Micro-scale flow cytometry-based and biochemical analysis of lipid signaling in primary B cell subpopulations

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ABSTRACT

B cell subpopulations in the spleen have been extensively characterized phenotypically; however, biochemical properties of these cell populations following B cell antigen receptor engagement have not been fully determined due to technical difficulties and limiting cell numbers. We therefore employed mini-scale protocols to assess lipid signaling, particularly that of diacylglycerol and inositol trisphosphate, with as few as 0.5×10^6 purified early (T1) and late (T2) transitional B cells. Additionally, utilizing flow cytometric techniques, we determined levels of phosphatidylinositol bisphosphate and calcium mobilization in T1 and T2 cells, as well as mature follicular and marginal zone B cells using less than 1×10^6 primary B cells. Thus, these biochemical and flow cytometric methodologies can be used to analyse signal-induced changes in phosphatidylinositol bisphosphate levels, diacylglycerol and inositol triphosphate production and calcium in each B cell population.

INTRODUCTION

The B cell compartment within the mouse spleen is comprised of at least five subpopulations, including immature transitional type 1 (T1), transitional type 2 (T2) and T2-preMZ, as well as mature Follicular (Fo) and marginal zone (MZ) B cells (1-4). Signaling through the B cell antigen receptor (BCR) is required for the generation of a functional mature B cell repertoire (3, 5), and multiple components of the BCR signalosome regulate biochemical events after BCR engagement. Upon activation by Btk, PLC-γ2 catalyzes the hydrolysis of phosphatidylinositol bisphosphate ($PtdInsP_2$ or PIP_2) into the two second messengers, inositol trisphosphate (IP_3), which activates Ca^{2+} signaling, and diacylglycerol (DAG), which activates protein kinase C (PKC) signaling pathways (6, 7). It is well established that both calcium and PKC signaling are important in B cell survival and development/maturation, as well as proliferation (8, 9). Thus, quantities of PIP_2, DAG and IP_3 are likely to direct the subsequent signaling events leading to the fate of developing B cells. Developing new protocols to study lipid signaling is therefore a prerequisite for understanding the molecular mechanisms underlying the generation of mature B cells.

Because only a small number of each B cell subpopulation can be obtained by flow cytometry-based cell sorting methods, we developed and optimized mini-scale DAG and IP_3 quantification protocols in order to analyze the levels of DAG and IP_3 within transitional B cell subsets following BCR cross-linking. These protocols allowed the quantification of lipid species utilizing as few as 0.5-5×10^6 purified B cells. Additionally, utilizing flow cytometry (FCM) techniques to identify B cell populations, we analyzed PIP_2 levels and calcium mobilization in transitional and mature B cell subsets, again using as few as 1×10^6 total B cells. Here we report the detailed experimental procedure of micro-scale flow cytometry and biochemical analysis of lipid signaling in primary B cells.

MATERIALS AND METHODS

Mice and B cell purification

Wild type C57BL/6 mice (Jackson Laboratories, Maine, USA) were used. All mice that were used as the source of splenocytes were treated humanely in accordance with federal and state government guidelines and their use was approved by the institutional animal committee.

Primary B lymphocytes were isolated from single cell suspensions pooled from the spleens of C57BL/6 mice. Cell suspensions were depleted of RBCs, and B lymphocytes were purified through magnetic sorting on an AutoMACS automated cell sorter (Miltenyi Biotech) by negative selection (CD43-depletion) to avoid B cell activation. The purity of B cells isolated in this manner was approximately 90–95% as verified by FCM analysis using anti-B220 and anti-IgM antibodies (Pharmingen, USA). FACS-sorting for highly purified transitional B cell subsets was performed as previously described (2).

Lipid analysis

PIP$_2$ quantitation: PIP$_2$ levels following anti-IgM stimulation were monitored by flow cytometry. MACS-enriched B cells were resuspended (10×10^6 cells/ml) in complete RPMI-1640, rested on ice for 1.5 hours, and incubated for 15 minutes at 37°C prior to assay. Cells were then stimulated with 20 μg/ml goat anti-mouse IgM F(ab')$_2$ antibodies (Jackson ImmunoResearch) for the indicated times, and immediately fixed (in order to preserve PIP$_2$ phosphorylation status) by harvesting 100 μl (containing 1×10^6 cells) into 1 ml cold PBS + 2% paraformaldehyde at each time-point. Cells were permeabilized utilizing the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions, and intracellularly labeled with a biotinylated antibody raised against PI(4,5)P$_2$ (Z-B045; Echelon Biosciences, Inc; 1:500 dilution) for 20 minutes on ice. Cells were then washed and labeled with APC-conjugated anti-CD19 (clone 1D3; 1:100 dilution), FITC-conjugated anti-HSA (clone M1/69; 1:500 dilution), and PE-conjugated anti-CD21 (clone 7G6, 1:400 dilution) for the identification of splenic B cell subsets, and PerCP-Cy5.5-conjugated streptavidin (1:300 dilution) to visualize PIP$_2$. Anti-C1qRp (AA4.1) and anti-CD23 antibodies were not utilized due to non-reactivity with B cells that have undergone fixation. Following a 20 minute incubation on ice, cells were washed, and resuspended in 100 μl FACS buffer (2% FBS in PBS), followed by 100 μl fix buffer (2% paraformaldehyde in FACS buffer) to preserve the cells. To test the specificity of the anti-PIP$_2$ antibody, freshly isolated MACS enriched B cells were fixed and permeabilized as described above, then intracellularly labeled with an anti-PIP$_2$-FITC antibody (Z-G045; Echelon Biosciences, Inc; 1:300 dilution) that had been pre-incubated with varying concentrations of PIP$_2$ liposomes (Y-P045 (biotinylated); Echelon Biosciences, Inc) for 15 minutes on ice. Data was acquired on a BD FACSCalibur or BD LSRII flow cytometer. Results are expressed as MFI and are representative of multiple experiments.

