CD19 differentially regulates BCR signalling through the recruitment of PI3K

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Abstract

CD19 is co-stimulatory surface protein expressed exclusively on B cells and serves to reduce the threshold for signalling via the B cell receptor (BCR). Co-ligation of CD19 with the BCR synergistically enhances MAP kinase activity, calcium release and proliferation, We recently found that these parameters were also enhanced in CD19-null primary murine B cells following BCR ligation, suggesting a regulatory role of CD19 in BCR signalling. Here we demonstrate that the enhanced BCR signalling in the absence of CD19 was not dependent on the src kinase Lyn, but linked to phosphoinositide 3-kinase (PI3K) activity. Consistent with this, we detect PI3K is associated with CD19 outside the lipid raft in resting B cells and that pre-ligation of CD19 to restrict its translocation with BCR into lipid rafts attenuated BCR-induced PI3K and MAP kinase activation and subsequent B cell proliferation. Thus we propose that CD19 can modulate BCR signalling in both a positive and negative manner depending on the receptor/ligand interaction *in vivo*.

Introduction

Like many cells in the immune system, B lymphocytes use co-stimulatory molecules to modulate the receptor signalling in response to foreign antigen. The CD19 co-stimulatory molecule has a well documented role in enhancing signalling events emanating from the B cell receptor (BCR). Biochemically, the nine conserved tyrosine residues (1) in the 240-amino acid cytoplasmic region of CD19 become rapidly phosphorylated following BCR ligation to generate functionally active SH2-recognition domains that mediate the recruitment to the plasma membrane of secondary effector molecules such as Vav, phosphoinositide 3-kinase (PI3K), Grb2, and PLC (2), which are important for the BCR-induced intracellular calcium flux and activation of mitogen-activated protein kinases (MAP kinases) (3). Indeed, co-ligation of the BCR and CD19 results in enhanced activation of three forms of MAP kinases, (Erk1/2, JNK and p38) and calcium release compared to BCR ligation alone (3-6). Interestingly, BCR-induced tyrosine phosphorylation of Lyn but not Syk was found to be greatly compromised in CD19-deficient primary B cells while phosphorylation of Fyn was abolished (7), suggesting a reduction in Src family PTK activity in the absence of CD19 (7, 8).

While the amplification function of CD19 on BCR signalling are well established, a negative role for CD19 in BCR signalling is also reported, in which cross-linking of CD19 was found to supress calcium release and/or proliferation following BCR ligation (9-12). Furthermore, transgenic mice overexpressing human CD19 gene have severely impaired development of immature B cells in the bone marrow and dramatically fewer mature B cells in the periphery (13). However, the biochemical mechanisms behind these discrepancies have not been elucidated. Here we defined a novel mechanism of CD19 in regulating BCR-mediated signal transduction. In the absence of CD19, primary murine B cells demonstrated elevated BCR signals in terms of MAP kinase activation, calcium flux and proliferation. These enhanced signalling parameters in the absence of CD19 were dependent on PI3K. Taken together, we proposed that CD19 functions as a molecular sinker, differentially regulating BCR signalling via sequestering PI3K away from lipid raft, depending on different immunological context.

Methods

Mice

The generation and characterization of Lyn-deficient and CD19-deficient mice have been described previously (14). Both mutations have been backcrossed onto the C57BL/6 background for >10 generations. Age and sex matched C57BL/6 mice were used as controls. All animal breeding and experimentation was conducted according to institutional guidelines, and approved by Walter and Eliza Hall Institute Animal Care and Use Committee.

Reagents

Abs used in this study were as follows: monoclonal antibody against phospho-tyrosine (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit serum against Lyn and Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), whereas rabbit anti-phospho-ERK, anti-ERK and anti-phospho-Akt were purchased from Cell Signalling (Beverly, MA). The PI3K specific inhibitor wortmanine was obtained from Calbiochem (San Diego, CA). Rabbit serum against CD19 was produced in this lab by immunising rabbit with peptides corresponding to the cytoplasmic domain of CD19. F(ab')2 goat anti-mouse IgM was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and intact goat anti-mouse IgM was from Southern Biotechnology Associates (Birmingham, AL).

