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Transitional B Lymphocyte Subsets Operate as Distinct Checkpoints in Murine Splenic B Cell Development

Thomas T. Su* and David J. Rawlings²†‡

Signaling through the Ag receptor is required for peripheral B lymphocyte maturation and maintenance. Defects in components of the B cell receptor (BCR) signalosome result in developmental blocks at the transition from immature (heat-stable Ag (HSA) high) to mature (HSA low) B cells. Recent studies have subdivided the immature, or transitional, splenic B cells into two subsets, transitional 1 (T1) and transitional 2 (T2) cells. T1 and T2 cells express distinct surface markers and are located in distinct anatomic locations. In this report, we evaluated the BCR signaling capacity of T1 and T2 B cell subsets. In response to BCR engagement, T2 cells rapidly entered cell cycle and resisted cell death. In contrast, T1 cells did not proliferate and instead died after BCR stimulation. Correlating with these results, T2 cells robustly induced expression of the cell cycle regulator cyclin D2 and the antiapoptotic factors A1/Bfl-1 and Bcl-xL and exhibited activation of Akt. In contrast, T1 cells failed to up-regulate these markers. BCR stimulation of T2 cells also led to down-regulation of CD21 and CD24 (HSA) expression, resulting in a mature B cell phenotype. In addition, T2 cells from Bruton’s tyrosine kinase-deficient Xid mice failed to generate these proliferative and survival responses, suggesting a requirement for the BCR signalosome specifically at the T2 stage. Taken together, these data clearly demonstrate that T2 immature B cells comprise a discrete developmental subset that mediates BCR-dependent proliferative, prosurvival, and differentiation signals. Their distinct BCR-dependent responses suggest unique roles for T1 vs T2 cells in peripheral B cell selection.


Postnatal B lymphocyte development begins in the bone marrow (BM) and ultimately leads to the generation of mature peripheral B cells capable of producing secreted IgGs. Based on the expression of cytosolic and surface markers, B cells in the BM can be divided into various stages, including pro-B, pre-B, immature, and recirculating B lymphocytes (1, 2). In mice, ~2 × 10⁷ BM immature B cells are produced daily (3). Only 10–20% of these cells survive to exit the BM and enter the spleen (4, 5). B lymphocytes in the spleen were initially categorized into mature (M) and immature, or transitional, cells. In contrast to immature B cells, mature cells are long-lived (15–20 wk vs 3–4 days for immature cells) and recirculate beyond the spleen to secondary lymphoid organs (6). Specific patterns of surface marker expression have been used to distinguish immature (heat-stable Ag (HSA) high IgM high IgD low B220 low) from mature (HSA low IgM low IgD high B220 high) splenic B cells (4, 7).

Signaling through the B cell receptor (BCR) is required for the development and maintenance of mature splenic B lymphocytes. Deletion of the Ig cytoplasmic tail leads to a severe block in the generation of peripheral B cells (8). Conditional ablation of the IgH chain in the periphery leads to rapid death in all splenic B cell populations (9), suggesting that an intact BCR is crucial for the survival of splenic B cells. Additional experiments, demonstrating a restricted BCR repertoire in mature splenic cells, suggest that signals from the Ag receptor drive B cell selection and differentiation into the pool of long-lived mature cells (10–15). Furthermore, mice defective in Bruton’s tyrosine kinase (Btk) (16) and several other BCR signaling molecules (including Syk, Lyn, phosphatidylinositol 3-kinase, BLNK, phospholipase Cγ2, and VAV) (17–20) each exhibit reduced numbers of mature splenic B cells. Together, these mouse models support two alternative, but not necessarily mutually exclusive, possibilities: 1) BCR signaling in immature B cells is required for their maturation into long-lived mature cells, and/or 2) BCR signaling in mature B cells is required for their ongoing survival.

Previous studies suggest that, in response to BCR cross-linking, HSA low mature B cells proliferate, whereas HSA high immature splenic B cells are relatively nonresponsive and die (7, 21). An implication of these results is that the splenic immature B cell stage acts as a target for negative selection in the periphery (21). However, recent reports have subdivided the immature (HSA high) splenic B cell population into two distinct subsets: transitional 1 (T1) and transitional 2 (T2) B cells (22). In vivo experiments indicate that T1 cells give rise to T2 cells, whereas T2 cells can further differentiate into HSA low mature follicular (M) B cells. Confocal microscopy reveals that T2 cells are situated within primary follicles adjacent to M cells, whereas T1 cells are located at the outer peritrabecular lymphoid sheath (PALS), outside of the follicle (22). Furthermore, a large fraction of T2 cells are in G2/M phase of the cell cycle, suggesting they are in a more activated state than the T1 immature subset. A recent report also indicates that T2 cells, but not T1 cells, actively proliferate in response to
the novel B cell growth factor BAFF (LYS, TALL-1, THANK, zTNF4) (23). These data suggest that immature splenic B cells are heterogeneous and that the T1 and T2 subsets may respond differentially to key developmental signals.

