Transitional B Cell Fate Is Associated with Developmental Stage-Specific Regulation of Diacylglycerol and Calcium Signaling upon B Cell Receptor Engagement

Kristen L. Hoek, Pierre Antony, John Lowe, Nicholas Shinners, Bhaskarjyoti Sarmah, Susan R. Wente, Demin Wang, Rachel M. Gerstein and Wasif N. Khan

*J Immunol* 2006; 177:5405-5413; doi: 10.4049/jimmunol.177.8.5405
http://www.jimmunol.org/content/177/8/5405

**References**
This article cites 75 articles, 40 of which you can access for free at:
http://www.jimmunol.org/content/177/8/5405.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Transitional B Cell Fate Is Associated with Developmental Stage-Specific Regulation of Diacylglycerol and Calcium Signaling upon B Cell Receptor Engagement

Kristen L. Hoek, Pierre Antony, John Lowe, Nicholas Shinners, Bhaskarjyoti Sarmah, Susan R. Wente, Demin Wang, Rachel M. Gerstein, and Wasif N. Khan

Functional peripheral mature follicular B (FoB) lymphocytes are thought to develop from immature transitional cells in a BCR-dependent manner. We have previously shown that BCR cross-linking in vitro results in death of early transitional (T1) B cells, whereas late transitional (T2) B cells survive and display phenotypic characteristics of mature FoB cells. We now demonstrate that diacylglycerol (DAG), a lipid second messenger, is produced preferentially in T2 compared with T1 B cells upon BCR cross-linking. Consistently, inositol 1,4,5-triphosphate is also produced preferentially in T2 compared with T1 B cells. Unexpectedly, the initial calcium peak appears similar in both T1 and T2 B cells, whereas sustained calcium levels are higher in T1 B cells. Pretreatment with 2-aminoethoxydiphenylborate, an inhibitor of inositol 1,4,5-triphosphate receptor-mediated calcium release, and verapamil, an inhibitor of L-type calcium channels, preferentially affects T1 B cells, suggesting that distinct mechanisms regulate calcium mobilization in each of the two transitional B cell subsets. Finally, BCR-mediated DAG production is dependent upon Bruton’s tyrosine kinase and phospholipase C-γ2, enzymes required for the development of FoB from T2 B cells. These results suggest that calcium signaling in the absence of DAG-mediated signals may lead to T1 B cell tolerance, whereas the combined action of DAG and calcium signaling is necessary for survival and differentiation of T2 into mature FoB lymphocytes. The Journal of Immunology, 2006, 177: 5405-5413.

The BCR plays a fundamental role in B lymphocyte development (1, 2). Surface IgM-expressing immature B cells that do not recognize self-Ag migrate from the bone marrow to the spleen as immature or transitional B cells. In the spleen, they then develop into mature follicular (Fo) and marginal zone (MZ) B cells (3). The transitional B cell population is heterogeneous and can be divided at least into three subsets based on variable expression of the cell surface markers (3–5). Cells of the earliest transitional (T1) stage display an IgM/iHSA/iHSA/iIgD/iCD21/iCD23 phenotype (3, 4). The late transitional (T2) B cells can be classified into two subsets; the T2 subset, which serves as a precursor for mature FoB cells (IgM/iIgD/iCD21/iCD23) originally defined by Allman et al. (4) and Loder et al. (3), and the newly defined T2-pre-MZB (IgM/iIgD/iCD21/iCD23), which serves as a precursor for mature MZB cells (5–7). The Loder et al. (3) T2 B cell population, which includes T2-pre-MZB cells, expresses AA4.1 and high levels of heat-stable Ag (HSA) (4, 8, 9). Thus, T1 B cells may give rise to T2 B cells, which either serve directly as a precursor for FoB cells or may transit through a T2-pre-MZ stage before becoming mature MZB cells. The molecular mechanisms that regulate the developmental transitions between immature transitional and mature B cells remain unclear.

According to the current model for FoB cell development, self-reactive T1 B cells are eliminated in response to strong BCR-induced signals (negative selection), whereas non-self-reactive T1 cells develop into T2 B cells via either a default pathway or tonic signaling, and then become resistant to BCR-induced apoptosis and ultimately require BCR-induced signals for survival and further differentiation into mature FoB cells (positive selection) (8–15). Analysis of the peripheral B cell repertoire, in both mice and humans, supports the hypothesis that peripheral immature to mature FoB cell differentiation results from positive selection (10, 16). Although the functional properties of transitional B cell subsets in vitro remain controversial (3, 4, 14, 15, 17), experimental evidence for the disparate biological outcomes of negative and positive selection, as well as the molecular mechanisms that underlie differential responses to BCR engagement within transitional B cell subsets, is now emerging. Recently, we and others have demonstrated that T1 and T2 B cell subsets exhibit distinct biological responses upon BCR ligation in vitro: T1 B cells die, whereas T2 B cells are resistant to apoptosis and display phenotypic characteristics of mature FoB cells (14, 15). However, the understanding of biochemical mechanisms that underlie the disparate biological responses of T1 compared with T2 B cells remains elusive.
BCR engagement leads to recruitment of multiple enzymes including Bruton’s tyrosine kinase (BTK) to the plasma membrane, forming a large multicomponent signaling complex termed the BCR signalosome (1, 18). We and others have previously demonstrated that BTK deficiency leads to a severe mature FoB cell deficiency, despite intact T2 and mature MZ B cell subsets, termed Xid (xid) in mice (19–22). An xid-like phenotype is also observed in animals with gene-targeted deletion of PLC-γ2, which is a direct target of BTK, and B cell linker protein (BLNK), an adaptor which facilitates BTK and PLC-γ2 interaction (23–27). These observations support an active role for BCR signaling, particularly the BTK/PLC-γ2 signaling axis, in the differentiation of T2 into mature FoB cells (3, 4, 15, 25, 26, 28).