DAG quantification: Purified B cells were labeled with [^{3}H]palmitic acid (2.5 mCi/ml), as a fatty acid precursor. Labeled B cells were washed twice with PBS, incubated for various time periods or stimulated through the BCR with goat anti-mouse IgM F(ab')$_2$ as indicated. The reactions were stopped by the addition of excess cold PBS. The cells were pelleted by centrifugation and used for lipid extraction. The pellet was suspended in 2 ml chloroform-methanol (2:1 v/v) and 0.4 ml of 0.5 N HCl was added. The mixture was shaken vigorously, centrifuged, and the upper (aqueous) phase was discarded while the lower (organic) washed twice with theoretical upper phase (10). The radioactivity of lipids was determined on an aliquot fraction by scintillation counting. Dipalmitin carrier standard was then added, the lipid phase evaporated gently under a N$_2$ stream and the residue dissolved in a minimum volume of chloroform-methanol (2:1 v/v). DAG was separated by thin layer chromatography on silica gel G plates using a double development system and detected under UV light after spraying the plates with primulin as previously described (11). The area containing DAG was scraped and the radioactivity determined as previously described (6, 11, 12).

[^{3}H]-Inositol labeled soluble inositol polyphosphate (IP) profiling: The soluble IP profile for B cells was determined by adapting a previously described protocol (13). Briefly, MACS-enriched total B cells or FACS-purified B cell subsets (10×10^6/ml) were cultured in inositol-free DMEM containing 60 mCi [^{3}H]Inositol/ml (Perkin Elmer) for 4 hours. Cells were harvested, washed twice with 1 ml PBS and suspended in PBS (10×10^6/ml). 100 μl cell aliquots (containing 1×10^6 cells) were treated with 25 μl PBS with or without 20 μg/ml goat anti-mouse IgM F(ab')$_2$. Treatments were stopped by adding 6.6 μl 10 N HCl and chilling cells on ice. Soluble IPs were extracted by adding 465 μl of chloroform/methanol (1:2 v/v). The mixture was vortexed for 2 minutes at maximum speed, followed by addition of 156 μl each of chloroform and 2 M KCl and another 2 minutes of vortexing. The lysate was spun at 13,000 g for 5 minutes and the supernatant recovered. Samples were analyzed by the HPLC (Shimadzu Corporation) connected to the Radio-HPLC Detector (IN/US Systems, Inc.). The IPs were resolved by a Whatman partisphere SAX strong-anion exchange column (4.6 x 125 mm) and a linear gradient from 10 mM to 1.7 M ammonium phosphate (pH 3.5) for 25 minutes, followed by elution with 1.7 M ammonium phosphate for 20 minutes.

Calcium mobilization in B cell subsets: MACS-enriched B cells were resuspended (10×10^6 cells/ml) in warm HBSS w/o phenol red, and loaded with indo-1-AM (5 μg/ml) for 30 minutes at 37°C. Cells were then washed, resuspended (20×10^6 cells/ml) in FACS buffer, placed on ice, and labeled for 20 minutes with PE-conjugated anti-HSA (clone M1/69; 1:750 dilution), biotinylated anti-CD23 (clone B3B4; 1:200 dilution) which was visualized by PerCP-Cy5.5-conjugated streptavidin (1:200 dilution), and APC-conjugated anti-C1qRp (clone AA4.1; 1:100 dilution) for B cell subset identification. Antibodies raised against B220,
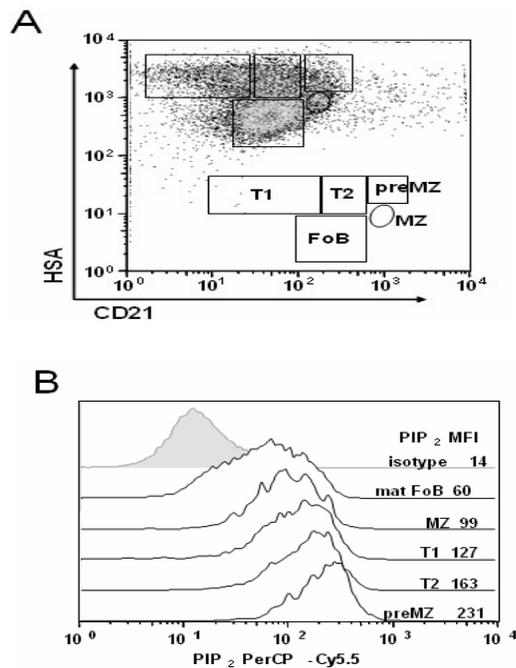
Fig. 1A & 1B: PIP$_2$ is hydrolyzed in all B cell subsets following anti-IgM stimulation. (A) : Freshly isolated MACS-enriched wt B cells were fixed with 2% paraformaldehyde, permeabilized and labeled with antibodies directed against CD19, CD21, and HSA for B cell subset identification. **(B):** B cells from (A) were examined for basal PIP$_2$ levels utilizing an anti-PI(4,5)P$_2$ antibody. Data is an example of multiple experiments.

CD19, and/or CD21 were not used in order to avoid inadvertent stimulation of B cells. Following labeling, cells were washed, resuspended (1×10^6/ml) in HBSS containing 1 mM calcium, and kept on ice until assay. Prior to acquisition, cells were warmed for 15 minutes in a 37°C water bath, and applied to a BD LSR II cytometer. Baseline, non-stimulated measurements were taken for 90 seconds, followed by the addition of 10 μg/ml goat anti-mouse IgM F(ab')$_2$ antibodies for the indicated time. Ionomycin (1 mM) was used as a positive control to ensure that cells were competent to release calcium from intracellular stores. Data is expressed as a ratio of Indo1-violet/Indo1-blue, and is a representative of multiple experiments.

Statistics

The data represent the mean of at least three experiments analyzed utilizing Student's *t*-test.

