SIGNALING REGULATION OF TRANSITIONAL IMMATURE TO MATURE B CELL DEVELOPMENT

By

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To my Mom and Dad, who have always been there for support and guidance

when I needed them

and

taught me to always follow my heart

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1. BCR→ B cell receptor
2. BTK→ Brutons Tyrosine Kinase
3. CD45-AP→ CD45-Associated Protein
4. DALPC→ Direct Analysis of Large Protein Comlpexes
5. IκB→ Inhibitor of kappa B
6. IKK→ Inhibitor of kappa B Kinase
7. M→ Mature
8. MZ→ Mariginal Zone
9. NF-κB→ Nuclear Factor kappa B
10. PLC-γ2→ Phospholipase C gamma 2
11. PKC-βII→ Protein Kinase C beta II
12. PP2A→ Protein Phosphotase 2A
13. PTP→ Protein Tyrosine Phosphotase
14. SKAP55→ Src Kinase Associated Protein 55
15. T1→ Transitional type 1
16. T2→ Transitional type 2

CHAPTER I

INTRODUCTION

Background and Research Objectives

Significance

The random nature of the B cell antigen diversification results in a substantial population of auto-reactive lymphocytes. To avoid autoimmunity, self-reactive B cell clones are eliminated by negative and positive selection during immature to mature B cell development. Although mutations in the B cell receptor (BCR) signaling components (e.g. Lyn, Syk, Btk, PLC-γ2, etc.)(2,8,11, 13-17) interrupt this transition; the molecular basis for this selection process remains unknown. The focus of these studies is to define the unique signaling molecules recruited to the BCR in transitional B cell subsets after BCR ligation and to characterize the gene expression profiles of T1 versus T2 B cell subsets as a means to determine whether stage specific BCR mediated outcomes in these B cell subsets are controlled by transcriptional and/or posttranslational modification of signaling proteins. Results from these studies will lead to the understanding of the mechanisms that drive negative versus positive selection processes of transitional B cells in the spleen.

B Cell Development

The production of functionally mature B lymphocytes requires the passage of precursor B cells through antigen-independent and antigen-dependent stages of development (Fig. 1). Early B cell development in the bone marrow culminates in the expression of IgM molecules or B cell antigen receptors (BCRs) on the surface of the

immature B cells (18, 19). Of the 10-20 million immature B cells (IgM^+) produced daily in the bone marrow, only 10% reach the periphery; of these, only 10-30% joins the mature B cell pool. Negative selection of self-reactive immature B cells can occur by multiple mechanisms, including deletion, anergy or receptor editing upon antigen encounter (22,31). Emigration of bone marrow derived immature B cells into the spleen as well as subsequent development into mature B cells is reliant upon successful cell surface expression of a functional BCR, as well as the functional biochemical signals emanating from the BCR. Targeted gene deletions of BCR signaling components (e.g. Lyn, Syk, Btk, PLC-γ2, etc.) interfere with the progression of immature to mature for maintenance of the mature B cell subsets (2,8,11, 13-17). Despite the requirement for BCR signaling during immature to mature B cell development, the specific roles and mechanisms for BCR signaling in this process remain poorly understood (21, 29-31). Previous studies have revealed that immature and mature B cell subpopulations display distinct responses to similar stimuli. For example, in response to IgM crosslinking, immature B cells die whereas mature B cells proliferate (2). Splenic immature B cells can be divided into at least two developmental subpopulations, Transitional type 1 (T1) and Transitional type 2 (T2) B cells (8). I hypothesize that T1 B cells, the most recent emigrants from the BM, develop into T2 B cells, which in turn are thought to develop into mature follicular (Fo) and marginal zone (MZ) B cells (8, Fig. 1) We and others have shown that T1 cells die whereas T2 cell survive, proliferate, and display a mature B cell phenotype following BCR engagement (2, 8-11). These observations suggest that the BCR may deliver distinct stage-specific signals within the immature B cell subsets that result in biologically distinct outcomes which in turn are indispensable for the generation of non-autoreactive mature B lymphocytes. However, the nature of these stage-specific signals is not completely understood.

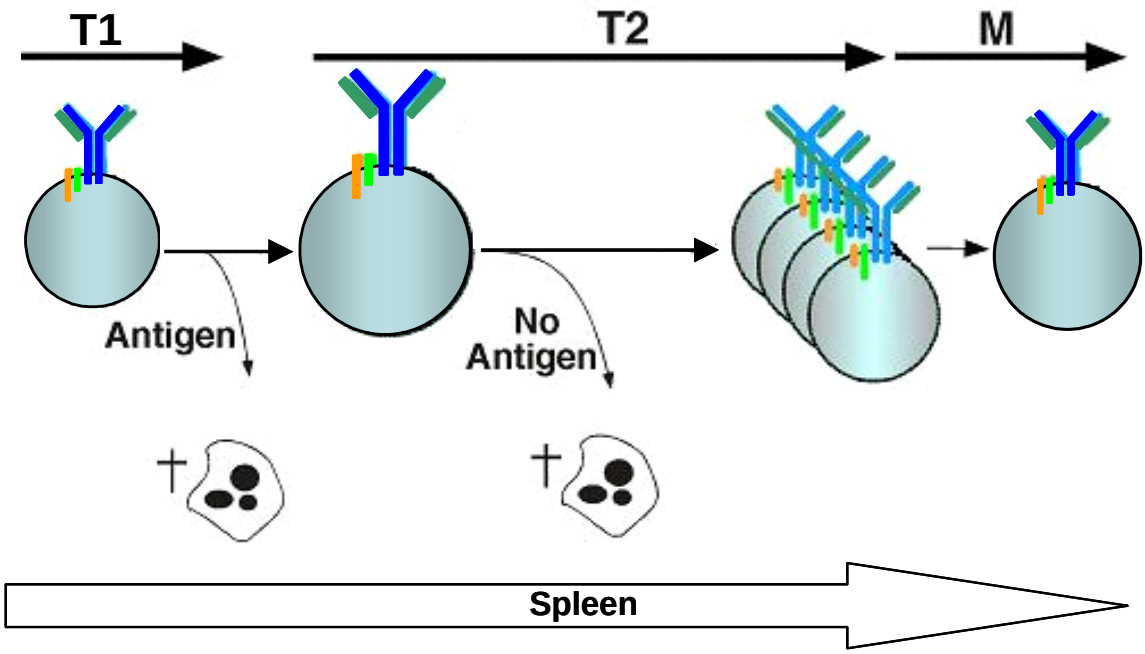

Figure 1. A Model for Splenic B cell Development.

BCR signaling and the BCR Signalosome

The BCR is a multi-protein receptor containing membrane immunoglobulin (mIgM), composed of a heavy and light chain that form the antigen binding pocket. The mIgM is associated with signal transducing chains Igα and Igβ (CD79a and CD79b), both of which contain one immunoreceptor tyrosine activation motif (ITAM) in their cytoplasmic tail (35, 36). The first membrane proximal event after BCR aggregation is activation of the Src family kinase Lyn, which is constitutively associated with lipid microdomains. Activated Lyn then phosphorylates ITAMs within the Igα and Igβ chains. In turn, the tyrosine phosphorylated ITAMs propagate signals via the recruitment and activation of cytoplasmic protein tyrosine kinases (PTKs) including Syk, Bruton's tyrosine kinase (Btk), Fyn and Blk (35). These kinases initiate the downstream signaling events that recruit adapter proteins BLNK (B cell linker protein), Grb2, guanine exchange factors (Vav1/2/3) and lipid metabolizers, PI3K (phosphatidylinositol 3 kinase) and PLC-γ2. Activation of PLC-γ2 results in the hydrolysis of phosphatidyl inositol 4,5 biphosphate (PIP2) to the lipid second messenger's diacylglycerol (DAG), which activates the serine/threonine kinase PKC, and inositol 3,4,5, triphosphate (IP3), which regulates intracellular calcium fluxes from endoplasmic reticulum. Together these molecules contribute to the formation of a large protein complex termed the BCR signalosome (15, 25, 28, 39). The biological significance of many of these signaling proteins including BTK, PLC-γ2 and PI3K in T2 to mature B cell transition have been demonstrated (3, 25, 28). Most of these, with the exception of Syk and Lyn appear to be dispensable for T1 to T2 cell development. This could either suggest that, signaling in T1 cells is quantitatively different from T2 cells and/or that the biochemical signals discharged from the BCR in T1 versus T2 cells are coupled differentially to the downstream effectors by the presence or absence of distinct components of the BCR signalosomes in the two B cell subsets.

Lipid Rafts and BCR Signaling

Current studies clearly establish that multiple signaling pathways operate in BCR mediated B cell survival and development. However, the mechanisms utilized by these signaling pathways in specific transitional B cell subsets remain undefined. One mechanism involves recruitment of the BCR and signaling proteins to the plasma membrane. The segregation of proteins and lipids in the plasma membrane is a key feature of antigen receptor signal transduction. Ordered membrane domains, termed lipid rafts, contain sphingolipids such as sphingomyelin, ganglioside GM1 and cholesterol (40, 41). Lipid rafts may serve as signal transduction platforms for the assembly of BCR signaling proteins (10, 41, 42,43). In resting cells the BCR is excluded from lipid rafts. Upon antigen encounter or BCR cross-linking, the receptor translocates into lipid rafts where the signal transducing molecules Igα and Igβ are phosphorylated by Lyn, which is constitutively associated with lipid rafts. In turn, recruitment of various signaling proteins responsible for the propagation of downstream signaling also takes place in lipid rafts. As such, recent studies have used membrane fractionation to identify signaling proteins in lipid rafts or confocal microscopy to show direct evidence of co-localization of the BCR with signaling proteins (40-43).

BCR Signaling NF-κB and Survival Genes

One of the main downstream targets of BCR signaling is the activation of a family of transcription factors known as NF-κB. NF-κB regulates survival, development and B cell activation. Upon activation of B cells by BCR engagement, dimeric NF-κB transcription factors translocate to the nucleus and regulate the expression of multiple cellular genes. There are two pathways that activate NF-κB, one is known as the classical pathway and the other is the alternative pathway. These pathways target five NF-κB subunits, which include c-Rel, RelA (p65), RelB, NF-κB1 (p105/p50) and NF-κB2

(p100/p52). The alternative pathway targets p100 by inducing its phosphorylation and leads to it's processing into p52. The classical pathway targets p50: RelA, p50: c-Rel or homodimers of these subunits. The activity of NF-κB through the classical pathway is primarily regulated by interactions with inhibitory IκB proteins. There are several IκB proteins, these include, IκBα, IκBβ, IκBγ, IκBε and IκBζ. This interaction blocks the ability of NF-κB to bind to DNA and results in the NF-κB complex being primarily in the cytoplasm due to a strong nuclear export signal in IκBs. It is known that BCR engagement only targets the classical pathway (3). BCR signals that lead to activation of NF-κB converge on the activation of a high molecular weight complex that contains serine-specific IκB kinases (IKKs). Lymphocytes contain at least three major distinct subunits: IKKα, IKKβ and IKKγ. In the classical pathway, activation of the IKK complex leads to the phosphorylation by IKKβ of IκBα, which targets IκBα for ubiquitination and degradation by the 26S proteasome allowing the unbound NF-κB to translocate into the nucleus. There are many families of anti- and pro-apoptotic genes. One of the better characterized is the Bcl-2 family, which is known to be a target of NF-κB. The relative expression and activity of various anti-apoptotic and pro-apoptotic Bcl-2 family proteins is a critical determinant of apoptosis sensitivity. Precisely how the functions of the separate subfamilies and individual Bcl-2 proteins are coordinated to control apoptosis is unclear.

Because one of the major differences between T1 and T2 B cells is their differential sensitivity to apoptosis, the studies in my thesis are particularly focused on determining stage specific expression of anti- and pro apoptotic genes and the components of the NF-κB signaling pathway.

CHAPTER II

PROTEOMIC ANALYSIS OF BCR SIGNALOSOMES IN T1, T2 AND MATURE B CELLS

Abstract

Our lab has shown that relative to T1, T2 cells more robustly activate certain signaling pathways under BCR control such as PLC-γ2/DAG, Ras/ERK and PI3K/Akt. These data suggest that T1 cells activate different signaling pathways compared to T2 cells. I hypothesize that BCR engagement recruits distinct signalosomes in T1 versus T2 B cells and leads to disparate biological responses in the two subsets. To test this hypothesis, I have investigated the composition of the BCR signalosomes recruited to lipid rafts isolated from T1, T2 and mature B cells using immunoblotting and mass spectrometric analyses. These studies identified the serine/threonine phosphatase PP2A, the CD45 associated protein (CD45-AP), and a never before identified protein in B cells, Src Kinase Associated Phosphoprotein (SKAP55). These molecules were identified selectively in lipid rafts isolated from T1 cells by a proteomics approach. I employed conventional biochemical analysis to verify these results with the longterm goal of defining the function of these proteins in peripheral B cell differentiation.

Introduction

Our current model to explain the disparate biological outcomes in T1 and T2 B cells is that differences in the composition of the BCR signalosomes activate distinct downstream signaling pathways and their target genes. Our lab has shown that T2 cells robustly induce the phosphorylation of ERK 1/2 and AKT in comparison to T1 cells. My long-term goal is to understand the molecular mechanisms controlling signal-induced apoptosis in T1 B cells and survival and differentiation in T2 B cells. The specific nature of these different BCR signaling mechanisms remains uncharacterized. Preliminary results demonstrating that T1 and T2 B cell populations respond distinctly to BCR engagement (Fig. 1)(2), support my hypothesis that the distinct biological outcomes following BCR signaling in T1 versus T2 cells results from alterations in the composition of the BCR signalosome. Activation of BCR induced signaling occurs primarily by protein-protein interactions and their membrane localization in proximity of the BCR and their substrates. One major mechanism of BCR signaling involves recruitment of BCR and the signaling proteins into detergent resistant membrane microdomians or lipid rafts (40-42). Therefore, I have examined the BCR signaling proteins that are recruited to lipid rafts in response to BCR crosslinking in transitional and mature B cell subsets.