In the current report, we directly evaluated the BCR-dependent signaling function of highly purified T1 vs T2 splenic immature B cells. Our data demonstrate important differences in BCR responsiveness between T1 and T2 cells. Most notably, the T2 subset of HSA$^{\text{high}}$ immature B cells generates proliferative, antiapoptotic, and differentiation signals in response to BCR engagement. In contrast, the T1 subset is relatively unresponsive to BCR stimulation. These observations argue against a requirement for BCR signaling in T1 cell development but indicate that BCR signaling likely plays a critical role in T2 B cell survival and maturation. Taken together with previously published data, the distinct BCR-dependent responses of these two immature subsets are consistent with T1 cells being a target for B lineage negative selection and T2 cells playing a unique role in Ag-driven positive selection.

Materials and Methods

Mouse strains

BALB/c and BALB/ldx mice were bred and maintained in the animal facilities of the MacDonald Research Laboratories and handled according to guidelines of the University of California (Los Angeles, CA) Animal Research Committee. Mice used in all experiments were between 6 and 12 wk old.

Cells and reagents

Single cell suspensions were prepared from splenocytes depleted of erythrocytes by lysis with ammonium chloride solution. Murine splenocytes were cultured in RPMI 1640 with 5% FCS plus supplement (glutamine, 2-ME, penicillin, streptomycin, 10 mM HEPES). Cells were stimulated with 10 μg/ml polyclonal goat F(ab)$ _{2}$ anti-mouse IgM Ab (The Jackson Laboratory, Bar Harbor, ME) or 1 μg/ml each of PMA plus ionomycin (Calbiochem, La Jolla, CA).

Flow cytometry

For cell surface staining, 5 × 10^5 cells per sample were incubated with appropriate Abs. Data were collected on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using CellQuest software (BD Biosciences). Anti-CD21-FITC (7G6), anti-CD24 (HSA)-PE (M1/69), anti-CD24 (HSA)-biotin (M1/69), anti-CD23-biotin (B3B4), anti-B220-PE (RA3-26), anti-CD21 and anti-CD24 (HSA) were purchased from BD Pharmingen (San Diego, CA). Anti-IgM-Cys $ _{5}$ Abs were purchased from The Jackson Laboratory, and anti-IgD-PE (11–26) were from Southern Biotechnology Associates (Birmingham, AL). All staining profiles were based on live-gated cells, as determined by forward and side scatter. For cell sorting, 5 × 10^5 cells per sample were incubated in 500 μl of staining medium (RPMI 1640 with 2.5% FCS plus supplement) with various Abs. Cells were sorted on a FACSVantage cell sorter (BD Biosciences) into 1 ml of collection medium (RPMI 1640 with 20% FCS plus supplement).

[3H]Thymidine uptake proliferation assay

Purified cells were incubated at 5 × 10^5/well in RPMI 1640 with 5% FCS plus supplement. Unless otherwise stated, cells were pulsed with 1 μCi [3H]thymidine for 12 h prior to harvesting. Cells were harvested and [3H]thymidine uptake was analyzed using a scintillation counter.

Immunoblotting

Total cell lysates were prepared by boiling in SDS-containing sample buffer for 10 min. Samples were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed using standard procedures. Lanes were loaded based on equal cell numbers. However, because certain cell populations (e.g., T2 cells) become more activated than others, we adjusted the loading volumes to approximate equivalent actin levels when necessary. Immunoblotting Abs used included: anti-actin D2 (M-20), anti-Bcl-xL (S-18; each from Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho Ser473 Akt, anti-Akt (New England Biolabs, Beverly, MA); anti-actin (A-2066; Sigma-Aldrich, St Louis, MO). Normalized densitometry readings were generated by subtracting the background and dividing by the respective control (actin) band intensities. Densitometry readings are presented as ratios relative to the normalized readings of the first lane of each blot.

