PKC-β controls IκB kinase lipid raft recruitment and activation in response to BCR signaling

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NF-κB signaling is required for the maintenance of normal B lymphocytes, whereas dysregulated NF-κB activation contributes to B cell lymphomas. The events that regulate NF-κB signaling in B lymphocytes are poorly defined. Here, we demonstrate that PKC-β is specifically required for B cell receptor (BCR)-mediated NF-κB activation. B cells from protein kinase C-β (PKC-β)-deficient mice failed to recruit the IκB kinase (IKK) complex into lipid rafts, activate IKK, degrade IκB or up-regulate NF-κB–dependent survival signals. Inhibition of PKC-β promoted cell death in B lymphomas characterized by exaggerated NF-κB activity. Together, these data define an essential role for PKC-β in BCR survival signaling and highlight PKC-β as a key therapeutic target for B-lineage malignancies.

Members of the NF-κB–Rel family of transcription factors are major transcriptional regulators of normal and pathologic immune cell function; they control the genes involved in cell activation and resistance to apoptosis[1]. NF-κB plays a central role in innate immunity: it mediates signals through inflammatory stimuli, such as tumor necrosis factor-α (TNF-α) and interleukin 1 (IL-1), and pattern recognition receptors, such as Toll-like receptors. In the adaptive immune response, NF-κB is a key effector for both B cell receptor (BCR) and T cell receptor (TCR) signaling. Mice that are deficient in specific NF-κB–Rel family members or inhibited with dominant-negative forms of IκB or IκB kinase (IKK) show defects in lymphocyte activation and development[2–6].

In contrast to its requirement in physiologic immune cell function, uncontrolled NF-κB activity is also a major hallmark of several types of lymphoid malignancies[7,8]. For example, exaggerated NF-κB activity and elevated expression of NF-κB–dependent prosurvival genes are observed in the certain types of B cell lymphomas, including a clinically refractory subset (40–50%) of non-Hodgkin's diffuse large B cell lymphomas (DLBCLs)[9–11]. Although the importance of NF-κB

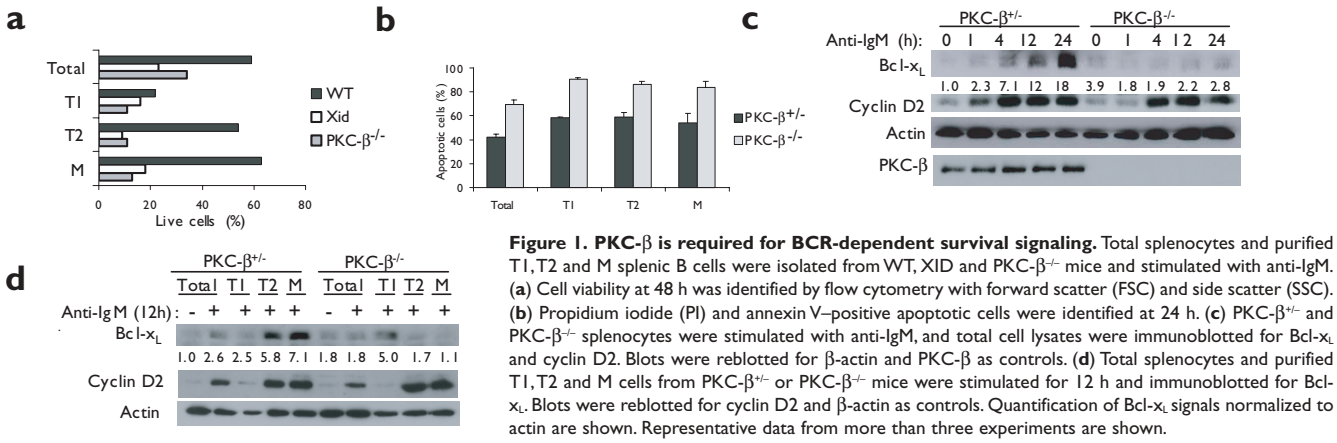

Figure 1. PKC-β is required for BCR-dependent survival signaling. Total splenocytes and purified T1, T2 and M splenic B cells were isolated from WT, XID and PKC-β$^{-/-}$ mice and stimulated with anti-IgM. (**a**) Cell viability at 48 h was identified by flow cytometry with forward scatter (FSC) and side scatter (SSC). (**b**) Propidium iodide (PI) and annexin V–positive apoptotic cells were identified at 24 h. (**c**) PKC-β$^{+/-}$ and PKC-β$^{-/-}$ splenocytes were stimulated with anti-IgM, and total cell lysates were immunoblotted for Bcl-x_L and cyclin D2. Blots were reblotted for β-actin and PKC-β as controls. (**d**) Total splenocytes and purified T1, T2 and M cells from PKC-β$^{+/-}$ or PKC-β$^{-/-}$ mice were stimulated for 12 h and immunoblotted for Bcl-x_L. Blots were reblotted for cyclin D2 and β-actin as controls. Quantification of Bcl-x_L signals normalized to actin are shown. Representative data from more than three experiments are shown.