Materials and methods

Mice

Dr. A. Takeda from Northwestern University graciously provided *CD45-AP*$^{-/-}$ mice. Cells used for B cell purification of subsets were from C57Bl6 mice. All mice that were used as the source of splenocytes were treated humanely in accordance with federal and state government guidelines, and their use was approved by the Institutional Animal Care and Use Committee (Vanderbilt University).

Cells and Isolation of Pure T1, T2 and Mature B cells

Primary B lymphocytes were isolated from spleens of C57Bl6 mice and purified by AutoMACS (Miltenyi Biotec) by a negative selection protocol as described previously **(29, 30).** The purity of B cells isolated in this manner was approximately 90–95% as verified by FACS analysis using anti-B220 and anti-IgM antibodies (Pharmingen, USA). To further separate the cells into purified subsets, Total splenocytes were stained using magnetic beads labeled with anti- AA4.1 and positively selected by AutoMACS (Miltentyi Biotech.) AA4.1 positive cells were then stained with FITC-conjugated anti-HSA (CD24), and biotinylated anti-CD23 (revealed by Cy-chrome-labeled streptavidin) antibodies to sort T1 ($CD23^{low}$/- HSA^{hi}) and T2 ($CD23^{Hi}$ HSA^{hi}). Cells were sorted on a FACStar^{TM} fluorescent cell sorter (Becton Dickenson). Re-analysis of the sorted B cell subsets by FCM revealed high purity for T1 (94%); T2 (77%) (Fig. 2) and mature (96.5%, data not shown) B cells. Mature B cells were sorted by CD43 depletion as described previously of the AA4.1 negative fraction, followed by an additional CD23 depletion by AutoMACS (Miltenyi Biotec) by a negative selection to remove and MZ B cells. Anti-IgM was not used for sorting to prevent inadvertent triggering of BCR signals. This protocol for B cell purification yields sufficient numbers of transitional and mature B cells to perform multiple functional and biochemical experiments described in the following sections. We routinely obtain ~$1x10^7$ T1 and 1-$2x10^7$ T2 B cells by FACS sorting.

Lipid Raft Isolation

Primary B-lymphocytes or purified T1, T2 and mature cells were stimulated with 20 μg/ml of anti-IgM (Jackson Laboratories) for 5 min or left unstimulated. Lysates from 2.5-$5x10^6$ cells were prepared in a non-ionic lysis buffer (Tris-HCl: 10 mM, pH 7.5, NaCl: 150 mM, Triton X-100: 1%, EDTA: 5 mM, PMSF: 1 mM, Aprotinin 1 μg/ml, Na_3VO_4:

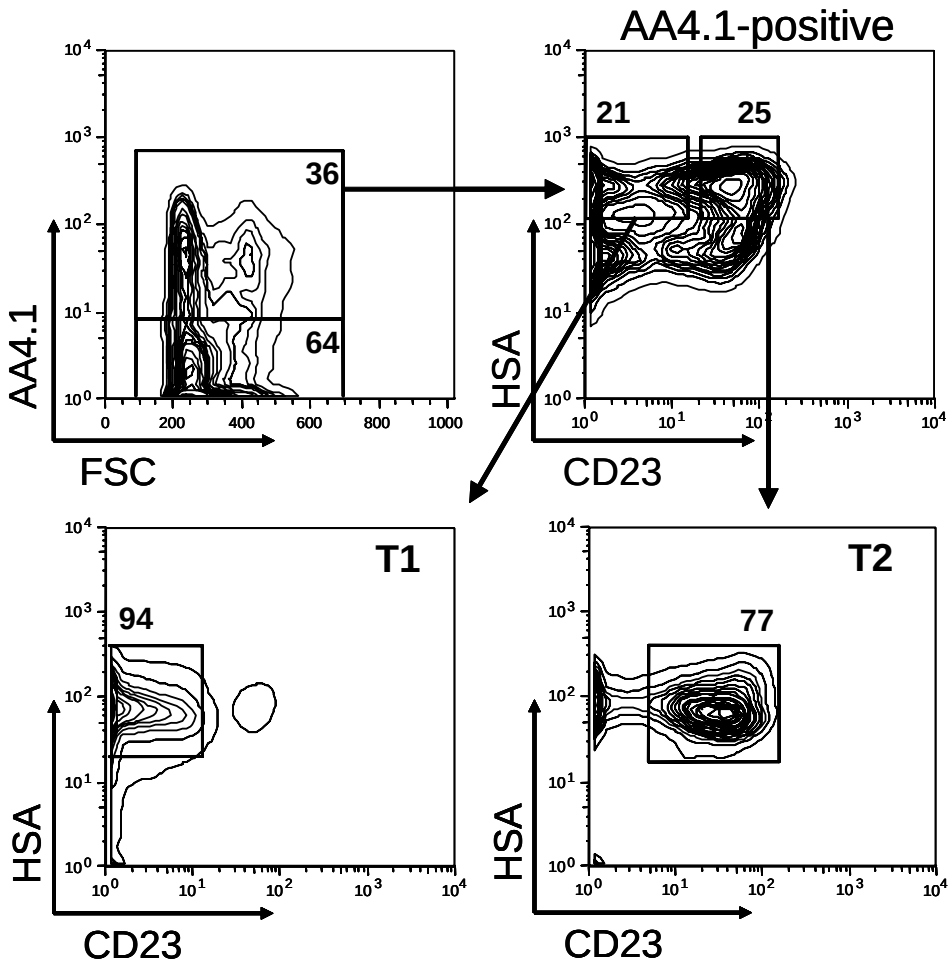

Figure 2. Purification of T1, T2 and M B cell populations. Splenocytes from 4 week old C57BL/6 nice were depleted of red blood cells and stained using anti-AA4.1, anti-CD23, and anti-HSA. The left top panel depicts the sorting gates used to fractionate AA4.1 positive from AA4.1 negative cells. The proportion of total cells analyzed within the gates is indicated. Only live cells were sorted. The upper left plot shows the gates used to sort T1 and T2 cells based on CD23 expression. The lower 2 plots depict re-analysis of the indicated sorted cell population, with the fraction of all cells that fall within each quadrant as indicated.

1 mM, NaF: 1 mM) for 10 min at 4ºC in 50 μl. Lysates were centrifuged at maximum speed for 10 min to remove cellular debris. The gradient was prepared with Optiprep/ Iodixanol (Nycomed through Pharmacia), which comes as a 60% solution and formed in a 2 ml Sorvall tube. To make the gradient, I prepared a 40% solution of Optiprep,1% TX-100 and a 5% solution of Optiprep, 1% TX-100. Lysates (50 μl) were mixed with 250 μl of 60% optiprep, 1% TX-100 to end up with a final volume of 300 μl of 50% optiprep in the Sorvall centrifuge tube. Next, 1.6 ml of 40% optiprep/TX-100 was layered on the 50% and finally 100 μl of the 5% was layered. The tubes were spun in an ultracentrifuge in an RP55A rotor at 55,000 RPM for 3 hours at 4ºC (Fig. 3A). Once centrifuged, 200 μl fractions were collected from each sample and subjected to SDS-PAGE or TCA precipitated to be submitted for proteomic analysis.

Western Blot Analysis

For raft recruitment studies approximately 10% (20 μl) of the isolated fractions in Iodixanol were resolved by 8-10% SDS–PAGE, and transferred on to Immobilon (PVDF, Millipore) membranes. The membranes were then blocked and subsequently probed in solutions containing 1x TBST (Tris-buffered saline and 0.1% Tween) and 5% nonfat dry milk or 3% BSA (Bovine Serum Albumin). The blots were probed with polyclonal antibodies for PKC-βII, PLC-γ2, Lyn and Bcl-2 (Santa Cruz). The $PP2A_C$ monoclonal antibody was from BD Transduction Laboratories and Syk from Cell Signaling Technology. The bound antibodies were revealed by horseradish peroxidase-conjugated goat anti-rabbit, or anti-mouse antibodies (Pierce) and detected by enhanced chemiluminescent detection (Pierce) on autoradiography film.

Immunoprecipations

Primary B lymphocyte lysates from $11x10^6$ cell were prepared in RIPA buffer (Tris-HCl: 50 mM, pH 7.4, NaCl: 150 mM, Triton X-100: 1%, SDS: 0.1%, EDTA: 1 mM, PMSF: 1 mM, Aprotinin 1 μg/ml, Na_3VO_4: 1 mM, NaF: 1 mM) for 10 min at 4ºC. Cell lysates were pre-cleared by incubating with either protein A-Sepharose or G-Sepharose beads for 1 h at 4ºC. The beads were pelleted and cellular extracts removed. Cell extracts were incubated with 2 μg of the PP2Ac monoclonal antibody (Transduction Lab) and protein G-Sepharose or with 2μg of the IKKγ polyclonal antibody (FL-419, Santa Cruz) and protein A-Sepharose for 12-14 h at 4ºC. Cell extracts were also incubated with the corresponding IgG as controls. The beads were pelleted and washed 3 times with ELB buffer. Bound proteins were eluted with SDS sample buffer and subjected to immunoblot analysis by SDS-PAGE**.**

Semiquantitative RT-PCR

100 ng of total RNA from FACS purified T1, T2 and mature B cells was subjected to semi-quantitative RT-PCR to detect transcript levels of SKAP55. RNA from T cells was used as a positive control. The amplified products were analyzed by TBE-polyacrylamide gel followed by ethidium bromide stain. GAPDH was used as a housekeeping gene. The conditions and primer sequences used were:

SKAP55 5' primer: TTGCAGAATGAGAACCTCAGCCCT
SKAP55 3' primer: CCCTCGTCCTGATAATCTGACCAA
GAPDH 5' primer: AACGACCCCTTCATTGAC
GAPDH 3' primer: TCCACGACATACTCAGCAC

94ºC 5'	1 cycle
94ºC 30''	32 cycles
60ºC 30''	
72ºC 1'	
72ºC 7'	1 cycle

Mass Spectrometric Analysis

MS analysis of lipid rafts was performed as previously described (48).

Results

Microscale Analysis of Lipid Rafts

Lipid rafts are thought to serve as platforms for the recruitment and formation of signaling complexes upon BCR engagement. One major objective of these studies is to determine the components of BCR signalosomes in the context of lipid rafts from highly purified T1, T2 and mature B cells. Due to relatively small numbers of T1 and T2 cells that can be purified using FACS, I optimized lipid raft preparation and analysis protocol that allowed proteomics and Western blotting analyses. These experiments utilize 2.0 ml tubes and centrifugation in a gradient in a Sorvall RP55A rotor. Triton X-100 cell lysates from $5x10^6$ purified B cells were passed through an iodixanol block gradient (50%/40%/5%)(1). To identify lipid raft fractions, 10% of each 0.2 ml fraction was analyzed by Western blot analysis with anti-Lyn antibodies to identify lipid raft fractions. The presence of Lyn revealed that the first two fractions were substantially enriched in lipid rafts (Fig. 3B). I also investigated whether PLC-γ2 and PKC-βII, which are expected to be recruited to lipid rafts upon BCR engagement, can be detected in the raft fractions. Indeed, upon stimulation, PLC-γ2 and PKC-βII as well as IgM are recruited to lipid rafts (Fig. 3C). Prior to proteomics analysis, I confirmed the presence of lipid raft associated proteins. The first two fractions from the iodixanol gradient that were enriched for lipid raft associated proteins were precipitated with TCA, fractionated by SDS-PAGE and silver stained (Fig. 3D). These results demonstrate my ability to enrich for lipid raft associated proteins from as few as $2.5x10^6$ B cells and detect signaling proteins

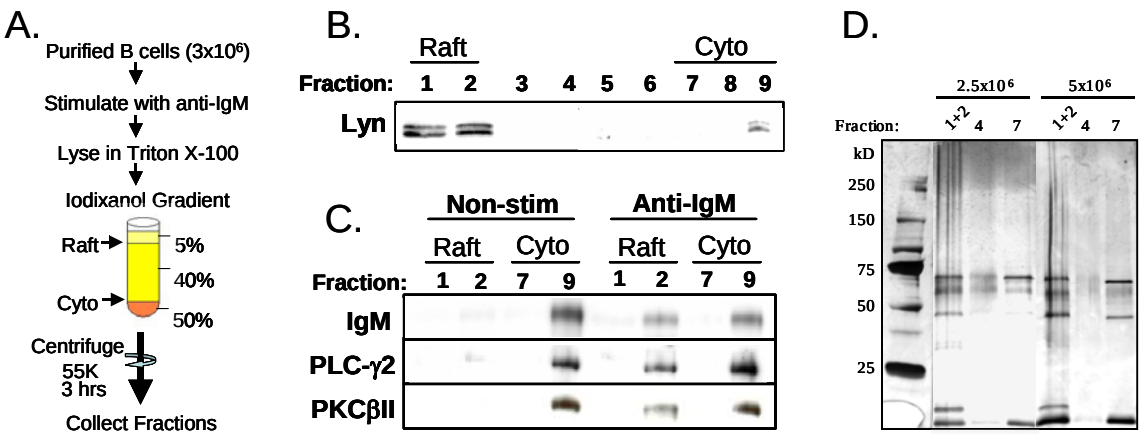

Figure 3. BCR engagement recruits signaling molecules to lipid rafts isolated from a small number of primary B cells. (A) Schematic representation of the experimental design. Lipid rafts were isolated from $5x10^6$ primary enriched total B cells that were stimulated or not with 20 μg/ml of anti-IgM $F(ab')_2$. Cells were lysed in a non-ionic detergent (Triton X-100) and separated by high-speed centrifugation through an iodixanol block gradient to enrich for lipid rafts. Fractions were collected and subjected to SDS-PAGE analysis. **(B)** $1/10^{th}$ of each fraction was separated by SDS-PAGE and analyzed by Western blotting. Detection of Lyn shows enrichment of lipid rafts. **(C)** Representative samples of raft and cytosol fractions were immunoblotted with antibodies for indicated signaling proteins. **(D)** Fractions 1 and 2 were pooled and TCA precipitated, denatured in SDS-PAGE buffer, subjected to SDS-PAGE and silver stained.

recruited to rafts following BCR stimulation. Additionally, these results established the feasibility of my microscale lipid raft preparations for further analyses including mass spectrometry.