RESULTS

PIP2 hydrolysis occurs in all B cell subsets as determined by FCM

Because PIP$_2$ serves as a substrate for PLC-γ2 in the production

of DAG (14), we wanted to determine basal and BCR-induced levels of PIP$_2$ in all B cell subsets. MACS-enriched splenic B cells were stimulated, harvested, and immediately fixed in 2% paraformaldehyde in order to preserve the phosphorylation status of PIP$_2$. Cells were then stained intracellular for PIP$_2$ and extracellular with a combination of anti-HSA, anti-CD21, and anti-CD19 antibodies for B cell subset identification. Utilizing flow cytometric analysis, CD19$^+$ cells were displayed with HSA and CD21 in order avoid inclusion of any remaining non-B cells in the analysis (Fig. 1A). CD21^{hi} MZ and pre-MZ were separated based on differential HSA expression, which corresponded to CD23 expression (preMZ are HSA^{hi}CD23$^+$, MZ are HSA^{mid}CD23^{lo}), as we previously reported (2). Basal PIP$_2$ levels were then measured in each subset (Fig. 1B). In addition, we determined the kinetics of BCR-induced PIP$_2$ production in each B cell subpopulation following stimulation with 20 μg/ml anti-IgM F(ab')$_2$ antibodies. In all splenic B cell subsets, PIP$_2$ levels were reduced immediately in response to stimulation with anti-IgM, indicative of PIP$_2$ hydrolysis (Fig. 1C).

Optimal lipid labeling with [^{3}H]palmitic acid is achieved in 6 hours

To analyze the lipid synthesis and BCR-directed DAG production in wild type primary B cells, we systematically assessed the numbers of primary B cells and the amount of radioactivity required for optimum labeling. MACS-enriched total splenic B cells or FACS-sorted B cell subsets were used immediately for labeling with [^{3}H]palmitic acid, a saturated fatty acid (6). Pilot experiments indicate that optimal lipid synthesis is achieved with a concentration of 2.5 mCi/ml (data not shown). Using this information, we discovered that the incorporation of radioactivity was detectable after 30 min, increased in a time-dependent manner reaching a plateau at 6 h, and remained unchanged through 24 h (Fig. 2A). The same profile was obtained when the results were expressed as cpm/mg protein indicating a dynamic process (data not shown).

BCR-induced DAG production is dose-dependent and can be detected with as few as 0.5×10^6 B cells

MACS-enriched total B cells were labeled for 6 h with [^{3}H]palmitic acid, then were stimulated for 5 minutes with various concentration of anti-IgM antibodies in order to determine the production of DAG (12). DAG production increases in a dose dependent manner and is optimal at 20 μg/ml of anti-IgM (Fab')$_2$ (Fig. 2B).

To test the feasibility of DAG quantification experiments with small numbers of primary B cells, we labeled MACS-enriched total B cells for 6 h with [^{3}H]palmitic acid, and determined its incorporation into whole lipids in various numbers of B cells. We observed a strong correlation between the total radioactivity detected in whole lipids with the number of B cells labeled (r = 0.998). It is noteworthy that the radioactivity was close to 50000 and 100000 cpm for 0.5×10^6 and 1×10^6 labeled cells respectively (Fig. 2C). Since the level of DAG represents 0.6% of total lipids

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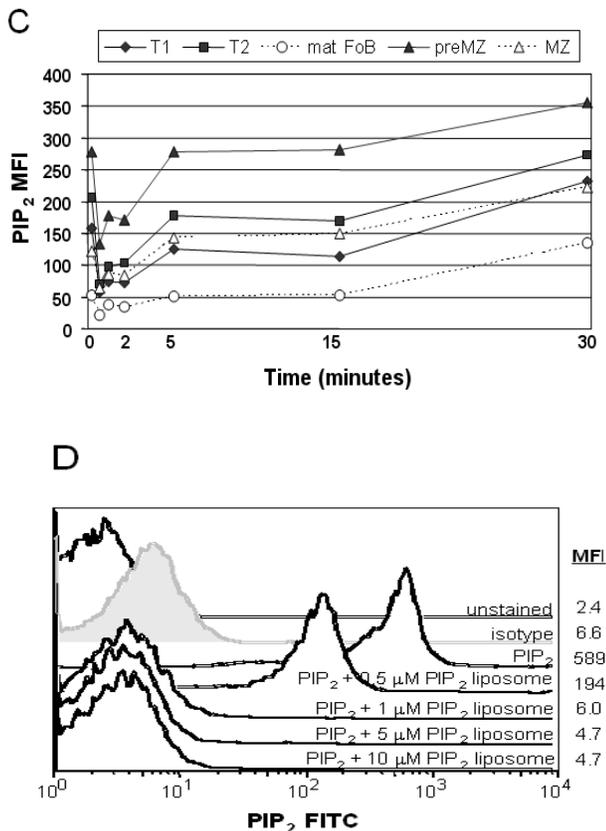
Fig. 1C & 1D: PIP₂ is hydrolyzed in all B cell subsets following anti-IgM stimulation. (C): B cells were stimulated for the indicated times with 20 µg/ml anti-IgM, immediately fixed and permeabilized, then labeled and examined for PIP$_2$ levels as in (B); T1 (filled diamonds), T2 (filled squares), mature FoB (open circles), preMZ (filled triangles), mature MZB (open triangles). Data is displayed as the average MFI of duplicate samples, and is a representative of at least 4 experiments. **(D):** The specificity of the anti-PIP$_2$ antibody was tested by pre-incubation of the antibody with PIP$_2$ liposomes for 15 min prior to intracellular labeling of MACS-enriched total B cells as in (B).

extracted from the mouse primary splenic B cells (2), the amount of [³H]DAG in 0.5×10^6 B cells should be close to 500 cpm which is more than 100 times greater than the background, and should therefore allow reliable estimation of DAG production.

We next determined the level of BCR-induced DAG production after 5 minutes of stimulation with anti-IgM (20 µg/ml) using various numbers of MACS-enriched total B cells ranging from 0.5×10^6 to 5×10^6. The results show that upon BCR ligation the profile of DAG production obtained from a small number (0.5×10^6) of primary B cells coincides precisely with that obtained from a large number (5×10^6) of B cells (Fig. 2D). When 0.5×10^6 B cells were labeled with [³H]palmitic acid, the radioactivity detected in DAG was similar to that calculated from the standard curve (450 cpm corresponds to 0.5% of total lipids). Therefore, lipid signaling in transitional B cell subsets may be investigated using [³H]palmitic acid as the radioactive

labeling precursor. In this regard, we previously demonstrated that BCR-mediated 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 non-stimulated control) and 30 min (2.8- ± 0.3-fold increase, and 4.8- ± 0.4-fold increase, respectively, compared with the non-stimulated control) stimulation with anti-IgM (2). In contrast, T1 B cells did not produce DAG in response to anti-IgM stimulation (2).