Results

T2, but not T1, splenic B cells proliferate in response to Ag receptor activation

The initial characterization of splenic T1 and T2 cells distinguished these two populations using the CD21 and CD24 (HSA) cell surface markers (22). T1 (CD21$^{\text{low}}$) and T2 (CD21$^{\text{high}}$) cells subdivide the immature (HSA$^{\text{high}}$) splenic B lymphocytes and are distinguished from mature splenic B cells, which are HSA$^{\text{low}}$. Surface IgM and IgD expression also distinguishes T1 (IgM$^{\text{high}}$IgD$^{\text{low}}$), T2 (IgM$^{\text{high}}$IgD$^{\text{high}}$), and M (IgM$^{\text{low}}$IgD$^{\text{low}}$) cells (data not shown) (22). For cell sorting, previous studies have isolated these splenic populations primarily using the CD21 and HSA markers to avoid potential preactivation with the anti-IgM staining Abs (22, 23). Following this same rationale, we stained primary murine splenocytes for CD21 and HSA and used FACS to isolate T1, T2, and M cells. The percentage of T1 (CD21$^{\text{low}}$HSA$^{\text{high}}$), T2 (CD21$^{\text{high}}$HSA$^{\text{high}}$), and M (CD21$^{\text{low}}$HSA$^{\text{low}}$) cells falling within their respective gates was similar to that reported in earlier studies (Fig. 1A) (22). After FACS isolation of these cells, reanalysis of the sorted samples consistently revealed a sorting purity of between 84 and 98% for each population (Fig. 1A). We further characterized the sorted cells by evaluating their B220, IgM, and IgD surface expression levels (Fig. 1B). These results confirm the phenotypes of the FACS-isolated T1, T2, and M cells and are consistent with previous reports of these cell populations (22).

To begin to evaluate the response of these developmental subsets to BCR engagement, equal numbers of T1, T2, and M cells were tested for BCR-mediated proliferation as assessed by [3H]thymidine uptake. In agreement with previous studies (7), HSA$^{\text{high}}$ mature B cells proliferated in response to BCR activation (Fig. 2A). The immature T1 subset did not proliferate after BCR stimulation (Fig. 2A). In striking contrast to T1 cells, the T2 subset of immature B cells proliferated robustly in response to BCR engagement. T2 cells proliferated to a similar extent as M cells over a series of time points (24, 36, and 48 h) (Fig. 2A). Of note, the FACS-isolated T1 and T2 cells expressed equivalent levels of surface IgM (Fig. 1B), excluding the possibility that differences in BCR responsiveness were secondary to differences in Ag receptor density.

In addition to T2 cells, marginal zone (MZ) B cells also exhibit a CD21$^{\text{high}}$HSA$^{\text{high}}$ surface phenotype (24). However, MZ cells can be further distinguished from the T2 subset by the lack of CD23 surface expression (i.e., CD21$^{\text{high}}$CD23$^{-}$ MZ cells vs CD21$^{\text{high}}$CD23$^{+}$ T2 cells) (22, 24). To determine whether the proliferative responses we observed in the CD21$^{\text{high}}$HSA$^{\text{high}}$ population were due to contaminating MZ cells, we used three-color FACS to separate MZ cells from T2 cells. CD21, HSA, CD23 staining of primary splenocytes reveals that...


\textbf{FIGURE 1.} Cell sorting of splenic B cell populations. A, In this representative experiment, BALB/c splenocytes were stained for CD21 and HSA surface markers with the CD21/HSA profile as shown (upper panel). T1 (CD21\textsuperscript{low}HSA\textsuperscript{high}), T2 (CD21\textsuperscript{high}HSA\textsuperscript{high}), and M (CD21\textsuperscript{low}HSA\textsuperscript{low}) cell gates were drawn according to previous reports (22). The percentage of splenocytes falling within T1, T2, and M gates are as shown. T1, T2, and M cells were FACS-isolated with CD21/HSA profiles and sorting purities of post-sort populations shown (lower panel). B, FACS-isolated T1, T2, and M populations were stained for B220, IgM, or IgD surface expression, with respective histogram plots as shown. C, BALB/c splenocytes were stained and sorted as in A, except for the additional use of anti-CD23 Abs to distinguish T2 (CD23\textsuperscript{−}) and MZ (CD23\textsuperscript{−}) cells within the CD21\textsuperscript{high}HSA\textsuperscript{high} population. Upper right panel, A histogram of CD23 surface expression, gated on the CD21\textsuperscript{high}HSA\textsuperscript{high} population (Pre-Sort). Cells were FACS sorted by CD21/HSA/CD23 markers to isolate T1, T2, MZ, and M cells using the gates shown (upper panels). CD21/HSA and CD23 profiles of post-sort T2 and MZ cells are depicted, with percentages in the CD23\textsuperscript{+} (T2) or CD23\textsuperscript{−} (MZ) gates as shown.

