Transitional Type 1 and 2 B Lymphocyte Subsets are Differentially Responsive to Antigen Receptor Signaling

James B. Petro\textsuperscript{1*}, Rachel M. Gerstein\textsuperscript{2*}, John Lowe\textsuperscript{1}, Robert S. Carter\textsuperscript{1}, Nicholas Shinners\textsuperscript{1} and Wasif N. Khan\textsuperscript{1#}

\textsuperscript{1}Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232-0146 and \textsuperscript{2}Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA 01655-0002

* These authors contributed equally to these studies.

Running Title:  BCR-signaling Responses of Transitional B Lymphocytes

\#Correspondence:  Wasif N. Khan, PhD.

Department of Microbiology and Immunology

Vanderbilt University School of Medicine

Nashville, Tennessee 37232-0146

Phone: 615-343-5632  Fax: 615-343-7392

Email: Wasif.khan@mcmail.vanderbilt.edu
Summary

Mature B-lymphocytes develop sequentially from transitional type 1 (T1) and type 2 (T2) precursors in the spleen. To elucidate the mechanisms that regulate the developmental fate of these distinct B cell subsets, we investigated their biochemical and biological responses following stimulation through the B-cell antigen receptor (BCR). As compared with the T1 subset, T2 cells are more responsive to BCR engagement as evidenced by their robust induction of activation markers, expression of the pro-survival protein Bcl-xL, and enhanced proliferation. BCR stimulation of T2 cells leads to the appearance of B cells with mature phenotypic characteristics, whereas T1 cells die. All of these T2 responses are dependent on the BCR signal transducer Bruton’s Tyrosine Kinase (BTK), which is dispensable for the T1 to T2 transition. Furthermore, the serine/threonine kinases extracellular signal-regulated kinase (ERK), p38 MAPK and Akt are predominantly activated in T2 compared to T1 B cells following BCR cross-linking. We conclude that T1 and T2 B cells respond differentially to BCR engagement via the induction of stage-specific signaling pathways. In turn, these signaling pathways likely govern the development and selection processes that are critical for the formation of the mature B cell compartment.

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1 T1, Transitional type 1; BCR, B cell antigen receptor; T, transitional; M, mature; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; BTK, Bruton’s tyrosine kinase; PTK, protein tyrosine kinase; BLNK, B cell linker protein; xid, x-linked immunodeficiency; FACS, flow cytometry
INTRODUCTION

B lymphocytes are generated throughout the life of most mammals. This process occurs in the fetal liver before birth and in the bone marrow (BM) thereafter. Production of functional B lymphocytes requires the normal progression of precursor B cells through both antigen-independent and antigen-dependent stages of development (1-3). The antigen-independent phase of B-cell differentiation that occurs in the fetal liver and the BM culminates in the expression of an assembled IgM molecule, which is displayed on the surface of immature B cells (3). Of the 10-20 million immature B cells (IgM+) daily produced from the BM, only 10% reach the periphery; of these, only 10-30% join the long-lived B cell pool (IgMloIgDhi) (4-7). Although the precise nature of this peripheral B cell loss is unclear, these observations suggest that immature B cells either compete with mature B cells for survival or they require selection signals to enter the long-lived mature B cell pool (6,8,9). Analysis of the peripheral B cell repertoire strongly suggests that the progression of immature B cells to the mature B cell stage is the result of positive selection (10-15). In this context, BCR-derived signals are indispensable for both the formation of the B cell repertoire and the long-term survival of mature B lymphocytes (6,16). Despite a critical requirement for the BCR during splenic B cell development, a specific role(s) for BCR signaling in this process remain(s) poorly understood (8,17,18).

Immature B cells undergo a maturation process in the periphery characterized by a sequential series of discrete stages that can be identified based upon the expression of developmental markers. Thus, splenic B cells can be divided into at least three developmental subpopulations: Transitional type 1 (T1) B cells, Transitional type 2 (T2) B cells, and Mature (M) B cells (19). T1 B cells can develop into T2 B cells, which in turn are thought to serve as the precursor to M B cells (19). Prior studies have revealed that immature and mature B cell
subpopulations display distinct responses to similar stimuli. For example, immature B cells undergo apoptosis in response to BCR engagement while M B cells proliferate under similar stimulatory conditions (2,4,5,8,17,20,21). These observations suggest that the BCR may deliver distinct signals at discrete stages of peripheral B cell development. Whether distinct BCR responses are developmentally regulated or stage-specific (T1, T2, and M) BCR signaling responses that determine the biological outcome are not known.

The BCR propagates biochemical signals via the activation of protein tyrosine kinases including the cytoplasmic PTKs Lyn, Syk, and Bruton’s tyrosine kinase (BTK) (22-25). These kinases in turn contribute to the formation and activation of a large protein complex termed the BCR signalosome (26-28). BCR signaling appears to control biological outcomes in part via activation of PLC-γ2, mitogen activated protein kinase (MAPK) and Akt signaling pathways (23,24,26-32). A naturally occurring point mutation (R28C) or gene-targeted deletion of btk results in the B cell deficiency disorder termed x-linked immunodeficiency (xid) in mice (33-36). Affected animals display a 50% reduction in the number of M splenic B cells (37-39). The M B cell deficiency in these mice is likely due to a failure of T2 B cell transition into M B cells. A similar M B cell deficiency has been observed in mice with gene-targeted deletions in several other components of the BCR signalosome including Syk, Vav1, Vav2, B cell linker protein (BLNK), Phosphatidylinositol 3-kinase (PI3K) and PLC-γ2 (27,40-45). In addition, a B cell activation factor (BAFF) has been shown to potentiate both the survival and differentiation of T2 B cells (46). These observations suggest that T2 B cells display signaling responses that are distinct from T1 and M B cells. These unique BCR responses may play a role in the progression of immature B cells into the long-lived M B cell compartment. However, little is known about the specific BCR signaling properties of T2 B cells.
Here we examine the BCR signaling responses of T2 B cells versus T1 and M B cells isolated from WT and BCR signaling defective mice (btk−/−). We demonstrate that T2 B cells display more potent responses to BCR stimulation than either T1 or M B cells. T2 B cells express higher basal and BCR-induced levels of activation markers, proliferate as well as M B cells, and a subset of them display a mature B cell phenotype in response to BCR stimulation in vitro. In contrast to T1, BCR stimulation of T2 B cells potently induces heightened expression of the pro-survival protein Bcl-xL. The btk−/− B cells were defective for these responses, further supporting an essential role for BCR signaling during peripheral B cell development. Furthermore, consistent with the distinct biological outcomes of T1 versus T2 B cells, BCR cross-linking induces phosphorylation of ERK1/2, p38 MAPK and Akt predominantly in T2 but only modestly in T1 cells. Together, these findings suggest that T2 B cells respond to BCR signals with greater potency than either T1 or M B cells. This enhanced response to BCR stimulation may contribute to the positive selection and progression of T2 B cells into the long-lived mature B cell pool.
Experimental Procedures

Mice

Four to 8 week old C57BL/6 mice were used for all the experiments. The generation of btk-deficient mice (null mutant; $btk^{-/-}$) has been described previously (34). These mice have a mixed genetic background of 129/SvxC57BL/6. For wild type controls, 129/SvxC57BL/6 or C57BL/6 mice (Jackson Laboratories, Maine) were used. All mice used as a source of cells were treated humanely in accordance with federal and state government guidelines and their use was approved by Vanderbilt’s and UMMS’s institutional animal committees.

