that MBC B7-Family subsets are genetically distinct from one another [26, 31]. Therefore, this experiment would require the isolation of MBCs both in terms of their B7-Family subset designation and surface expression of IgD. These six populations could then be compared to each other and to MZ and FO NBC gene profiles.

Finally, while we have made the case for IgD-defined MBC subsets in mice, we have yet to determine what role these cells play in the generation and maintenance of humoral memory in mice. We propose using a modified version of our adoptive transfer system to determine the functions of these subsets. Using techniques outlined in Zuccarino-Catania's paper [31], we would sort out NIP^{+} MBC and NBC IgD^{Hi} and IgD^{Lo} cells (MBCs from a previous adoptive transfer, NBCs from un-stimulated B18 Jκ KO mice) and transfer them into naïve AM14 $V\kappa8R^{+/1}$ mice. These mice would then be stimulated with NP-CGG and assayed for their ability to generate GCs, ASCs and NIP-specific antibodies. Additionally, the ability of each IgD subset (whether memory or naïve) can be assessed for its ability to generate MBCs of each IgD and B7-Family subset.

APPENDIX

Table 1: Abbreviations

Abbreviation	Definition
ASC	Antibody secreting cell
BCR	B cell receptor
Btk	Burkett's tyrosine kinase
B1-8	mVh186.2 B cell receptor heavy chain
DAG	diacylglycerol
DN	Double negative ($CD80^-$ PD-$L2^-$) memory B cells
DP	Double positive ($CD80^+$ PD-$L2^+$) memory B cells
FO	Follicular
GC	Germinal center
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based action motif
MBC	Memory B cell
MZ	Marginal Zone
NBC	Naïve B cell
NP	nitrophenol
NP-CGG	NP_{33}-chicken γ-globulin (4hydroxy-3-nitrophenyl) acetyl chicken γ-globulin
NP-Ficoll-FITC	NP_{33}-Fluroscein-AECM-Ficoll
PD-1	Programmed Death protein 1
PD-L2	Programmed Death Ligand 2
PLCγ2	1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-2
pPLCγ2	phospho- PLCγ2
pS6	phospho- ribosomal protein S6
pSyk	phospho-Syk
S6	Ribosomal protein S6
SP	Single positive ($CD80^-$ PD-$L2^+$) memory B cells
Syk	Spleen tyrosine kinase
T_{fh}	T-follicular helper cell
Tg	Transgene/transgenic
TI	T-cell independent