HPLC analysis of BCR-induced IP₃ production in splenic B cell subsets

Because we observed significant differences in BCR-mediated DAG production in transitional B cell subsets, we also investigated the production of IP$_3$ in response to BCR stimulation utilizing HPLC. Using MACS-enriched total B cells, we first performed a kinetics study and determined that 1 minute of anti-IgM stimulation was the optimum time-point for IP$_3$ detection (Figure 3A and B). We then utilized 1×10^6 FACS-purified transitional T1 and T2 B cells to investigate IP$_3$ production following anti-IgM stimulation. T2 B cells produced significantly more IP$_3$ than T1 B cells (1.52 ± 0.5-fold increase and 3.09 ± 0.2-fold increase, respectively; n = 3) (Figure 3 C-E), consistent with the previously observed DAG production profile (2).

FCM analysis of intracellular Ca²⁺ mobilization in B cell subsets

Because the generation of IP$_3$ subsequently induces Ca^{2+} mobilization in primary *wt* B cells (15), we next wanted to investigate calcium mobilization in B cell subsets. To precisely define stage-specific calcium mobilization within B cell subsets, we utilized FCM techniques. 1×10^6 MACS-enriched B cells were loaded with indo-1-AM and labeled with a combination of anti-C1qRp (AA4.1), anti-HSA and anti-CD23 antibodies for B cell subset identification (Fig. 4A). Antibodies directed against B220, CD19, and CD21 were not used in order to avoid inadvertent B cell activation. Cells were applied to the cytometer, baseline measurements taken for 60-90 seconds, then stimulated with anti-IgM for the indicated times. From the AA4⁺ gate, T1 (HSA^hiCD23⁻) and T2 (HSA^hiCD23⁺) cells were gated for calcium analysis. The vast majority of T2-preMZ cells do not express AA4.1; therefore they are excluded from T2 cells gated in this manner. From the AA4- gate, mature FoB (HSA^loCD23⁺) and MZ (HSA^midCD23^lo) were gated for calcium analysis. Our results demonstrate that calcium mobilization can be monitored in all B cell subsets following anti-IgM stimulation, using as few as 1×10^6 MACS-enriched, FACS-stained total B cells (Fig. 4B).

DISCUSSION

The goal of this study was to develop protocols to assess
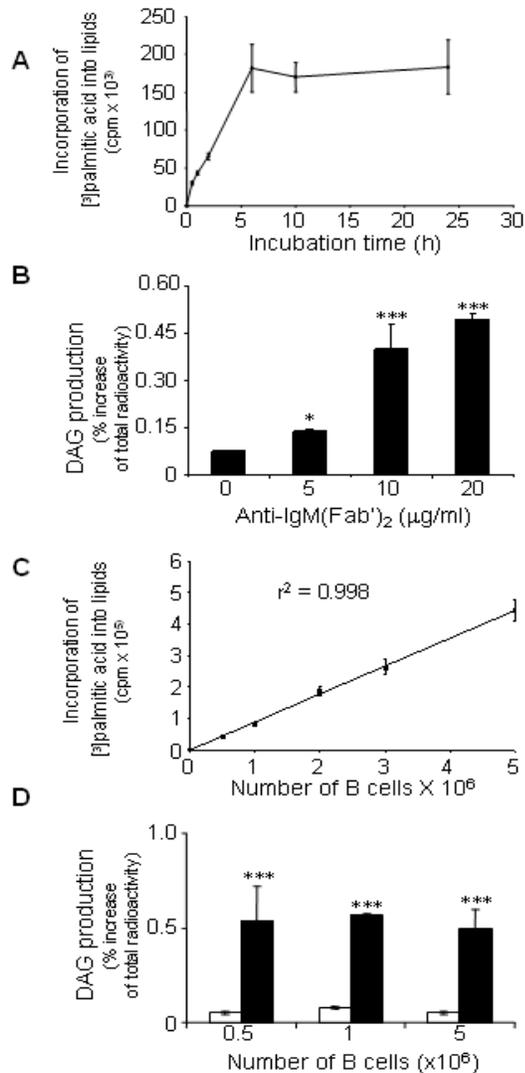
Fig. 2: Lipid synthesis and DAG production in small numbers of primary B cells. (A): Kinetics of lipid synthesis in primary B cells. Incorporation of [^{3}H]palmitic acid (2.5 mCi/ml) into lipids was determined in purified wt primary B cells. Lipid extraction was performed according the protocol of Folch (10). Lipid synthesis is expressed in cpm and represents the average + SEM of three independent performed in triplicate. **(B):** BCR-induced DAG production is dose dependent. 5×10^6 purified B cells were labeled for 6h with [^{3}H]palmitic acid (2.5 mCi/ml) and stimulated for 5 min with various doses of anti-IgM. Lipids were extracted and separated as described (12) and DAG was quantified. DAG is expressed as percentage increase of DAG of total radioactivity and represents the average + SEM of three independent performed in triplicate. *$P<0.05$; ***$P<0.001$. **(C):** [^{3}H]palmitic acid is incorporated into whole lipids in small numbers of primary B cells. The indicated numbers of non-stimulated B cells from wt mice were labeled for 6h with [^{3}H]palmitic acid (2.5 mCi/ml) and total incorporation of [^{3}H]palmitic acid into lipids (r=0.998) was monitored as described (6). **(D):** BCR-induced DAG production can be measured in as few as 0.5×10^6 primary B cells. The same protocol as in (B) was applied to 0.5-5×10^6 primary B cells that were stimulated for 5 minutes with 20 µg/ml of anti-IgM. ***$P<0.001$.

dynamic changes in lipid signaling in each B cell subset following antigenic stimulation. By utilizing FCM techniques to assess both PIP$_2$ levels and calcium mobilization in individual B cell subsets, we eliminated the necessity for purification of B cell subsets in these analyses. Additionally, we defined optimal conditions to rapidly analyze DAG and IP$_3$ production utilizing numbers of total primary B cells similar to the quantities of transitional B cells that can be obtained by magnetic bead and FCM based cell sorting protocols (2, 16).