\textbf{T2, but not T1, B cells up-regulate cyclin D2 after BCR engagement}

Cyclin D2 is a critical cell cycle regulator that is expressed in splenic B cells after BCR engagement (26). Therefore, we tested the ability of splenic T1, T2, and M cell populations to up-regulate cyclin D2. Strikingly, after 12 h of BCR stimulation, cyclin D2 expression was predominantly induced in CD21\textsuperscript{high}HSA\textsuperscript{high} T2 cells (Fig. 3A). In contrast, T1 cells only minimally induced cyclin D2 (Fig. 3, A and B). Interestingly, the cyclin D2 up-regulation in M cells was also significantly less robust than in T2 cells at all time points evaluated (Fig. 3, A and B, and data not shown). This difference was consistent with the early proliferative response of T2 cells (Fig. 2C). The marked differences in cyclin D2 up-regulation between T1, T2, and M cells persisted for at least 48 h post-BCR engagement (data not shown).

Though MZ cells do not proliferate after BCR activation, BCR stimulation of MZ cells does elicit early signaling events, including an exaggerated calcium response (24). To test whether the pronounced cyclin D2 induction in CD21\textsuperscript{high}HSA\textsuperscript{high} cells was due to MZ vs T2 cells, we again used three-color FACS to separate MZ and T2 cells as described in Fig. 1B. BCR cross-linking led to no significant induction of cyclin D2 expression in CD23\textsuperscript{+} MZ cells (Fig. 3B). In contrast, BCR-activated CD23\textsuperscript{−}CD21\textsuperscript{high}HSA\textsuperscript{high} T2 cells strongly up-regulated cyclin D2 expression (Fig. 3B). In summary, the inability to fully up-regulate cyclin D2 in T1 and MZ cells correlates with their inability to proliferate in response to BCR cross-linking. The rapid and

\textbf{~60% of CD21\textsuperscript{high}HSA\textsuperscript{high} cells are CD23\textsuperscript{+} (T2), whereas 40% are CD23\textsuperscript{−} (MZ) (Fig. 1C). After FACS isolation using all three surface markers, reanalysis of the sorted populations revealed a purity of 88% for T2 cells and 95% for MZ cells (Fig. 1C). Equal numbers of purified T2 and MZ cells, along with T1 and M cells, were assessed for BCR-mediated proliferation. Strikingly, CD23\textsuperscript{−} T2 cells proliferated robustly, whereas CD23\textsuperscript{−} MZ cells responded minimally to BCR activation (Fig. 2B). These results are consistent with previous reports showing that MZ cells do not proliferate in response to BCR cross-linking and instead undergo apoptotic cell death (24). Using this more stringent purification strategy, T2 cells exhibited greater proliferation than M cells (Fig. 2B), indicating that the presence of contaminating MZ cells in the CD21\textsuperscript{high}HSA\textsuperscript{high} population led to an underestimation of the T2 proliferative response in Fig. 2A.

Earlier reports indicated that T2 cells, compared with T1 or M cells, contain a higher percentage of cells basally in the G\textsubscript{0}/M phase of cell cycle (22). Cell cycle analysis using propidium iodide revealed a similar increase in cycling T2 cells (data not shown). We further investigated this early activation of T2 cells using a proliferation assay at very early time points (6–24 h) after BCR engagement. Interestingly, T2 cells entered cell cycle much more rapidly than either T1 or M cells (Fig. 2C). Though the significance of this early BCR responsiveness is unclear, these observations highlight the unique BCR response of T2 cells and distinguish them from both T1 and M cells.

We also evaluated the capacity of T1, T2, and M cells to up-regulate surface expression of the early activation marker CD69. BCR stimulation led to an equivalent up-regulation of CD69 expression on the viable cells in all three subpopulations (Fig. 2D). Of note, the capacity of splenic T1 cells to up-regulate CD69 is distinct from a previous report indicating that phenotypically similar immature B cells in the BM fail to up-regulate this marker (25). Thus, the T1, T2, and M splenic B cell populations each exhibit the ability to generate at least a subset of early BCR-dependent signals, despite having distinct proliferative responses.

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A robust cyclin D2 induction in T2 cells further correlates with their early proliferative capacity.