Flow Cytometric Analysis

For flow cytometric analyses, splenocytes were harvested from WT and $btk^{-/-}$ mice and depleted of red blood cells (RBCs). $1 \times 10^6$ cells/sample were stained for surface expression of cell surface antigens in FACS buffer (PBS with 2% fetal bovine serum) with indicated combinations of FITC-conjugated anti-CD21, PE-conjugated anti-CD23, PE-conjugated anti-IgM, Cyochrome-conjugated anti-B220, FITC-conjugated anti-CD25, PE- or APC-conjugated anti-CD5, and biotin-conjugated anti-HSA (M1/69, Pharmingen) antibodies revealed by Cyochrome-conjugated streptavidin (Pharmingen). Live cells were analyzed based on their FSC/SSC properties. In some cases (Figure 3), cells were stained in FACS buffer (deficient RPMI (Irvine [Cat#9826-10L]) with 10 mM Hepes, 3% newborn calf serum, 1 mM EDTA and 0.02% sodium azide), with indicated combinations of FITC anti-CD21, PE-conjugated anti-CD24 (HAS; clone 30F1), and biotin-conjugated anti-CD23, anti-CD25 or biotin anti-IgD, revealed with Cyochrome-conjugated streptavidin (Pharmingen, except Southern Biotech Assoc.
for PE-30F1 and Ebioscience for anti-IgD). After washing in stain media, cells were resuspended in 1 mg/ml propidium iodide to exclude dead cells.

For the detection of intracellular Bcl-xL protein levels, splenocytes were incubated for 16 hours in the presence or absence of anti-IgM. 1x10^6 cells/sample were stained for surface expression of CD21 and HSA. Cells were then fixed in 4% paraformaldehyde and permeabilized in 0.3% saponin. Intracellular staining for Bcl-x_L was achieved by incubating the permeabilized cells with FITC-conjugated anti-Bcl-x_L antibodies in the presence of 15% mouse serum. Samples were stained in parallel with a FITC-conjugated IgG3 antibody as a background control. All flow cytometric samples were assayed on a FACSCalibur™ or FACScan flow cytometer (Becton Dickinson) and the data was analyzed using CELLQuest™ (Becton Dickinson) or FlowJo (TreeStar Inc) software.

**In Vitro Differentiation Assay**

Splenic B cell subsets were isolated via a two-step process. First, B cells were purified from the spleens of WT and *btk*^-/-^ mice by a process of negative selection using an auto-MACS automated cell sorter (Milentyi Biotechnology). Briefly, pooled splenocytes were depleted of RBCs, incubated with anti-CD43 antibodies coupled to magnetic beads to deplete CD43-bearing leukocytes, thereby excluding B cells. In some experiments, T cells were depleted using biotinylated anti-CD3, -CD4, -CD8 antibodies followed by streptavidin-magnetic beads. Purified B cells were then stained using FITC-conjugated anti-CD21, PE-conjugated anti-CD23, and biotinylated anti-HSA (revealed by Cychrome-labeled streptavidin) antibodies or FITC-conjugated anti-CD21, PE-conjugated anti-HSA (CD24), and biotinylated anti-CD23 (revealed by Cychrome-labeled streptavidin) antibodies to sort T1, T2, and M B cell populations as
described in previous reports (19,46). Anti-IgM was specifically not used to avoid pre-activation through the BCR. Cells were sorted on a FACStar™ fluorescent cell sorter (Becton Dickinson) at the Department of Veterans Affairs Medical Center, Nashville, TN or at UMass Medical School, Worcester, MA. To assess their purity the sorted cells were reanalyzed by FACS. The purity for T1, T2, and M B cells was 90-95%, 70-95%, and 95-98%, respectively.

**In Vitro Proliferation Assays**

2-5x10⁴ purified T1, T2, M and total (pre-sort) B cells were dispensed in 96-well microtiter plates (in triplicates) at 100 µl/well, and then cultured in complete RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 µM 2-ME, and 2 mM L-glutamine. Plates were incubated as indicated in the presence of either 20 µg/ml anti-IgM (Jackson ImmunoResearch), or PMA/Iono, 1.0 µM each (Calbiochem-Novabiochem corporation, La Jolla, CA). At the end of the incubation period, the cells were pulsed with 1.0 µCi of [³H]thymidine (Amersham, Arlington Heights, IL) per well for 12 hours. Cells were then harvested with a cell harvester (Tomtec orange), and [³H]thymidine uptake was measured with a beta plate counter (Wallac, Gaithersburg, MD).

**Western Blotting**

For western blot analysis of Bcl-xL, purified T1, T2 and, total B cells (as described in Fig. 2.) were used. Cells were washed and resuspended in complete RPMI (supplemented with 10% FCS, 50 mM 2-ME and 100 µg/ml penicillin and 100 µg/ml streptomycin) at a concentration of 1x10⁶ cells/ml and cultured for 16 hours with or without 10 µg/ml F(ab’)₂ goat anti-mouse IgM antibodies (Jackson ImmunoResearch). For western blot analysis of MAP Kinases, cells were
resuspended in PBS at a concentration of 5x10^6 cells/ml and incubated with or without 20 µg/ml F(ab’)2 for indicated times as described above. After stimulation, whole cell extracts were prepared and resolved by 4-20% gradient denaturing SDS-PAGE, and blotted on to Immobilon (Millipore) membranes. The membranes were probed with rabbit anti-Bcl-xL (Pharimgen) or anti-phospho p38 (Thr180/Tyr182), anti-phospho-ERK1/2 (Thr202/Tyr204) anti-phospho-Akt (Ser473) or antibodies to ERK1/2, p38, and Akt (Cell Signaling, MA) and anti-β actin antibodies (Santa Cruze Biotechnology Inc.) for protein loading control according to the manufacturer’s instructions. The bound antibodies were revealed by HRP-conjugated goat anti-rabbit or rabbit anti-goat IgG antibodies, followed by enhanced chemiluminescent detection (Pierce, Rockford, IL) on autoradiography film.
RESULTS

An Intact BCR Signaling Pathway is Required for the Development of Transitional Type 2 B cells into Mature B cells

In prior studies, we and others have demonstrated that the M B cell population is reduced in \( btk^{-/-} \) mice and that the majority of the remaining B cells display an immature phenotype (\( \text{IgM}^{\text{bright}}\text{IgD}^{\text{low}} \) and \( \text{IgM}^{\text{bright}}\text{IgD}^{\text{bright}} \)) \( (19,33,34,47) \). BTK is an integral component of the BCR signal transduction network. Therefore, a block in B cell ontogeny at an immature B cell stage in \( btk^{-/-} \) mice suggests an important role for BCR signaling at this stage of B cell development.