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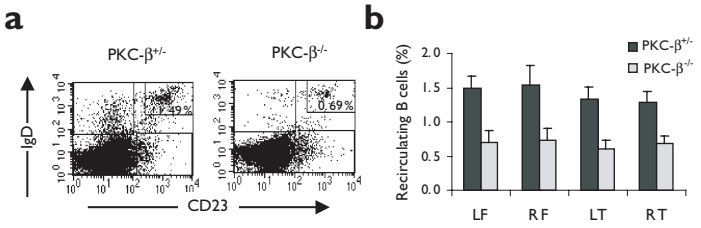

Figure 2. PKC-β$^{-/-}$ mice have a reduced percentage of recirculating BM B cells. (**a**) Representative cell staining of BM cells from a pair of age-matched PKC-β$^{+/-}$ and PKC-β$^{-/-}$ mice with anti-CD23 and anti-IgD. Cells were gated on live cells by FSC and SSC; the percentages of CD23^{+}IgD^{+} cells are indicated. CD23^{+}IgD^{+} cells were >99% B220^{+}. (**b**) The mean ± s.e.m. percentages of CD23^{+}IgD^{+}B220^{+} recirculating BM cells from five pairs of age-matched PKC-β$^{+/-}$ and PKC-β$^{-/-}$ mice are shown. BM cells were isolated from individual leg bones and stained for flow cytometry. Left femur, LF; right femur, RF; left tibia, LT; and right tibia, RT. Representative data from one of two experiments are shown.

in lymphocyte maintenance and activation is evident, the mechanism of antigen receptor–dependent NF-κB activation remains incompletely defined.

Bruton's tyrosine kinase (Btk) is required for BCR-mediated NF-κB survival signaling. Btk-deficient mice show a B cell survival defect and loss of BCR-induced NF-κB activation[12,13]. Upon BCR engagement, Btk functions to regulate sustained calcium signaling and diacylglycerol (DAG)-dependent events[14]. X-linked immunodeficiency (XID) mice, with a mutant *Btk* gene, show B cell developmental defects that include reduced numbers of follicular mature and peritoneal B1 cells and poor responses to T-independent type 2 (TI-2) antigens[15,16].

Mice that are deficient in a single member of the protein kinase C (PKC) superfamily, PKC-β, also show a loss of peritoneal B1 cells and poor TI-2 responses[17]. Despite this B cell phenotype, the specific role played by PKC-β in BCR signaling remains unknown. PKC-β depends on both DAG and intracellular calcium for its activation[18], which suggests that PKC-β may be downstream of Btk activation. Consistent with this view, FcεRI-mediated activation of PKC-β is abolished in Btk-deficient mast cells[19]. The PKC isoform PKC-θ is required for TCR-mediated NF-κB survival signaling in mature T cells, but this isoform is minimally expressed in B cells[20]. Inhibitor studies have implicated another PKC isoform, PKC-δ, in BCR-dependent NF-κB signaling[21]. However, PKC-δ$^{-/-}$ B cells show normal NF-κB survival signaling[22,23]. Together, these findings suggested that the conventional-type PKC member PKC-β might regulate NF-κB activity in B cells.

Here, we directly tested the requirement for PKC-β in BCR-mediated NF-κB survival signaling. Upon BCR stimulation, PKC-β-deficient B cells failed to activate NF-κB or up-regulate Bcl-x_L expression and subsequently died. Both PKC-β and the IKK complex were recruited into lipid raft microdomains upon BCR stimulation and these events were completely dependent upon PKC-β activity. The specific requirement for PKC-β in B lymphocyte NF-κB activation also suggested that PKC-β might be an attractive target for treating mature B cell malignancies, including the refractory subset of non-Hodgkin's lymphomas, which is characterized by either constitutive NF-κB activation or increased PKC-β expression[10,24]. Consistent with this idea, small molecule PKC-β inhibitors were highly effective in treating such cancers *in vitro*.