A BCR-dependent survival signal is generated in T2 but not T1 B cells

In addition to proliferative signals, BCR engagement leads to antiapoptotic signals in splenic B cells (27–29). After 48 h of in vitro culture in the absence of any stimulation, the majority of primary murine splenocytes undergo cell death (30–32% viable) as assessed by forward and side scatter (Fig. 4A). However, in the presence of BCR cross-linking Abs, splenocyte viability remained nearly as high (75–80%) as that observed in freshly isolated cells (80% viable; Fig. 4A). These results suggest that a BCR-mediated signal can protect at least some splenic B cells from death.

To determine differences in BCR-dependent survival between the immature T1 and T2 subsets, we cultured purified T1, T2, and M cells for 48 h in the presence or absence of BCR cross-linking Abs and assessed their viability. In the absence of stimulation, all three populations exhibited significant cell death (Fig. 4B). However, in the presence of BCR stimulation, T2 (78% viable) and M (85% viable) cells were rescued from death (Fig. 4B). In contrast, BCR stimulation did not rescue T1 cells from death and, instead, reproducibly enhanced cell death in this developmental subset (<10% viable).

A1/Bfl-1 is an antiapoptotic factor in mature B lymphocytes (30–32). A1 is also highly expressed in long-lived mature splenic B cells (33). Expression array analysis indicates that A1 is a rapidly induced, BCR-dependent, splenic B cell gene product (T. T. Su and D. J. Rawlings, manuscript in preparation). To assess the ability of T1, T2, and M cells to up-regulate A1, we used RT-PCR to quantitate the levels of A1 mRNA after BCR engagement. After 12 h of BCR stimulation, A1 was markedly induced in T2 and M cells (Fig. 4C). In contrast, A1 was only minimally induced in T1 cells (Fig. 4C).

Like A1, Bcl-xL is an additional, inducible B cell survival factor (27, 28, 31). Furthermore, Bcl-xL−/− mice exhibit a dramatic loss of the peripheral lymphoid system (34). Bcl-xL induction in T1,
T2, and M cells was assessed by Western blot analysis. Similar to the results with A1, Bcl-xL expression was consistently (≥50%) higher in T2 cells than in T1 cells (Fig. 4D).

Akt/PKB is a proto-oncogene important for a range of cellular growth and survival signals (35, 36). Akt is a serine/threonine kinase known to phosphorylate and inactivate the proapoptotic protein Bad (37, 38). Recent evidence has also implicated Akt in NFκB-dependent survival signaling (39–41). Both A1 and Bcl-xL are NFκB-dependent genes (30–32, 42), and Akt activity is induced upon Ag receptor engagement in total splenic B cells and mature B cell lines (data not shown) (43). Therefore, we investigated the ability of T1, T2, and M cells to activate Akt. Akt activity, as measured by site-specific phosphorylation at serine 473, peaks at 2 min after BCR stimulation and gradually decreases after 5, 10, and 20 min (data not shown). At the peak time point, Akt activity was predominantly induced in T2 cells after BCR activation (Fig. 4E). In contrast, little or no Akt activation was observed in T1 cells (Fig. 4E). In addition, M cells exhibited a significantly lower level of Akt activation than T2 cells, suggesting that Akt-independent mechanisms of NFκB activation may exist in long-lived mature B cells. The difference in Akt activation may also be exaggerated, in part, by the early BCR responsiveness of T2 cells. Together, these data demonstrate that the immature T1 and T2 subsets clearly differ in their ability to generate a BCR-dependent survival signal as revealed by both cellular and molecular analysis.

**BCR engagement drives the differentiation of T2 B cells into a mature B cell phenotype**

The data presented demonstrate clear differences in BCR responsiveness between the T1 and T2 immature B cell subsets. T2 cells uniquely proliferate and survive in response to BCR cross-linking, consistent with a potential role in positive selection. However, in addition to these molecular events, the process of positive selection requires that the selected subset also be capable of differentiating into the subsequent developmental stage.

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**FIGURE 3.** T2, but not T1, cells up-regulate cyclin D2 upon BCR activation. A, Total or CD21/HSA FACS-isolated T1 (CD21^low^HSA^high^), T2 (CD21^high^HSA^high^), and M (CD21^low^HSA^low^) cells from BALB/c mice were stimulated for 12 h with anti-IgM (10 μg/ml) Abs and immunoblotted for cyclin D2. B, Total or CD21/HSA/CD23 FACS-isolated T1 (CD21^low^HSA^high^CD23^), MZ (CD21^high^HSA^high^CD23^), and M (CD21^low^HSA^low^) splenocytes were stimulated for 12 h and immunoblotted for cyclin D2 as in A. Unstimulated samples are included to show the absence of cyclin D2 prior to BCR stimulation. All blots were stripped and reprobed for β-actin as a control for relative protein loading. Data are representative of more than three experiments.