Immature splenic B cells can be divided into at least two developmentally distinct subpopulations: T1 B cells (\( \text{IgM}^{\text{bright}}\text{CD21}^{\text{low/-}} \) or \( \text{HSA}^{\text{bright}}\text{CD21}^{\text{low/-}} \)) and T2 B cells (\( \text{IgM}^{\text{bright}}\text{CD21}^{\text{bright}} \) or \( \text{HSA}^{\text{bright}}\text{CD21}^{\text{bright}} \)). T2 B cells are believed to be the immediate precursors of M B cells (\( \text{IgM}^{\text{int}}\text{CD21}^{\text{int}} \) or \( \text{HSA}^{\text{int}}\text{CD21}^{\text{int}} \)) \( (19) \). To begin to understand the effects of BCR signaling in the immature B cell subpopulations, we characterized the phenotype of WT splenic B cells using flow cytometry (FACS) and compared it with splenocytes isolated from \( btk^{-/-} \) mice. FACS analysis using anti-CD21 and anti-IgM antibodies defined a block in \( btk^{-/-} \) mice at the T2 stage to M stage of B cell development as there is a significant reduction in the numbers of M B cells \( (3.9\times10^{6} \text{ in } btk^{-/-} \text{ vs } 19.2\times10^{6} \text{ in WT mice (Figure 1)}) \). The proportion and number of T2 B cells in \( btk^{-/-} \) animals compared to the WT mice was slightly increased \( (9.6\times10^{6} \text{ in } btk^{-/-} \text{ mice vs } 8.3\times10^{6} \text{ in WT mice}) \) (Figure 1). This increase might be due to the presence of marginal zone (MZ) B cells that develop normally in \( btk^{-/-} \) mice and constitute approximately 2-4\% of the cells shown as T2 in Figure 1 (data not shown). Although some of the T2 B cells expressed slightly decreased levels of IgM, most of the \( btk^{-/-} \) B cells continued to express higher levels of IgM and HSA compared to controls (Figure 1 and data not shown). In addition, the T1
and T2 subsets were not detectable in lymph nodes (LN), data not shown). Together, these results suggest that T2 B cells are found in the spleen but not LNs and that BTK is required for the developmental transition from T2 to M B cell stage. These findings are consistent with a previous study that examined T1 and T2 B cell populations in CBA/N (xid) mice (19).

Because T2 B cells are found exclusively in the spleen, where they then develop into M B cells, positive signals mediated by the BCR (provided, in vivo, by the splenic microenvironment) may play an important role in this process. Therefore, we asked whether stimulation via the BCR can induce the maturation of T1 and T2 B cells into M B cells. For these experiments, T1, T2 and M B cells were sorted based on their surface expression of HSA, CD21, and CD23 as described by Loder et. al. (19). Anti-IgM was not used for sorting to prevent inadvertent triggering of BCR signals prior to the initiation of the experiment (19,46). Re-analysis of the sorted B cell subsets by FACS revealed high purity for T1 (97.7%) and M (96.5%) B cells; however, in purified T2 B cells a low level contamination with M B cells was observed (84.5% pure) (Figure 2). We believe that the presence of the M B cells did not significantly interfere with our differentiation assay because the rate of M B cell proliferation is equal to that of T2 B cells in response to BCR stimulation (Figure 3A and Figure 5). Thus, the ratio of T2/M B cells (11:1) should not decrease significantly during the course of the experiment.

Purified T1, T2 and M B cells were incubated for up to 96 hours with or without anti-IgM antibodies. The fraction of live and dead cells within each sample was determined and then the cells were then stained with fluorescent-tagged antibodies that recognize CD21, HSA, or IgD to define the ratio of T2/M B cells. The percent of the surviving cells after various incubation times is shown in Figure 3A. Very few cells were found at 72 and 96 hrs in T1 cell cultures and,
therefore, are not included in the analysis shown in Figure 3A. Although cell death was also observed in T2 and M B cell cultures, a significantly higher proportion of anti-IgM stimulated T2 and M B cells survived compared to similarly treated T1 B cell cultures (Figure 3A). Analysis of live cells revealed that up to 40 percent of T2 B cells down-regulated the expression of both CD21 and HSA to intermediate levels (HSA_{\text{int}} CD21_{\text{int}}), a phenotype consistent with their transition into the M B cell compartment (Figure 3B). Furthermore, after anti-IgM treatment, the majority of input T2 cells express IgD on their cell surface at higher levels than input M cells, as the geometric mean fluorescence intensity (MFI) was 242 on enlarged T2 cells in contrast to a MFI of 85 on M cells (Figure 3C). These results suggest that BCR delivers a signal that facilitates T2 to M B cell maturation.

*Enhanced Expression of Activation Markers by Transitional 2 B cells*

The ability of T2 B cells to develop into M B cells prompted us to investigate their activation responses following BCR stimulation. For these studies, splenocytes were cultured with or without anti-IgM antibodies as indicated. FACS analyses revealed that T2 B cells displayed heightened expression of the activation markers CD25, CD5, CD95, and CD86 compared to either T1 or M B cells prior to BCR stimulation. Following activation, the expression levels of these cell surface antigens increased on both M and T2 B cells, however T2 B cells displayed a greater increase than did M B cells. Interestingly, when input T2 cells were analyzed 72 hrs after anti-IgM treatment, the cells that retain T2 phenotype expressed more CD25 than the cells with a M phenotype (Figure 4A). In contrast, the expression of these activation markers on untreated T1 cells was low or undetectable and did not significantly increase in response to treatment with anti-IgM (Figure 4B). BCR signaling-defective \( btk^{-} \) B
cells were defective for the up-regulation of these cell surface antigens upon anti-IgM stimulation (data not shown). Together, these findings suggest that the activation response of T2 B cells to BCR engagement relative to T1 B cells may be the result from distinct signaling program of the T2 B cell subset. Alternatively, the robust BCR responses of the T2 B cells may result from a developmental switch during T1 to T2 transition that enhances BCR-dependent responses of T2 B cells.

**T2 B cells Proliferate More Efficiently than T1 B cells in Response to BCR Stimulation**

Previous studies have shown that unlike mature B cells, immature splenic B cells (IgM$^{\text{bright}}$HSA$^{\text{bright}}$) do not proliferate but instead undergo apoptosis (4,5,8,20,21,48). These findings are supported by the observation that xid and btk$^{-/-}$ B cells, which typically display an immature phenotype, undergo excessive cell death in response to treatment with anti-IgM (49-51). The recent discovery that splenic immature B cells are heterogeneous and comprised of two distinct subsets (19) prompted us to investigate the proliferative responses of these individual B cell populations. Splenocytes were stained with anti-CD21 and anti-HSA antibodies and sorted into T1 (CD21$^{\text{low/-}}$HSA$^{\text{high}}$), T2 (CD21$^{\text{bright}}$HSA$^{\text{bright}}$), and mature B cell (CD21$^{\text{int}}$HSA$^{\text{int}}$) populations via FACS. To determine the proliferative responses of T1, T2, and M B cell subsets to BCR stimulation, equivalent numbers of sorted cells were incubated with anti-mouse IgM F(ab’)$_2$ antibodies, for the indicated lengths of time. Cultured cells were pulsed with [$^{3}$H]-thymidine to monitor DNA synthesis. As shown in Fig. 5A, M B cells, but not T1 B cells, proliferated in response to anti-IgM treatment. In sharp contrast to the T1 B cells, the T2 subset of immature B cells proliferated nearly as well as M B cells.
The T2 B cell fraction used in these experiments likely contains marginal zone (MZ) B cells that, like T2 cells, express high levels of HSA and CD21. Although MZ B cells proliferate poorly in response to anti-IgM (52), they could influence the magnitude of overall proliferation observed for the T2 B cell fraction. Therefore, the T2 cell fraction was purified from MZ B cells by staining for CD23, which is expressed by T2 but not by MZ B cells. MZ B cell-depleted T2 B cells proliferated equally well (Figure 5B) or better (Figure 5D) than M B cells at a range of time points (12 to 72 hours, Figure 5B). In fact, T2 B cells proliferated better than M B cells at early time points (Figure 5C & D and Figure 3A). Furthermore, the corresponding \( btk^{-/-} \) B cell subsets failed to proliferate in response to BCR stimulation at all time points tested (Figure 5C and D). The proliferation of \( btk^{-/-} \) B cells was comparable to WT when treated with PMA and ionomycin (Figure 5E). Taken together, the observed lack of proliferation of T1 B cells in response to BCR stimulation is in agreement with previous reports (4,5,20,48). Importantly, the T2 subset of immature B cells responded to anti-IgM treatment with rapid and strong proliferation (Figure 5). These results indicate that within the immature B cell subpopulations, BCR stimulation leads to the survival and differentiation of T2 B cells whereas T1 B cells do not proliferate.