Results

BCR-induced NF-κB maintenance signal requires PKC-β

Signaling through the BCR is essential for the maintenance of peripheral B cells[25]. In the spleen, B cell development proceeds through the transitional 1 (T1), transitional 2 (T2) and mature follicular (M) B cell stages[26]. Although T1 cells are relatively nonresponsive to BCR stimulation, T2 cells generate proliferative, pro-survival and differentiation signals upon antigen-receptor engagement[27]. Specific pro-survival signals observed in T2 and M cells include BCR-dependent *in vitro* survival and induction of the anti-apoptotic genes encoding Bcl-x_L and A1 (also known as Bfl-1).

We first tested the requirement for PKC-β in BCR-dependent maintenance by evaluating its role in B cell survival. We isolated T1, T2 and M splenic B cells from wild-type (WT), XID or PKC-β-deficient mice by fluorescence activated cell sorting (FACS) using the CD21 and CD24 (also known as HSA) cell surface markers, as previously described[26]. Consistent with published results, BCR engagement promoted cell survival in T2 and M cells from WT but not XID mice (**Fig. 1a**)[27]. Similar to the XID phenotype, PKC-β$^{-/-}$ T2 and M cells showed reduced cell survival compared to WT controls (**Fig. 1a**). These findings correlated directly with increased apoptosis in both unstimulated and stimulated PKC-β$^{-/-}$ splenic B cells (**Fig. 1b**).

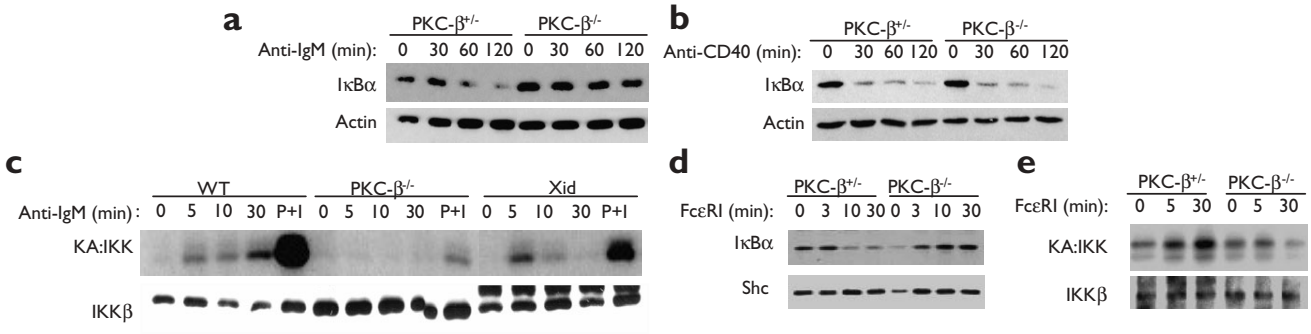

Figure 3. PKC-β is required for BCR- and FcεRI-dependent NF-κB activation. Splenocytes were stimulated with (**a**) anti-IgM or (**b**) anti-CD40 in the presence of cycloheximide (to inhibit protein synthesis). IκBα degradation was assessed by immunoblotting. (**c**) Splenocytes from WT, PKC-β$^{-/-}$ and XID mice were stimulated with anti-IgM or with PMA + ionomycin (P+I) and IKK kinase activity (KA) was assessed. Cell lysates were immunoblotted for IKKβ as a control. (**d,e**) PKC-β$^{+/-}$ and PKC-β$^{-/-}$ BMMCs were stimulated with FcεRI stimulation. (**d**) Cell lysates were immunoblotted for IκBα and reprobed with anti-Shc as a loading control or (**e**) assessed for IKK kinase activity as in Fig. 2c. Representative data from one of three or four experiments are shown.