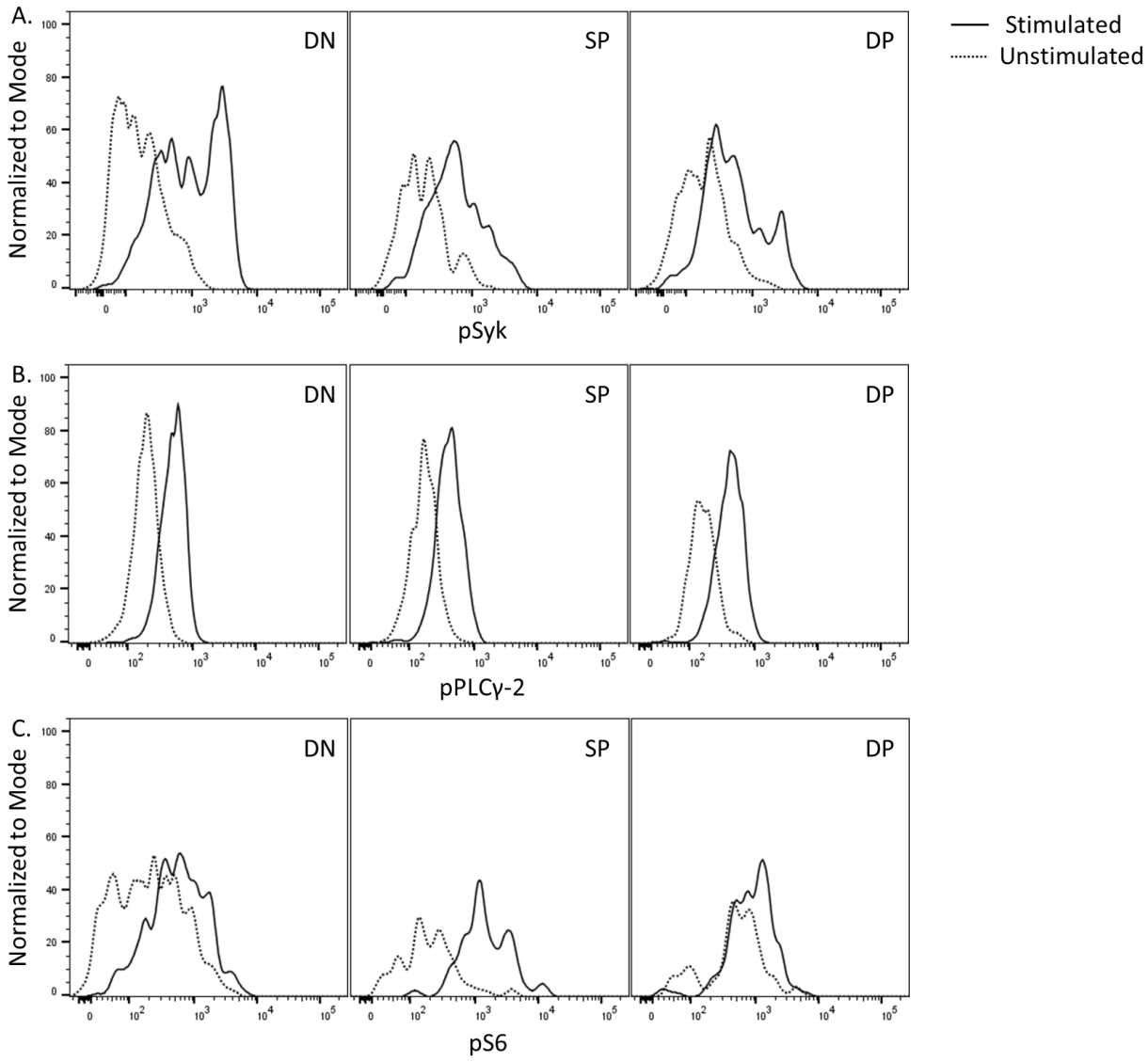

Figure 7: Sample Phospho-Protein Staining in B7-Family MBC Subsets. *Representative histograms of phospho-Syk (A), phospho-PLCγ-2 (B) and phospho-S6 (C) expression before (dotted line) and after (solid line) stimulation with anti-IgM. Cell counts were normalized to the mode.*