**BCR Activation Leads to Heightened Expression of Bcl-X\(_L\) in T2 B Cells**

Although many T2 B cells die in response to BCR engagement (Figure 3A), a significant number survive as evidenced by both the \([^{3}\text{H}]\)-thymidine incorporation and *in vitro* differentiation experiments (Figures 5 and 3A). In contrast, none of the \( btk^{-/-} \) T2 B cells appear to survive (Figures 5). One factor that may contribute to the excessive cell death of BTK-mutant B cells is their inability to up-regulate expression of the pro-survival gene Bcl-x\(_L\) (49,51).
therefore evaluated whether BCR-directed signals elicit differential expression of Bcl-x<sub>L</sub> in these B cell subpopulations. Thus, anti-IgM treated B cells from WT and <i>btk<sup>−/−</sup></i> mice were stained with fluorescent-tagged antibodies that recognize HSA and CD21, and Bcl-x<sub>L</sub> expression was analyzed by intracellular staining to reveal the levels of Bcl-x<sub>L</sub> expression in the B cell subpopulations (Figure 6). We observed that Bcl-x<sub>L</sub> was induced to the highest level in WT but not <i>btk<sup>−/−</sup></i> T2 B cells and at a significantly lower level in T1 and M B cells (Figure 6B; left panel). In contrast, <i>btk<sup>−/−</sup></i> B cells failed to induce the expression of Bcl-x<sub>L</sub> in response to BCR activation (Figure 6B; right panel). Further analysis, based upon HSA and CD21 expression, clearly showed that T1 and M B cells each weakly induced Bcl-x<sub>L</sub> upon BCR treatment, while T2 B cells strongly executed this response (Figure 6C; lower three panels).

To confirm this interpretation, we initiated further studies to evaluate the upregulation of Bcl-x<sub>L</sub> protein levels by Western blotting. Cellular extracts were prepared from FACS-sorted and BCR-stimulated T1, T2, and M B cell populations (as in Figure 2). Consistent with the higher expression levels of Bcl-x<sub>L</sub> observed by FACS analysis (Figure 6A-C), the absolute levels of Bcl-x<sub>L</sub> protein markedly increased in T2 B cells stimulated with anti-IgM antibodies (Figure 6D, compare lanes 5 and 6). In contrast, BCR stimulation did not significantly change the levels of Bcl-x<sub>L</sub> protein in T1 cells (Figure 6D, compare lanes 1 and 2). These results indicate that the increased expression of Bcl-x<sub>L</sub> following engagement of the BCR is most pronounced for the T2 B cell subset. These observations are consistent with a role for Bcl-x<sub>L</sub> in promoting B cell survival to a sufficient extent such that B cells are able to initiate additional biological outcomes necessary for maturation and activation. Thus, the inability of T1 B cells to induce Bcl-x<sub>L</sub> to high levels may contribute in part to their enhanced apoptosis in response to BCR crosslinking (5,20,48). In contrast, induction of Bcl-x<sub>L</sub> by T2 cells may play a critical role in mediating their
survival and subsequent development into M B cells. In this respect, the inability of \( btk^- \) T2 B cells to induce Bcl-x\( _L \) in response to BCR engagement may account in part for the impaired T2 to M development and B cell survival observed in \( xid \) and \( btk^- \) mice.

**BCR Stimulation Induces Activation of Serine/threonine kinases predominantly in T2 B Cell subset**

BCR signals direct genetic reprogramming, activation, and mitogenesis by initiating the activation of multiple biochemical signaling pathways involving tyrosine kinases and serine/threonine kinases, including extracellular signal regulated protein kinase (ERK), p38 MAPK and Akt. To investigate whether MAPK signaling pathways are differentially activated in T1 versus T2 B cells, we first determined the kinetic profiles of MAPK phosphorylation in primary B cells. Anti-IgM treatment resulted in the increased phosphorylation of ERK1/2 within 5 minutes which returned to basal levels within 30 minutes (Figure 7A, top panel). Non-stimulated B cells showed significant phosphorylation of p38, which initially decreased upon BCR stimulation (first 2 minutes), peaked within 5 minutes to a level only slightly higher than non-stimulated cells, and then gradually decreased to undetectable levels between 10 to 30 minutes (Figure 7A, middle panel). These results are in agreement with the only published report in which the authors studied BCR-induced p38 phosphorylation in mouse primary B cells (53). The authors did not detect p38 phosphorylation when measured after 10 minutes of incubation with anti-IgM antibodies. Akt phosphorylation in response to BCR engagement occurred within 2 minutes and was barely detectable after 10 minutes (Figure 7A, middle panel).

Upon stimulation of transitional B cell subsets, we found that BCR engagement induced phosphorylation of ERK1/2 in T2 at a significantly higher level than in T1 or M B cells (Figure
Similarly, phosphorylation of p38 was also induced mainly in T2 B cells albeit modestly (Figures 7C and D). Interestingly, T1 cells showed higher levels of phosphorylated p38 in non-stimulated state than T2 B cells (Figures 7C and D, and data not shown). Like ERK1/2 and p38, BCR stimulation lead to the phosphorylation of Akt predominantly in T2 B cells (Figure 7E, compare lanes 5 and 6 with 2, 3 and 8). Induction of serine/threonine kinases, ERK1/2, p38 and Akt, in T2 but not in T1 B cells demonstrate differential regulation of these signaling pathways in the two subsets of immature B cell populations. These observations suggest that developmental stage-specific signaling may play a role in the transitions from T1 to T2 and M B cells.
DISCUSSION

In these studies, we have compared the effects of BCR signaling within peripheral B cell populations at discrete stages of development. Our results clearly establish that the two populations that comprise the immature splenic B cell compartment (T1 and T2) react distinctly to BCR stimulation. Specifically, T1 cells die in response to BCR signals, whereas T2 cells are stimulated to express activation markers and the pro-survival gene Bcl-xL, and to proliferate. Moreover, T2 but not T1 cells display a M B cell phenotype when cultured in the presence of anti-IgM antibodies that induce BCR signaling. The T2 responses to BCR stimulation differ from M B cells as they induce higher levels of Bcl-xL than M B cells. Moreover, MAPK ERK1/2, p38 and Akt are preferentially activated in T2 B cells in response to BCR engagement in vitro whereas T1 B cells did not induce significant phosphorylation of these BCR signal transducers. The dramatically different responses of T1 versus T2 B cells to BCR engagement indicate that the response to BCR signals within these immature B cell subpopulations are developmentally regulated and may play a critical role in peripheral B cell development.

Prior studies have suggested that BCR cross-linking leads to cell cycle arrest and apoptosis of splenic immature B cells (characterized by higher cell surface expression of HSA and IgM) by a process termed negative selection (4,5,48,54). Further analyses demonstrated that the immature B cells both in the BM and those which then migrate to the spleen, termed peripheral transitional immature B cells by Carsetti et. al., die upon BCR cross-linking (20). A subsequent report demonstrated that immature B cells are subject to BCR-induced cell death even upon further maturation (i.e., acquisition of IgD expression) (55). The immature B cell populations studied in these two reports may represent T1 and T2 B cell subsets. Our results have further defined the immature B cell population that is the likely target of negative selection.
by examining B cell subsets at discrete stages of peripheral B cell development. We found that T1 B cells are subject to BCR-induced cell death, which is consistent with this immature B cell subset as the target of negative selection (22). However, in contrast to previous studies (55) we found that T2 B cells (that may be equivalent to IgD+ immature B cells analyzed by Norvell et al and T3 subset analyzed by Allman et al) (55,56) proliferate in response to BCR stimulation. These differences may arise from heterogeneity within the IgD+ immature B cell subset or they could be due to the experimental protocol employed. Norvell et al (55) used irradiated and auto-reconstituted mice for their experiments while we used FACS-purified “steady-state” B cell subsets. Based on our findings, it can be hypothesized that T1 B cells that encounter self-antigen are eliminated, whereas those that do not encounter self-antigen in the BM, in transit to, or within the spleen may develop into T2 B cells. This hypothesis is supported by the recent findings of Loder et al., which suggest that T1 B cells can differentiate into T2 B cells in vivo (20).