Cell preparation and activation

Single cell suspensions were prepared as described and splenic B cells were enriched by direct purification using MACS magnetic beads directly conjugated to anti-B220 (RA3-6B2), following the manufacturer's directions (Miltenyi Biotec, Bergisch Gladbach, Germany) and using a MACS LS column. B cell purity was determined by flow cytometry to be >98%. Purified B cells (20–30 x 10^6) were resuspended in media without FCS at 37°C before stimulated with F(ab')2 goat anti-mouse IgM at a final concentration of 40 μg/ml for various times and subsequently lysed on ice for 30 min in Triton X-100 buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) or RIPA buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM

Tris, pH 7.5), both in the presence of 1x complete protease inhibitor (Roche Diagnostics, Mannheim, Germany) plus Na_3VO_4 and PMSF. Co-ligation of the BCR with CD19 was performed by staining B cells with biotinylated mAbs specific for Igκ and CD19, either alone or together in the presence of saturating concentrations of 2.4G2. Pre-ligation of CD19 was achieved by staining B cells with biotinylated mAb specific for CD19 in the presence of saturating concentration of 2.4G2 and cross-linked with streptavidin at 37 C for 3 min before stimulated with F(ab')2 goat anti-mouse IgM at indicated concentration for indicated time. For PI3 kinase inhibition, purified B cells were treated with DMSO or wortmainne to a final concentration of 50nM for 20 min at 37°C before BCR stimulation using F(ab')2 anti-IgM at 40 μg/ml.

Lipid raft isolation

100 x 10^6 B cells were lysed in 1 ml of TNEV Buffer (10 mM Tris-HCl, pH7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4), plus 1% Triton X-100 on ice for 30 min. After centrifugation for 10 min at 3000 rpm on a bench top centrifuge at 4 ^oC, the cleared lysates were diluted 1 : 1 with 85% (w/v) sucrose in TNEV. 6 ml of 35% and 3.5 ml of 5% sucrose in TNEV were then in turn overlayed on top of the diluted lysates. Lipid rafts from the cells were floated toward top by ultracentrifuging the sample at 33,500 rpm (200,000 g) for 16 hours at 4 ^oC in SW40Ti rotor. 1 ml fractions from the top to the bottom were collected into eppendorf tubes for analysis of protein composition.

Biochemistry

For immunoprecipitation assays, cell lysates were incubated with the optimal concentration of mAb specific for CD19 or CD22 for 2 h at 4°C, followed by Protein G Sepharose beads (Amersham Phamacia Biotech, Uppsala, Sweden) for an additional hour. The immunoprecipitates were washed with lysis buffer three times and eluted by addition of an equal volume of 2x SDS sample buffer containing 5% 2-ME and heating for 5 min at 95°C. Precipitated proteins or total cell lysates were separated by electrophoresis through either 4–20% gradient or 8% constant gels (Gradipore, Sydney, Australia) and subsequently transferred to nitrocellulose membranes (Amersham Pharmacia Biotech). Blots were blocked with buffer containing 3% BSA at room temperature for 1 h and then

incubated with primary Abs against the proteins of interest overnight at 4°C. After extensive washing, primary Abs were revealed by incubation with HRP-conjugated anti-rabbit serum (Silenus, Hawthorn, Australia) or anti-mouse IgG serum (Southern Biotechnology Associates). To verify equivalent protein loading, blots were stripped and re-probed with appropriate Abs. For quantitation of band intensities, non-saturating exposures were scanned and the density of the band was determined using a Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

Calcium flux

Spleen cells were loaded with indo-1 acetoxymethyl (Molecular Probes, Eugene, OR) as described. After loading, cells were stained with fluorochrome-labeled anti-B220 and biotinylated anti-alone or together with either biotinylated anti-CD22 or biotinylated anti-CD19. Staining was conducted in the presence of saturating concentrations of 2.4G2 to prevent FcR binding. After washing and re-suspension at10^7cells/ml, baseline fluorescence was established using a MoFlo (Cytomation, Fort Collins, CO). BCR ligation alone or co-ligation with either CD22 or CD19 was initiated by addition of avidin to a final concentration of 20 μg/ml. The subsequent calcium flux was followed for the indicated times on B cells identified by their fluorescence.