In this report we used a combination of the most current staining schemes to identify B cell subpopulations in the spleen by FCM (1-4, 16, 17). We have combined extracellular staining for B cell subset identification with intracellular staining protocols in order to determine PIP$_2$ levels in all B cell subsets following stimulation with anti-IgM (Fig. 1). It is important to note that following an initial period of BCR-induced PIP$_2$ hydrolysis, BCR-induced PIP$_2$ production occurs in all the B cell subpopulations (Fig. 1C). This increase in PIP$_2$ over time may result from DAG conversion into PIP$_2$ or PIP$_2$ synthesis by PIP5K following BCR stimulation (18, 19). Thus, a PIP$_2$ cycle is activated in B cells in response to BCR cross-linking. The lower production of DAG and IP$_3$ in T1 B cells suggests developmental stage-specific mechanisms of PIP$_2$ cycling in splenic B cell subpopulations. Additionally, it is noteworthy that the B cell subset with the lowest baseline (mature FoB) had the greatest changes in PIP$_2$ MFI while the B cell subsets with the highest baselines (T2 and preMZ) had the smallest changes in PIP$_2$ MFI (Fig. 1C), indicating that the inducible production of PIP$_2$ through BCR stimulation is tightly associated with its basal level. As we have previously shown for PKC phosphorylation (2), this protocol can be extended to study other intracellular biochemical signaling events following antigenic stimulation.

In addition, we have employed FCM techniques in order to assess calcium mobilization in B cell subsets. Again, employing a unique combination of antibodies, which identify B cell subsets but will not activate cells inadvertently, we monitored calcium mobilization in each B cell subset following antigenic stimulation using as few as 1×10^6 MACS-enriched B cells and found that all B cell subsets mobilize calcium following anti-IgM stimulation (Fig. 4). These FCM-based techniques use only minimal numbers of primary B cells, and eliminate the need for FACS-based cell sorting of individual B cell populations.

We also report that ^{3}[H]palmitic acid can be used to efficiently label lipids in primary splenic B cells. We observed that peak radioactivity is reached after 6 h of labeling (Fig. 2A). Since splenic B cells die rapidly *in vitro* in the absence of B cell growth factors and/or agonists, the relatively short duration of labeling (6 h) with ^{3}[H]palmitic acid minimizes the loss of B cells (16, 17). Our results also show that lipid levels can be determined with small numbers of B cells, and that the radioactivity incorporated into lipids in 0.5×10^6 B cells is close to 5×10^4 cpm (8000 times higher than the background) which should allow easy quantification of lipid species (Fig. 2C). These data are in agreement with studies performed with primary B cells labeled with [^{3}H]arachidonic acid (15), both in
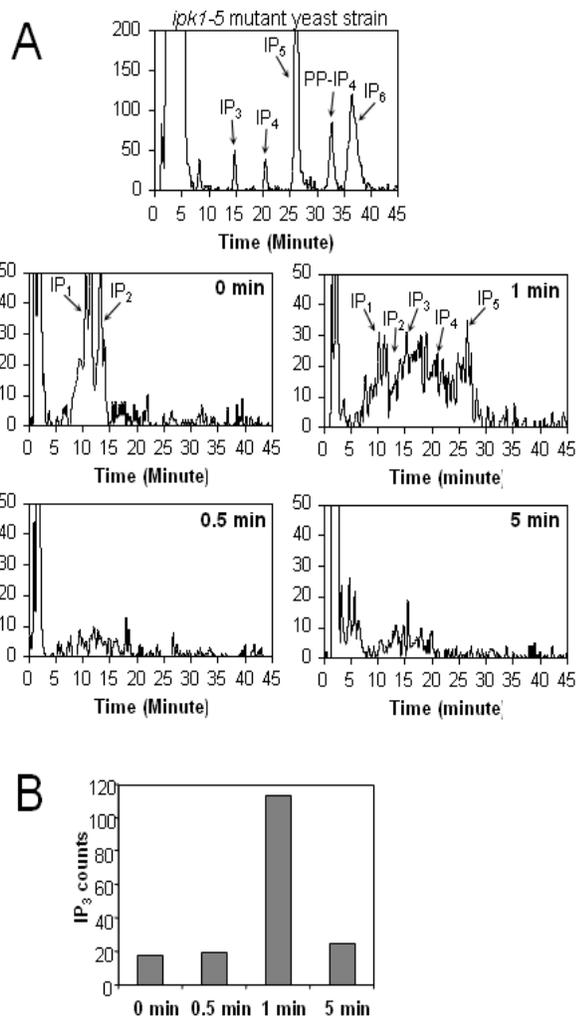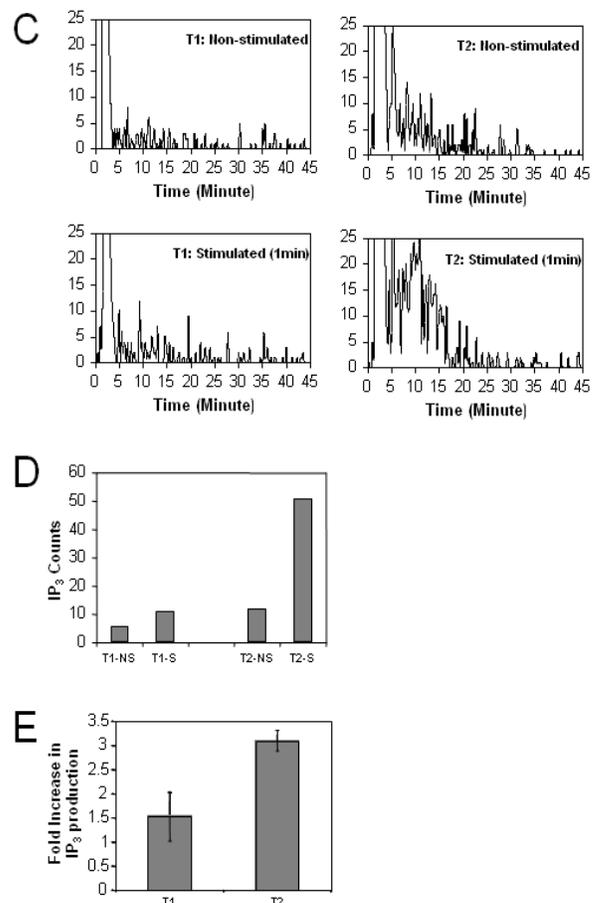
Fig. 3A & 3B: Generation of IP₃ in primary B cells: A) Timecourse of IP$_3$ production in primary B cells. 3×10^6 MACS-enriched B cells were labeled with ^{3}[H]inositol (60 mCi/ml) for 4 h, then incubated with PBS (non-stimulated) or stimulated with 20 µg/ml anti-IgM for the indicated times. Production of soluble IP species was measured by HPLC and the position corresponding to each species is indicated. IP$_3$ production was measured as the total counts detected within the 14.25-15.25 minute time window. HPLC profile of an *ipk1-5* mutant yeast strain (26) serves as reference. **(B):** IP$_3$ production from the B cells in (A) is expressed as the counts in the position corresponding to IP$_3$.