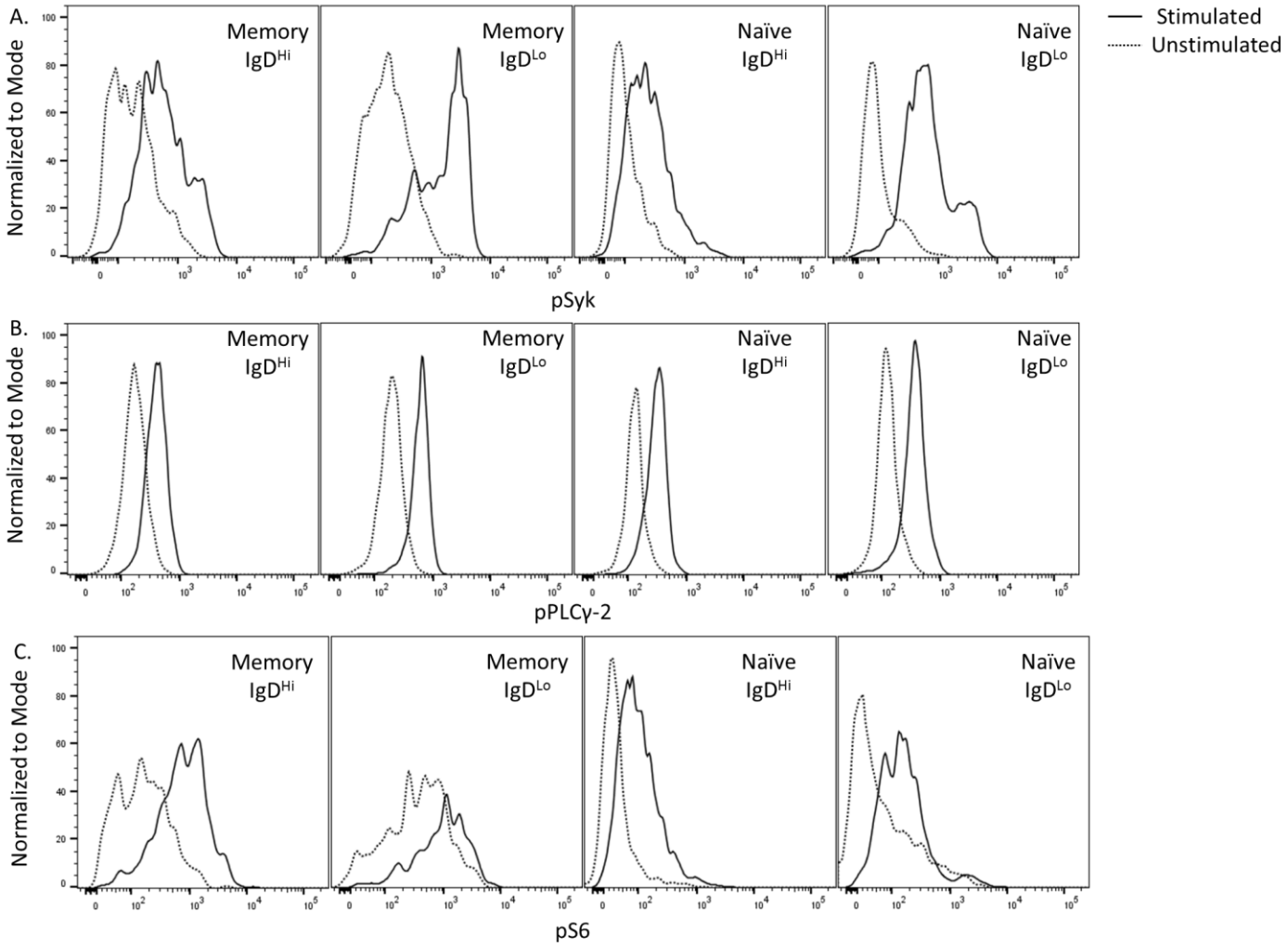

Figure 8: Sample Phospho-Protein Staining in IgD MBC and NBC Subsets. *Representative histograms of phospho-Syk (A), phospho-PLCγ-2 (B) and phospho-S6 (C) expression before (dotted line) and after (solid line) stimulation with anti-IgM. Cell counts were normalized to the mode.*

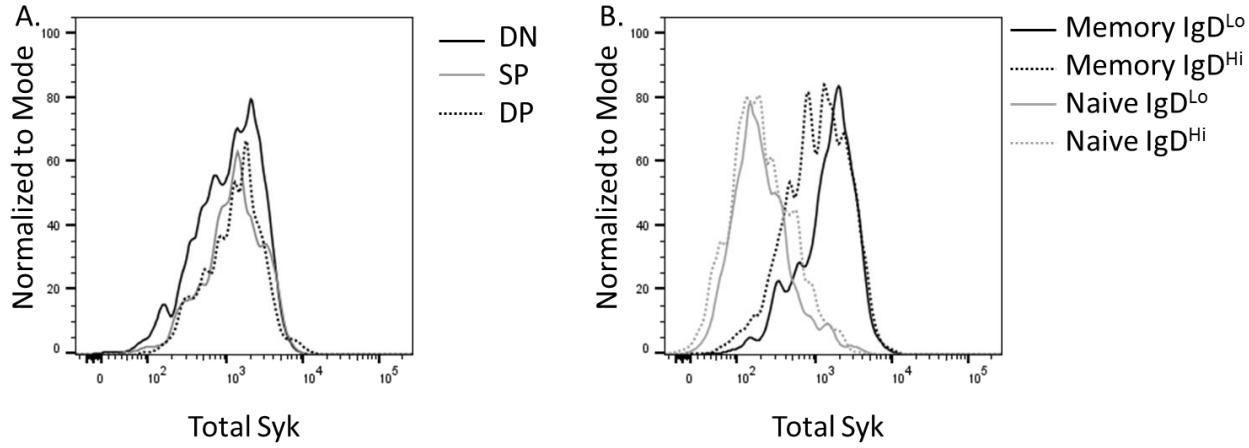

Figure 9: Sample Total Syk Staining in B7-Family and IgD Subsets. *Representative histograms of total Syk in unstimulated B7-Family MBC subsets (A) or IgD MBC and NBC subsets (B). Cell counts were normalized to the mode.*

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