Our in vitro experiments with the T1 B cell subset did not recapitulate the appreciable differentiation into T2 cells observed in vivo (20) because of the massive death of T1 B cells in vitro even in the absence of BCR stimulation. Despite this cell loss, very small numbers of T2 cells were derived from T1 cells (data not shown). Thus, differentiation of T1 B cells to the T2 stage in vivo may require the physiological milieu of the spleen to provide discrete growth and survival signals that facilitate B cell development. These signals may be delivered by basal “BCR tickling” and by other cellular receptors. The recently discovered B cell activation factor (BAFF) of the TNF family may provide some of the signals necessary for B cell survival during the T1 to T2 transition (46,57). This proposal is in agreement with the observed B cell developmental block at the T1 stage in BAFF−/− mice (57), suggesting that the T1 to T2 B cell
transition and subsequent development may require BAFF. Further studies of the intrinsic B cell signaling program and signals delivered by extracellular stimuli will be required to elucidate the molecular processes involved in the development of T1 into T2 B cells.

We demonstrate that in contrast to T1 B cells, T2 B cells respond to BCR stimulation by increasing the expression of Bcl-xL, proliferating robustly, and ultimately displaying a phenotype similar to M B cells. Consistent with this idea, we noted that enlarged IgD+ cells in the spleen tend to have very high levels of IgD (as did in vitro-activated T2 cells) and are preferentially found in the T2 subset (Fig. 3C). It seems possible that these cells may be recent recipients of positive selection signals in vivo. Therefore, T1 and T2 B cell stages may represent two distinct stages during peripheral B cell development: The T1 stage may provide an opportunity to eliminate self-reactive B cells while the T2 stage may serve as the target for positive selection. In this context, prior studies suggest that the positive selection influences the formation of the M B cell repertoire (10-13). For example, only a limited number of antigen specificities are represented among antigen receptors of the M B cells when compared with the available repertoire at the immature B cell stage (11,13) suggesting a role for BCR directed clonal selection. The observed positive responses of T2 B cells to BCR engagement (this study) may contribute to the formation of the antigen-specific M B cell repertoire.

The T2 B cell subpopulation proliferate in response to BCR stimulation (Fig.5). Although the significance of this proliferation remains unclear, an increase in the T2 B cell population may be necessary to accommodate the cell loss that occurs during the recruitment of immature B cells into the pool of mature B cells (4-6,8,21). This is supported by the observation that a significant fraction of T2 B cells exposed to anti-IgM in vitro undergo apoptosis while only some cells mature to display a M B cell phenotype (Fig. 3). These results suggest that a
limited set of immature B cells is selected into the M B cell compartment. This observation is consistent with prior studies that demonstrated a loss of 70-90% of the immature B cells during development and the concurrent maturation of only 10-30% to the long-lived pool of B cells (4-6).

The proliferative and apoptotic responses of T2 versus T1 cells may reflect differences in their intracellular programs. The specific nature of these different programs remains largely uncharacterized. We observe that in contrast to WT, T2 B cells that lack BTK (isolated from btk<sup>-/-</sup> mice) showed profound defects in the induction of activation markers, Bcl-x<sub>L</sub>, and proliferation following BCR engagement. In separate studies, we have shown that at least two components of the BCR signalosome, BTK and PLC-γ2, are required for the activation of NF-κB and transcriptional up-regulation of the bcl-x gene [(58) and JBP, NPS and WNK, manuscript in press] further supporting an involvement of the BCR signaling in T2 B cell responses. Like BTK and PLC-γ2, other components of the BCR signalosome including PI3K, Vav, and BLNK are involved in the transition of immature B cells to more mature stages (27,28,40,42-45). The opposite biological responses of T1 vs T2 and an involvement of the BCR signaling components in this process is also supported by a recent study published during the preparation of this manuscript. Thus coupling of the BCR with downstream signaling pathways via the BCR signalosome plays an important role at the T2 stage of B cell development.

Results in figure 7 show that BCR stimulation activates serine/threonine signaling pathways in T2 at a much higher level than in T1 B cells (Figure.7). The ERK1/2, p38 and Akt signaling pathways appear to regulate cell growth and differentiation in response to cellular stimulation (29,30,59-61). Our findings suggest that activation of ERK1/2, p38 and Akt in T2 B cells may promote B cell survival and proliferation and may contribute to the molecular signals
underlying the T2 to M transition. In this regard, increased ERK1/2 phosphorylation has been shown to play a role at the checkpoint between pro- to pre-B cell transition (62). However, the mechanism of how the BCR is coupled to the downstream pathways in T1 versus T2 cells remains unclear. One possible reason for the observed signaling differences between T1 and T2 B cells may arise from differences in the components that comprise the BCR signalosome within these B cell subpopulations. Alternatively, robust signals generated at the T2 stage may simply be the consequence of the efficiency with which the BCR signaling components are recruited to the sphingolipid-rich membrane microdomains known as lipid rafts. These structures have been suggested to serve as platforms for initiating downstream BCR signaling cascades (63). Indeed, the BCR is poorly co-localized with lipid rafts in immature B cells compared to M B cells (64).

Taken together, these findings support a model in which T1 and T2 B cells may assemble distinct BCR signaling complexes and/or sequester similar complexes with differential efficiency into lipid rafts following BCR cross-linking. Clearly, more investigation is required to elucidate the genetic and biochemical reprogramming that must occur to induce the radically different biological outcomes observed in T1 and T2 B cells in response to activation through the same receptor. Regardless of the specific nature of such a mechanism, based on the results presented here, we propose that the T2 subset contains B cells that display a state of “heightened alertness” to more efficiently execute both mitogenic and differentiation signals mediated by the BCR as compared with either T1 or M B cells. The distinct functional role for the BCR signaling in the regulation of proliferation and differentiation is also supported by a recent study showing positive responses of T2 B cells compared to T1 B cell subset (65). This higher responsiveness of T2 B cells to BCR signals may ensure their positive selection into the long-lived mature B cell pool and play an important role in shaping the mature B cell repertoire.
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**Abbreviations:** T, transitional; BCR, B cell antigen receptor; M, mature; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; BTK, Bruton’s tyrosine kinase; PTK, protein tyrosine kinase; BLNK, B cell linker protein; xid, x-linked immunodeficiency; FACS, flow cytometry;
References