Proteins Associated with Cellular Organelles Are Not Detected in Lipid Raft Preparation

My objective is to identify the T1 and T2 specific BCR signaling proteins recruited into lipid rafts. When isolating lipid rafts, one of the concerns that arise is the contamination of non-plasma membranes in the fractions. To address this I used a non-ionic detergent to solubilize the plasma membrane and isolated lipid rafts through fractionation by density gradient ultracentrifugation at a microscale. The purity of raft preparations was determined by assessing the presence of non-raft proteins of lysosomal (LAMP1), Golgi (GM130), ER (GRP78) and mitochondrial (Bcl-2) origin. Results in Fig. 4 show that these non-raft proteins are not detected in raft fractions isolated from either non-stimulated or BCR stimulated cells by immunoblotting. These results demonstrate that my lipid rafts preparations are not contaminated with detectable amounts of non-raft proteins and are therefore suitable for mass spectrometric analysis of known and novel proteins recruited to lipid rafts in response to BCR engagement.

Recruitment of IgM, Syk, PLC-γ2 and PKC-βII in T1 and T2 B Cell Lipid Rafts

It has previously been shown that T1 cells are capable of raft independent signaling and therefore unable to cap upon BCR crosslinking. Since one of my major goals is to identify molecules recruited into lipid rafts upon BCR engagement in each particular subset I wanted to address this concept. Therefore I showed through confocal microscopy that the BCR colocalizes with GM1 in T1 B cells just as well an in T2 B cells (Fig. 5A). To investigate BCR signaling proteins that are known to be recruited into lipid rafts in B cells, I probed lipid raft fractions from T1, T2 and mature B cells for the

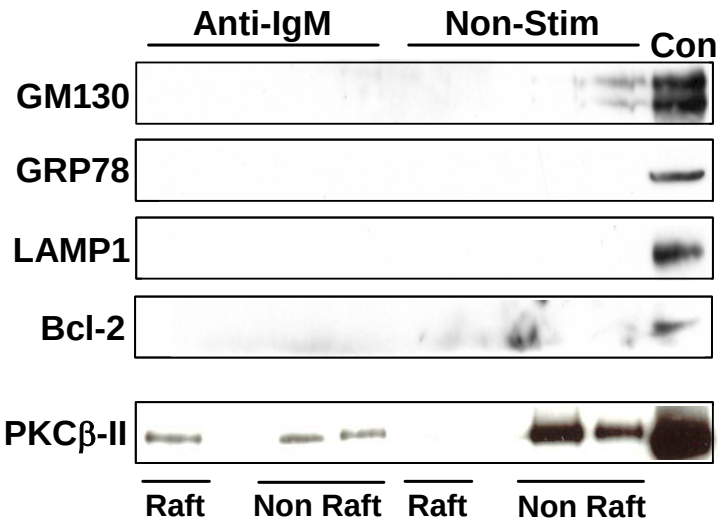

Figure 4. Proteins associated with other cellular organelles are not detected in our lipid raft preparations. Lipid rafts were isolated from $5x10^6$ primary enriched B cells that were stimulated or not with 20 μg/ml of anti-IgM $F(ab')_2$. Cells were lysed in a non-ionic detergent (Triton X-100) and separated by high-speed centrifugation through an iodixanol block gradient to enrich for lipid rafts. Fractions were collected and subjected to SDS-PAGE analysis. $1/10^{th}$ of the raft and non-raft fractions were separated by SDS-PAGE and immunoblotted with antibodies to GM130 (Golgi), GRP78 (ER), LAMP1 (lysosome) and Bcl-2 (mitochondrial) proteins. Fraction 2 was most enriched for lipid rafts as determined by immunoblotting for Lyn and Flotillin-2 (data not shown).

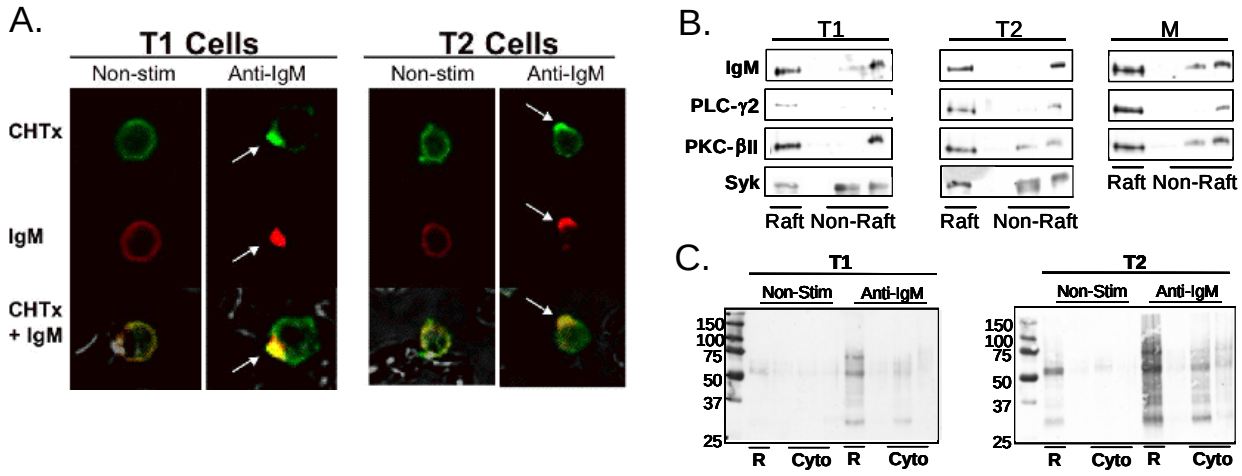

Figure 5. BCR engagement recruits phosphorylated signaling proteins into lipid rafts in T1, T2 and mature B cell subsets. (A) GM1 and BCR colocalize in both T1 and T2 cells upon anti-IgM stimulation. T1, and T2 cells were FACS purified as described in Fig. 4. Cells were stimulated with Cy5-labeled anti-IgM F(ab')$_2$ fragment or vehicle control as indicated. Cells were stimulated for 5 min and stained with FITC-labeled Cholera toxin to visualize lipid rafts. FITC-labeled anti-HSA or anti-CD40 antibodies were used as a negative control for capping (data not shown). Non-stimulated samples were fixed before staining with Cy5-labeled F(ab')$_2$ anti-IgM for 1 h at 4°C, washed twice, and stained with FITC-labeled Cholera toxin. All cells were examined by confocal microscopy using argon and helium-neon lasers and visualized at mid-plane at 100 fold magnification. **(B and C)** Purified T1, T2 and mature B cells (4x10^6) were stimulated for 5 min with 20 μg/ml of anti-IgM F(ab')$_2$. Samples were processed as in Fig. 3. **(B)** 1/10th of raft and cytosolic fractions were separated by SDS-PAGE and immunoblotted with antibodies for indicated proteins or **(C)** anti-pY. Presence of Lyn and Flotillin-2 were used to identify raft-enriched fractions (data not shown).

presence of multiple BCR signaling proteins. Fig. 5B shows that Syk, PLC-γ2 and PKC-βII are recruited to lipid rafts in T1 as well as T2 and mature B cell subsets. Analysis of total protein tyrosine phosphorylated proteins indicates that BCR stimulation leads to anincrease in tyrosine phosphorylated proteins in lipid rafts isolated from T2 compared to T1 cells (Fig. 5C). These results suggest higher BCR signaling activity in T2 versus T1 B cells.

Proteomic Analysis of Proteins in Lipid Rafts in T1, T2 and Mature B cells in response to BCR Engagement

I employed mass spectrometry to identify previously unrecognized proteins specifically in BCR signalosomes that associate with lipid rafts of transitional versus mature B cell subsets. This approach allowed the detection of proteins in an unbiased method from BCR complexes of T1, T2 and mature B cells, thus independently verifying results obtained by Western blot analysis, and allowing the identification of novel proteins. To this end I successfully employed mass spectrometry to identify proteins in lipid rafts isolated from BCR stimulated T1, T2 and mature B cell subsets (Fig. 6 and Table 1).

Lipid rafts were prepared from FACS purified T1, T2 and mature B cells ($3x10^6$), as previously described (2,4) and stimulated with anti-IgM. The enriched fractions for lipid rafts in T1 and T2 B cells were concentrated by TCA precipitation and analyzed by a state of the art proteomics method termed Direct Analysis of Large Protein Complexes, or DALPC (48). The DALPC approach uses multidimensional microcapillary liquid chromatography and tandem mass spectrometry to separate and sequence peptide mixtures. Denatured and reduced proteins are first trypsin digested to generate a mixture of peptides, The mixture is applied to a strong cation exchange chromatography column followed by reverse phase column, resulting in a high-resolution two dimensional

separation of peptides prior to injection to a tandem mass spectrometer. A software algorithm SEQUEST is used to correlate each sequencing spectrum of the fragmented peptides with predicted amino acid sequences in translated genomic and protein databases (50). This software reassembles the most significantly identified peptides into a list of proteins in the original sample (48). The approach allows the unbiased identification of proteins at or below the sensitivity of silver stained gels. A large number of publications have demonstrated that the approach can identify novel components in biological complexes that were missed in earlier studies (48, 52).

In order to profile the composition of proteins in lipid rafts a new software application to display and compare the profiles of proteins in complex mixtures was developed by Dr. Andrew Link. This software is termed BioInformatic Graphical Comparative Analysis Tools (BIGCAT) (50). This new software greatly expands the options and statistical tools for interpreting, analyzing and comparing the composition of proteins in complex mixtures. Using this proteomic approach, I have obtained data from T1, T2 and mature B cell subsets (Fig. 6 and Table 1). Peptide sequences obtained from lipid rafts isolated from anti-IgM stimulated T1, T2 and mature B cells identified 195 distinct proteins in T1, 114 in T2 and 172 proteins in mature B cells. Of these, 91 were unique to T1, 38 were unique to T2 and 70 unique to mature B cells (Fig. 6A). Proteins known to be associated with other cellular organelles, as well as structural proteins were excluded from the analysis. I identified proteins covering several functional classes involved in signal transduction, as well as products of genes only reported as ESTs or considered as hypothetical proteins.

A select set of proteins identified in T1, T2 and mature cells is shown in Table 1. Identification of CD45-associated protein (CD45-AP) and two subunits of protein phosphatase 2A (PP2A), as well as a novel adaptor protein Src Kinase Associated Phosphoprotein (SKAP55) specifically in T1 B cell subsets was particularly intriguing.

CD45 is a receptor-like protein tyrosine phosphatase (PTP), which is critical for B cell development and selection (Nature, 381:325, 1996). The peptide sequence and MS spectrogram that identified this molecule, as well as its domain structure are shown in Figure 6B. A recent report indicates that CD45-AP enhances the phosphatase activity of CD45 by inhibiting its dimerization during T cell activation (Blood, 103:3440, 2004). Consequently, gene deletion of CD45-AP reduces PTP activity of CD45. Recruitment of CD45-AP in response to BCR stimulation preferentially in T1 may therefore increase PTP activity and dephosphorylate BCR induced tyrosine phosphorylation of BCR signaling proteins. This hypothesis is consistent with a reduction in total protein tyrosine phosphorylation in T1 B cells (Fig. 5C)

PP2A is a serine/threonine phosphatase with multiple functions in cellular growth (Blood, 92:539, 1998). It inhibits MAPK and AKT activity and activates pro-apoptotic proteins of the Bcl-2 family. Recruitment of PP2A into lipid rafts together with BCR in T1 cells may contribute to the observed T1 cell death in response to BCR engagement. However, I cannot draw any conclusions until these results are verified in multiple experiments with non-stimulated and BCR stimulated T1, T2 and mature B cell subsets.