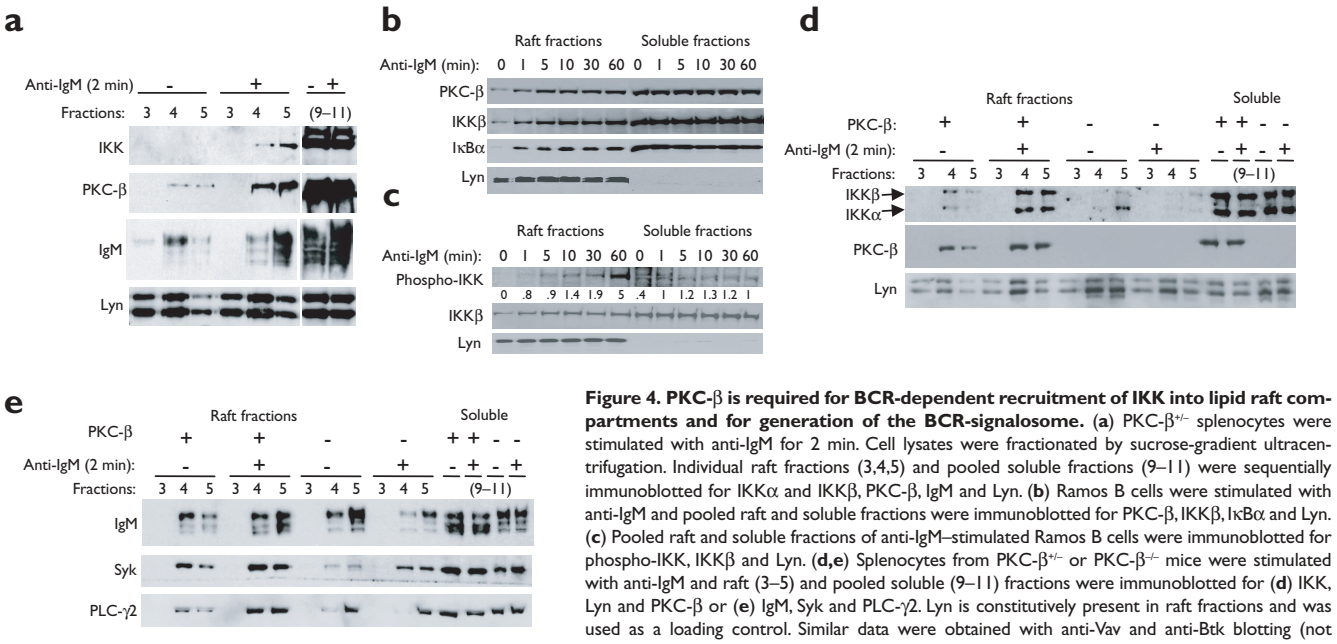

Figure 4. PKC-β is required for BCR-dependent recruitment of IKK into lipid raft compartments and for generation of the BCR-signalosome. (**a**) PKC-β$^{+/-}$ splenocytes were stimulated with anti-IgM for 2 min. Cell lysates were fractionated by sucrose-gradient ultracentrifugation. Individual raft fractions (3,4,5) and pooled soluble fractions (9–11) were sequentially immunoblotted for IKKα and IKKβ, PKC-β, IgM and Lyn. (**b**) Ramos B cells were stimulated with anti-IgM and pooled raft and soluble fractions were immunoblotted for PKC-β, IKKβ, IκBα and Lyn. (**c**) Pooled raft and soluble fractions of anti-IgM–stimulated Ramos B cells were immunoblotted for phospho-IKK, IKKβ and Lyn. (**d,e**) Splenocytes from PKC-β$^{+/-}$ or PKC-β$^{-/-}$ mice were stimulated with anti-IgM and raft (3–5) and pooled soluble (9–11) fractions were immunoblotted for (**d**) IKK, Lyn and PKC-β or (**e**) IgM, Syk and PLC-γ2. Lyn is constitutively present in raft fractions and was used as a loading control. Similar data were obtained with anti-Vav and anti-Btk blotting (not shown). Representative data from one of three experiments are shown.

The XID survival defect has been attributed to an inability to up-regulate the anti-apoptotic protein Bcl-x_L[28,29]. Bcl-x_L induction is reduced in XID cells, whereas transgenic overexpression of Bcl-x_L rescues the XID survival defect[30]. Therefore, we tested whether the survival defect in PKC-β$^{-/-}$ mice could also be attributed to a failure in Bcl-x_L induction. Immunoblotting indicated that Bcl-x_L protein expression was progressively up-regulated after BCR stimulation in unfractionated splenocytes: expression increased 18-fold at 24 h (**Fig. 1c**). In contrast, PKC-β$^{-/-}$ cells failed to completely induce Bcl-x_L expression, showing little or no appreciable increase over basal expression at any time point (**Fig. 1c**) The amount of Bcl-x_L at 24 h in PKC-β$^{-/-}$ splenocytes was one-sixth that observed in PKC-β$^{+/-}$ splenocytes. Consistent with the survival defect observed in PKC-β$^{-/-}$ T2 and M cells, FACS-purified T2 and M cells from PKC-β$^{-/-}$ mice also failed to up-regulate Bcl-x_L expression, yet showed normal cyclin D2 induction in response to BCR activation over 12–36 h (**Fig. 1d** and data not shown). Consistent with the survival defect *in vitro*, PKC-β$^{-/-}$ mice showed slightly reduced numbers of mature, recirculating B220$^+$IgD$^+$CD23$^+$ bone marrow (BM) B cells (**Fig. 2**).