Biological significance of the BTK/PLC-γ2 signaling axis in B cell survival and/or differentiation is further supported by studies suggesting that one of the molecular defects in BTK- and PLC-γ2-deficient B cells is their inability to activate IκB kinase and NF-κB (4, 26, 28–30). Consistently, deletion of IκB kinase results in a reduction of the mature FoB cell compartment (31, 32). We have also shown that the transcription factor NF-κB up-regulates the antiapoptotic protein Bcl-xL in response to BCR cross-linking in a BTK/PLC-γ2-dependent manner (33). Moreover, BCR-induced NF-κB activation proceeds via protein kinase C (PKC) (34–36). Together, these studies suggest that the BTK/phospholipase C (PLC)-γ2 signaling axis regulates B cell survival by activation of PKC/NF-κB signaling pathways.

Phosphatidylinositol-4,5-bisphosphate (PIP2) is at a critical nexus in BCR signaling because it serves as a substrate for both PLC-γ2 and PKC, which in turn activate NF-κB, PI3K, and MAPK (36, 37). Phosphatidic acid (PA) is in turn synthesized in response to PLC-γ2 activation and serves as a substrate for PI3K, which in turn activates PKC and MAPK (36, 38). Together, these studies suggest that the BTK/PLC-γ2 signaling axis regulates B cell survival by activation of PKC/NF-κB signaling pathways.

Materials and Methods

Mice

The generation of Btk-deficient (btk−/−), PLC-γ2-deficient (plc-γ2−/−) and Notch2 haploinsufficient (notch2+/−) mice has been previously described (19, 25, 51). For wild-type controls, 129/Sv × C57BL/6 or C57BL/6 mice (The Jackson Laboratory) were used. All mice were ~6 wk of age. Mice were treated humanely in accordance with federal and state government guidelines and their use was approved by the institutional animal committee.

B cell purification and transitional B cell sorting

Primary B lymphocytes were isolated using MiniMACS magnetic sorting by negative selection (CD43 depletion) to avoid inadvertent activation of B cells as previously described (14). The purity of B cells isolated in this manner was ~90–95% as verified by flow cytometry (FCM) analysis using anti-B220 and anti-IgM Abs (BD Pharmingen). All purifications were performed at 4°C, and cells were used immediately. Splenic B cell subsets were purified based on a combination of the schemes described previously (3–5) (Table I). To avoid use of anti-IgM Abs, which could influence BCR signaling under study, HSA and CD21 were used for cell sorting and to exclude T2-pre-MZ B from T2 cells. T1, T2, and mature FoB cell subsets for DAG studies were FACS purified using Abs directed against HSA, CD21, and CD23, and the purity of each subset was between 85 and 95% as previously described (14). In some experiments, DAG production in T1 B cells was compared with a pool of T2/mature FoB cells, and T1 and T2 B cells were purified using a series of MACS depletions and positive selections, which included CD43 depletion followed by CD23 depletion and AA4.1-positive selection. The purity of T1 (CD43−CD3−AA4.1+) and T2/mature FoB cells (CD43+CD3+) was ~95% as determined by FCM. T1 and T2 B cells for use in IP studies and phospho-PKC immunoblotting were obtained by MACS enrichment for AA4.1−transitional B cells, followed by FACS purification using Abs directed against HSA and CD23 to sort AA4.1−/HSA−/CD23+ (T1) and AA4.1+HSA−/CD23+ (T2) B cells. The purity of the T1 subset was ~95%, and the purity of the T2 subset was ~75–90% as determined by FCM. Mature FoB cells were MACS-enriched from the AA4.1-negative fraction by CD23 depletion followed by CD9 depletion (to remove mature and precursor MZ B cells). The purity of the mature FoB subset was ~85% as determined by FCM.