Proteomic analysis revealed various unidentified proteins covering several functional classes involved in signal transduction, such as genes only reported as ESTs or by Riken Cell Bank (Japan). A significant number of these proteins contained domains that are known “signatures” of signaling proteins (kinase, SH2, SH3, PH and C1 domains), fulfilling my criteria for selection for further characterization. A database search of a 26 amino acid peptide sequence obtained from BCR stimulated T1 B cells revealed that this peptide sequence was derived from a cDNA clone reported by Riken Cell Bank. The deduced amino acid sequence contained a PH domain and an SH3 domain. Further analysis with the deduced amino acid sequence revealed that this sequence corresponds to Src Kinase Associated Phosphoprotein (SKAP55). SKAP55 is

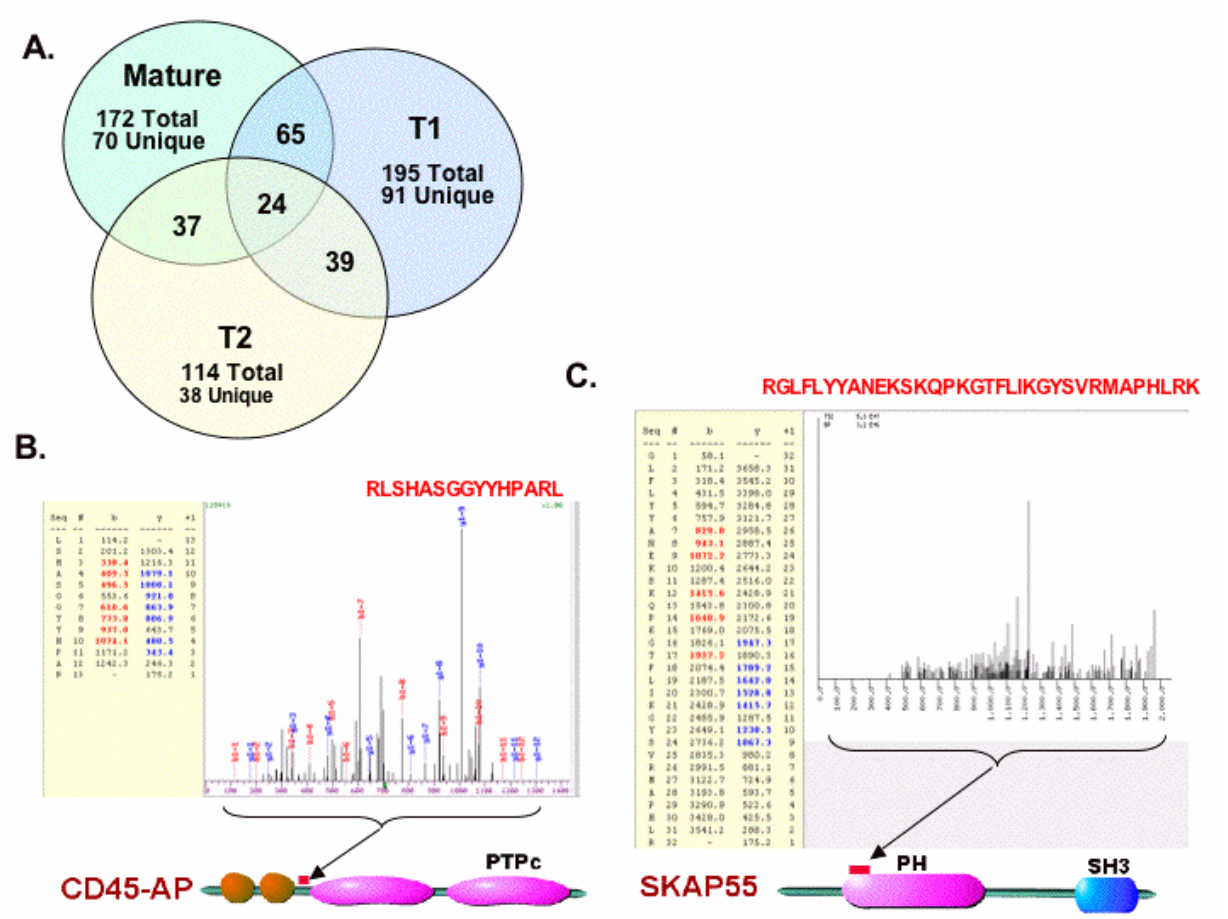

Figure 6. MS analysis of lipid rafts isolated from BCR-stimulated T1, T2 and mature B cell subsets. (A) Comparison of proteins identified in T1, T2 and mature B cells. Peptides obtained from DALPC that identified **(B)** CD45-AP, and **(C)** SKAP55. The MS/MS spectrums which significantly correlated with peptides to CD45-AP and SKAP55 are shown.

Table 1. Select examples of the proteins identified by DALPC in lipid rafts isolated from anti-IgM stimulated T1, T2 and mature (M) B cells

B Cell	Protein Name	Description
T1	PAK2	Activated by CDC42/p21
	Tyrosine-protein Kinase CSK	Tyrosine phosporylates SRC Kinase
	CD45-associated protein	**Interacts with CD45 and augments PTP activity**
	Calmodulin	Regulates enzymes in a Ca2+ dependent manner
	Protein phosphatase 2A, structural A subunit	**Plays a role en cell survival signaling**
	Protein phosphatase 2A, regulatory B subunit (PTPA)	**Plays a role en cell survival signaling**
	Rho GDP-dissociation inhibitor 2	Regulates GDP/GTP dissociation of GDP from Rho proteins
	Rho-related GTP-binding protein RhoC	Regulates receptor induced signal transduction
	GDP binding protein, bete polypeptide 2-like 1	Receptor, Kinase activity
	Proteasome activator complex subunit 2	Required for efficient antigen processing
	SKAP55	**Adapter protein involved in CD45 T-cell signaling**
	CDP-diacylglycerol-inositol 3- phosphatidyltransferase	Phospholipid biosynthesis
T2	Serine/Threonine kinase 10	Ste20 familiy member, involved in cell adhesion and regulates Plk1
	Indian hedgehog	Involed in osteogenesis and epithelial cell differentiation
	Retinoblastoma binding protein 6	May be involved in growth control and differentiation
	Ubiquitin specific proetease 16	ubiquitin-dependent protein catabolism
	CD37	Plays a role in T-B cell activation
	Glycosyltransferase 28 domain containing 1	Lipid glycosylation, carbohydrate binding
M	MAP3K12 binding inhibitory protein 1	Inhibits MAP3K12 activity to induce activation of JNK/SAPK pathway
	Cdc42 homolog	Small GTPase, signal transduction
	Transforming protein RhoA	Regulates receptor induced signal transduction
	Proteasome subunit alpha type 1	Protein Degradation
	Ubiquitin-activating enzyme E1 X	Activates ubiquitin
	Protein tyrosine phosphatase S	Possibly a cell adhesion molecule.
	Inositol 1 (or 4) monophosphatase	Phosphatidylinositide metabolism
	Lymphocyte specific protein 1	Thought to be involved in signal tranduction by calcium binding

a 55 kD protein, which is comprised of a unique N-terminal region, a PH domain, followed by three tyrosine residues and a SH3 domain (Fig. 6C). Studies in human T cell lines have shown that SKAP55 binds to the catalytically active site of CD45.

SKAP55 a substrate of CD45 (67, 73) also binds to Src family kinases (SFKs) via their SH2 domains. Prior studies have also shown that, in T cells, the SH3 domain of SKAP55 binds to the central proline rich region of other adapters SLAP-130 and ADAP, thereby connecting SKAP55 to signaling pathways that control cell adhesion and migration (61). However, SKAP55 function in B cells remains entirely unknown.

CD45-AP Deletion enhances BCR induced Tyrosine Phosphorylation and Affects B Cell Developmental Progression

Through proteomics and mass spectrometry, I identified CD45-AP in lipid rafts isolated from T1 cells. CD45-AP is an adaptor molecule that binds to CD45 and enhances its phosphotase activity by inhibiting the dimerization of CD45 on the cell membrane. Previous studies on CD45-AP deficient (*CD45-AP*$^{-/-}$) mice showed that these mice had a similar phenotype but significantly weaker, to CD45 knockout mice in regards to T cell activation. These studies showed that *CD45-AP*$^{-/-}$ had no apparent effect in B cell development. I wanted to examine whether CD45-AP had an effect on B cell signaling and further characterize its effect on B cell development. Lipid rafts were isolated from *wt* and *CD45-AP*$^{-/-}$ cells as described in materials and methods. Cells were left unstimulated or stimulated with 20 μg/ml of anti-IgM. Fractions were collected, run on SDS-PAGE and probed with 4G10 (anti-phosphotyrosine). What I observed was that *CD45-AP*$^{-/-}$ cells had more detectable tyrosine phosphorylation in lipid rafts as compared to *wt* cells (Fig. 7). Surprisingly, *wt* type cells had more tyrosine phosphorylation in the cytoplasmic fractions than the cytoplasmic fractions of the *CD45-AP*$^{-/-}$ cells (Fig. 7). This

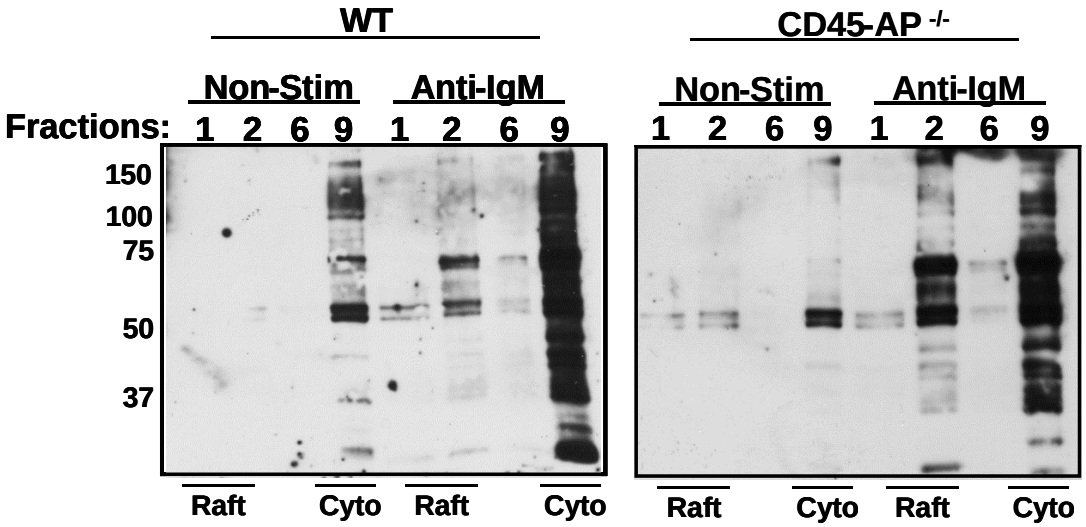

Figure 7. CD45-AP deficiency affects tyrosine phosphorylation in lipid rafts after BCR engagement. Lipid rafts were isolated from $5x10^6$ primary enriched B cells from wt or CD45-AP$^{-/-}$ mice. Cells were stimulated or not with 20 μg/ml of anti-IgM F(ab')$_2$. Cells were lysed in a non-ionic detergent (Triton X-100) and separated by high-speed centrifugation through an iodixanol block gradient to enrich for lipid rafts. Fractions were collected and subjected to SDS-PAGE analysis. 1/10th of the raft and non-raft fractions were separated by SDS-PAGE and immunoblotted with 4G10 (anti-tyrosine phosphorylation).

suggests a possible role for CD45-AP would be to regulate the threshold of CD45 signaling required for B cell activation.

The observed effect of loss of CD45-AP on BCR induced tyrosine phosphorylation led me to investigate if CD45-AP absence affected B cell development. Previous studies did not detect any apparent phenotypic alterations in B cell development in *CD45-AP*$^{-/-}$ mice (68, 70). To determine if B cell development is affected, *wt* and *CD45-AP*$^{-/-}$ cells from two different ages were stained with antibodies to identify populations as previously described. Upon examination of the data, I observed that at 4 weeks, *CD45-AP*$^{-/-}$ mice had a higher percentage of immature cells as compared to *wt* mice (Fig. 8). At 16 weeks *CD45-AP*$^{-/-}$ had a lower percentage of immature B cells when compared to *wt* cells (Fig. 8). This data suggest that loss, CD45-AP may accelerate B cell development, which is only apparent with ageing. Consistent with my hypothesis, I found that at 16 weeks there was an increase in the percentage of marginal zone B cells (16%) when compared to *wt* cells (4%). These data demonstrate that CD45-AP deficiency does affect B cell development in a stage specific manner with age.

Differential Expression of SKAP55 in T1 versus T2 B cell subsets

My mass spectrometry results that SKAP55 is found in lipid rafts of BCR stimulated T1 cells prompted me to investigate the expression of this protein within the two transitional B cell subsets. RT-PCR was performed with primers flanking exon 2 and 5 on the RNA isolated from FACS purified T1 and T2 cells as described (Fig. 2) and from murine thymocytes. As expected, T cells express SKAP55 mRNA (Fig. 9A). SKAP55 mRNA expression was also clearly detected in T1 cells (Fig. 9A). In contrast, PCR amplicon was barely detectable in T2 cells (Fig. 9A). Consistent with mRNA expression profile, SKAP55 protein was also detected in T1 cells (Fig. 9B). This protein was detected in total purified B cells at significantly reduced levels probably due to a small T1

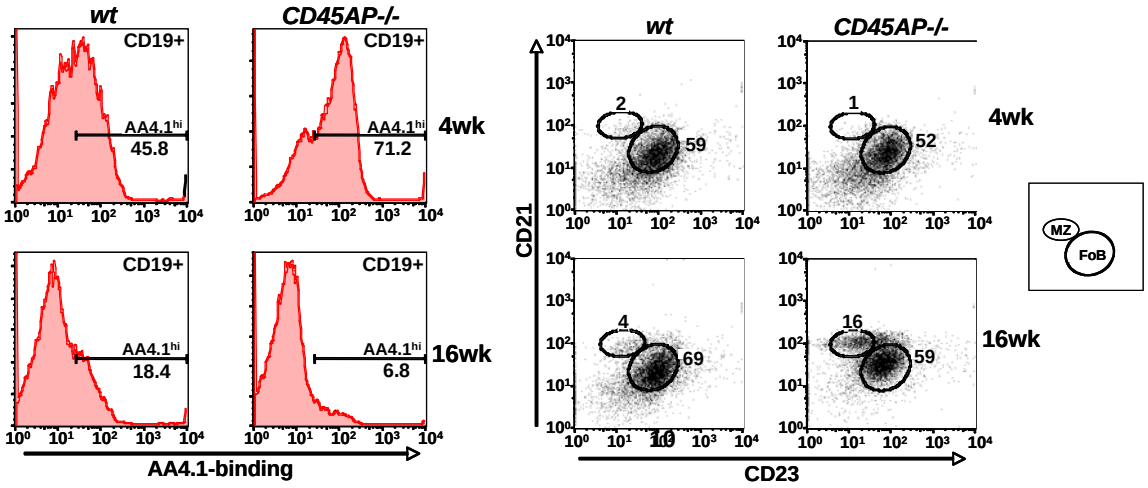

Figure 8. Age-dependent accumulation of transitional Vs. MZ B cells in CD45-AP$^{-/-}$ Mice. Freshly isolated *wt* (1x10^6) and *CD45-AP$^{-/-}$* splenocytes were labeled with the indicated B cell phenotypic markers, and analyzed by FCM. **(A)** CD19+ B cells. Transitional and mature B cell subsets are plotted based on AA4.1 expression. **(B)** CD19+ B cells. Mature (FoB) and marginal zone (MZ) B cell subsets are plotted based on CD23 and CD21 expression.