BCR-dependent NF-κB activation requires PKC-β

Because BCR-dependent survival and Bcl-x_L induction are both NF-κB–mediated events[31], we next evaluated the requirement for PKC-β in NF-κB activation. NF-κB is regulated by the inhibitory IκB molecule, which sequesters the NF-κB transcription factor in the cytosol. Upon phosphorylation by the IKK complex, IκB becomes targeted for ubiquitination and is subsequently degraded[32].

We first tested for NF-κB activation by evaluating BCR-dependent IκB degradation. Upon BCR stimulation of PKC-β$^{+/-}$ splenocytes, the amount of IκBα protein progressively decreased after 60 and 120 min (**Fig. 3a**). In contrast, BCR stimulation of PKC-β$^{-/-}$ cells resulted in little or no detectable degradation of IκBα. In contrast to its requirement in BCR signaling, PKC-β was not required for CD40-mediated IκB degradation (**Fig. 3b**).

Because degradation of IκB is dependent on phosphorylation by the IKK complex, we next tested whether BCR-mediated IKK activation was reduced in PKC-β$^{-/-}$ mice. Using an IKK *in vitro* kinase assay, we observed that BCR stimulation of WT splenocytes lead to early (5 min) and sustained (30 min) IKK activation (**Fig. 3c**). In contrast, engagement of PKC-β$^{-/-}$ splenocytes resulted in no detectable IKK activity (**Fig. 3c**). In addition, treatment with the DAG analog phorbol 12-myristate 13-acetate (PMA) + the calcium ionophore ionomycin did not rescue IKK activation in PKC-β$^{-/-}$ cells, which was consistent with PKC-β acting downstream of DAG and calcium signaling.

In agreement with published studies[12,13], BCR-dependent IKK activation at 30 min was defective in XID splenocytes (**Fig. 3c**). But, in contrast to PKC-β$^{-/-}$ cells, IKK activation in XID splenocytes was rescued by PMA + ionomycin treatment. However, despite the absence of sustained IKK activity, early (5-min) IKK activation was intact in XID cells. This finding was consistent with the established role for Btk in sustained DAG and calcium signaling and the intact early calcium signal in Btk-deficient human B cells[33]. These data suggested that PKC-β is essential for BCR-mediated NF-κB activation and that Btk regulates NF-κB *via* sustained activation of PKC-β.

FcεRI-induced NF-κB activation requires PKC-β

Another PKC isoform, PKC-θ, is required for TCR-dependent NF-κB activity[20]. PKC-θ is virtually undetectable in B lymphocytes, which raises the possibility that PKC-β may be required for activating NF-κB only in cells lacking PKC-θ[34]. To test this possibility, we evaluated NF-κB activation in BM-derived mast cells (BMMCs) that express both PKC-β and PKC-θ. Upon FcεRI activation, control PKC-β$^{+/-}$ BMMCs rapidly degraded IκB. In contrast, PKC-β$^{-/-}$ BMMCs showed a clear defect in IκB degradation, with minimal changes to IκB protein content after receptor engagement (**Fig. 3d**). Stimulation of PKC-β$^{+/-}$ BMMCs over a 30 min time-course progressively activated IKK, whereas PKC-β$^{-/-}$ cells failed to do so (**Fig. 3e**). Thus, despite the presence of PKC-θ in mast cells, PKC-β plays a nonredundant role in controlling FcεRI-mediated NF-κB activation.