Lipid analysis

DAG quantification. Purified B cells or B cell subsets were labeled for 6 h with palmitic acid (2.5 μCi/mL), as a fatty acid precursor. Lipid synthesis was monitored at 37°C, and plp-γ2−/− B cells, and the production of DAG was quantified as previously described (34, 48). Labeled B cells were washed twice with PBS and stimulated through the BCR with 20 μg/mL goat anti-mouse IgM F(ab′)2 for the indicated times. The reactions were stopped by the addition of cold PBS. Cells were centrifuged, and the pellet was used for lipid extraction following suspension in 0.5 mL of chloroform/methanol (2:1 v/v) and addition of 0.4 mL of 0.5 N HCl. The mixture was used for lipid extraction following suspension in 2 mL of chloroform/methanol (2:1 v/v) and addition of 0.4 mL of 0.5 N HCl. The mixture was shaken vigorously, centrifuged, and the upper (aqueous) phase was discarded while the lower (organic) phase was washed twice with 1 mL of PBS, and suspended in 200 μL of PBS. One hundred microliters of cells were treated with 25 Ci of [3H]inositol (PerkinElmer) for 4 h. Cells were harvested, and DAG was quantified as previously described (34, 48). Labeled B cells were washed twice with PBS and stimulated through the BCR with 20 μg/mL goat anti-mouse IgM F(ab′)2 for the indicated times. The reactions were stopped by the addition of cold PBS. Cells were centrifuged, and the pellet was used for lipid extraction following suspension in 0.5 mL of chloroform/methanol (2:1 v/v) and addition of 0.4 mL of 0.5 N HCl. The mixture was shaken vigorously, centrifuged, and the upper (aqueous) phase was discarded while the lower (organic) phase was washed twice with a total of 2 mL of PBS. One hundred microliters of cells were treated with 25 μL of PBS with or without 20 μg/mL goat anti-mouse IgM F(ab′)2. Treatments were stopped by adding 0.6 μL of 0 N HCl and chilling cells on ice. Soluble IPs were dissolved in a minimum volume of chloroform/methanol (2:1 v/v) and the mixture was vortexed for 2 min at maximum speed, followed by addition of 156 μL each of [3H]inositol-labeled soluble insoluble polyphosphatase profiling. The soluble immunoprecipitation (IP) profile for B cells was determined by adapting a described previously protocol (54). Briefly, FACS-purified B cell subsets (~5 × 10^6) were cultured in 0.5 mL of inositol-free DMEM containing 30 μCi of [3H]inositol (PerkinElmer) for 4 h. Cells were harvested, washed twice with 1 mL of PBS, and suspended in 200 μL of PBS. One hundred microliters of cells were treated with 25 μL of PBS with or without 20 μg/mL goat anti-mouse IgM F(ab′)2. Treatments were stopped by adding 0.6 μL of 0 N HCl and chilling cells on ice. Soluble IPs were dissolved in 465 μL of chloroform/methanol (1:2 v/v). The mixture was vortexed for 2 min at maximum speed, followed by addition of 156 μL each of Table I. Expression levels of surface markers on splenic B cell subsets

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>FoB</th>
<th>pre-MZ</th>
<th>MZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA4.1</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–/+</td>
<td>–</td>
</tr>
<tr>
<td>IgM</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+/++</td>
</tr>
<tr>
<td>IgD</td>
<td>–/flow</td>
<td>+/flow</td>
<td>+</td>
<td>++/–</td>
<td>+/++</td>
</tr>
<tr>
<td>CD21</td>
<td>–/–</td>
<td>–/–</td>
<td>+</td>
<td>++/–</td>
<td>+/++</td>
</tr>
<tr>
<td>CD23</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
</tr>
<tr>
<td>CD24(HSA)</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
<td>++/++</td>
</tr>
</tbody>
</table>

Downloaded from http://www.jimmunol.org by guest on October 4, 2017
chloroform and 2 M KCl and another 2 min of vortexing. The lysate was spun at 13,000 × g for 5 min, and the supernatant was recovered. Samples were analyzed by HPLC, with the IPs resolved by a Whatman partisphere SAX strong-anion exchange column (4.6 × 125 mm) and a linear gradient from 10 mM to 1.7 M ammonium phosphate (pH 3.5) for 25 min, followed by elution with 1.7 M ammonium phosphate for 20 min.

**Intracellular staining**

Total splenocytes or MACS-enriched B cells were stimulated with 20 μg/ml goat anti-mouse IgM (Fab′), Abs for the indicated times, and then immediately fixed with 2% paraformaldehyde. After centrifugation, cells were permeabilized (BD Cytofix/Cytoperm), and were intracellularly labeled with a biotinylated Ab raised against PI(4,5)P2 (Z-B045; Echelon Biosciences), or a rabbit polyclonal Ab raised against phosphorylated PKCδ (9374S; Cell Signaling). Cells were labeled with Abs directed against CD19, HSA, and CD21 for the identification of splenic B cell subsets. PIP2 was visualized by PerCP-Cy5.5-conjugated streptavidin. Phospho-PKCδ was visualized by anti-rabbit Alexa 488. Data were acquired on a BD Biosciences FACS Calibur flow cytometer. Results are expressed as mean fluorescence intensity and are representative of multiple experiments.