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**FIGURE 4.** BCR activation generates a survival signal in T2, but not T1, cells. A, Total BALB/c splenocytes were cultured for 48 h with (+IgM) or without (−IgM) anti-IgM (10 μg/ml) Abs. The percentage of viable cells (% Live) were identified by forward and side scatter using flow cytometry, as previously described (23). Splenocytes immediately isolated from mice are included as a control (0 h). B, Total or CD21/HSA FACS-isolated T1, T2, and M splenocytes were cultured with (+IgM) or without (−IgM) anti-IgM Abs for 48 h. Cell viability was assessed as above and depicted in graphical form, as shown. C, FACS-isolated T1, T2, and M cells (3 × 10^6^) were cultured for 12 h with (+) or without (−) anti-IgM stimulating Abs. RT-PCR was performed to assess mRNA expression of the A1/Bfl-1 gene. G3PDH mRNA levels were also assessed on the same samples as a control. All A1 lanes or G3PDH lanes were run on the same gel. Immunoblotting for A1 could not be done due to the poor quality of commercially available Ab reagents (data not shown). D, Total, T1, T2, and M splenocytes were stimulated for 12 h as in C and were immunoblotted for Bcl-xL. Blots were then stripped and reprobed for actin. E, Total, T1, T2, and M splenocytes were stimulated for 2 min at 37°C with anti-IgM stimulating Abs and were immunoblotted for phospho Ser^733^ Akt. Blots were stripped and reprobed for total Akt as a control. Data are representative of two to five experiments.
To begin to address the developmental capacity of these B lineage populations, the ability of T2 cells to differentiate into M cells was evaluated. For these studies, we used an in vitro assay similar to that previously described in studies of the B cell growth factor, BAFF (23). T1, T2, and M cells were FACS isolated using CD21/HSA surface markers as above (Figs. 1A and 5A). The cell populations were cultured in the presence or absence of BCR stimulation and stained again for CD21/HSA after 48 or 96 h. Consistent with our earlier results, the majority of T1 cells died within 48 h of receptor engagement (Fig. 4B). However, the CD21/HSA profile (gated on the live population) remained essentially unchanged in both T1 and M populations (data not shown). In contrast, BCR stimulation of purified T2 cells led to a progressive decrease in CD21^{high}HSA^{high} T2 cells (Fig. 5, A and B) and a time-dependent increase in CD21^{low}HSA^{low} M cells (Fig. 5, A and C). Ag receptor activation of T2 cells led to the generation of 34 and 54% of cells exhibiting a mature cell phenotype by 48 and 96 h, respectively. This increase in M cells is not likely a result of preferential outgrowth or selective survival of a small fraction of contaminating M cells, because T2 cells proliferate and survive similarly to M cells (Figs. 2 and 4B). In addition, because MZ cells do not proliferate in response to BCR activation, contaminating MZ cells within the sorted CD21^{high}HSA^{high} T2 gate are also unlikely to contribute to the production of M cells in this assay. Consistent with this prediction, CD21/CD23 staining in the T2 cell differentiation assay revealed a specific loss of CD21^{high}CD23^{-} MZ cells upon BCR stimulation (Fig. 5D).

Finally, we also tested whether the ability of T2 cells to differentiate was a consequence of general cellular activation or a specific response to BCR engagement. We compared the ability of anti-IgM Abs vs the B cell mitogen, LPS, to drive T2 cell differentiation. Strikingly, although LPS generates both strong proliferative and survival signals in splenic B lymphocytes (data not shown), LPS stimulation failed to drive the T2 to M shift by CD21/ HSA staining (Fig. 5E). Thus, the ability of purified T2 cells to differentiate into an M cell phenotype appears to be mediated uniquely through the B cell Ag receptor.

**BCR-dependent responses in T2 cells require Btk**

To begin to address the molecular requirements for T2 cell signaling, we used Xid mice, mutated in the Btk gene (16). Btk is a critical component of the BCR signalosome required for the generation of both 1,2-diacylglycerol and calcium-dependent signals (44). Xid mice have reduced numbers of M cells but an intact T1 and T2 pool (22). We purified T1, T2, and M cells from BALB/c (wild-type (WT)) or BALB/xid (Xid) mice using CD21/HSA cell sorting, as in Fig. 1 (data not shown). In contrast to WT cells, T2 and M cells from Xid mice failed to proliferate after 48 h of BCR activation (Fig. 6A). In addition, after 12 and 36 h of BCR stimulation, Xid T1, T2, and M cells exhibited significantly enhanced cell death compared with WT controls (Fig. 6B). Notably, Xid T2 cells were only 15% viable, compared with 69% in WT T2 cells after 36 h. At the molecular level, both total and purified T2 splenocytes from Xid mice exhibited only minimal BCR-induced