Fig. 3C – 3E: Generation of IP$_3$ in primary B cells: (C): 1×10^6 FACS-purified *wt* T1 and T2 B cells were labeled with ^{3}[H]inositol as in (A) and stimulated with 20 µg/ml anti-IgM for 1 minute. IP$_3$ production was detected as in (A). Data is a representative of four independent experiments. **(D):** IP$_3$ production from the T1 and T2 B cells in (C) is expressed as the counts in the position corresponding to IP$_3$. **E)** Fold change in IP$_3$ level from T1 and T2 B cells following stimulation with anti-IgM was calculated from the counts measured in the position corresponding to IP$_3$ as in (C and D) (n=3).

the profile of DAG accumulation and the quantity of radioactivity.

An alternate enzymatic method for the quantification of DAG employed an Escherichia coli DAG kinase which catalyzes the production of [^{32}P]phosphatidic acid from DAG substrate (20). This radiosensitive assay was successfully developed for sn-1,2 diacylglycerols quantification. Moreover, the DAG kinase assay was already applied to Epstein-Barr virus negative Burkitt's lymphoma Ramos cells to quantify ceramide (21). Here, our lipid separation method allows the separation of sn-1,2 diacylglycerol from sn-1,3 diacylglycerol species, and the DAG level reflects that of both isomers (12). In addition, we were able to quantify other neutral lipid species (palmitic acid, mLtriglycerides, and monoglycerides) in one single experiment (data not shown).

Development of immunocompetent B-lymphocytes is critically dependent on signaling through the BCR (5). Our results show that while 20 µg/ml of anti-IgM (Fab')₂ antibodies induces maximal production of DAG, lower doses also lead to significant production of DAG (Fig. 2B).

We also demonstrate that in response to BCR cross-linking, an increase in DAG production is clearly detectable with as few as 0.5×10^6 B cells (Fig. 2D). We have previously employed the methods described in this report to determine DAG production
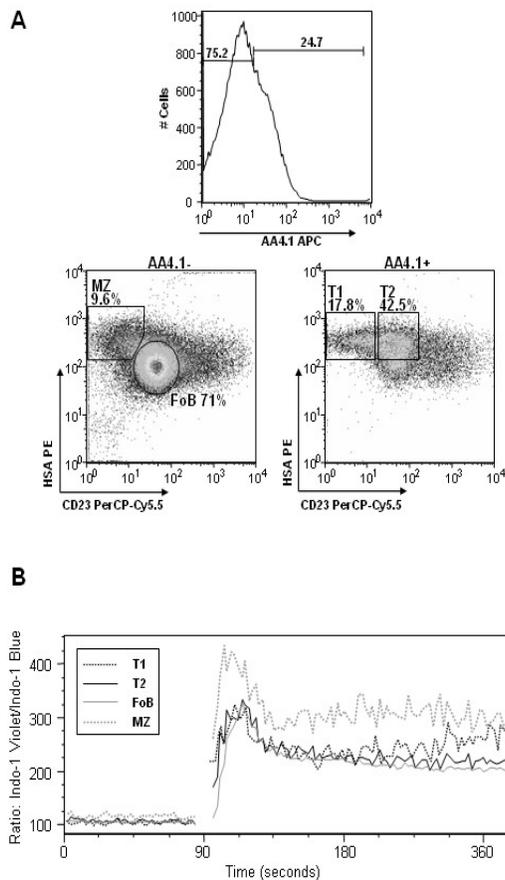
Fig. 4: Calcium mobilization in B cell subsets. (A): MACS-enriched B cells were loaded with Indo-1-AM (5 μg/ml), and labeled with AA4.1, anti-CD23, and anti-HSA for B cell subset identification. **(B):** Cells were applied to a cytometer, basal Ca^{2+} levels were monitored for 90 s, then cells were stimulated with 10 μg/ml anti-IgM (indicated by the arrow) and monitored for 4-6 min; T1 (dotted black line), T2 (black line), mature FoB (gray line), MZ (dotted gray line). Data is a representative of 4-6 independent experiments.

following anti-IgM stimulation in 1×10^6 FACS-purified T1, T2 and mature FoB cells (2). In that study, we reported that T2 and FoB cells rapidly produce detectible levels of DAG following anti-IgM stimulation and that increased DAG levels are maintained for a significant period of time, while T1 B cells do not produce DAG at any time-point following anti-IgM stimulation (2).

After we observed that DAG was differentially produced in transitional B cells subsets, we wanted to confirm that IP_3 production followed a similar pattern, as these two lipids are both by-products of PIP_2 hydrolysis. Consistent with DAG production, IP_3 was detected by HPLC in total B cells and in purified T2 B cells, but not in purified T1 B cells (Fig. 3 A and C). In addition to IP_3, we found that levels of other IPs (i.e., IP_1, IP_2, IP_4, and IP_5) also change in all B cell populations, however, these changes are most apparent in total B cells (Fig. 3A). This observation may reflect more robust changes in mature follicular B cells, which is the predominant population

(approximately 60-70%) in splenic B cells. Thus, the magnitude of BCR-induced IP_3 production is likely to be underestimated because IP_3 can undergo additional phosphorylations by kinases to generate IP_4, IP_5 IP_6. These findings also raise the possibility that other IPs may also play distinct roles in modulating B cell physiology. Indeed, a previous report showed that the PH domain of Btk functions as an inositol 1,3,4,5-tetrakisphosphate (IP_4), inositol 1,3,4,5,6-pentakisphosphate (IP_5), and inositol 1,2,3,4,5,6-hexakisphosphate (IP_6) binding domain and that *xid* mutations (R28C) found in the Pleckstrin Homology domain result in a dramatic reduction of IP_4 binding activity (22). Consistent with these results, BCR-induced DAG/IP_3 production is Btk dependent, suggesting that B cell differentiation is likely to be correlated with the IP_4 binding capacity of the PH domain of Btk (2, 22, 23). Additionally, a recent report revealed that inositol 1,4,5-triphosphate 3-kinase and its reaction product IP_4 are both required to mediate survival signalling in B cells via a Ras-ERK signalling pathway that controls the expression of the proapoptotic protein, Bim (24).