**Immunoblotting**

FACS-sorted T1 and T2 B cells and MACS-enriched mature FoB cells were rested on ice in complete RPMI 1640 for 3 h, at room temperature for 15 min, and then warmed to 37°C for 15 min before stimulation with anti-IgM Fab′ (for T1) or anti-IgM Fab′, for 5 min, or left nonstimulated. For immunoblot analysis of phospho-pan-PKC and phospho-PKCδ (Cell Signaling), cytoplasmic extracts were prepared in radioimmunoprecipitation assay buffer containing protease inhibitor (Roche), and the cytoplasmic fractions were cleared by centrifugation at 14,000 rpm for 20 min. Protein was quantified by Bradford assay and 20 μg of total protein per point was denatured in Laemmli reducing buffer by boiling at 100°C for 5 min. Proteins were separated by SDS-PAGE and were electrotransferred onto polyvinylidine difluoride membrane (Immobilon; Millipore). The membranes were sequentially probed with Abs specific for p-PKCδ (pan), p-PKCδ (Cell Signaling), and actin (Santa Cruz). Bound Abs were revealed by HRP-conjugated goat anti-rabbit IgG Ab (Pierce), followed by ECL detection (Pierce) on autoradiography film.

**Calcium mobilization**

Purified B cells (10 × 10⁶ cells/ml in HBSS) were loaded with 5 μg/ml indo-1-AM for 30 min at 37°C. Cells were then washed, placed on ice, and labeled with PE-conjugated anti-HSA, biotinylated anti-CD23 (visualized by PerCP-Cy5.5-conjugated streptavidin), and allophycocyanin-conjugated anti-ClqRp (clone AA4.1) for B cell subset identification. Abs raised against AA4.1 and CD21 were not used to avoid inadvertent stimulation of B cells. Before acquisition, cells were resuspended by 1 × 10⁶/ml in HBSS containing 1 mM calcium, warmed to 37°C for 15 min, and applied to a BD LSR II cytometer. Baseline, nonstimulated measurements were taken for 90 s, followed by the addition of 10 μg/ml goat anti-mouse IgM Fab′ (for T1) or Abs for the indicated time. In some experiments, cells were preincubated with 100 μM 2-aminoethoxydiphenylborate (2-APB) (Calbiochem 100065) or 100 μM verapamil (Calbiochem 676777) for 15 min before application to the cytometer.

**Results**

**Flow cytometric analysis of B cell subsets**

For our studies, B cell subsets were gated using a combination of Loder et al. (3) and Allman and colleagues (4, 5) schemes to classify five major splenic B cell subsets: immature (AA4.1−) T1 and T2 B cells, mature (AA4.1+) FoB and MZB cells, and the recently described MZ precursors (AA4.1+CD19−CD23+CD21high) (Table I) (55). B cell subsets were visualized using HSA and CD21 (Fig. 1A), allowing for clear visual separation of T2 B cells from MZ and MZ precursors (T2-pre-MZ), which both express high levels of CD21. MZ and T2-pre-MZ can be further distinguished from each other by expression of CD23 and AA4.1 (Fig. 1A, right panel). The HSA-CD21 MZ B cell gate was based on low reactivity to CD23; however, it is important to note that, although >80% of the cells in the MZ gate are AA4.1-negative, a significant proportion (~50%) of these cells do retain reactivity to CD23. To ascertain that the T2 cells we are analyzing do indeed belong to the immature splenic B cell compartment, we investigated the expression of CD93/ClqRp (recognized by the mAb AA4.1), which is exclusively expressed on immature B cells (4, 56). FCAM analysis revealed that neither mature FoB nor MZ B cells exhibit significant levels of reactivity to AA4.1 (Fig. 1B). In contrast, reactivity to AA4.1 is clearly detected in T1 as well as T2 B cell subsets. We observe that T1 B cells react higher to AA4.1 than T2 (∼0.5-±0.8-fold higher and 3.6-±0.5-fold higher relative to mature B cells, respectively), and that AA4.1 is detected on the T2-pre-MZ subset (2.8-±0.7-fold higher relative to mature B cells). Additionally, all of the transitional B cell populations express high levels of HSA. Thus, all transitional B cells demonstrate characteristics of immature B cells. Additionally, disparate biological
outcomes between T1 and T2 B cells are not due to differences in the cell surface expression levels of surface IgM (Fig. 1C). We have also found that there is comparable up-regulation of the activation marker CD69 in both T1 and T2 B cells following anti-IgM F(ab\''\')2 stimulation (Fig. 1D), revealing that both T1 and T2 B cell subsets are capable of transducing BCR-mediated signals.

BCR-induced DAG production occurs preferentially in T2 compared with T1 B cells

Using FACS-purified T1, T2, and mature FoB cell populations (Fig. 2A), we measured [\[^3\]H\]DAG production in response to stimulation with anti-IgM F(ab\''\')2 Abs (Fig. 2B). Our studies reveal that BCR-induced DAG production occurs in T2 and mature B cells following both 5 min (2.1±0.1-fold increase, and 5.1±0.3-fold increase, respectively, compared with the nonstimulated control) and 30 min (2.8±0.3-fold increase, and 4.8±0.4-fold increase, respectively, compared with the nonstimulated control) stimulation with anti-IgM. In sharp contrast, T1 B cells do not produce detectable levels of DAG under similar stimulatory conditions. To rule out the possibility of an early transient production of DAG in T1 B cells, purified T1 and a mixture of T2/mature FoB cells were stimulated through the BCR for 0.5 and 1 min (Fig. 2C). DAG production in response to BCR cross-linking was not detectable in T1 B cells at either of the early time points, whereas the mixture of T2/mature FoB cells exhibits DAG production upon BCR engagement. The kinetic profile of DAG production that we observed is comparable to previous studies performed with total B cells that were stimulated through the BCR (57). In addition to DAG, PIP\(_2\) hydrolysis by PLC-\(\gamma\)2 produces IP\(_3\) in primary wt B cells (57). Therefore, using FACS-sorted T1 and T2 B cells (Fig. 2D), we also investigated the production of IP\(_3\) in response to BCR stimulation (Fig. 2E). Consistent with DAG generation, we found that IP\(_3\) is preferentially produced in T2 relative to T1 B cells following anti-IgM stimulation (3.0±0.2-fold increase, and 1.3±0.5-fold increase, respectively; \(n=4\)). Because both T1 and T2 B cells up-regulate the activation marker CD69 following anti-IgM stimulation (Fig. 1D), reduced DAG/IP\(_3\) production is not likely due to a general inability of T1 B cells to signal via the BCR. We