1. Hardy, R. R., and Hayakawa, K. (2001) *Annu Rev Immunol* **19**, 595-621
2. Monroe, J. G. (2000) *Curr Top Microbiol Immunol* **245**(2), 1-29
3. Willerford, D. M., Swat, W., and Alt, F. W. (1996) *Curr Opin Genet Dev* **6**(5), 603-9
4. Allman, D. M., Ferguson, S. E., and Cancro, M. P. (1992) *J Immunol* **149**(8), 2533-40
5. Allman, D. M., Ferguson, S. E., Lentz, V. M., and Cancro, M. P. (1993) *J Immunol* **151**(9), 4431-44
6. Rajewsky, K. (1996) *Nature* **381**, 751-758
7. Rolink, A. G., Andersson, J., and Melchers, F. (1998) *Eur J Immunol* **28**(11), 3738-48
8. Meffre, E., Casellas, R., and Nussenzweig, M. C. (2000) *Nat Immunol* **1**(5), 379-85.
9. Rolink, A. G., Schaniel, C., Andersson, J., and Melchers, F. (2001) *Curr Opin Immunol* **13**(2), 202-7.
10. Coutinho, A. (1993) *Immunol Today* **14**(1), 38-40.
11. Forster, I., and Rajewsky, K. (1990) *Proc Natl Acad Sci U S A* **87**(12), 4781-4
12. Gu, H., Tarlinton, D., Muller, W., Rajewsky, K., and Forster, I. (1991) *J Exp Med* **173**(6), 1357-71
13. Levine, M. H., Haberman, A. M., Sant'Angelo, D. B., Hannum, L. G., Cancro, M. P., Janeway, C. A., Jr., and Shlomchik, M. J. (2000) *Proc Natl Acad Sci U S A* **97**(6), 2743-8.
14. Melchers, F., ten Boekel, E., Seidl, T., Kong, X. C., Yamagami, T., Onishi, K., Shimizu, T., Rolink, A. G., and Andersson, J. (2000) *Immunol Rev* **175**, 33-46.
15. Viale, A. C., Coutinho, A., Heyman, R. A., and Freitas, A. A. (1993) *Int Immunol* **5**(6), 599-605.
16. Lam, K. P., Kuhn, R., and Rajewsky, K. (1997) Cell 90(6), 1073-83
17. King, L. B., and Monroe, J. G. (2000) Immunol Rev 176, 86-104.
18. Monroe, J. G. (2000) Clin Immunol 95(1 Pt 2), S8-13.
19. Loder, F., Mutschler, B., Ray, R. J., Paige, C. J., Sideras, P., Torres, R., Lamers, M. C., and Carsetti, R. (1999) J Exp Med 190(1), 75-89.
20. Carsetti, R., Kohler, G., and Lamers, M. C. (1995) J Exp Med 181(6), 2129-40
21. Sandel, P. C., and Monroe, J. G. (1999) Immunity 10(3), 289-99.
22. Campbell, K. S. (1999) Curr. Opin. Immunol. 11(3), 256-64
23. Kurosaki, T. (2000) Curr. Opin. Immunol. 12, 276-281
24. Kurosaki, T., and Tsukada, S. (2000) Immunity 12(1), 1-5
25. Reth, M., and Wienands, J. (1997) Annu Rev Immunol 15, 453-79
26. Benschop, R. J., Brandl, E., Chan, A. C., and Cambier, J. C. (2001) J Immunol 167(8), 4172-9.
27. Fruman, D. A., Satterthwaite, A. B., and Witte, O. N. (2000) Immunity 13(1), 1-3
28. Lewis, C. M., Broussard, C., Czar, M. J., and Schwartzberg, P. L. (2001) Curr Opin Immunol 13(3), 317-25.
29. Aman, M. J., Lamkin, T. D., Okada, H., Kurosaki, T., and Ravichandran, K. S. (1998) J Biol Chem 273(51), 33922-8.
30. Downward, J. (1998) Curr Opin Cell Biol 10(2), 262-7
31. Buhl, A. M., Nemazee, D., Cambier, J. C., Rickert, R., and Hertz, M. (2000) Immunol Rev 176, 141-53.
32. Marshall, A. J., Niiro, H., Yun, T. J., and Clark, E. A. (2000) Immunol Rev 176, 30-46.
33. Kerner, J. D., Appleby, M. W., Mohr, R. N., Chien, S., Rawlings, D. J., Maliszewski, C. R., Witte, O. N., and Perlmutter, R. M. (1995) *Immunity* 3(3), 301-12
34. Khan, W. N., Alt, F. W., Gerstein, R. M., Malynn, B. A., Larsson, I., Rathbun, G., Davidson, L., Muller, S., Kantor, A. B., Herzenberg, L. A., and et al. (1995) *Immunity* 3(3), 283-99
35. Rawlings, D. J., Saffran, D. C., Tsukada, S., Largaespada, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Bazan, J. F., Howard, M., Copeland, N. G., and et al. (1993) *Science* 261(5119), 358-61
36. Thomas, J. D., Sideras, P., Smith, C. I., Vorechovsky, I., Chapman, V., and Paul, W. E. (1993) *Science* 261(5119), 355-8
37. Satterthwaite, A. B., and Witte, O. N. (2000) *Immunol Rev* 175, 120-7
38. Scher, I. (1982) *Adv Immunol* 33, 1-71
39. Sideras, P., and Smith, C. I. (1995) *Adv Immunol* 59, 135-223
40. Doody, G. M., Bell, S. E., Vigorito, E., Clayton, E., McAdam, S., Tooze, R., Fernandez, C., Lee, I. J., and Turner, M. (2001) *Nat Immunol* 2(6), 542-7.
41. Fruman, D. A., Snapper, S. B., Yballe, C. M., Davidson, L., Yu, J. Y., Alt, F. W., and Cantley, L. C. (1999) *Science* 283(5400), 393-7
42. Fu, C., Turck, C. W., Kurosaki, T., and Chan, A. C. (1998) *Immmunity* 9(1), 93-103
43. Tedford, K., Nitschke, L., Girkontaite, I., Charlesworth, A., Chan, G., Sakk, V., Barbacid, M., and Fischer, K. D. (2001) *Nat Immunol* 2(6), 548-55.
44. Wang, D., Feng, J., Wen, R., Marine, J. C., Sangster, M. Y., Parganas, E., Hoffmeyer, A., Jackson, C. W., Cleveland, J. L., Murray, P. J., and Ihle, J. N. (2000) *Immunity* 13(1), 25-35
45. Xu, S., Tan, J. E., Wong, E. P., Manickam, A., Ponniah, S., and Lam, K. P. (2000) *Int Immunol* **12**(3), 397-404.

46. Batten, M., Groom, J., Cachero, T. G., Qian, F., Schneider, P., Tschopp, J., Browning, J. L., and Mackay, F. (2000) *J Exp Med* **192**(10), 1453-66.

47. Khan, W. N., Nilsson, A., Mizoguchi, E., Castigli, E., Forsell, J., Bhan, A. K., Geha, R., Sideras, P., and Alt, F. W. (1997) *Int Immunol* **9**(3), 395-405

48. Norvell, A., Mandik, L., and Monroe, J. G. (1995) *J Immunol* **154**(9), 4404-13

49. Anderson, J. S., Teutsch, M., Dong, Z., and Wortis, H. H. (1996) *Proc Natl Acad Sci U S A* **93**(20), 10966-71.

50. Brorson, K., Brunswick, M., Ezhevsky, S., Wei, D. G., Berg, R., Scott, D., and Stein, K. E. (1997) *J. Immunol.* **159**(1), 135-43

51. Solvason, N., Wu, W. W., Kabra, N., Lund-Johansen, F., Roncarolo, M. G., Behrens, T. W., Grillot, D. A., Nunez, G., Lees, E., and Howard, M. (1998) *J Exp Med* **187**(7), 1081-91

52. Oliver, A. M., Martin, F., Gartland, G. L., Carter, R. H., and Kearney, J. F. (1997) *Eur J Immunol* **27**(9), 2366-74

53. Wu, H. J., Venkataraman, C., Estus, S., Dong, C., Davis, R. J., Flavell, R. A., and Bondada, S. (2001) *J Immunol* **167**(3), 1263-73.