Cell Proliferation

Splenic B cells were positively sorted by MoFlo (Cytomation, Fort Colins, CO) using B220 as a marker then cultured in 96 well plates at 0.5-1 x 10^5 cells/well and stimulated with of $F(ab')_2$ anti-IgM at indicated concentration for 40 h at 37^0C. [^{3}H] thymidine was added at 1 μCi/well and the culture continued for an additional 6 h. The uptake of radioactivity was measured by gas phase scintillation counter.

Results

BCR signalling is enhanced in the absence of CD19

To elucidate the contribution of CD19 to BCR signal transduction, we investigated the consequence of BCR crosslinking in the complete absence of CD19. Given that proximal signalling events following BCR engagement converge on the activation of Erk MAP kinase (15), we firstly measured the Erk activation in CD19-deficient primary B cells following BCR ligation. In contrast to the synergistic activating effects attributed to CD19 in Erk activity following BCR ligation (4, 14), CD19-deficiency resulted in increased Erk activation compared with wild-type primary B cells stimulated in an identical manner (Fig. 1A). This result was consistent with various degrees of BCR stimulation and also when BCR was ligated for various lengths of time (Fig. 1B). We next examined another important parameter of BCR signalling, calcium flux, in the context of CD19 deficiency. Primary B cells loaded with the Ca^{++} sensitive dye indo-AM were stimulated with $F(ab')_2$ anti-IgM. Consistent with our MAPK data, *cd19*$^{-/-}$ B cells displayed an exaggerated Ca^{++} flux compared to wild-type B cells (Fig. 1C). Collectively, these data suggest B cell hypersensitivity following antigen receptor ligation in the absence of the CD19 co-receptor.

Enhanced proliferation of CD19 deficient B cells

We next determined the biological consequence of the observed biochemical hypersensitivity in the absence of CD19. B lymphocytes undergo robust proliferation following BCR ligation, which can be measured by both [^{3}H] incorporation and cellularity in vitro. Splenic B cells were FACS sorted using the surface antigen CD45R (B220) and then cultured for 2 days in the presence of $F(ab')_2$ anti-IgM at 37^0C. [^{3}H] thymidine was added at the last 6 hour of culture before the measurement of radioactivity uptake was performed by gas phase scintillation counter as index of cell proliferation. Simultaneously, identical cultures were harvested and cellularity was enumerated by flow cytometry. In the absence of CD19, B cells proliferated faster, but also enter the first division earlier (Fig. 2, and data not shown), supporting an inhibiting role for CD19 on B cell proliferation in the context of $F(ab')_2$ anti-IgM stimulation.

CD19 does not require Lyn kinase to modulate BCR signalling

The hyper-activated Erk MAP kinase, enhanced calcium flux and increased proliferation *in vitro* induced by BCR crosslinking in CD19 deficient primary B cells is reminiscent of the phenotype of Lyn-deficient B cells, suggesting that Lyn might be involved in this pathway. Indeed, CD19 is proposed to amplify the role of Lyn (7), thus it is possible that the negative regulatory role of Lyn in Erk is compromised in the absence of CD19, resulting in the elevated Erk MAP kinase. In light of this, we observed no evidence linking Lyn kinase to the hyper-activated Erk MAP kinase in the absence of CD19. Indeed MAP kinase activity was further increased rather than unaltered by the loss of Lyn (Fig. 3A), indicating a Lyn-independent inhibition of Erk by CD19. Similarly, CD19 does not appear to function via Lyn kinase in attenuating BCR-induced calcium flux since the BCR-induced tyrosine phosphorylation of CD22, a substrate of Lyn kinase (Fig.3B) is normal in CD19-deficient primary B cells (Fig. 3C). Thus, Lyn kinase is not the mediator of the observed negative role of CD19 in proximal BCR signalling.