---

**FIGURE 2.** BCR-induced DAG production occurs preferentially in T2 relative to T1 B cells. A, FACS-sorting scheme for the separation of T1, T2, and mature FoB cells for DAG studies. We estimate that T2 B cells sorted in this manner contain ~14% T2-pre-MZ. **B**, wt T1, T2, and mature FoB cells, FACS-purified as in A were labeled with [\[^3\]H\]palmitic acid (2.5 Ci/ml) for 6 h, and then incubated with PBS (□) or stimulated with 20 \(\mu\)g/ml anti-IgM (■) for the indicated time periods. Following lipid extraction, DAG production was quantified and expressed as percentage increase of DAG of total radioactivity. Results represent the average ± SEM of three independent experiments performed in triplicate. C, MACS-sorted T1 and a pool of T2/mature wt B cells (1.5 to \(10^6\)) were labeled as in B, and then stimulated with 20 \(\mu\)g/ml anti-IgM for the indicated time period; T1 (solid line); T2/mature pool (dashed line). The production of DAG is expressed as percentage increase of DAG of total radioactivity and represents the average ± SEM of three independent experiments performed in triplicate. D, FACS-sorting scheme for the separation of T1 and T2 cells for IP\(_3\) studies. We estimate (based on independent FCM stainings) that T2 B cells sorted in this manner contain ~9% T2-pre-MZ. E, FACS-purified wt T1 and T2 B cells were labeled with [\[^3\]H\]inositol (60 \(\mu\)Ci/ml) for 4 h, and then incubated with PBS (□) or stimulated with 20 \(\mu\)g/ml anti-IgM (■) for 1 min. IP\(_3\) production was measured by HPLC. IP\(_3\) level is expressed as the counts measured in the peak corresponding to IP\(_3\), and is representative of four independent experiments.
estimate that the T2 cells used for the analysis of DAG may contain ~14% T2-pre-MZ B cells, whereas <9% of the T2 cells used in the IP_{3} assay were identified as potential T2-pre-MZ B cells. Because DAG and IP_{3} are the products of PIP_{2} hydrolysis, and we obtained similar profiles for DAG and IP_{3} production, we believe that T2-pre-MZ B cells did not contribute significantly to the levels of DAG in T2 B cells, although we cannot formally exclude this possibility. Together, these data indicate that distinct stage-specific signaling mechanisms are likely to be responsible for differential lipid metabolism in the T1 and T2 B cell subsets.

**PIP_{2} levels are lower in T1 compared with T2 B cells**

To understand the mechanisms underlying differences in BCR-induced DAG production within the T1 and T2 B cell subsets, we first examined the levels of PIP_{2}, which serves as a substrate for PLC-γ2 in the production of DAG (58). To determine both basal and BCR-induced levels of PIP_{2}, we performed FCM analysis using an anti-PI(4,5)P_{2} Ab. Our results demonstrate that basal levels of PIP_{2} are lower in T1 relative to T2 B cells (Fig. 3A). Although the kinetics of PIP_{2} upon BCR cross-linking are similar in T1 and T2 B cells (Fig. 3A), the magnitude of change in PIP_{2} was again lower in T1 relative to T2 B cell subset. It is also noteworthy that both basal and BCR-induced levels of PIP_{2} were higher in T1 B cells than in mature FoB cells. Additionally, the T2-pre-MZ cells possessed the highest basal and BCR-induced level of PIP_{2} (Fig. 3A). The higher PIP_{2} level in T2 B cells is unlikely to result from contaminating T2-pre-MZ cells because similar results were obtained with B cells depleted of CD9^{+} cells, which should eliminate the majority of MZ and pre-MZ B cells (Fig. 3B) (59, 60), as well as with B cells isolated from notch2^{-/-} mice, which are severely impaired in MZ and pre-MZ B cell development (Fig. 3C) (7, 51, 61).

**Intracellular Ca^{2+} mobilization occurs via distinct mechanisms in T1 and T2 B cell subsets**

The generation of IP_{3} induces Ca^{2+} mobilization in primary wt B cells (57). The duration and magnitude of calcium flux is likely to modulate B cell survival and differentiation by the activation of specific transcription factors including NFAT and NF-kB (62). To precisely define stage-specific Ca^{2+} mobilization within transitional B cell subsets, we stimulated purified wt B cells with anti-mouse IgM F(ab')_{2} Abs and determined Ca^{2+} flux in each splenic B cell subset by FCM. Our results demonstrate that despite differential IP_{3} production, the initial Ca^{2+} peak is similar in T1 and T2 B cells following BCR engagement (Fig. 4, top panel).