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**FIGURE 5.** BCR engagement drives T2 cells to exhibit a mature follicular B cell phenotype. A, T2 (CD21^{high}HSA^{high}) splenocytes were FACS-isolated using CD21/HSA markers with pre- and postsort staining profiles as shown (left panel). Purified T2 cells (sorted T2) (5 × 10^5) were cultured with (+IgM) or without (−IgM) anti-IgM (10 μg/ml) Abs for 48 or 96 h. Cells were then stained again for CD21/HSA markers with live-gated profiles as shown. The percentage of FACS-separated T2 cells still lying within the CD21^{high}HSA^{high} T2 gate (B) or within the CD21^{low}HSA^{low} M gate (C) after 48 or 96 h of culture in the presence (+IgM) or absence (−IgM) of BCR stimulation are graphically depicted. D, T2 cells were isolated and stimulated for 48 h as in A. Cells were then stained for CD21, HSA, and CD23. Live-gated CD21/CD23 profiles are as shown with the percentage of MZ (CD21^{high}CD23^{-}) cells as indicated. E, FACS-isolated T2 cells were stimulated for 48 h with anti-IgM (10 μg/ml) Abs or LPS (1 μg/ml). Cells were then stained for CD21/HSA markers as in A. The percentages of M cells are as indicated. IgM and LPS each induced cells to blast (as determined by forward and side scatter) and proliferate (by [3H]thymidine incorporation) (data not shown). Data are representative of two to five experiments.
cyclin D2 up-regulation (Fig. 6C). However, stimulation with PMA (a 1,2-diacylglycerol analog) plus ionomycin (a calcium ionophore) rescued cyclin D2 expression, indicating that signaling components downstream of Btk are unaltered in Xid cells (Fig. 6C). Furthermore, live-sorted (by propidium iodide and annexin V FACS sorting just before immunoblotting) Xid splenocytes still exhibited reduced cyclin D2 levels (data not shown), suggesting that the Xid cyclin D2 defect was independent of the survival defect. Together, these data demonstrate a direct requirement for Btk in the BCR responsiveness of T2 cells.

These results are consistent with previous data suggesting that Xid splenocytes have both proliferative and survival defects (27, 28, 44, 45). Because Xid mice have a reduced M cell pool, it had been unclear whether the Xid proliferative defect was truly a primary cycling defect or secondary to the loss of BCR-responsive M cells (46, 47). Previous analysis of unmatched cell populations likely explains the conflicting data regarding the Btk dependence for cyclin D2 induction (28, 29, 48). Our results directly address these questions and demonstrate a primary requirement for Btk in cell cycle entry and cyclin D2 up-regulation in transitional 2 B lymphocytes.

**Discussion**

In this report, we tested the ability of T1 and T2 transitional B cells to respond to Ag receptor engagement and demonstrate significant differences between these immature B cell subsets. Our data indicate that, whereas the T1 subset of immature B cells is relatively nonresponsive to Ag receptor engagement, the T2 cell stage is likely a unique BCR-responsive checkpoint in peripheral B cell development. The observation that certain transitional B cells actively respond to BCR stimulation suggests that BCR signaling in immature B cells may play an important role in the generation of long-lived mature B lymphocytes. Mice defective in Btk, Lyn, phosphatidylinositol 3-kinase, BLNK, phospholipase Cγ2, or VAV1 and VAV2 each have reduced numbers of mature B cells but an intact T2 cell pool (data not shown) (17–20). Consistent with this hypothesis, T2 cells purified from Btk-deficient Xid mice fail to generate proliferative and survival signals (Fig. 6). Together, these data suggest that BCR engagement and intact BCR signaling at the T2 cell stage are imperative for the development of mature B cells.

**Differences in BCR responsiveness suggest distinct roles for the T1 and T2 immature B cell subsets**

Of the 2 × 10^7 IgM^+ B cells that are generated from the BM daily, 10% enter the spleen and only 1–3% enter the mature B cell pool (49, 50). Studies done in α-HEL BCR transgenic mice indicate negative selection is responsible for at least part of this process (51). Several lines of evidence demonstrate that B lymphocyte negative selection occurs at the splenic immature B cell stage (21). Our data demonstrate that the T1 immature subset does not proliferate in response to BCR cross-linking, but rather dies (Figs. 2, A and B, and 4B). Furthermore, T1 cells do not up-regulate genes required for cell proliferation and survival (Figs. 3 and 4, C and D), Together, these data are consistent with the T1 subset of immature B cells being the target for BCR-induced negative selection in the periphery.