54. Goodnow, C. C., Crosbie, J., Adelstein, S., Lavoie, T. B., Smith-Gill, S. J., Brink, R. A., Pritchard-Briscoe, H., Wotherspoon, J. S., Loblay, R. H., Raphael, K., and et al. (1988) *Nature* **334**(6184), 676-82

55. Norvell, A., and Monroe, J. G. (1996) *J Immunol* **156**(4), 1328-32.
56. Allman, D., Lindsley, R. C., DeMuth, W., Rudd, K., Shinton, S. A., and Hardy, R. R. (2001) *J Immunol* **167**(12), 6834-40.

57. Schiemann, B., Gommerman, J. L., Vora, K., Cachero, T. G., Shulga-Morskaya, S., Dobles, M., Frew, E., and Scott, M. L. (2001) *Science* **293**(5537), 2111-4.

58. Petro, J. B., and Khan, W. N. (2001) *J Biol Chem* **276**(3), 1715-1719.

59. Glassford, J., Holman, M., Banerji, L., Clayton, E., Klaus, G. G., Turner, M., and Lam, E. W. (2001) *J Biol Chem* **276**(44), 41040-8.

60. Ichijo, H. (1999) *Oncogene* **18**(45), 6087-93.

61. Sakata, N., Kawasome, H., Terada, N., Gerwins, P., Johnson, G. L., and Gelfand, E. W. (1999) *Eur J Immunol* **29**(9), 2999-3008.

62. Fleming, H. E., and Paige, C. J. (2001) *Immunity* **15**(4), 521-31.

63. Cherukuri, A., Dykstra, M., and Pierce, S. K. (2001) *Immunity* **14**(6), 657-60.

64. Chung, J. B., Baumeister, M. A., and Monroe, J. G. (2001) *J Immunol* **166**(2), 736-40.

65. Su, T. T., and Rawlings, D. J. (2002) *J Immunol* **168**(5), 2101-10.
Figure Legends

Fig. 1. B cell development is impaired at Transitional stage 2 in **btk**⁻/⁻ mice. Freshly isolated WT and **btk**⁻/⁻ spleen and LN cell suspensions were stained for CD21 and IgM to reveal T1, T2 and mature B cell populations. Cells within the live lymphocyte gate were analyzed. The percent of T1, T2, and M of the total B cell population is shown within the respective regions.

Figure 2. Purification of T1, T2 and M B cell populations. Splenocytes from 4 week old C57Bl/6 mice were depleted of RBC and of T cells (MACS) and stained using anti-CD23, anti-CD21 and anti-HSA (clone 30F1). The middle top panel depicts the sorting gates used to fractionate CD23-negative from CD23-positive cells. The proportion of total cells analyzed within the gates is indicated. Only live (Propidium iodide-negative) cells were sorted. Upper gates for forward and side scatter were used to exclude cell doublets. The plots are shown as 5% probability plots. The upper left plot shows the gate used to sort T1 cells, and indicates the proportion of CD23-negative cells that fall within that gate. The upper right plot shows the gates used to sort T2 and M cells, and the fraction of CD23-negative cells within these gates. The lower 3 plots depict re-analysis of the indicated sorted cell population, with the fraction of all cells that fall within each quadrant indicated.

Figure 3. T2 B cells display characteristics of M B cells upon anti-IgM treatment in *vitro*. (A). Proportion of live cells after culture in the presence or absence of anti-IgM antibodies. 5 x 10⁵ FACS-sorted T1, T2 or M cells (depicted in Figure 2) were cultured with or without 10 µg/ml anti-IgM F(ab’)₂ harvested at the indicated time, stained with acridine orange and ethidium bromide, and enumerated using a fluorescent microscope and a hemocytometer.
The number of live cells (acridine orange-positive) and the number of dead cells (ethidium bromide-positive) were used to calculate the fraction of live and dead cells within each sample. Since most of the T1 cells died during culture after 24 hours the percent of T1 live cells is not shown for 72 and 96 hours. (B). Phenotypic analyses of T2 B cell differentiation. Cells (from Figure 2) were placed in culture with or without anti-IgM F(ab’)_2 (10 µg/ml), harvested at the indicated time, stained with anti-HSA and anti-CD21 and analyzed by FACS. Propidium iodide was also included to exclude dead cells. Plots are shown as 5% probability plots. Gates for T1, T2 and M cells were drawn using comparably stained samples from freshly harvested spleen. The fraction of live cells that falls within the indicated gates is shown. Sorted M cells were analyzed in parallel and did not substantially change in phenotype except for a general trend to up-regulate HSA, independent of IgM stimulation. (C). Phenotypic analyses of IgD expression on T1, T2 and M cells. Left panel: analysis of purified cells after culture. 5 x 10^5 FACS-sorted cells (depicted in figure 2) were placed in culture for 96 hrs as in (A), stained with anti-IgD and analyzed by FACS. Propidium iodide was also included to exclude dead cells. The fraction of live cells that is IgD-positive and enlarged (higher FSC) is indicated within the gate. The number above the gate corresponds to the MFI for IgD of the cells within the gate. The number in the lower corner of the plot corresponds to the number of live cells depicted in the plot; in the case of untreated cells, very few live cells were left in culture. Sorted T1 cells were analyzed in parallel but are not included due to extensive cell death. All plots are shown as 5% probability plots. Right panel: analysis of freshly isolated spleen cells stained for IgD, HSA and CD21. Gates for T1, T2 and M cells were drawn using all live cells, then imposed upon the indicated subsets. The fraction of live cells that fall within the indicated gates are shown for the FSC by IgD plot. The upper right plot depicts the HSA and CD21 profile for the high FSC high IgD
subset indicated (numbers given are the percent of the subset), whereas the lower left plot shows fraction of the IgD-positive, low FSC subset that correspond to T1, T2 or M cell populations.

**Figure 4. T2 B cells express higher levels of activation antigens than T1 or M B cells.** (A). Phenotypic analyses of CD25 expression on purified M and T2 cells after culture. 5 x $10^5$ FACS-sorted cells M or T2 cells (depicted in Figure 2) were placed in culture with or without anti-IgM F(ab\')$_2$ (10 µg/ml), harvested at the indicated time, stained with anti-HSA, anti-CD21 and anti-CD25 and analyzed by FACS. Propidium iodide was also included to exclude dead cells. Plots are shown as 5% probability plots. For cells derived from cultured input T2 cells, gates for T2 and M cells were drawn using a comparably stained (using anti-HSA and anti-CD21) sample from freshly harvested spleen. The gated population is indicated by the T2 or M designation within the plot. The fraction of live cells that fall within the indicated gates are shown in the plots. The number below the gate is the MFI of CD25 expression for the gated population. For treated input T2 cells, the MFI of all live cells is 343 at 24 hrs and 462 at 72 hrs. For the sorted M cells, all live cells are depicted in the FSC by CD25 plots, and the gate reflects the fraction of live cells with activation of CD25 above autofluorescence background. In the case of input T2 cells, the FSC versus CD25 plot depicts the fraction of the T2 or M subset that express elevated CD25. Sorted T1 cells were analyzed in parallel but are not included due to extensive cell death. Analysis of freshly isolated splenocytes revealed that 20% of T2 cells express CD25 (MFI= 812) whereas only 3.4% of M cells express CD25 (MFI= 132). (B). T2 B cells express higher levels of CD5 that is induced at a greater level than T1 or mature B cells following BCR stimulation. Splenocytes from WT mice were incubated with anti-IgM (10 µg/ml) or left untreated. Following 48 hours incubation, cells were stained for CD21, HSA, and
CD23 in combination with anti-CD5. Vertical lines indicate levels of antigen expression on non-stimulated cells.