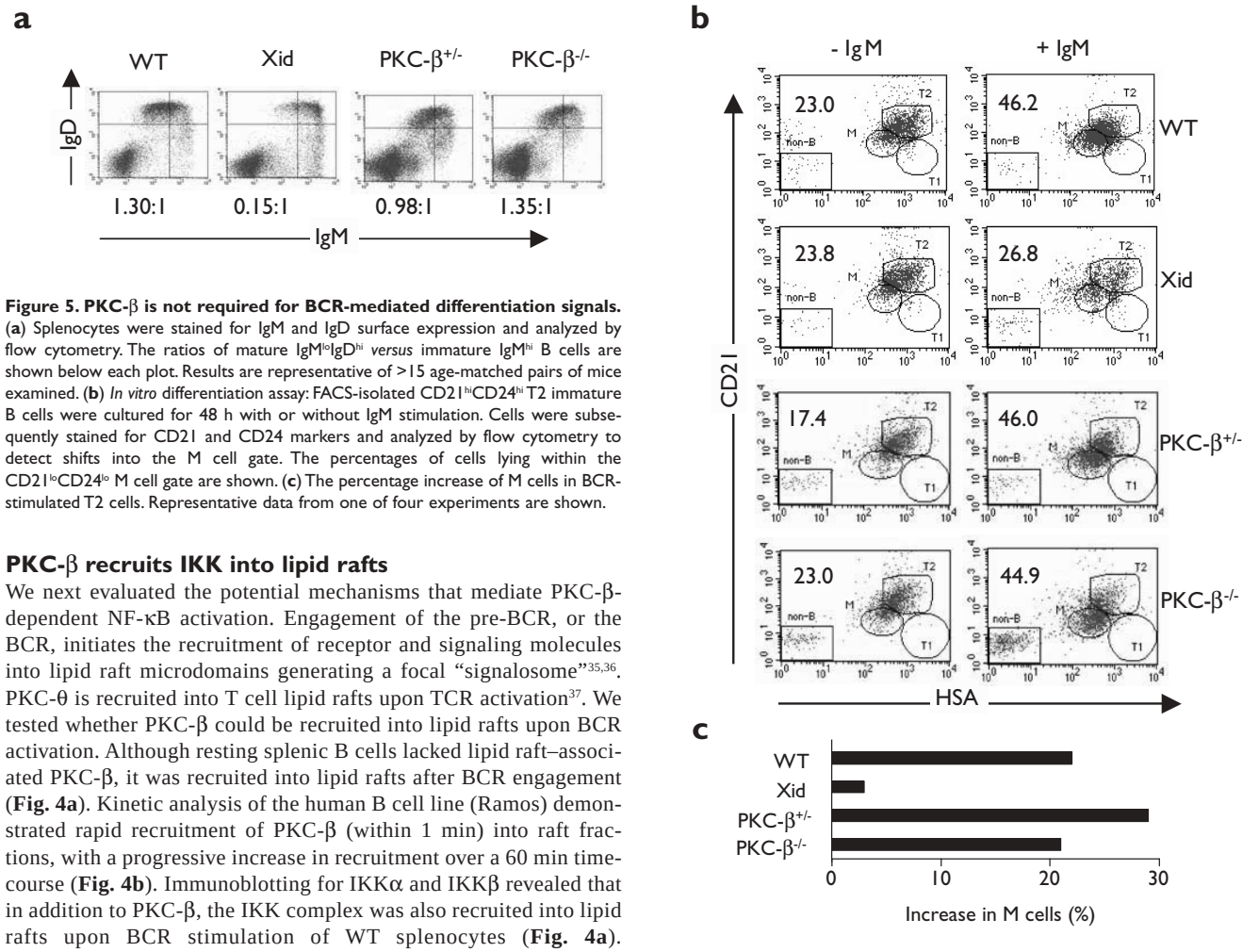

Figure 5. PKC-β is not required for BCR-mediated differentiation signals. (**a**) Splenocytes were stained for IgM and IgD surface expression and analyzed by flow cytometry. The ratios of mature IgM^{lo}IgD^{hi} *versus* immature IgM^{hi} B cells are shown below each plot. Results are representative of >15 age-matched pairs of mice examined. (**b**) *In vitro* differentiation assay: FACS-isolated CD21^{hi}CD24^{hi} T2 immature B cells were cultured for 48 h with or without IgM stimulation. Cells were subsequently stained for CD21 and CD24 markers and analyzed by flow cytometry to detect shifts into the M cell gate. The percentages of cells lying within the CD21^{lo}CD24^{lo} M cell gate are shown. (**c**) The percentage increase of M cells in BCR-stimulated T2 cells. Representative data from one of four experiments are shown.

PKC-β recruits IKK into lipid rafts

We next evaluated the potential mechanisms that mediate PKC-β-dependent NF-κB activation. Engagement of the pre-BCR, or the BCR, initiates the recruitment of receptor and signaling molecules into lipid raft microdomains generating a focal "signalosome"[35,36]. PKC-θ is recruited into T cell lipid rafts upon TCR activation[37]. We tested whether PKC-β could be recruited into lipid rafts upon BCR activation. Although resting splenic B cells lacked lipid raft–associated PKC-β, it was recruited into lipid rafts after BCR engagement (**Fig. 4a**). Kinetic analysis of the human B cell line (Ramos) demonstrated rapid recruitment of PKC-β (within 1 min) into raft fractions, with a progressive increase in recruitment over a 60 min time-course (**Fig. 4b**). Immunoblotting for IKKα and IKKβ revealed that in addition to PKC-β, the IKK complex was also recruited into lipid rafts upon BCR stimulation of WT splenocytes (**Fig. 4a**). Recruitment of IKK into raft fractions was detectable within 1 min and increased gradually over 60 min after BCR activation in both primary B cells and human B cell lines (**Fig. 4b** and data not shown). Recruitment of PKC-β and IKK into lipid rafts coincided with the presence of the BCR, immunoglobulin M (IgM) and thus these proteins are likely key components of the raft-associated BCR signalosome (**Fig. 4a**).