In addition to negative selection, mounting evidence suggests that positive selection also plays a role in peripheral B cell development. Whereas MHC molecules are the selecting ligands for T cell positive selection, analogous ligands for the BCR remain largely unknown. This has made it difficult to directly evaluate positive selection in B lymphocytes. Recent work using transgenic BCR models, however, has provided clear evidence for positive selection for two unique B cell lineages, peritoneal B1 cells and MZ cells (52, 53). In addition, analysis of Ag receptor diversity revealed that, in comparison to HSA^high immature B cells, HSA^low mature B cells have a much more restricted repertoire of expressed H-L chain pairs (10, 11). The generation of mature cells expressing only a very limited subset of H-L chain pairs (in several independent transgene models) is most consistent with the positive selection of a minority of immature B cells (11, 14, 15). Notably, a subsequent study using a combination of transgenic H and dysfunctional L chain pairs clearly indicates that positive selection...
operates in this oligoclonal receptor model (54). In that model, the level of H chain expression and the nature of the L chain expressed coordinately controlled generation of the mature peripheral B cell pool. These combined observations suggest that the repertoire restriction occurring at the immature to mature splenic B cell transition is primarily due to positive selection. Despite these supportive data, however, an appropriate target population for positive selection in the periphery has not yet been described.

The immature T2 subset is an immediate precursor to mature B cells in vivo, presenting T2 cells as a potential target population for selection into the mature B cell pool (22). The current report presents significant further data supporting this hypothesis. In response to BCR stimulation, T2 cells enter cell cycle rapidly and proliferate (Fig. 2, A–C), correlating with early and robust cyclin D2 induction (Fig. 3). BCR engagement of T2 cells also activates survival signals (Fig. 4) and leads to a change in surface phenotype consistent with a mature follicular B cell (Fig. 5). Taken together, these results strongly support the hypothesis that T2 cells are a likely target for B lineage positive selection into long-lived mature B cells.

Together, our findings suggest that T1 and T2 cells may act as distinct checkpoints for selection into the mature B cell pool. The T1 subset of immature B cells transits from the BM, through the bloodstream, to the splenic PALS (22). In contrast, the T2 subset is primarily located in the splenic follicle, adjacent to mature B cells (22). Their distinct anatomic locations and distinct responses to BCR engagement suggest a model whereby T1 cells with receptor specificities for blood-borne self-Ags are deleted by negative selection, whereas T2 cells with specificities for alternative follicular Ags become positively selected into the mature B cell pool (Fig. 7). This model, however, does not exclude the possibility that additional selection steps may also occur within the mature B cell pool itself.

Differences in BCR responsiveness suggest that distinct signaling complexes are present in T1 vs T2 cells
Consistent with the short lifespan (3–4 days) of immature splenic B cells, in vivo transfer studies indicate that T1 cells differentiate...
into T2 cells within 48 h (22). The clear differences in BCR responsivity between T1 and T2 cells suggest that during this relatively brief time period, important changes rapidly occur to transform T1 cells, which die upon BCR activation, into T2 cells, which proliferate, survive, and mature upon receptor engagement (Fig. 7, lower panel). It is unclear what drives this T1 to T2 transition. However, recent evidence indicates BAFF-deficient mice have a developmental block at the T1 stage, suggesting that this novel B cell activator may play a key role in the T1 to T2 transition (55). In addition, signal transduction through Syk may also be required for this developmental step. Syk−/− B cells do not enter the splenic follicle, the precise anatomic restriction separating T1 from T2 cells (56).

In addition to identifying the signals driving this T1 to T2 step, it will be equally important to identify the molecular changes induced at this transition. What is it about the BCR signaling machinery of T2 cells that allows this population to respond so differently to T1 cells? The very similar surface phenotype and chaperone of T2 cells that allows this population to respond so differently to T1 cells? The very similar surface phenotype and chaperone of T2 cells vs only 30% of HSAhigh immature splenic B cells (57). We are currently evaluating the hypothesis that the subtraction of HSAhigh immature B cells capable of colocalizing the BCR to the lipid rafts is the BCR-responsive T2 population. Further studies comparing the specific signaling components present in the lipid rafts of T1 vs T2 cells will be important in confirming our predictions and further understanding the molecular differences between these two closely related, yet functionally distinct, B lymphocyte populations.

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