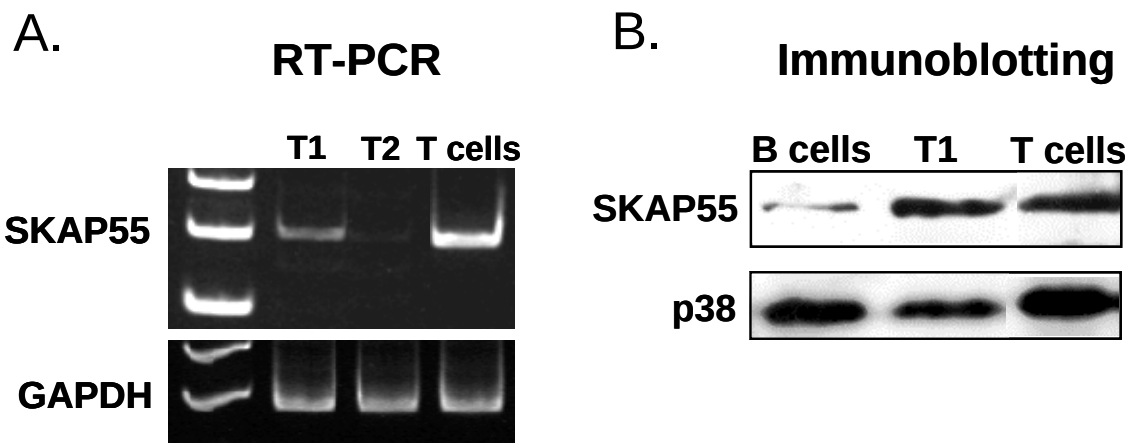

Figure 9. SKAP55 is specifically expressed in T1 cells. (A) SKAP55 mRNA is selectively expressed in T1 but not T2 B cells. Total RNA (100 ng) purified from FACS-sorted T1 and T2 B cells as well as primary T cells (positive control) was subjected to semiquantitative RT-PCR for SKAP55 transcript. The amplified products were analyzed by TBE-polyacrylamide gel followed by ethidium bromide stain. GAPDH was used as a housekeeping gene. **(B)** SKAP55 protein is detected exclusively in T1 cells. Total cell lysates from $1.5x10^6$ primary enriched total B cells; T cells and FACS-sorted T1 B cells were run on SDS-PAGE and immunoblotted with anti-SKAP55. p38 was used as a positive control for protein loading.

cell contribution. These results confirm that SKAP55 is expressed preferentially in the T1 cell subset within the B cell compartment. SKAP55 is known to be expressed in T cells, where it serves as an adapter protein that couples Fyn and other Src kinases to the PTPase of CD45 (73). The expression of SKAP55 preferentially in T1 cells and its known function in T cells clearly warrant investigation of its interactions with Src family kinases in T1 cells, in particular Lyn, and determination of its function in T1 cell selection and maturation process. Together, these results verify the reliability of the proteomic approach to identify molecules involved in stage-specific BCR signaling events.

Recruitment of PP2A Subunits to Lipid Rafts in Total B Cells

Protein phosphotase 2A (PP2A) is a multimeric protein that has been described in multiple signaling pathways involved in cell growth, differentiation and apoptosis among others. Two subunits of PP2A were identified by proteomic analysis in rafts isolated from highly purified T1 B cells (Table 1). To investigate the role that PP2A plays in BCR signaling I verified the recruitment of PP2A subunits to lipid rafts isolated from total B cells. I probed lipid raft fractions from total B cells for the catalytic subunit of PP2A (PP2A/c) and for one of the regulatory subunits (PTPA/PR53) that was identified by proteomic analysis. Fig. 10A shows that PP2A/c and PTPA/PR53 are recruited to lipid rafts upon BCR crosslinking in total B cells. PKC-βII was used as a control to demonstrate recruitment into lipid rafts. Prior studies suggest that PP2A plays a role in NF-κB signaling. In collaboration with Dr. Brian Wadzinski investigated the role of PP2A in NF-κB activation (65). Previous studies suggest a possible interaction between PP2A and the IKK complex. To validate, I performed coimmunoprecipitations to determine if PP2A interacts with the IKK complex. As shown in Fig. 10B, PP2A or IKKγ coimmunoprecipitate with both IKKα and PP2A. Since IKKγ was used for the

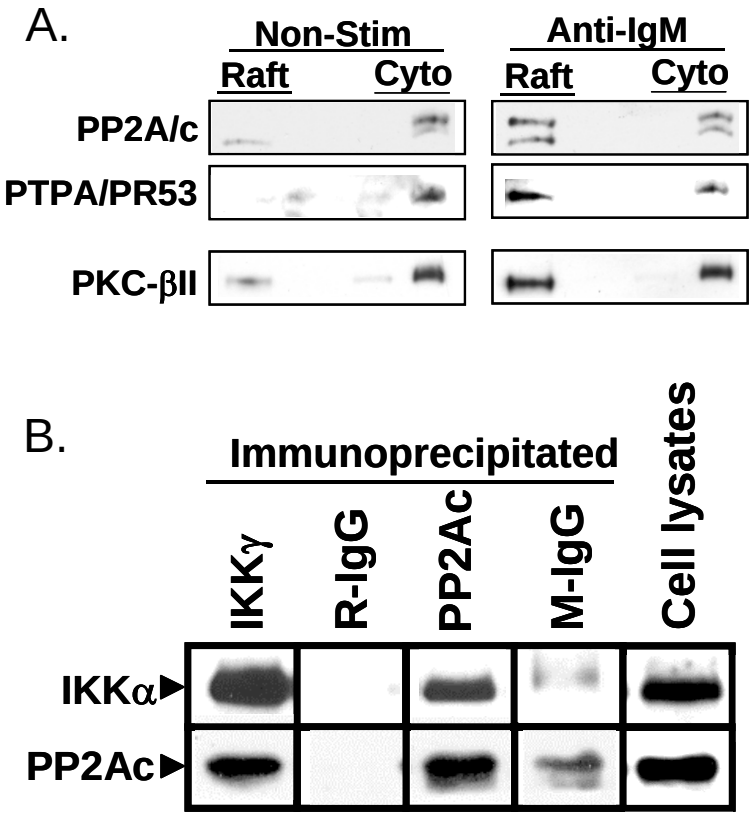

Figure 10. BCR engagement recruits PP2A/c into lipid rafts and PP2A Co-immunoprecipatates of IKK subunits in total B cells. Enriched B cells ($4x10^6$) were stimulated for 5 min with 20 μg/ml of anti-IgM $F(ab')_2$. Samples were processed as in Fig. 3. **(A)** $1/10^{th}$ of raft and cytosolic fractions were separated by SDS-PAGE and immunoblotted with antibodies for the indicated proteins. Presence of Lyn and Flotillin-2 were used to identify raft enriched fractions (data not shown). **(B)** Cell extracts from purified primary B lymphocytes were incubated with the PP2Ac monoclonal antibody (Transduction Lab) and protein G-Sepharose or the IKKγ polyclonal (FL-419) antibody and protein A-Sepharose and the corresponding IgG controls. The resulting immune complexes were washed, eluted with SDS sample buffer and subjected to SDS-PAGE and probed with antibodies against IKKα and PP2Ac.

immunoprecipitation, this suggests that PP2A is associated with the classical NF-κB pathway which is the pathway targeted by BCR engagement. While a function for PP2A/NF-κB pathway in T1 B cells remains to be determined, these results verify the reliability of the proteomic approach to identify molecules involved in stage-specific BCR signaling events.

Discussion

Splenic B cells can be divided into at least three developmental subpopulations, Transitional type 1 (T1), Transitional type 2 (T2) B cells and mature B cells. Previous studies have revealed that T1 and T2 B B cell subpopulations display distinct responses to similar stimuli. For example, in response to IgM crosslinking, T1 B cells die whereas T2 B cells proliferate and differentiate into a mature B cell phenotype. My hypothesis was that the composition of the BCR signalosome is altered in T1 to T2 B cell transition. To test this hypothesis I determined the effects of the BCR stimulation on the translocation of signaling proteins into lipid rafts (Figs. 3C and 5B). My results showed that T1, T2 and mature B cells were capable of recruiting known BCR associated molecules such as IgM, Syk, PLC-γ2 and PKC-βII (Fig. 5B). The ability of all subsets being able to recruit these molecules leads me to examine whether activation of these molecules was occurring to the same extent. When I inspected the levels of tyrosine phosphorylation in lipid rafts isolated from stimulated T1, T2 and mature B cells, I found that T1 cells had less detectable tyrosine phosphorylation when compared to T2 cells. These data suggest that even though all these B cell subsets recruit known major molecules involved in BCR signaling to lipid rafts, they are being differentially regulated, possibly within the lipid rafts.

This outcome could be due to the recruitment of unsuspected and/or unknown molecules. Therefore, I employed a novel mass spectrometric approach known as Direct

Analysis of Large Protein Complexes (DALPC), to characterize and identify known and novel proteins specifically in BCR signalosomes that associate with lipid rafts of T1, T2 and mature B cell subsets. A number of known proteins as well as novel proteins were identified (Fig. 6 and Table 1). An abundance of proteins that are involved in phosphotase activities were selectively identified in T1 B cells (Table 1). The serine/threonine phosphatase PP2A, CD45 associated protein (CD45-AP) and a never before identified protein in B cells, SKAP55. These were of particular interest, due to our previous results showing decreased tyrosine phosphorylation in lipid rafts isolated from T1 B cells (Fig. 5C) and also the differential activation of AKT and MAPK between T1 and T2 B cells (2).

PP2A accounts for a majority of the serine–threonine phosphatase activity in cells and has been implicated in the regulation of many signaling pathways and phenotypes. Typically, the PP2A heterodimer complex is comprised of a scaffolding A subunit with an apparent molecular-mass of 65 kDa and the 36 kDa catalytic subunit (PP2Ac). This A/C subunit heterodimer interacts with a regulatory B subunit yielding the PP2A holoenzyme. My studies show that PP2A is recruited to lipid rafts upon BCR engagement in total B cells (Fig. 10A). Prior studies have shown that PP2A can also interact with IKKs, dephosphorylates specific residues and possibly have a positive regulatory role NF-κB activation. My data shows that PP2A does interact with the IKK complex as it co-immunoprecipates with IKKγ (Fig. 10B). These studies were recently published in collaboration with Dr. Brian Wadzinski (65).

CD45 is one of the main regulators of lymphocyte signaling. It is known that upon BCR stimulation CD45 dephosphorylates tyrosine residues on Src kinases, leading to their activation. These Src kinases then initiate the downstream signaling cascades induced by BCR crosslinking. CD45-AP has been shown to augment the tyrosine

phosphotase activity of CD45 by preventing CD45 dimerization. Previous studies have shown that CD45-AP deficient mice, phenotypically resemble CD45 knockout mice, but to a less severe extent. In addition, I observed that upon BCR crosslinking *CD45-AP*$^{-/-}$ B cells show more detectable tyrosine phosphorylation of proteins in lipid rafts to *wt* cells (Fig. 7). Also, even though there was more tyrosine-phosphorylation in lipid rafts from *CD45-AP*$^{-/-}$ cells, *wt* cells have increased tyrosine phosphorylation in the cytoplasmic fractions (Fig. 7). This increased phosphorylation in rafts from the *CD45-AP*$^{-/-}$ could be that in the absence of CD45-AP the initial membrane proximal events (activation of Src kinases) is unaffected, but the downregulatory (downregulation of BCR signaling) role of CD45 affected. This would lead to an accumulation of phosphorylated proteins in lipid rafts (Fig. 7). Another possibility is that CD45-AP is required for the activation of specific membrane proximal signaling events while dispensable for others. If so, then upon BCR crosslinking, CD45 is able to activate certain signaling molecules, thereby allowing the activation of downstream signaling pathways independent of CD45-AP. In this case, a possible role for CD45-AP would be to regulate the threshold of CD45 signaling required for B cell activation. A recent report suggested that BCR signaling threshold determines B cell fate into FoB (mature B cells) or MZ (marginal zone) B cells. If the absence of CD45 does influence BCR signaling threshold, that would explain the accumulation of MZ B cells with age in *CD45-AP*$^{-/-}$ (Fig. 8).

Adapter proteins primarily mediate protein-protein interactions and control proper targeting of signaling molecules to downstream effectors by facilitating the formation of specific protein complexes. Proteomic analysis identified a novel adaptor protein, specifically in T1 and not in T2 or mature B cells. This protein was identified as SKAP55. Conventional methods confirmed its presence and specificity to T1 cells by RT-PCR and by immunoloblotting (Fig. 9). However, these results do not reveal whether SKAP55 is constitutively associated with rafts or is recruited there in response to BCR engagement.

SKAP55 is a 55 kD (359 amino acid) protein, which is comprised of a unique N-terminal region, a PH domain, followed by three tyrosine residues and a SH3 domain (Fig. 6). Studies in human T cell lines have shown that SKAP55 binds to the catalytically active site of CD45. SKAP55 a substrate of CD45 (73) also binds to Src family kinases (SFKs) via their SH2 domains including Lyn. Prior studies have also shown that, in T cells, the SH3 domain of SKAP55 binds to the central proline rich region of other adapters SLAP-130 and ADAP, thereby connecting SKAP55 to signaling pathways that control cell adhesion and migration (61). However, SKAP55 function in B cells remains entirely unknown.