We asked whether lipid raft recruitment led to IKK activation. We evaluated the relative amount of activated IKK in raft *versus* nonraft fractions in BCR-stimulated Ramos B cells using an antibody that specifically recognizes phosphorylated activation loop serine residues on activated IKK: phospho-ser^{180} on IKKα and phospho-ser^{181} on IKKβ[1] (**Fig. 4c**). We observed a progressive increase in the content of activated IKK only in raft fractions. At 60 min, the activated IKK content was fivefold higher in the raft fractions compared to soluble fractions. The peak in IKK phosphorylation (60 min) was consistent with the time for peak IKK kinase activity observed in primary B cells (30–60 min) (**Fig. 4c** and data not shown). The major IKK substrate, IκBα, was also coordinately recruited into the lipid raft signalosome upon BCR engagement (**Fig. 4c**) Thus, raft-associated IKK was enzymatically active and likely positioned so that it was in direct proximity with its substrate, IκBα. Phosphorylation of this substrate led to its subsequent degradation and the deinhibition of NF-κB.

We directly tested whether PKC-β was required for IKK recruitment into lipid rafts. BCR stimulation of PKC-β$^{+/-}$ splenocytes led to IKKα and IKKβ accumulation in lipid raft fractions (**Fig. 4a,d**). In contrast, BCR engagement in PKC-β$^{-/-}$ cells led to little or no detectable increase in IKKα and IKKβ proteins in raft fractions (**Fig. 4d**). To determine whether loss of PKC-β expression lead to a more general defect in signalosome formation, we also evaluated the recruitment of additional BCR signaling molecules. The recruitment of the BCR, Syk, phospholipase C-γ2 (PLC-γ2) and other proximal signaling molecules were all reduced in PKC-β$^{-/-}$ B cells (**Fig. 4e** and data not shown).

PKC-β activity is specific to BCR-mediated maintenance

Btk, like PKC-β, is associated with lipid rafts upon receptor stimulation[35], consistent with a common requirement in NF-κB activation. Despite similarities with respect to NF-κB survival signaling, PKC-β$^{-/-}$ and XID mice show a marked difference in the generation of mature naïve splenic B cells. Staining for surface IgM and IgD expression demonstrated that the mature (IgM^{lo}IgD^{hi}) B cell pool was severely reduced in XID mice, but intact in PKC-β$^{-/-}$ animals (**Fig. 5a**); this was in agreement with published reports[15,17]. The intact mature B cell pool in PKC-β$^{-/-}$ mice, despite their defect in NF-κB activation, suggested that additional pathways that activate NF-κB (for example, CD40) are active in these mice.