Elevated BCR signalling in the absence of CD19 requires PI3K

In addition to the tyrosine kinase Lyn, the lipid kinase PI3K is also reported to mediate CD19 signalling (4, 16). We next investigated the possibility that CD19 works through PI3K to install its observed inhibitory role in BCR signalling. To this end, we treated primary B cells from both wild-type and *cd19*$^{-/-}$ mice with or without wortmannine, a specific PI3K inhibitor, prior to BCR crosslinking. Co-ligation of CD19 with the BCR induced higher PI3K activity, as determined by pAkt, and higher levels of Erk activation, than BCR stimulation alone (Fig. 4A). This suggests a role for PI3K in CD19 signalling. Interestingly, in the absence of CD19, BCR ligation by itself also led to a similar enhanced PI3K and MAPK activation compared to their wild-type counterparts (Fig. 4A). Furthermore, we observed that the enhanced Erk activation signal in the absence of CD19 is attenuated by wortmannine, as was the case when CD19 was co-ligated with BCR (Fig. 4A), suggesting that PI3K is involved in the signalling hypersensitivities displayed by CD19-deficient B cells. Consistent with the enhanced PI3K/Erk activation, BCR-induced calcium flux was also elevated in CD19-deficient B cells (Fig. 1C, Fig. 4B), and could be

suppressed by PI3K inhibitor wortmannine (Fig. 4B). Together these data support both a positive and negative regulatory role for CD19 in BCR signalling via PI3K activity.

CD19 resides outside lipid raft in resting B cells and constitutively recruits PI3K

The micro-domains referred to as lipid rafts, are proposed to serve as platforms within the plasma membrane for receptor signalling in immune cells. We next investigate the spatial relationship between CD19, BCR and Src kinase in lipid raft in order to gain insight into a mechanistic explanation for the negative role of CD19. Lipid rafts were isolated from resting B cells according to their solubility in non-ionic detergents, as lipid rafts are defined as cholesterol-rich membranes. Following solubilisation the insoluble membranes can be separated from the soluble material based on their buoyant density on sucrose gradients. The presence of lipid raft-specific component GM1 in fraction 4, but absent in fractions 11/12 defines the identities of these membranes (Fig.5A, top panel). In resting B cells, the BCR detected by anti-IgM blot is located outside the lipid raft, separated from the Src kinase Lyn inside the raft. Interestingly, CD19 constitutively resides outside the signalling platform, lipid raft (Fig. 5A, bottom panel), although its co-ligation with BCR translocated the complex into the lipid raft (17). As a surface molecule, the signalling functions of CD19 derive primarily from its cytoplasmic tail in recruiting cytosolic signalling component for activation. To examine the physical evidence of PI3K involvement in the negative CD19 signalling, we immuno-precipitated CD19 from the splenic B cells before and after BCR stimulation and found small amounts of PI3K constitutively bound to CD19 at steady state which was further increased following BCR engagement (Fig. 5B). Given the constitutive sequestration of CD19 from lipid raft in resting B cells (Fig.5A, bottom panel), the PI3K that is bound to CD19 in steady state are spared from involvement in the initial BCR signalling events.