I hypothesize that SKAP55 contributes to BCR signaling in T1 cells and promotes survival and selection, and that it facilitates migration during T1 cell maturation. This hypothesis predicts that SKAP55 enhances Lyn activation by coupling it to CD45 protein tyrosine phosphotase (PTP) activity, by means of dephosphorylating a regulatory tyrosine residue(s). This increase in Lyn activity, leads to the enhanced activation and association with other regulatory molecules, such as CD22 and SHP-1, thus down regulating BCR signaling in T1 B cells (Fig. 11). The absence of SKAP55 in T2 cells eliminates this enhanced association of CD45 and Lyn, allowing typical activation and signal transduction to occur upon BCR engagement.

Also SKAP55 may play a role in T1 development is through its interaction with ADAP and SLAP-130 to induce cytoskeletal reorganization and migration. Migration of T1 cells into B cell follicles coincides with their transition into T2 cells. The mechanisms that control T1 cell adhesion and migration are unclear. A recent report demonstrated that SKAP55 could increase T cell-APC conjugation, adhesion to fibronectin and intercellular adhesion molecule-1 (ICAM-1), and enhances integrin- adhesion, raising the tantalizing possibility that SKAP55 may facilitate T1 migration into B cell follicles.

Taken together, these results are consistent with my hypothesis that distinct signaling pathways are activated in a stage specific manner and that this is responsible for the disparate biological outcomes seen upon BCR crosslinking.

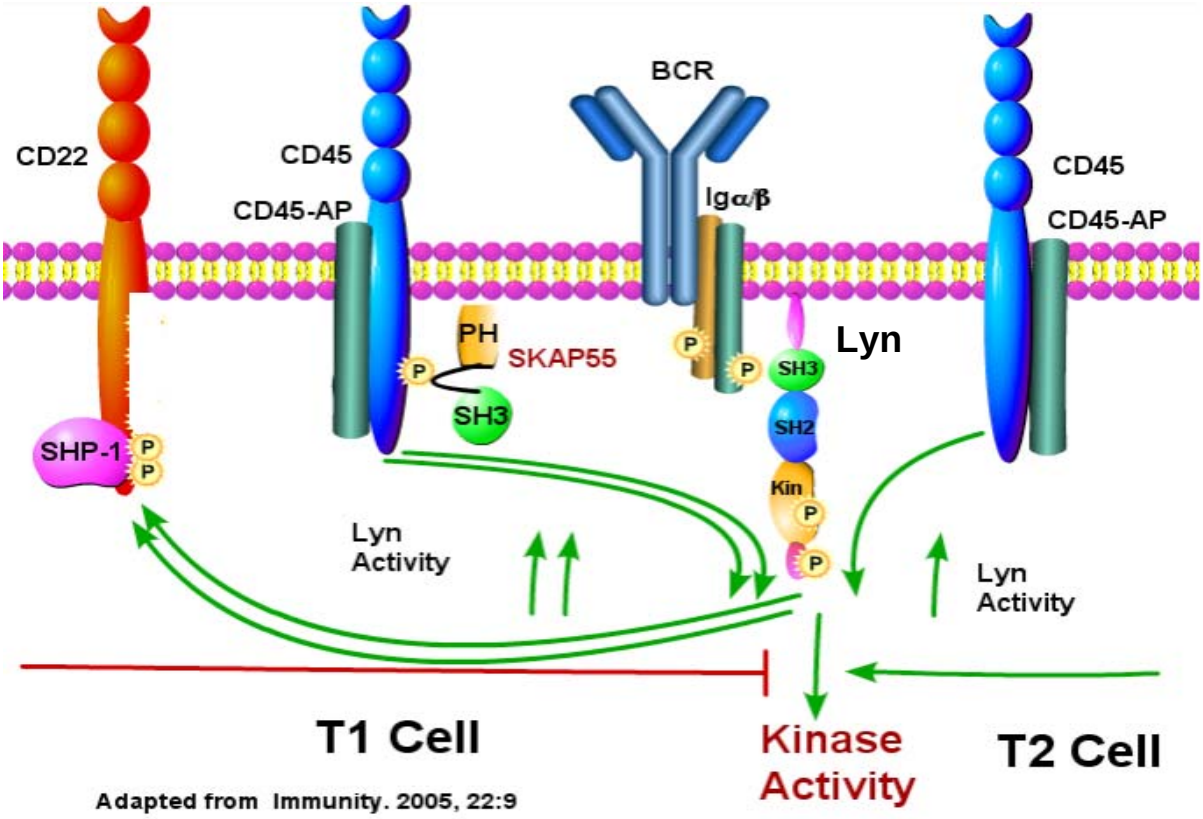

Figure 11. A Model for SKAP55 Regulation of BCR Signaling.

CHAPTER III

EXPRESSION PROFILING OF GENES EXPRESSED IN T1, T2 AND MATURE B CELL SUBSETS BY MICROARRAY ANALYSIS

Abstract

The immature splenic B cell subsets termed Transitional Type 1 (T1) and Transitional Type 2 (T2) respond differentially to B cell antigen receptor (BCR) crosslinking; T1 cells die whereas T2 cells proliferate and differentiate into a mature B cell phenotype. To determine whether genetic reprogramming underlie T1 and T2 B cell maturation I employed cDNA microarray to profile gene expression. The observed main difference between T1 and T2 B cells is differential sensitivity to cell death, therefore I investigated if this was due to changes in the balance of pro- and anti-apoptotic genes as well as the NF-κB family of transcription factors which are known to regulate many of these survival genes. Expression of pro-apoptotic genes did not show any significant difference among T1, T2 and mature B cells, but T1 cells showed lower expression of two anti-apoptotic genes, A1 and Bcl-2 both of which are under NF-κB control. These results suggest that reduced survival of T1 cells may be due to lower expression of NF-kB dependent anti-apoptotic genes. Consistent with this I found that T1 B cells had lower levels of NF-κB1 and NF-κB2 than T2 and mature B cells. Together, these results suggest that gene expression profiling is a feasible approach to help define the unique characteristics of the transitional B cell subsets that may underlie their differentiation.

Introduction

Immature B cells progress through at least two transitional stages (T1 and T2) within the spleen before developing into a mature B cell phenotype. Our lab has shown that purified T1 cells undergo apoptosis in response to IgM cross-linking, whereas T2 cells survive, proliferate and display a mature B cell phenotype. Currently available data suggest that multiple BCR signal transducers are preferentially activated in T2 relative to T1 B cells. A crucial step during the differentiation of many cell types is genetic reprogramming (51-53). In turn, *de novo* synthesis of new gene products regulates specific molecular processes including changes in the hardwiring of cells as they differentiate. Thus, the genetic architecture of each differentiating B cell population is likely to contribute to the specific biochemical code at each developmental stage and may control distinct cellular responses. An alternative hypothesis to the differences in the recruitment of distinct proteins in T1 versus T2 BCR signalosomes would be that the distinct biological outcomes in T1 versus T2 cells arise from differences in the gene expression profile of BCR signaling proteins that accompany T1 to T2 development. To gain insights into the genetic reprogramming that accompanies the differentiation of T1 and T2 B cells, I determined gene expression profiles in each B cell subset by cDNA microarray analysis.

A DNA microarray is typically used to detect gene expression in RNA samples. Total RNA is extracted from the sample tissue. RNA is prepared from the desired tissue and primed with anchored oligo-dT, reverse transcribed, and labeled with a fluorescent molecule. This labeled target is then allowed to bind to its complement on the microarray, and the amount of bound probe is detected by the fluorescence intensity. If the probe has been properly designed and the hybridization is stringent enough, the probe will bind only to the specific message, and the relative fluorescence of the probe can be used to infer the level of gene expression. Therefore, I have used microarray to determine if

stage specific biological outcomes in B cell subsets are controlled by transcriptional regulation of genes that regulate BCR signaling and cell survival during B cell development.

Materials and Methods

Cells and Isolation of Pure T1, T2 and Mature B cells

Refer to Materials and Methods section in chapter 2.

Affymetrix Microarray Analysis

Approximately 1.5-5x10^6 T1, T2 and mature B cells were isolated on a FACStar™ fluorescent cell sorter as mentioned previously. Total RNA was extracted from these samples utilizing the QIAGEN RNA mini prep. The samples were quantified in 10mM Tris on the Nanodrop ND-1000 Spectrophotometer by the Vanderbilt Microarray Shared Resource center (VMSR). In addition to concentration, this instrument reports the 260/280 absorption ratios, which can be an important indicator of hidden contaminants. The samples were then analyzed on an Agilent 2100 Bioanalyzer. The electropherogram it produces makes it easy to spot certain contaminants or degradation, and to calculate the 28s:18s ratio. All samples were hybridized to Affymetrix Mouse 2.0 arrays. This array provides coverage of the transcribed mouse genome on a single array, including 39,000 transcripts, representing, 34,000 well characterized mouse genes.

PIQOR™ Microarray Analysis

1.5x10^6 T1, T2 and mature B cells were isolated on a FACStar™ fluorescent cell sorter as mentioned previously. The cells were then frozen in liquid nitrogen and

prepared for shipment to Miltenyi Biotec laboratories. RNA was prepared using the M&N RNA II Kit. The samples were then analyzed on an Agilent 2100 Bioanalyzer system. There was not enough material available for T7 based linear amplification, therefore SuperAmplification was performed according to Miltenyi's undisclosed protocol. The samples were quantified on the Nanodrop ND-1000 Spectrophotometer. Probe labeling was performed according to Miltenyi Biotec's undisclosed protocol. 250 ng each of Cy3 or Cy5 fluorescently labeled probes were subsequently hybridized on PIQOR™ Immunology Mouse Microarrays and subjected to overnight hybridization using a hybridization station. Cy5 (red) was used to probe the experimental samples and Cy3 (green) the control samples. Two hybridizations were performed, one comparing T1 (experimental) vs. T2 (control) and the other mature (experimental) vs. T2 (control).

Results

Gene Expression Profiles of Stimulated T1, T2 and Mature B Cell Subsets by Affymetrix

Others and I have shown that T1 and T2 B cells respond differently to BCR stimulation. The major observable difference is cell death, T1 cells die where as T2 proliferate and resemble a mature B cell phenotype. To determine if these disparate responses are due to changes in the BCR signaling proteins and balance of pro- and anti-apoptotic genes, I performed a whole genome microarray analysis. RNA was isolated from T1, T2 and mature B cells that were either left unstimulated or stimulated with 20 μg/ml of anti-IgM for 1 hour. Additional samples of only T2 and mature B cells were also stimulated for 18 hours. All the samples were hybridized to Affymetrix Mouse 2.0 arrays. The data analysis is focused on comparisons where all three subsets are

included, with a particular focus on BCR signaling related genes and both anti- and pro-apoptotic genes.

Known BCR signaling molecules where analyzed (Syk, BTK, PLC-γ2, etc.) but showed no difference between T1, T2 and mature B cells (data not shown). Table 2 shows the anti- and pro-apoptotic genes that were at least 2 fold different among the B cell populations when compared to each other. The highlighted genes represent anti-apoptotic genes. In non-stimulated samples, T1 cells had lower levels of anti-apoptotic genes when compared to either T2 or mature B cells. Of particular interest were A1 and Bcl-2, which are anti-apoptotic genes known to play an important in role B cell survival. These genes were consistently lower in T1 compared to T2 and mature B cells, both in non-stimulated samples and after 1 hour stimulations. Additionally I found that T1 B cells did not show an increase in the amount of pro-apoptotic genes when compared to T2 and mature B cells.

I also compared survival genes that were either upregulated or downregulated 2 fold within each particular subset, to determine the ability of each subset to regulate these genes, comparing non-stimulated and 1 hour stimulations. Table 3 represents the genes that were identified in this analysis. I observed that all three B cell subsets were cable of upregulating and down regulating both anti- and pro-apoptotic genes to the same degree. Taken together the information from this analysis with one where each subset was compared to another, these suggest that the main difference in T1 B cell may be lower expression of the anti-apoptotic genes.

NF-κB Related Gene Expression Profiles of Stimulated T1, T2 and Mature B Cells by Affymetrix

It is known that expression of survival genes is targeted by BCR engagement. Because survival genes are targeted by NF-κB upon BCR engagement, therefore I

Table 2. Survival genes differentially expressed among non-stimulated and stimulated T1, T2 and mature B cells by Affymetrix

	Time	Name	Fold	Accesion
T1 fold relative to T2	**0h**	Pim1	5.28	AI323550
T2 fold relative to T1	**0h**	A1	6.5	L16462.1
		Bcl-2	55.72	BM119782
		Bcl-2	2.14	BI664467
		Bim	8	BE686465
T1 fold relative to M	**0h**	Nip2 *	388.02	AK017785.1
		PUMA*	42.22	NM_008611.1
M fold relative to T1	**0h**	A1	5.28	L16462.1
		c-IAP1*	16	NM_134141.1
		Bcl-2	90.51	BM119782
		Bcl-2	5.28	AI505544
		Bcl-2	4.92	BB030997
		Bcl-2	2.46	BI664467
		Birc2	2.83	NM_007465.1
		Bid	2.83	BC002031.1
		Nip3L	2.14	AK004667.1
		Nip3L	2	AK018668.1
		Nip2	2.83	NM_016787.1
		Bnip1	4.59	BG073508
		Bim	7.46	BE686465
T1 fold relative to T2	**1h**	c-IAP5	2.83	BC004702.1
		PUMA	34.3	NM_008611.1
T2 fold relative to T1	**1h**	A1a	3.48	AI326167
		A1c *	27.86	NM_007535.1
		Pim3*	2	AV309401
T1 fold relative to M	**1h**	c-IAP5	4.59	BC004702.1
		PUMA*	3.48	NM_008611.1
M fold relative to T1	**1h**	Bcl-2	2	BI664467
		A1a	6.5	AI326167
T2 fold relative to M	**0h**	Diva	25.99	NM_013479.1
		Nip2	6.06	AK014659.1
M fold relative to T2	**0h**	Bcl-2	2.14	AI505544
		Bcl-2	3.73	BB030997
		c-IAP1	2.14	NM_134141.1
		c-IAP1	3.48	NM_134141.1
		Bax	2.46	BC018228.1
		Bid	2.64	BC002031.1
		Nip1	2.83	BG073508
M fold relative to T2	**1h**	A1a	2	AI326167

The highlighted areas represent Anti-apoptotic genes.