**Figure 5. T2 B cells proliferate while T1 cells die in response to BCR stimulation.** (A). Splenocytes from WT mice were purified (FACS sorted) for T1, T2 and mature B cells based on their expression levels of CD21 and HSA and used in proliferation assays (5x10^5 cells/well). Cells were incubated with 10 µg/ml anti-IgM or left non-stimulated for the specified periods. B. Kinetics of T2 B cell proliferation (50x10^5 cells/well) was compared with mature B cell population with cells purified as in (A). (C) Proliferation assays were performed on T1, T2, mature and total B cells isolated from WT and btk-/− mice using CD21, HSA and CD23 triple staining protocol as described in figure 2. This protocol eliminates contaminating MZ B cells from T2 isolation. Cells (2x10^5 cells/well) were incubated for (C). 48 hours or (D). 72 hours with anti-IgM as in A. The relative [³H]-thymidine incorporation in the anti-IgM treated samples indicates that, like mature B cells, T2 B cells can proliferate with similar (A & B) or even better (C & D) amplitude and kinetics in response to the BCR stimulation. The enhanced proliferation of T2 B cells is more evident in T2 preparations depleted of MZ B cells (C &D). None of the btk−/− B cell subpopulations responded to anti-IgM treatment but did respond to (E) PMA and ionomycin by proliferation.

**Figure 6. BCR Activation Leads to Heightened Expression of Bcl-XL in T2 B Cells Relative to T1 and Mature B Cells and this response is absent in btk−/− B cells** (A). FACS analysis of intracellular Bcl-XL levels in WT (left panel) and btk−/− B cells (right panel). Purified B cells were cultured in the presence or absence of 10 µg/ml anti-IgM for 16 hrs prior to staining.
with biotin-conjugated anti-HSA and FITC-conjugated anti-CD21 antibodies. Cells were then fixed, permeabilized, and stained with PE-conjugated anti-Bcl-xL antibodies as described in Materials and Methods. Bcl-xL expression of CD21+ and HSA+ B cells is shown. (B) FACS analysis of Bcl-xL expression within specific B cell subpopulations from WT (left panel) and btk-/- mice (right panel). Bcl-xL expression in anti-IgM-treated T1, T2, and M B cells [as in (A)] are displayed. Subpopulations were identified based upon the relative expression levels of HSA and CD21, respectively. (C) FACS analysis of Bcl-xL in purified B cell subpopulations. B cells from WT mice were activated with either anti-IgM or PMA and ionomycin (P/I) for 16h prior to staining as in (A). Expression of Bcl-xL in total B cells (upper panel), T1 B cells (second panel), T2 B cells (third panel), and Mature B cells (M; lower panel) is presented. B cell subpopulations were identified based upon their relative expression of HSA and CD21. (D) Western blot analysis of Bcl-xL in sort-purified T1 and T2 B cell subpopulations. Cells were stimulated with anti-IgM as in (A). Equal amounts of total protein extracts (1.5 x 10^6 cell equivalents/lane) were subjected to immunoblot analysis with anti-Bcl-xL antibodies.

**Figure. 7. BCR engagement induces greater phosphorylation of Serine/Threonine kinases, ERK1/2, p38, and Akt in T2 than in T1 B cells.** A) Kinetics of ERK1/2, p38 and Akt phosphorylation in primary B cells in response to BCR stimulation. Purified B cells were incubated with 20 µg/ml of anti-IgM for indicated times and total cellular extracts equivalent to 3.0 x10^6 cells for each lane were analyzed for phosphorylation of ERK1/2, p38 and Akt by SDS-PAGE immunoblotting. The blots were probed sequentially with anti-phospho-ERK1/2 (top panel), anti-phospho-p38 (second from top panel) and anti-phospho-Akt (third from top panel). Total p38 blot is shown for protein loading control (bottom panel). B) BCR mediated induction
of ERK1/2 phosphorylation. Splenic T1, T2, and M B cell subpopulations were FACS-purified as in Fig. 2 and stimulated with 20 µg/ml of anti-IgM for 5 minutes or left non-stimulated. Total cellular extracts from equal number of cells within each subset (0.75-1.0 x10^6, lanes 1-8; 3.0 x10^6, lanes 9-11) were subjected to SDS-PAGE and immunoblotting. Cellular extracts from total B cells were used as control for anti-IgM mediated activation of BCR signaling. Immunoblots were sequentially probed with anti-phospho-ERK1/2 (Top panel) and β-actin for protein loading control (bottom panel). C) BCR mediated phosphorylation of p38 MAPK. Cells were prepared and analyzed as in (B) and sequentially probed with anti-phospho-p38 (top panel) and β-actin for protein loading control (bottom panel). D) Kinetics of BCR mediated phosphorylation of p38 MAPK. Cells were stimulated for indicated time periods and total cellular extracts equivalent to 0.7 x10^6 (T1, T2, and M) and 2.0 x10^6 (4X B cells) were subjected to SDS-PAGE and immunoblotting as in (C). (E) BCR mediated phosphorylation of Akt. Cells were prepared, stimulated and analyzed as in (D) except the blots were probed sequentially with anti-phospho-Akt and anti-Akt. Results presented in this figure are representative of at least three experiments.
Fig. 1
Figure 3A
Figure 3B
Figure 3C
Figure 4A
Fig. 4B
Fig. 5
Fig. 6
Fig. 6 C
Fig. 6D
A. Time following anti-IgM stimulation (min)

|     | 0 | 1 | 2 | 3 | 4 | 5 | 10 | 15 | 30 | P/I |
|-----|---|---|---|---|---|---|----|----|----|-----|
| Phos-ERK1/2 |   |   |   |   |   |   |    |    |    |     |
| Phos-p38     |   |   |   |   |   |   |    |    |    |     |
| Phos-AKT     |   |   |   |   |   |   |    |    |    |     |
| Total p-38   |   |   |   |   |   |   |    |    |    |     |

B. Anti-IgM (5 min):

|     | T1 | T2 | M  | 1X B cells | 3X B cells |
|-----|----|----|----|------------|------------|
| Phos-ERK1/2 |   |   |    |     |            |

| Fold change | 1.0 | 1.7 | 1.0 | 11.0 | 1.0 | 5.0 | 1.0 | 13.0 | 1.0 | 12.0 |
|-------------|-----|-----|-----|------|-----|-----|-----|------|-----|------|
| Actin       |     |     |     |      |     |     |     |      |     |      |

Fig. 7
C. Anti-IgM (5 min):

|          | T1 | T2 | M | B cells | B cells |
|----------|----|----|---|---------|---------|
| Phos-p38 | 1.0| 0.8| 1.0| 2.0     | 1.0     |
|          | 1.0| 1.2| 1.0| 1.4     | 1.0     |
|          | 1.0| 0.98| |

Fold change

Actin

D. Anti-IgM (min):

|          | T1 | T2 | M | 3X B cells |
|----------|----|----|---|------------|
| Phos-p38 | 1.0| 0.8| 1.0| 0.9        |
|          | 1.0| 2.9| 4.3| 1.5        |
|          | 1.0| 1.0| 1.0| 0.9        |
|          | 1.0| 2.9| 4.3| 1.5        |

Fold change

Actin

E. Anti-IgM (min):

|          | T1 | T2 | M | 3X B cells |
|----------|----|----|---|------------|
| Phos-AKT | 1.0| 4.0| 8.0| 1.0        |
|          | 1.0| 8.5| 13.0| 4.0       |
|          | 1.0| 1.0| 4.0| 2.0       |

Fold change

AKT

Fig. 7
Transitional type 1 and 2 B lymphocyte subsets are differentially responsive to antigen receptor signaling
James B. Petro, Rachel M. Gerstein, John Lowe, Robert S. Carter, Nicholas Shinners and Wasif N. Khan

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