Tethering CD19 attenuates BCR induced Akt/Erk activation and proliferation

The observation that PI3K binds to CD19 constitutively at steady state outside lipid raft suggests that it is possible for CD19 to function as a molecular sinker in isolating signalling competent molecules away from the BCR complex. To verify this hypothesis, we bound surface expressed CD19 with biotinlated antibody to restrict their recruitment

with BCR into the lipid raft by tethering them (pre-ligation) with streptavidin prior to anti-IgM stimulation. Compared with un-ligated samples, CD19 pre-ligation resulted in diminished PI3K/Akt activation and Erk activation following BCR cross-linking (Fig. 6A). To further confirm that CD19 isolates BCR signalling molecule specifically, CD19 pre-ligation was performed with increasing concentration of ligating antibody, and the BCR-induced Erk activation was found to be reduced in a dose dependent manner (Fig. 6B). Finally, B cell proliferation was examined using the same CD19 pre-ligation strategy (Fig. 6C). Collectively, these data suggest that CD19 attracts a critical level of positive signalling molecules such as PI3K to its cytoplasmic tail and these signalling proteins are only activated if CD19 was co-ligated with BCR into lipid raft. In the absence of CD19, these positive signalling molecules were available to bind to additional adaptor proteins that either reside in the lipid raft or are translocated into the lipid rafts in response to BCR cross-linking augmenting signalling.

Discussion

In this study, we present data suggesting that three of the major signalling pathways following BCR ligation are hyper-activated in the absence of CD19, indicating a regulatory role of CD19 in BCR signalling. Similarly to its positive roles, negative regulatory roles of CD19 were not Lyn-, but PI3K- dependent. Given our findings of PI3K constitutively associated with CD19 outside the lipid raft, CD19 appears to function as molecular sinker to sequester positive signalling molecules such as PI3K away from BCR complex in steady state. These findings are attractive in that they offer an explanation for previous results reporting that the loss of CD21 (complement receptor 2) in CD21/CD19/CD81/leu13 complex correlated with autoimmune disease progression in mice (18-20).

The hyper-responsive behaviour of CD19-deficient primary B cells in response to BCR stimulation is somewhat unexpected, since CD19 has been historically regarded as positive modulator of BCR signalling (21). However, the initial report of CD19-deficient mice did detect slight increase in B cell proliferation in response to anti-IgM+IL-4, which also responded to surface immunoglobulin crosslinking by increasing intracellular calcium (22). Likewise, anti-IgM-mediated Akt phosphorylation was shown to accumulate more rapidly in $CD19^{-/-}$ splenic B cells and A20-$CD19^{neg}$ cells (23), which is in contrast to the reduced Akt phosphorylation observed in A20 (16) and splenic B cells in response to anti-Ig β (24). These inconsistencies may have arisen from variations in A20 lines and IgM vs Igβ stimulation of splenic B cells. Nonetheless, the baseline PI3K activity did appear to be ~50% higher in $CD19^{-/-}$ B cells compared to wild-type counterparts and further increased following BCR ligation with anti-IgM (23). In addition, splenic B cells in the absence of CD19 were found to generate elevated or prolonged late phase calcium responses following IgM ligation (7, 23, 25), which is consistent with our results. Interestingly, BCR-induced hyper-activation of Erk MAP kinase in CD19-deficient primary B cells has not been previously reported. To our knowledge, the data presented in this current study is the first evidence for CD19 acting to inhibit Erk activation in the absence of BCR co-ligation.

As a hallmark antigen of the B cell lineage, CD19 is a well-known co-receptor for BCR signalling (21). Upon BCR stimulation, the cytoplasmic tails of CD19 undergo phosphorylation at multiple tyrosine residues to recruit several signalling molecules that further augment intracellular signalling. Amongst these, Lyn and PI3K were two kinases frequently reported to mediate CD19 co-stimulation (4, 8, 12, 16). We have previously shown that the positive functions of CD19 in BCR signalling do not depend on Lyn (14). Here we provided the evidence that the negative effects of CD19 in BCR signalling were also Lyn independent, as the absence of Lyn further increase the hyper-responsiveness of CD19-null splenic B cells. Furtermore, BCR induced phosphorylation of the Lyn target, CD22, is normal in the absence of CD19 (Fig. 3). Instead, we observed that the elevated signalling induced by BCR ligation in the absence of CD19, was PI3K dependent (Fig. 4).