Genes indicated by asterisks (*) are genes whos signal intensity were below the minimum used to determine reliability. These genes have to be confirmerd through additional methods.

Table 3. Survival genes differentially expressed within non-stimulated and stimulated T1, T2 and mature B cells by Affymetrix

	Time	Name	Fold	Accesion
T1 Fold Increase	**1h**	A1	5.66	L16462.1
		Mcl1	2	BC003839.1
		BimL	19.7	AF032460.1
		Bcl-2 *	29.86	BM119782
		c-IAP1*	24.25	NM_134141.1
		c-IAP2	2.3	NM_007465.1
		c-IAP5	2	BC004702.1
		Bim	27.86	BE686465
		Bim	5.66	BM120925
		Bim	4	BB667581
		Bim	3.48	BB667581
		Bim *	6.06	AW411692
		Nip1	2.3	BG073508
T1 Fold Decrease	**1h**	Nip2 *	181.02	AK017785.1
		PUMA *	5.28	NM_008611.1
		Bcl2-l14	9.85	BC025541.1
T2 Fold Increase	**1h**	A1a	4.29	AI326167
		c-IAP1	2.46	NM_134141.1
		c-IAP1	4	NM_134141.1
		Bax	2.46	BC018228.1
		BimL *	24.25	AF032460.1
		Bim	3.25	BE686465
		Bim	6.5	BM120925
		Bim	2.3	BB667581
		Bim	5.28	BB667581
		Bim	3.25	AW411692
		Bcl2-l13	2.64	BC027307.1
T2 Fold Decrease	**1h**	Mcl1	2	AV318494
		Bcl-2	3.25	BC027249.1
		Bcl-w *	3.25	BE991142
		Nip2 *	4	AK014659.1
		Bcl2- l12 *	6.96	BB227384
M Fold Increase	**1h**	A1a	7.46	AI326167
		Bcl-x *	2	BM228788
		Bcl-x short *	2.46	U10100.1
		BimL *	6.96	AF032460.1
		Bim	3.03	BE686465
		Bim	8	BM120925
		Bim	3.25	BB667581
		Bim	5.66	BB667581
		Bim	11.31	AW411692
M Fold Decrease	**1h**	Bcl-2	3.48	BC027249.1
		Bcl-2	3.73	AI505544
		Bcl-2	5.66	BB030997
		Nip2	2	AV144704
		Bad	2.14	NM_007522.1
		Bid	2	BC002031.1
		Nip2	2.3	AK014659.1

The highlighted areas represent Anti-apoptotic genes.

Genes indicated by asterisks (*) are genes whos signal intensity were below the minimum used to determine reliability. These genes have to be confirmerd through additional methods.

determined whether these transcription factors and their upstream regulators were expressed differentially among the B cell populations.

T1 and T2 B cells had similar levels of most the inhibitors of κB (IκBs) and Inhibitors of κB kinases (IKKs) in both stimulated and non-stimulated samples (Table 4). The most important difference was that non-stimulated T1 cells had lower levels of NF-kB1 (p105/p50) and NF-kB2 (p100/p52) when compared to both T2 and mature B cells. Interestingly I found that most of these genes were consistently higher in non-stimulated mature B cells, when compared to both T1 and T2 cells. However, these differences were not observed after BCR stimulation. This suggest that both T1 and T2 B cells are capable of upregulating these genes at similar levels as mature B cells, or that after stimulation, mature cells, downregulate these genes to the same level as T1 and T2 B cells. In either case, it appears BCR stimulation similarly regulates components of NF-κB signaling both in T1 and T2 B cells as well as in mature B cells. A higher level of NF-κB subunits in the mature B cells suggests increasing dependence of B cells on NF-κB with maturation.

Gene Expression Profile Analysis of Freshly Isolated T1, T2 and Mature B cells PIQOR

One question that is frequently asked is if the response of purified cells after culture is representative of the *in vivo* response, especially in the context of development. To address this question I preformed microarray analysis of freshly isolated B cell subsets. T1, T2 and mature B cells were purified as shown in figure 2. This B cell purification scheme excludes a T2 B cell population which is thought to serve as precursors for MZ B cells. The cells were submitted to Miltenyi Biotec laboratories to perform focused gene expression analysis with PIQOR^{TM} Microarrays. These microarrays are designed to cover genes in select groups including, signaling and cell

Table 4. NF-κB and related gene expression in non-stimulated and stimulated T1, T2 and mature B cells by Affymetrix.

	Fold	Gene
Higher in T1 non Vs T2 non		
	3.03	IκBα
Higher in T2 non Vs T1 non		
	2.64	NF-κB1 (p105)
Higher in M non Vs T1 non		
	2.83	NF-κB2 (p100)
	2.46	IκBζ (zeta)
	2	IκBα
	3.73	IκBβ
	2.14	IκBε
	2	IκBε
	4	NF-κB1 (p105)
	2	IKKβ
	2.3	IKKγ
Higher in M 1h Vs T1 1h		
	2.3	IKKε
Higher in M non Vs T2 non		
	4.92	NF-κB2 (p100)
	2	IκBζ (zeta)
	2.14	IκBα
	6.96	IκBβ
	2.14	IκBε
	2.14	IκBε
	12.13	IKKβ
	2.3	IKKβ
Higher in M 1h Vs T2 1h		
	2.64	IKKε

death among others. The Immunology microarray includes 1,076 key genes for immune response, cell death, extracellular matrix, and signal transduction helping to understand the network and the underlying gene regulation of signaling pathways. Two hybridizations were performed, one comparing T1 (experimental) vs. T2 (control) and the other mature (experimental) vs. T2 (control). A focus of these studies was to investigate differences among genes that encode proteins involved in the regulation of BCR signaling. Additionally, since the major difference among T1, T2 and mature B cells is survival, I also analyzed the expression of anti- and pro- apoptotic genes and components of the NF-κB signaling pathways.

Consistent with the data obtained with Affymetrix, analysis of these data demonstrated that there is no significant difference in the expression levels of BCR associated signaling molecules in resting T1, T2 and mature B cells (data not shown). However examination of the anti-apoptotic genes revealed that Bcl-2 and A1 transcripts were expressed approximately 2 fold less in T1 compared to T2 B cells (Table. 5, highlighted)(These data are consistent with the results obtained from the Affymetrix microarray, Table. 2). These genes were not differentially expressed in T2 and mature B cells when compared. T1 B cells also express 2 fold lower levels of Bcl-2 and A1 when compared to mature B cells. Surprisingly another anti-apoptotic gene, Pim-1 was expressed 2 fold higher in T1 cells when compared to T2 and mature B cells Table. 5). There are no detectable differences in the expression of pro-apoptotic genes within these subsets. These data are also consistent with the results obtained from the Affymetrix array. Prior knockout studies have shown that loss of Bcl-2 deficiency results in an age-dependent decline of B lymphocytes. Whether modest lower levels of A1 and Bcl-2 in T1 relative to T2 cells have a developmental stage specific biological significance remains to be elucidated.

Table 5. List of genes differentially expresses in freshly isolated T1 and T2 B cells from the PIQOR microarrays.

Gene	Fold	Accesion
IL18	2.25	P70380
IFN-GAMMA	3.27	P01580
BCL-2	2.04	P10417
TNFRSF11A (RANK) (RECEPTOR ACTIVATOR OF NF-KB)	2.44	Q8VCT7
LT-ALPHA (TNF-BETA)	1.92	P09225
A1 (BFL-1)	2.17	Q07440
CD87	2.38	P35456
THY1 (CD90)	5.56	P01831
CD96	2.32	Q99M67
CD21	1.74	P19070
KI67 (MKI67)	2	Q61769
KIAA0042: (KIAA0042 OR KIF14) KINESIN-LIKE PROTEIN KIF14.	2.44	O35064
BMP-8 (OP 2).	2.38	P34821
CYR61 (IGFBP10 OR GIG1 OR CCN1)	2.04	P18406
TGF-BETA 3	2.27	P17125
GREM2	2.32	O88273
DOC2 BETA.	2.7	Q6NXK3
APXL	2.7	Q8CDZ0
MPP2 (MAGUK P55 SUBFAMILY MEMBER 2)	3.03	Q9WV34
FAK1: (FAK1 OR FAK OR PTK2)	2.7	P34152
CCR8: (CCR8 OR CMKBR8 OR CKRL1 OR TER1)	2.22	P56484
STMN1 (STMN1 OR LAP18 OR OP18)	2.63	P54227
COL9A2 (ALPHA-2 IX COLLAGEN)	2.5	Q07643
LAMB1 (LAMININ B1 CHAIN)	2.13	P02469
FIP (FOS-INTERACTING PROTEIN)	2.22	Q64705
SMALL INDUCIBLE CYTOKINE A17	2.78	Q9WUZ6
CXCL13 (BLC OR BCA1)	2.32	O55038
CD118 (IFN-ALPHA-REC)	2.38	P33896
GRL (GRL OR NR3C1)	2.04	P06537
TRAF1	2.22	P39428
SMAD5	2.78	P97454
FLAP: (ALOX5AP)	5.32	P30355
PIM1	2.13	P06803
MGST1 (MGST OR GST12)	1.9	Q91VS7
AOC3 (VAP1)	2.22	O70423
DR6 (TNFRSF21)	2.27	Q91XH9
FCP1 (CTDP1)	1.92	Q7TSG2
HHEX (PRHX OR PRH OR HEX)	2.02	P43120
LGALS3 (LECTIN L-29)	2.75	P16110
RGS2 (G0/G1 SWITCH REGULATORY PROTEIN 8).	1.73	O08849
CD44	2.56	Q05732
TGFBI (BIGH3)	1.89	P82198
SB135 (MYADM OR MUG)	2.01	O35682

Green indicates a 1.7 fold downregulation of a certain gene in comparison to the control sample, while red indicates a more than 1.7 fold increase of the respective gene in comparison to the control. T2 cells are the control and T1 cells are the experiment.

Contrary to the data obtained from Affymetrix, none of the NF-κB or related genes was differentially expressed between T1, T2 and mature B cells.

In addition to known BCR signaling and apoptosis related genes I also analyzed data of genes known to play a roles in immunological responses. Tables 5 and 6 list genes that were either upregulated or downregulated 1.7 fold. Green indicates a 1.7 fold downregulation of a certain gene in comparison to the control sample, while red indicates a more than 1.7 fold increase of the respective gene in comparison to the control. These genes may lead to previously unstudied pathways and molecules that may play a role in transitional B cell survival and differentiation.

Discussion

In studies presented here, two different platforms of microarray analysis were employed. A whole genome analysis utilized the Affymetrix Mouse 2.0 arrays. This array provides coverage of the transcribed mouse genome on a single array, including 39,000 transcripts, representing, 34,000 well characterized mouse genes. In addition I also used a new platform from Miltenyi Biotec laboratories, which uses a topic-defined array, in order to focus the analysis. These platforms were used to characterize the genetic architecture of each differentiating B cell population which is likely to contribute to the specific biochemical code at each development stage and may control distinct cellular responses. To simplify the analysis, I focused our attention on the regulation of genes involved in BCR signaling, survival and the genes responsible for regulating the transcription of these survival genes, with a focus on the NF-κB pathway.

In both the Affymetrix and PIQOR microarray analyses, known BCR molecules were not differentially expressed in any B cell subset. This is most likely due because the membrane proximal signaling events in BCR signaling are important in the

Table 6. List of genes differentially expresses in freshly isolated T2 and mature B cells from the PIQOR microarrays.