---

**FIGURE 3.** PIP_{2} levels in are higher in T2 relative to T1 B cells. A. Freshly isolated MACS-enriched wt B cells were labeled with Abs directed against CD19, CD21, and CD24 (HSA) for B cell subset identification as in Fig. 1A, and then permeabilized and examined for basal PIP_{2} levels using an anti-PI(4,5)P_{2} Ab (left panel). MACS-enriched B cells were stimulated for the indicated times with 20 μg/ml anti-IgM, immediately fixed, and then labeled and examined for PIP_{2} levels as in A (right panel); T1 ( ), T2 ( ), mature FoB ( ), pre-MZ ( ), mature MZB ( ). Data are displayed as mean fluorescence intensity (MFI), and are a representative of at least four experiments. B. Freshly isolated MACS-enriched wt B cells depleted of CD9^{+} B cells were labeled for B cell subset identification as in A, and then permeabilized and examined for basal (left panel) and BCR-induced (right panel) PIP_{2} levels. Data are representative of three experiments. C. Freshly isolated MACS-enriched notch2^{+/-} B cells were labeled for B cell subset identification as in A, and then permeabilized and examined for basal (left panel) and BCR-induced (right panel) PIP_{2} levels. Data are representative of two experiments.

**FIGURE 4.** BCR-mediated calcium mobilization occurs via distinct mechanisms in T1 and T2 B cells. MACS-enriched B cells were loaded with Indo-1-AM (5 μg/ml), and labeled with AA4.1, anti-CD23, and anti-HSA for B cell subset identification; T1 (dotted black line), T2 (black line), mature FoB (gray line). Upper panel. No pretreatment; middle panel, pretreatment for 15 min with 100 μM 2-APB; lower panel, pretreatment for 15 min with 100 μM verapamil. Cells were applied to a cytometer, basal flux in each splenic B cell subset was measured for 90 s, and then cells were stimulated with 100 μg/ml anti-IgM (indicated by the arrow) and monitored for 4–6 min. Data are representative of four to six independent experiments.
However, T1 B cells display higher sustained levels of Ca\(^{2+}\) relative to T2 and mature FoB cells. The initial Ca\(^{2+}\) peak appears to depend, at least in part, on IP\(_3\)Rs because pretreatment of the cells with 2-APB, a inhibitor of IP\(_3\),R-mediated Ca\(^{2+}\) release, reduced the initial Ca\(^{2+}\) peak in all B cell subsets (Fig. 4, middle panel). However, the initial Ca\(^{2+}\) peak in T1 B cells appears to be more sensitive to pretreatment with 2-APB than T2 or mature FoB cells. In contrast, sustained Ca\(^{2+}\) influx in T1 B cells appears to be less sensitive to 2-APB treatment than in T2 and mature FoB cells. Additionally, inhibition of L-type channels with verapamil led to a more severe reduction in calcium influx in T1 relative to T2 and mature FoB cells.

PKC phosphorylation is higher in T2 relative to T1 B cells

DAG targets multiple signaling pathways including PKCs, chimaerins, and RasGRPs (42, 44). Our results demonstrate a reduction in BCR-mediated DAG production in T1 compared with T2 B cells. A consequence of reduced DAG production should be reduced activation of PKC in T1 relative to T2 B cells upon BCR engagement. We therefore monitored the magnitude of BCR-induced phosphorylation of DAG-responsive PKCs in transitional B cell subsets using immunoblotting and FCM. Surprisingly, BCR-induced phosphorylation of conventional PKCs and nonconventional PKC\(\delta\) was detected in T1 B cells, albeit at a modestly reduced level compared with T2 as well as FoB cells (Fig. 5A). Because sufficient numbers of FACS-sorted T2-pre-MZ and MZ B cells for immunoblotting could not be obtained, BCR-induced phosphorylation of PKC\(\delta\) in T2-pre-MZ and MZ B cells was examined using FCM. Again, an increase in PKC\(\delta\) phosphorylation was clearly detectable in all B cell subsets (Fig. 5B); however, both basal and BCR-induced phosphorylation of PKC\(\delta\) was higher in T2-pre-MZ and MZ B cells relative to T1, T2, and mature FoB cells. Overall, the differences in PKC phosphorylation between T1 and T2 B cells are modest when compared with the observed differences in the production of DAG (Fig. 2), suggesting that very small amounts of DAG, which may be undetectable in our assay system, are sufficient to induce PKC phosphorylation. Together, these results suggest that phosphorylation of PKCs is not the major DAG-dependent mechanism responsible for differential responses of T1, T2, and mature B cells to BCR stimulation.