The current model of BCR signalling suggest that following contact of BCR with antigen, kinetic segregation excludes bulky phosphatases from close contact zones, resulting in activation of protein tyrosine kinases and accumulation of phosphorylated BCR through localized cytoskeleton re-organisation (26). CD19 becomes only transiently associated with BCR micro-clusters following stimulation with membrane-bound antigen (27), and the mediators recruited through CD19, such as PI3K, are laterally segregated from those recruited solely through the BCR in the B cell membrane (28). We found that CD19 is constitutively located outside the lipid raft, and associated with PI3K. Therefore, it seems plausible to suggest that CD19 attracts signalling components a molecular sinker, which will be released for BCR signalling only when it is located in BCR micro-clusters or lipid raft to stimulate cytoskeleton re-organization. In the absence of CD19, however, such signalling molecules like PI3K, will bind to other adaptor molecules that are located within BCR micro-cluster during antigen stimulation.

Indeed, other adaptors for PI3K that also contain YxxM motifs to recruit the p85α subunit of PI3K, such as BCAP (29) and TC21 exist (30-31). Interestingly, TC21 is a small GTPase encoded by Rras2, which interacts constitutively with BCR and displayed receptor specific activation properties (31). Therefore, one can speculate that more PI3K

association with these molecules would occur in the absence of CD19 following BCR ligation.

Our current finding of inhibitory roles for CD19 in BCR signalling adds diversity to the signalling functions of this surface molecule. Indeed, immune signalling needs to be plastic to cope with various stimulatory scenarios. Alternative stimulation of such may arise from soluble versus membrane-bound antigens. Our study has significant implication in both physiological and pathological conditions. As a ligand for CD21, circulating complement could tie up or exclude CD19 from BCR complex to attenuate unnecessary signalling. In the case of pathogen invasion, however, complement coated pathogenic antigens co-ligate CD19 with BCR to achieve maximum immune responses. Indeed, the association of signalling molecules with CD19 may "prime" the B cell for activation via the BCR. This "priming" effect of CD19 may occur under conditions where CD19 is recruited into BCR complexes by binding antigens associated with complements or via ligands that are physically associated with antigen such as IgM and stromal cells (32).

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Declaration of Interest

The authors have no financial conflict of interest.

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Figure Legends

Figure 1. Hypersensitivity of CD19-deficient B cells to BCR ligation. Purified splenic B cells from wild-type and CD19 deficient mice were stimulated with F (ab')2 portion of goat anti-IgM either for 5 min at different concentration (A), or for different length of time at 20 μg/ml (B). Total cell lysates were separated by SDS-PAGE, transferred into filters and subjected to anti-phospho-Erk probing. Filters were then subsequently re-probed with anti-actin without stripping for loading controls. (C) Splenic B cells from wild type and $CD19^{-/-}$ mice were loaded with the calcium-sensitive dye indo-1-AM. After establishing baseline fluorescence, BCR stimulation was initiated by addition of F(ab')2 anti-IgM and the analysis of calcium flux was performed using a flow cytometry for the time indicated. All results are representative of 2 -3 independent experiments.

Figure 2. BCR-induced proliferation is enhanced in CD19-deficient primary B cells. Purified splenic B cells from wild-type and CD19-deficient mice were stimulated with F(ab')2 anti-IgM, cultured up to 2 days as indicated before pulsed with [^{3}H] thymidine for additional 6 hours. Incorporation of [^{3}H] as a measure of proliferation is displayed as counts per minute (c.p.m.) for each sample. The histograms represent the average of triplicate wells with s.d. indicated. This result is representative of two independent experiments.