Together, these results demonstrate our ability to perform experiments to determine BCR-dependent lipid signaling utilizing the small numbers of B cells that are obtained with any of the B cell subpopulation purification protocols we have previously published (2, 16). Additionally, while these protocols were established in cells that were stimulated through the BCR, it is reasonable to believe that these protocols will be useful when monitoring B cell activation by many different cellular stimuli; in fact, we have already utilized these protocols to monitor DAG and IP_3 production, as well as calcium mobilization, in B cells stimulated with BAFF (25). Thus, the protocols that we describe in this report should allow a comprehensive determination of differential lipid signaling and signal transduction in primary B cell populations in response to various stimuli.

ACKNOWLEDGMENTS

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PROTOCOLS

B cell purification by negative selection

Materials:

       FACS buffer (PBS + 2% FBS)
       MACS buffer (PBS + 0.5% BSA and 1mM EDTA)
       Complete RPMI (RPMI + 10% FBS, 4mM L-glut, 100U P/S, 0.1% 2-ME, 10mM HEPES)
       * anti-mouse CD43 magnetic beads (130-049-801 Miltenyi Biotech)
       * AutoMACS automated cell sorter or MiniMACS columns (Miltenyi Biotech)
       ** B-cell enrichment kit (557792 BD Biosciences) and manual IMAG magnet

1) Harvest the spleen from a wt C57BL/6 mouse.
2) Isolate splenocytes by smashing spleen in a 70 mM cell strainer with 5ml PBS. Transfer cells to a 15ml conical tube and rinse strainer 2X with 5ml PBS. Transfer washes to the tube with cells and centrifuge for 6 minutes at 1600 rpm at 4°C.
3) Aspirate supernatant and lyse RBC, while vortexing, by the addition of 900 μL sterile filtered ddH$_2$0 followed immediately by the addition of 100 μL 10x PBS. Remove floating fat, and add FACS buffer to 10 ml.
4) Count cells.
5) Centrifuge cells for 6 minutes at 1600 rpm at 4°C. Resuspend cells at 100×10^6/ml in MACS buffer.
6) *Add anti-CD43 beads and incubate according to the manufacturers instructions.
7) *Sort cells on the AutoMACS using the DelpeteS cycle or on the MiniMACS magnetic columns according to the manufacturers directions.
8) Resuspend CD43-negative cells in 1-2 ml of complete RPMI and count cells.
9) Stain a small aliquot for purity with anti-B220 or anti-CD19, and analyze by FCM.

       ** 6-7) Alternatively, B cells can be enriched using the B cell enrichment kit and manual magnet from BD according to the manufaturers instructions.

PIP$_2$ quantitation by FCM

Materials:
       Stop buffer (PBS + 2% paraformaldehyde)
       BD Cytofix/Cytoperm kit
       Fix buffer (FACS + 2% paraformaldehyde)
       *Biotinylated anti-PIP$_2$ antibody (Z-B045; Echelon Biosciences)
       Anti-HSA-FITC antibody (BD clone M1/69)
       Anti-CD21-PE antibody (BD clone 7G6)
       *SAv-PerCP-Cy5.5 (BD 551419)
       Anti-CD19-APC antibody (BD clone 1D3)
       *anti-PIP$_2$-FITC (Z-G045; Echelon Biosciences)

1) Resuspend MACS-enriched B cells in RPMI (10×10^6/ml) and rest on ice for 1hr.
2) Warm cells in a 37°C water bath for 15 minutes
3) Prepare "Stop" tubes (1ml stop buffer) for each time point
4) Harvest Time 0 sample by collecting 100 μl (1×10^6 cells) into the appropriate stop tube (harvest an extra sample if staining with an isotype control)
5) Stimulate cells for desired time with anti-IgM F(ab')$_2$
6) Harvest cells by collecting 100 μl at each desired time point into appropriate stop tubes
7) Centrifuge 1700 rpm for 4 minutes
8) Fix/permeabilize using BD Cytofix/cytoperm kit according to manufacturer's instructions
9) *Intracellular stain with anti-PIP$_2$-biotin (1:500) in 100 μl perm-wash buffer/1×10^6 cells for 20 minutes. Wash with 1ml perm-wash, and spin at 1700 rpm for 4 minutes.
10) *Stain intracellular with SAv-PerCP-Cy5.5 (1:300), and extracellular with anti-HSA (1:500), anti-CD21 (1:250), and anti-CD19 (1:100) in 100 μl perm-wash buffer/1×10^6 cells for 20 minutes. Wash with 1ml perm-wash, and spin at 1700 rpm for 4 minutes.
11) Resuspend cells in 100μL FACS + 100 ml Fix.

12) Acquire data on FACS machine.