BCR-induced DAG production requires BTK and PLC-\(\gamma\)2

Although the BTK/PLC-\(\gamma\)2 signaling axis appears to be dispensable in the differentiation of T1 to T2 B cells, functional integrity of this signaling axis is essential for the transition from T2 to mature B cells (14, 15, 25, 26, 28, 63). To test the hypothesis that BTK and PLC-\(\gamma\)2 control this process by mediating DAG production, \(wt\), \(btk^{-/-}\), and \(plc-\gamma2^{-/-}\) B cells were stimulated with anti-IgM (Abs) for the indicated times and DAG production was monitored (Fig. 6). Consistent with previous reports (57), an increase in DAG production in \(wt\) B cells is observed in response to BCR cross-linking (5.1- \pm 0.9-fold increase at 5 min, and 6.8-\pm 1.0-fold increase at 30 min compared with the nonstimulated B cells). In contrast, BCR stimulation did not induce DAG in either \(btk^{-/-}\) or \(plc-\gamma2^{-/-}\) B cells. These results demonstrate that BTK and PLC-\(\gamma\)-2 are required for BCR-induced production of DAG. They also extend our previous findings, which indicate a requirement for BTK and PLC-\(\gamma\)-2 in BCR-directed DAG production in DT40 B cells (34, 48).

Discussion

Because DAG is a strong inducer of cell survival and differentiation (34, 64), our finding that DAG is preferentially produced in T2

![FIGURE 5. PKC phosphorylation is modestly higher in T2 compared with T1 B cells.](http://www.jimmunol.org/

![FIGURE 6. BCR-mediated DAG production requires BTK and PLC-\(\gamma\)-2.](http://www.jimmunol.org/)
relative to T1 B cells (Fig. 2, B and C) correlates with the observed BCR-induced survival and differentiation of T2 B cells but not T1 B cells (9, 14, 15). It is noteworthy that similar BCR-induced DAG production is observed in T2 and mature FoB cells, suggesting that DAG plays an important role in the survival of both T2 and mature FoB cells. We also observed that T2 B cells display higher constitutive levels of DAG and IP<sub>3</sub>, as well as PIP<sub>2</sub> and phosphorylated PKCs, compared with T1 cells (Figs. 2, A and C; 3, and 5). These data suggest an active BCR signaling process, which is consistent with T2 B cell survival and positive selection into the mature FoB cell pool. We also demonstrated that both btk<sup>−/−</sup> and plc-y2<sup>−/−</sup> B cells, which are impaired in the development of mature FoB cells, are unable to generate DAG following BCR engagement (Fig. 6). Coupled with previous genetic evidence demonstrating that the BTK/PLC-γ2 signaling axis is critical for T2 to mature FoB cell development, our results support the hypothesis that DAG plays an important role in T2 B cell survival and differentiation into mature FoB cells. It is unlikely that BTK/PLC-γ2-dependent DAG production plays a significant role in T2-pre-MZ to MZ B cell maturation because MZ B cells develop normally in BTK- and PLC-γ2-deficient mice (3, 26, 65). Thus, the BTK/PLC-γ2/DAG signaling axis may promote the differentiation of T2 into mature FoB cells; however, this pathway appears dispensable for the differentiation of T2-pre-MZ into MZ B cells, and may even inhibit the development of MZ B cells as previously proposed (65).

Although DAG has been implicated in cell survival and differentiation, the significance of PIP<sub>2</sub> in these processes is unknown. Despite having the lowest basal and BCR-induced levels of PIP<sub>2</sub>, we found that mature FoB cells produce substantial levels of DAG upon BCR cross-linking. These results suggest that differences in PIP<sub>2</sub> levels between T1 and T2 B cells may not be responsible for the disparate DAG production observed upon BCR stimulation. Moreover, BCR signal-induced changes in PIP<sub>2</sub> levels may not reflect the generation of lipid second messengers (DAG and IP<sub>3</sub>). Thus, higher levels of DAG in mature FoB relative to transitional B cells may reflect differentiation stage-specific metabolism of DAG rather than the levels of available substrate (PIP<sub>2</sub>).

It was recently proposed that DAG-dependent activation of conventional PKCβ initiates a survival, but not a differentiation, program in response to BCR cross-linking through the activation of NF-κB and induction of Bcl-x<sub>L</sub> (33, 36). Thus, DAG at least in part may regulate T2 B cell survival via activation of the PKC pathway. In this model, survival of T2 cells may be regulated by PKCβ, and their differentiation by nonconventional PKCs. Multiple nonconventional PKC isoforms are expressed in B cells and are activated in response to BCR cross-linking, but their specific roles in splenic B cell maturation remain largely unresolved (66). However, our data showing only minor differences in the phosphorylation of both conventional and nonconventional PKCs in T1 and T2 B cells (Fig. 5) argues against a central role for PKC-mediated signaling in transitional B cell differentiation processes. Consistently, mice deficient for either conventional and nonconventional PKCs display a milder B cell deficiency than that observed in BTK- or PLC-γ2-deficient mice (9, 19, 36, 67, 68), suggesting that other DAG-dependent and PKC-independent pathways likely contribute to T2 B cell survival and differentiation. Alternatively, DAG may regulate T2 B cell survival and differentiation via activation of the Ras-MAPK pathway. In this regard, we have previously shown that ERK, a downstream effector of Ras, is preferentially activated in T2 compared with T1 B cells (14). The Ras/ERK pathway has also previously been shown to control early B cell development, indicating its role in B cell differentiation (69, 70). Thus, DAG may activate survival via the PKCβ/NF-κB/Bcl-x<sub>L</sub> signaling axis and differentiation via the Ras pathway (34).