Gene	Fold	Accesion
MAPK11 (P38 BETA)	2.06	Q9WUI1
TNFRSF9 (CD137 ANTIGEN).	4.3	P20334
TNFRSF11A (RANK) (RECEPTOR ACTIVATOR OF NF-KB)	4.35	Q8VCT7
CD62L (LY-22)	1.79	P18337
THY1 (CD90)	4.35	P01831
CD23	1.86	P20693
ITGA4 (INTEGRIN ALPHA-IV)(CD49D)	2.02	Q61740
CD5	5.88	P13379
GPCR25	1.78	Q921N3
KIAA0042: (KIAA0042 OR KIF14) KINESIN-LIKE PROTEIN KIF14.	2.44	O35064
BMP-8 (OP 2).	2.38	P34821
CYR61 (IGFBP10 OR GIG1 OR CCN1)	2.7	P18406
TGF-BETA 3	2.32	P17125
GREM2	2.38	O88273
DOC2 BETA.	2.32	Q6NXK3
UBIQUITIN-PROTEIN LIGASE NEDD-4	3.57	O08758
NEDD-4-LIKE E3 UBIQUITIN-PROTEIN LIGASE WWP2	2	Q8BTG4
APXL	2.32	Q8CDZ0
MPP2 (MAGUK P55 SUBFAMILY MEMBER 2)	2.63	Q9WV34
(PROTEIN KINASE C INHIBITOR PROTEIN-1) (KCIP-1)	1.75	P62259
FAK1: (FAK1 OR FAK OR PTK2)	2.5	P34152
HCK	1.81	P08103
PTPN12: (PROTEIN-TYROSINE PHOSPHATASE G1)	1.92	P35831
CCR6 (CCR6 OR CMKBR6 OR STRL22 OR GPR29 OR CKRL3)	3.68	O54689
CCR8: (CCR8 OR CMKBR8 OR CKRL1 OR TER1)	2.27	P56484
STMN1 (STMN1 OR LAP18 OR OP18)	2.56	P54227
COL9A2(ALPHA-2 IX COLLAGEN)	2.5	Q07643
ITGB7 (INTEGRINB7)	2.15	P26011
LAMB1 (LAMININ B1 CHAIN)	1.92	P02469
FOSB (G0/G1 SWITCH REGULATORY PROTEIN 3).	3.03	P13346
C-FOS)	2.12	P01101
FOXG1A-FOXG1B (TRANSCRIPTION FACTOR BF-2).	1.96	Q80VP3
FIP (FOS-INTERACTING PROTEIN)	2.22	Q64705
SMALL INDUCIBLE CYTOKINE A17	2.5	Q9WUZ6
CD118 (IFN-ALPHA-REC)	2.04	P33896
GRL (GRL OR NR3C1)	2.17	P06537
TRAF1	2.32	P39428
C-MYB	7.14	P06876
SMAD5	2.38	P97454
MGST2 (GST2)	3.45	Q8R032
AOC3 (VAP1)	2.27	O70423
DR6 (TNFRSF21)	2.27	Q91XH9
FCP1 (CTDP1)	2	Q7TSG2
HHEX (PRHX OR PRH OR HEX)	2	P43120
IRAK-M (IRAK3)	1.78	Q8CE40
LMO-4	1.75	P61969
RGS2 (G0/G1 SWITCH REGULATORY PROTEIN 8).	3.85	O08849
CD44	2.04	Q05732
AIF1 (IBA1)	1.72	Q9EQW9
CAPN2 (CAPN2)	2.04	O35518
KLF6: (COPEB OR BCD1 OR CPBP)	1.81	-
TLR-3	1.87	Q99MB1

Green indicates a 1.7 fold downregulation of a certain gene in comparison to the control sample, while red indicates a more than 1.7 fold increase of the respective gene in comparison to the control. T2 cells are the control and mature cells are the experiment.

maintenance and survival of immature and mature B cells. It is unlikely that these genes would vary significantly between T1, T2 and mature B cell.

There are many families of anti- and pro-apoptotic genes. One of the better characterized is the Bcl-2 family. Bcl-2-related proteins can act as cellular bodyguards or assassins to positively or negatively control apoptosis (55). Bcl-2 family proteins are characterized by the presence of up to four relatively short sequence termed Bcl-2 homology (BH) domains. More than 20 family members have been identified and these can be divided into three subfamilies, based on structural and functional features. The anti-apoptotic subfamily contains the Bcl-2 and Bcl-X_L proteins, which suppress apoptosis and contain all four BH domains, designated BH1–4. Mcl-1 is another Bcl-2-related survival protein, but is somewhat structurally distinct and lacks a BH4 domain. Pro-apoptotic proteins, such as Bax and Bak contain BH1–3 domains and are termed 'multidomains', whereas other pro-apoptotic proteins, such as Bim, Bad and Bid, contain only the BH3 domain and are termed 'BH3-only'. The targeting of Bcl-2 family proteins to mitochondrial membranes is thought to play an important role in apoptotic cell death (55).

The relative expression and activity of various anti-apoptotic and pro-apoptotic Bcl-2 family proteins is a critical determinant of apoptosis sensitivity. Precisely how the functions of the separate subfamilies and individual Bcl-2 proteins are coordinated to control apoptosis is unclear. I performed microarray analysis on non-stimulated and BCR stimulated T1, T2 and mature B cells. Upon examination of the survival genes that showed at least a 2 fold difference, I found that T1 cells had consistently lower levels of anti-apoptotic genes, when compared to T2 and mature B cells, both in non-stimulated and stimulated samples. Of these genes, A1 and Bcl-2 were of particular interest due to their role in B cell survival and these genes are currently being studied in our lab. A1 was found to be 6.5 fold lower in non-stimulated T1 when compared to T2, and 5.28 fold less than non-stimulated mature B cells. After 1 hour stimulations with anti-IgM, T1 cells

still had significant lower levels of A1, 3.5-27.86 as compared to T2 cells and 6.5 when compared to mature B cells. Bcl-2 showed an almost identical trend as A1. Inspection of the pro-apoptotic genes revealed no significant difference between T1, T2 and mature B cell subsets; therefore they are not shown in table 2.

Interestingly, upon examination of number of genes that are different between T1, T2, and mature B cells, I observed that non-stimulated mature B cells had more genes expressed at higher levels, than T1 or T2 B cells. This difference decreases upon stimulation. This suggests that in non-stimulated B cells, mature cells have a higher relative expression of both anti- and pro-apoptotic genes, than T1 and T2 B cells. The decrease after stimulation could imply that T1 and T2 B cells upregulate many of these survival genes to similar levels as mature B cells. On the other hand, this could also mean, that mature B cells, downregulate the relative expression of these genes after BCR engagement. In response to BCR stimulation all three subsets were capable of down- and up-regulating both anti- and pro-apoptotic genes to the same extent. In addition, there was no significant difference in the number of genes that were identified as altered in each transitional and mature B cell subset.

The relative expression of NF-κB subunits, as well as its upstream regulators inhibitors of κB (IκBs), and inhibitors of κB kinase (IKKs) revealed significant differences in the levels of only some genes in the NF-κB pathway. The most appreciable difference was that non-stimulated T1 cells had lower levels of NF-κB1 (p105/p50) and NF-κB2 (p100/p52) when compared to both T2 and mature B cells. NF-κB1 is important in controlling lymphocyte and macrophage function in immune and inflammatory responses. NF-κB1 deficient mice have impaired B cell proliferation (56). Interestingly I found that most of these genes were consistently higher in non-stimulated mature cells, when compared to both T1 and T2 cells, but that these differences are not observed after one

hour stimulations. This suggest that both T1 and T2 B cells are both capable of upregulating these genes at similar levels to mature B cells, or that after 1 hour stimulations, mature cells, downregulate these genes to the same level as T1 and T2 B cells. This observation is similar to that of the survival genes (Table 2). Additionally, a lower expression of NF-κB1 and NF-κB2 in T1 relative to T2 and mature B cells correlates well with the observed lower expression of survival genes in this B cell subset and suggest that these differences may mediate sensitivity of T1 B cells to apoptosis.

To address the *in vivo* significance of our gene expression profiling, I performed additional microarray analysis of freshly isolated T1, T2 and mature B cells using an immunology topic defined microarray (Miltenyi Biotec). This array contains over a thousand genes that represent genes for immune response, cell death, extracellular matrix, and signal transduction. Initially I focused our analysis on survival genes, as I did previously with the Affymetrix microarray. I found that most survival genes were not differentially expressed within T1, T2 and mature B cells. Consistent with previous experiments closer examination revealed that A1 and Bcl-2 were expressed at lower levels (2 fold) in T1 when compared with T2 (Table5, highlighted) and mature B cells. As previously mentioned, pro-apoptotic genes showed no difference among T1, T2 and mature B cells. These data, in addition to the results from the Affymetrix microarray, support the idea that it is the levels of anti-apoptotic genes, may contribute to the differential responses seen in T1 and T2 B cells upon BCR crosslinking.

Analysis of the NF-κB genes in the PIQOR array revealed that there was no difference in the level of NF-κB1 or NF-κB2. These results are contradictory to those obtained in the Affymetrix array. Our lab has studied through the use of Real Time PCR (TaqMan) that T1 cells do have lower levels of both NF-κB1 and NF-κB2 compared to

T2 and mature B cells. This suggests that the PIQOR microarray is possibly less sensitive in detecting changes in the levels of transcripts.

There are a number of signaling pathways other than survival genes that likely play a role in the survival and differentiation of immature B cells. These signals could be derived from other cell surface receptors involved in any number of cellular events, ranging from integrin signaling, Toll-like receptors and chemokine receptors among many others. The previous analyses of BCR stimulated T1, T2 and mature B cells, allows us to elucidate those genes that are affected by BCR signaling. Studies performed with freshly isolated cells provide information of the signaling events including those (independent of BCR signaling) *in vivo*. The genes that were identified in tables 5 and 6 represent genes involved in other cellular events.

Together, these studies provide a molecular basis for understanding the stage-specific genetic programs and signaling pathways that regulate B cell responses of cell death versus survival, proliferation and differentiation.

CHAPTER IV

CONCLUDING REMARKS

The immature B cell compartment in the spleen comprises of two subsets termed Transitional Type 1 (T1) and Transitional Type 2 (T2). Our laboratory has shown that T1 and T2 B cell subsets respond differently to crosslinking of the BCR; T1 cells die upon BCR stimulation whereas T2 cells proliferate and differentiate into a mature B cell phenotype. The signaling mechanisms that control these disparate biological outcomes in T1 versus T2 cells remain elusive. I employed genomic and proteomic approaches to define the global genetic programs and biochemical signaling programs that control the differential BCR responses of T1, T2 and mature B cells.

Through proteomics I identified SKAP55 in lipid rafts from BCR stimulated T1 cells, and RNA and protein analysis verified its preferential expression in T1 cells (Figs. 9). I also identified CD45-AP specifically in lipid rafts from stimulated T1 cells. In this regard, I have found that like CD45-deficient mice, CD45-AP-deficient mice also have an increase in transitional B cells suggesting a defect in BCR-induced cell death and negative selection (Fig. 8). Previous studies have shown a link between SKAP55 and CD45 in T cells, and their role in T cell receptor signaling. Prior studies have also shown that SKAP55 is able to bind various Src-kinases, including Lyn. Together these data suggest that SKAP55 may couple CD45/CD45-AP to BCR signaling through its interaction with Lyn. This proximity may concentrate CD45 activity on the negative regulatory tyrosine (Y508) enhancing Lyn activity. In future experiments we will investigate this hypothesis by immunoblotting with site-specific phosphorylation Abs to Lyn in *CD45-AP*$^{-/-}$ in comparison with *skap55*$^{-/-}$ T1 B cells. Currently *skap55*$^{-/-}$ mouse is not available, but our lab is planning to generate this knockout strain. To determine

whether SKAP55 gene deletion affects B cell maturation, we will employ in-depth flowcytometry (FCM) analysis to characterize B cell subsets. Additionally, *skap55*$^{-/-}$ BM cells would be transferred into sublethally irradiated *wt* mice and cells would be recovered and analyzed each week for 6-8 weeks for FCM analysis for B cell developmental phenotype to determine whether any observed defects in *skap55*$^{-/-}$ mice are B cell intrinsic

I also hypothesize that SKAP55 may regulate BCR signaling as shown previously in T cells. To test this hypothesis we will assess the effects of SKAP55 deletion on BCR signal-dependent responses of T1 relative to other B cell subsets. I anticipate that loss of SKAP55 will affect peripheral B cell maturation at the T1 stage due to its selective expression in this B cell subset (Fig. 9). In Addition if we find a defect in cell survival we will define the underlying molecular mechanisms by assaying for the induction of anti-apoptotic proteins such as A1, Bcl-2, and Bcl-X_L. The roles of these anti-apoptotic genes in B cell survival are currently being studied in our lab.

It is critical to investigate SKAP55 and CD45/CD45-AP interactions in T1 specific signaling pathways as well as the impact of SKAP55 in the regulation of T1 B cell development. This question can be addressed by comparing the phenotype of *skap55*$^{-/-}$ mice with *CD45-AP*$^{-/-}$ or *CD45*$^{-/-}$ mice. If we find similar B cell defects in these knockout models, these experiments would suggest a functional link between SKAP55 and CD45/CD45-AP during B cell development and would be followed up by interbreeding of *skap55*$^{-/-}$ mice with *CD45-AP*$^{-/-}$ or *CD45*$^{-/-}$ mice. We would employ in-depth flowcytometry (FCM) analysis to characterize any defect in development and B cell subsets.

Migration of T1 cells into B cell follicles coincides with their transition into T2 cells. The mechanisms that control T1 cell adhesion and migrations are unclear. A recent report demonstrated that SKAP55 could increase T cell-APC conjugation, adhesion to fibronectin and intercellular adhesion molecule-1 (ICAM-1), and enhances integrin-

adhesion. These results suggest that SKAP55 may facilitate T1 migration into B cell follicles. Therefore, it would be important to investigate whether localization of B cell subsets is altered in the *skap55*$^{-/-}$ mice. If an alteration in the localization of B cell subsets in *skap55*$^{-/-}$ mice is observed, the effects of SKAP55 deficiency on T1 cell adhesion and migration could be assessed.

I used two different microarray platforms to characterize the genetic architecture of each B cell population as a way to define the biochemical signals that may play a role in their distinct biological outcomes. I focused our analysis on survival genes, anti- and pro-apoptotic, and genes that regulate the transcription of these survival genes. These analyses showed that levels of pro-apoptotic genes did not vary between T1, T2 and mature B cells. In freshly isolated and cultured cells that were un-stimulated and stimulated, I consistently found that A1 and Bcl-2 were expressed in lower levels in T1, than in T2 and mature B cells. These data suggest that it is a quantitative difference of these survival genes that may lead to the disparate outcomes seen in T1 and T2 B cells.

Even though microarrays offer a powerful approach to the analysis of gene expression many of the genes we focused on are regulated post-translationaly. These microarray studies allow us to identify candidate genes that may play an essential role in the survival and differentiation of transitional and mature B cells. Our lab is currently working on characterizing, at a stage specific level, the protein levels of these and other anti-apoptotic genes. In addition, we are studying the signals that are required for the transcription of these genes. These studies will establish the contribution of these survival genes in the development of T1, T2 and mature B cells.

These studies also revealed differential expression other genes involved in multiple signaling pathways. These genes may lead to previously unthought of pathways that may play a role in development and differentiation of T1 and T2 B cells.

Together, these studies provide a molecular basis for understanding the stage-specific signaling pathways that regulate B cell responses in T1, T2 and mature B cells.

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