** 9-10) Alternatively, anti-PIP$_2$-FITC (1:300) can be used to study PIP$_2$ levels in total B cells (for subset identification, use in combination with HSA-PE (1:500), CD19-PerCP-Cy5.5 (1:200), and CD21-APC (1:400); all from BD)

DAG quantification by TLC

1) Resuspend purified B cells in complete RPMI, and label cells with 2.5 mCi/ml [^{3}H]palmitic acid (Sigma) for 6 hours at 37°C, 5% CO$_2$.
2) Spin cells at 2000 rpm for 5 minutes.
3) Remove supernatant
4) Wash cells twice with RPMI without serum
5) Resuspend cells (3x10^6/ml) in PBS in glass tubes and warm to 37°C .
6) Incubate cells with 20 μg/ml anti-IgM F(ab')$_2$ for appropriate times at 37°C.
7) Stop reactions by adding 1 ml cold PBS
8) Spin the cells at 3000 rpm for 5 minutes and wash twice with PBS
9) Remove supernatant
10) Extract lipids with 2 ml of a mixture of cold chloroform:methanol (2:1, v:v)
11) Place the samples at 4 °C overnight
12) Add 400 μl HCl (0.5N) to the 2 ml sample
13) Vortex tubes for 10 seconds
14) Spin at 3000 rpm for 5 minutes at 4°C
15) Take 10 μl of the upper phase and count the radioactivity in scintillation counter to ensure that after washing the cells no radioactivity remains in the aqueous phase. Presence of radioactivity in the aqueous phase will indicate poor lipid labelling and the experiment should be discarded.
16) Remove the upper phase
17) Transfer 1 ml of the organic phase to a new glass tube
18) Remove 10 μl of the organic phase and count the radioactivity in scintillation counter
19) Add 40 μl of cold carrier lipids (mixture of phosphatidic acid, monoglycerides, 1-2 dipalmitin, palmitic acid, tripalmitin, each at 10 mg/ml; Sigma)
20) Evaporate the organic phase gently under an N$_2$ stream
21) Resuspend the residue in 40 μl chloroform-methanol (2:1, v:v)
22) Load on a 20x20 cm Silica Gel plate (Merck)
23) Separate neutral lipids from the other lipids in the upper phase with a mixture of ethylacetate:isooctane:acetic acid:water (130:20:30:100)
24) Allow the TLC to run half-way on the plate (25-30 minutes) in the first dimension
25) Dry the plate gently with a hair dryer for 10 minutes
26) Further separate the neutral lipids with a mixture of isopropylether:acetic acid (96:4, v:v) in the second dimension
27) Dry the plates gently with a hair dryer for 10 minutes
28) Spray the plates with primulin (in acetone-water 8:2, v:v)
29) Using the carrier lipids as markers, identify the lipid species, and scrape the desired lipids (in silica) into scintillation vials
30) Add 150 μl of water and 3 ml of scintillation fluid
31) Vortex each tube for 20 seconds
32) Count radioactivity

IP$_3$ quantification by HPLC

Soluble Inositol Polyphosphate (IP) Extraction

1) Incubate MACS-enriched total B cells or FACS-purified B cell subsets (10x10^6/ml) in inositol-free DMEM containing 60 mCi [^{3}H]Inositol/ml (Perkin Elmer) for 4 hours.
2) Harvest cells, wash twice with 1 ml PBS and suspended in PBS (10x10^6/ml) in a glass tube.
3) Dispense 100 μl aliquots (1x10^6 cells) in 2.0 ml screw-cap polypropylene tubes.
4) Warm cells to 37°C for 5 min. Treat cells with 25 μl PBS with or without 20 μg/ml goat anti-mouse IgM F(ab')^2 for desired time.
5) Stop treatments by adding 6.6 μl 10 N HCl and chilling cells instantly on ice.

6) Extract soluble IPs by adding 465 μl of chloroform/methanol (1:2 v/v). Vortex the mixture at maximum speed for 2 minutes.
7) Add 156 μl each of chloroform and 2 M KCl and vortex for another 2 minutes.
8) Spin the lysate at 13,000 g for 5 minutes and the collect the supernatant in a screw-cap tube. Store at -20°C if needed.

Separation of IPs through HPLC

Analyze the samples by the HPLC (Shimadzu Corporation) connected to the Radio-HPLC Detector (IN/US Systems, Inc.).

Sample preparation: Add ammonium phosphate (pH 3.5) to the supernatant (10 mM final). Filter through an Amicon Ultrafree-MC 0.45 mm filter unit (Millipore).

HPLC: Inject sample (500 ml) onto a Whatman partisphere SAX strong-anion exchange column (4.6 x 125 mm). Resolve the IPs by a linear gradient from 10 mM to 1.7 M ammonium phosphate (pH 3.5) for 25 minutes, followed by elution with 1.7 M ammonium phosphate for 20 minutes. For detection by an online Radio-HPLC detector, use Tru-Count (IN/US System) scintillation counting solution in 1:1 ratio.

Calcium mobilization by FCM

Materials needed:
      HBSS w/o phenol red (containing 10% FCS, 1mM Mg^{++}, with/without 1mM Ca^{++})
      Indo-1-AM (Molecular probes I-1223), resuspended at 1 mg/ml in DMSO
      Anti-HSA-PE antibody (BD clone M1/69)
      Anti-CD23-biotinylated antibody (BD clone B3B4)
      AA4.1-APC (e-bioscience 17-5892)
      Non-indo-labeled cells to use for single stain controls for FCM

1) Rest MACS-enriched B cells on ice in complete RPMI for 1-2hrs
2) Wash cells, resuspend (10×10^6 cells/ml) in warm HBSS (without calcium), and load with indo-1-AM (5μg/ml) for 30 minutes in a humidified 37°C incubator.
3) Wash cells twice with HBSS
4) Suspend cells (20×10^6 cells/ml) in FACS, and place on ice (reserve a small aliquot of indo-labeled cells to serve as the indo-1 single stain control for FCM)
5) Label remaining cells with biotinylated-CD23 (1:200 dilution) for 20 minutes (label single stain control cells as well)
6) Wash cells, and suspend (20×10^6 cells/ml) in FACS
7) Label cells with HSA-PE (1:750 dilution), SAv-PerCP-Cy5.5 (1:200 dilution), and AA4-APC (1:100 dilution) for 20 minutes (label single stain control cells as well)
8) Wash cells, and suspend (1×10^6 cells/ml) in HBSS (with calcium)
9) Keep on ice until assay
10) Warm cells for 15 minutes in a 37°C water bath prior to assay
11) Compensate the flow cytometer with single stain control cells
12) Load sample onto flow cytometer set to measure the ratio of Indo-1-violet/Indo-1-blue
13) Take baseline (non-stimulated) measurement for 30-90 seconds
14) Stimulate cells, and measure calcium ratio for 4-10 minutes

** To monitor calcium efflux from the ER without influx of extracellular calcium, use calcium-free HBSS when acquiring. Calcium can be added back in to monitor calcium influx.