Despite differential IP<sub>3</sub> production (Fig. 2E), both T1 and T2 B cells mobilize Ca<sup>2+</sup> in response to BCR cross-linking (Fig. 4, top panel). This observation is in agreement with the study by Yellen et al. (50), which demonstrated that intracellular Ca<sup>2+</sup> is mobilized similarly in both immature and mature B cell populations despite the lack of IP<sub>3</sub> production in immature B cells. Together, these data suggest that the Ca<sup>2+</sup> mobilization observed in T1 B cells may occur independently of IP<sub>3</sub> hydrolysis, which leads to DAG and IP<sub>3</sub> generation. Alternatively, T1 B cells may be more sensitive to IP<sub>3</sub>, and may thus be able to respond to lower levels of IP<sub>3</sub> than T2 B cells. In this regard, we observed that the initial Ca<sup>2+</sup> peak in T1 B cells is more sensitive to treatment with 2-APB, an inhibitor of IP<sub>3</sub>-mediated calcium release, whereas sustained calcium influx in T1 B cells is less sensitive to 2-APB than in T2 and mature FoB cells (Fig. 4, middle panel). Sustained Ca<sup>2+</sup> influx via calcium release activated channels has been attributed to sustained high levels of IP<sub>3</sub> (45); however, we have demonstrated that T1 B cells produce significantly less IP<sub>3</sub> compared with T2 B cells. This suggests that T1 cells may use other channels for sustained Ca<sup>2+</sup> influx, such as L-type or L-type-like channels, as has been reported for B cells and B cell lines (46, 47). Consistent with this hypothesis, inhibition of L-type channels with verapamil led to a more severe reduction in calcium mobilization in T1 relative to T2 and mature FoB cells (Fig. 4, bottom panel). Taken together, these results suggest that the mechanisms by which both intracellular calcium release and sustained calcium influx occur may be different in each transitional B cell subset. Additionally, we have previously reported that verapamil inhibits the induction of NFAT, whereas 2-APB is an inhibitor of NF-κB activation (34). Differential responsiveness of transitional B cell subsets to these inhibitors (Fig. 4) suggests that following BCR engagement, T1 B cells may preferentially use NFAT, whereas T2 B cells may use both NFAT and NF-κB for the transcription of genes necessary for survival and/or differentiation. Furthermore, activation of NFAT in the absence of its binding partner, AP-1 (which is DAG/PKC dependent), has been shown to induce tolerance in T lymphocytes, and apoptosis in B cell lines (71, 72). Thus, it is possible that induction of NFAT following BCR engagement may promote apoptosis specifically in T1 B cells.

**FIGURE 7.** Model for peripheral B cell development. In this model, calcium mobilization in the relative absence of DAG/IP<sub>3</sub> production following BCR cross-linking leads to T1 cell death, whereas in the absence of BCR cross-linking, T1 cells develop into T2 B cells via a default pathway or via tonic BCR signaling. In T2 B cells, BCR cross-linking leads to production of DAG/IP<sub>3</sub> as well as calcium mobilization, both of which are required for T2 B cell survival and differentiation into mature FoB cells. Consistent with this model, a block in B cell development at the T2 stage is observed in cells that are unable to produce DAG, such as BTK- or PLC-γ2-deficient B cells.
In contrast to T2 B cells, T1 B cell survival and differentiation into T2 B cells likely occurs independently of DAG. Consistent with this hypothesis, the atypical DAG/Ca^2+ -insensitive PKCa in pre-B cells mediates NF-κB activity through Src family protein tyrosine kinases including Fyn, Lyn, and Btk independently of Btk, PLC-γ2, Vav, or CD19 (73). Moreover, T1 B cell death may occur by an active process that involves production of ceramide, which triggers a proapoptotic program as observed in the immature B cell line WEHI 231 (74). Additionally, a recent report indicated that cholesterol levels are similar in T1 relative to T2 immature B cells, but lower than that of mature B cells (75). This difference correlates with developmental differences in surface BCR compartmentalization and signaling, underscoring the importance of the quality and quantity of lipid species in splenic B cell subsets.

Regardless of the specific lipid-mediated mechanisms that control T1 vs T2 B cell fate, we postulate that mobilization of calcium without the balancing DAG-mediated signals may promote apoptosis in T1 B cells, whereas balanced calcium and DAG-mediated signals may promote T2 B cell survival and differentiation into mature FoB cells (Fig. 7).

Acknowledgments
We thank Dr. Yoshio Hamada (Tissue and Cell Culture Laboratory, National Institute for Basic Biology, Japan) for the generous gift of notch2<sup>−/−</sup> mice (provided by James Thomas, Vanderbilt University, Nashville, TN), and the Vanderbilt University Medical Center Flow Cytometry Core (Jim Higginston and Michele Nadej) for assistance with FACS sorting and calcium mobilization experiments.

Disclosures
The authors have no financial conflict of interest.

References