Figure 3. Lyn-independent regulation of BCR signalling in CD19-deficient B cells. (A) Purified splenic B cells from wild-type, CD19-deficient, or Lyn- CD19- double deficient mice were stimulated with F(ab')2 portion of goat anti-IgM at 40 μg/ml for 5 min and lysed. Total cell lysates were processed as in Fig. 1a, and probed with anti-phospho-Erk antibody. Filters were then subsequently re-probed with anti-actin without stripping for loading controls. (B-C) Purified splenic B cells from wild-type, Lyn-deficient-, and CD19-deficient mice were stimulated with F(ab')2 portion of goat anti-IgM for indicated time and lysed. CD22 was immunoprecipitated from the different samples, and western blotted with anti-phosphotyrosine antibody (4G10). The filters were subsequently

stripped and re-probed with anti-CD22. All results are representative of 2 -3 independent experiments.

Figure 4. BCR-induced hypersensitivities in the absence of CD19 are suppressed by PI3K inhibitor. (A) Splenic B cells from both wild-type and CD19-deficient mice were pre-treated with either DMSO or Wortmannin (Wtm) at 50nM before the cells were stained with biolinlated anti-CD19 mAb in the presence of saturated concentration of 2.4G2 to block the Fcγ receptor binding. The cells were then stimulated with F(ab')2 anti IgM for 5 min and whole cell lysates were probed with anti- phospho-Erk and anti- phospho-Akt antibodies before the filter was stripped and re-probed with anti-Erk2 Ab as loading control. The signal intensities of pAkt and pErk in each sample were measured with ImageJ software and plotted into histogram against that of Erk proteins (bottom panel). (B) Splenic B cells from wild type and $CD19^{-/-}$ mice were loaded with the calcium-sensitive dye into-1 and then treated with or without 50nM Wortmannin. After establishing baseline fluorescence, BCR stimulation was initiated by addition of F(ab')2 anti-IgM and the analysis of calcium flux under the flow cytometry continued for the time indicated. All results are representative of 2 - 3 independent experiments.

Figure 5. CD19 resides outside the lipid rafts and constitutively associates with PI3K.
(A). $1x10^8$ B cells were lysed with 1% Triton X 100 buffer, and the solubilized supernatant laid over a sucrose gradient. After centrifugation at high speed, 1 ml fractions from top to bottom of the sucrose gradient were collected, either directly subjected to western blotting with the different antibodies indicated (top three panels), or immunoprecipitated for CD19 (bottom panel). (B) Splenic B cells from wild-type mice were stimulated with $F(ab')_2$ portion of goat anti-IgM for 5 min or not, and lysed with cold 1% Triton X100 lysis buffer on ice for 30 min. After spinning down at top speed at 4 ºC, the supernatant of samples were subjected to CD19 immunoprecipitation, and western blotted sequentially with anti-p85 subunit of PI3K (top panel) or anti-CD19 (bottom panel). All results are representative of 2 independent experiments performed.

Figure 6. Pre-ligation of CD19 attenuates BCR-induced activation in primary B cells. (A) Purified splenic B cells from wild-type mice were stained with biotinlated anti-CD19 mAb at 0.2μg/ml in the presence of saturating concentration of 2.4G2, and tethered with streptavidin at 20μg/ml for 3 min before they were stimulated with F(ab')2 portion of goat anti-IgM at 40μg/ml for 5 min. Whole cells lysates were separated by SDS-PAGE, transferred into filter and probed sequentially with anti-phospho-Akt (upper panel), anti-phospo-Erk (middle panel), and anti-actin (bottom panel) without stripping of the filter. (B) Experiment was performed as in (A), except stained with indicated concentration of anti-CD19, and probed with anti-phospho-Erk (upper panel) and anti-actin (lower panel). The signal intensities of pErk of each sample were measured by ImageJ software and plotted into histogram against that of Actin (left panel). (C) Cells were treated with biotinlated anti-CD19 or anti-B220 before cross-linked and stimulation with $F(ab')_2$ anti-IgM at indicated concentration for 40 h at 37^0C. The proliferation of stimulated splenic B cells was measured as in Fig. 2. All results are representative of 2 -3 independent experiments.