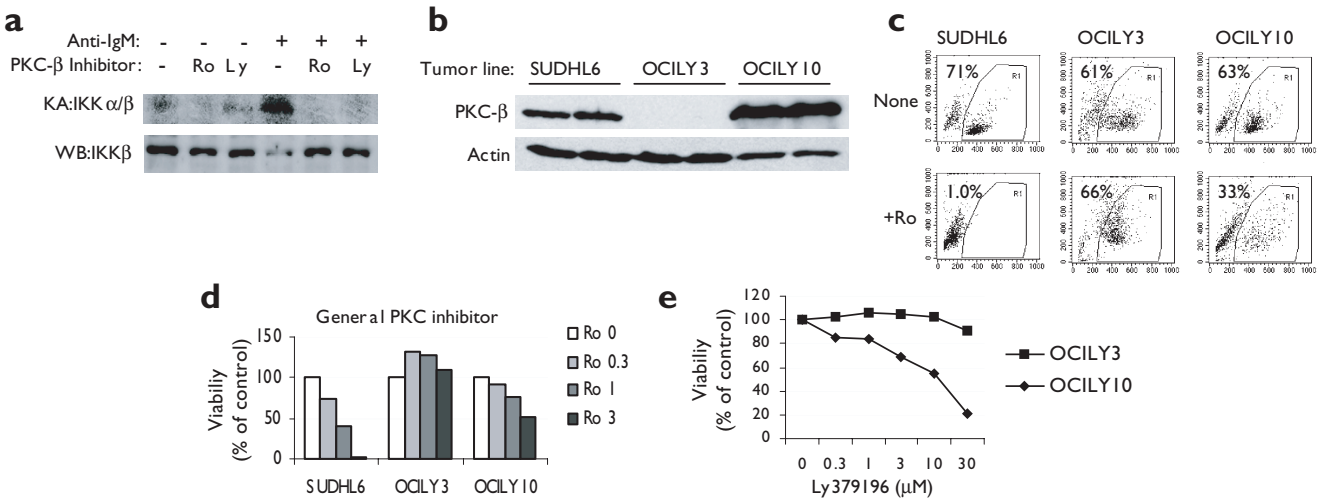

Figure 6. PKC-β inhibition leads to cell death in PKC-β–expressing DLBCL lines. (**a**) WT splenocytes were stimulated with anti-IgM for 10 min, with a 5 min preincubation with the general PKC inhibitor Ro318425 (Ro) or the PKC-β–specific inhibitor Ly379196 (Ly) (both at 5 μM). IKK kinase activity was assessed. Cell lysates were immunoblotted for IKKβ as a control. (**b**) Total cell lysates from three different DLBCL lines were immunoblotted for PKC-β expression. Blots were reprobed for actin as a loading control. Duplicate samples for each cell line are shown. (**c**) Three DLBCL lines were cultured (5×10^5/ml in 200 μl) with various doses of Ro318425. After 48 h, cells were analyzed by flow cytometry. Scatter profiles are shown for the untreated and 3 μM Ro318425-treated samples with the percentage of viable-gated cells indicated. (**d**) The dose-response profile is shown: viability is indicated as a percentage of the respective control (0 μM Ro318425) cells. (**e**) Dose-response profiles of two activated B cell–type DLBCL lines to increasing doses of Ly379196 (a PKC-β–specific inhibitor) are shown as in **d**. Representative data from one of three experiments are shown.

It is unclear whether the loss of mature B cells in XID mice is due to a defect in differentiation from immature to mature B cells or merely a defect in mature B cell survival[38,39]. An *in vitro* differentiation assay to demonstrate that BCR engagement, but not general cellular activation, drives T2 immature B cells to differentiate into the mature B cell pool[27]. Using this assay, we examined the requirement for PKC-β and Btk in BCR-dependent T2 B cell differentiation. Although BCR stimulation of WT T2 cells led to differentiation into the M cell pool, XID T2 cells failed to do so (**Fig. 5b,c**). In contrast, both PKC-$\beta^{+/-}$ and PKC-$\beta^{-/-}$ T2 cells clearly differentiated into M cells. These results suggested that whereas Btk is required for a BCR-mediated differentiation signal in these cells, PKC-β is not. Consistent with these findings and in agreement with published reports[27,28,40], BCR stimulated XID splenocytes, T2 and M cells failed to up-regulate both the cell cycle regulator cyclin D2 and the anti-apoptotic protein Bcl-x_L (data not shown). PKC-β–deficient cells also failed to up-regulate Bcl-x_L but, in contrast to XID cells, showed normal cyclin D2 expression (**Fig, 1c,d**).

PKC-β inhibitors block DLBCL cell survival

The specific role for PKC-β in regulating B cell survival suggested that it might provide an attractive target in the treatment of non-Hodgkin's B cell lymphoma. We therefore tested whether small molecule inhibitors of PKC-β could block the survival of non-Hodgkin's DLBCL tumor cell lines[10] (**Fig. 6**). Addition of either a general PKC inhibitor (Ro318425) or a PKC-β–specific inhibitor (Ly379196) inhibited BCR-dependent IKK activation, demonstrating their functional activity in blocking the PKC-β–dependent NF-κB pathway (**Fig. 6a**). Addition of either of these PKC-β inhibitors to DLBCL tumor lines for 48 h led to a dose-dependent reduction in cell viability of the SUDHL6 and OCILY10 lines (**Fig. 6c–e**). OCILY10 cells expressed higher amounts of PKC-β than SUDHL6 cells and required a higher dose of inhibitor to cause cell death (**Fig. 6b–d**). Thus, the amount of cellular PKC-β was directly correlated with the dose of PKC inhibitor required to mediate cell death.

PKC-β expression was undetectable in the OCILY3 tumor line (**Fig. 6b**) and neither PKC-β inhibitor caused cell death in OCILY3 cells (**Fig. 6c–e**). Thus, whereas an NF-κB–dependent survival signal contributes to the tumorigenicity of OCILY3 cells[10], NF-κB activation in this line occurs independently of PKC-β. Evaluation of five additional B cell lymphoma lines, two of which were PKC-β–deficient, similarly demonstrated the efficacy of PKC-β inhibitors in killing PKC-β–expressing lines (**Fig. 7**). These findings demonstrated the relative specificity of PKC-β inhibitors and their capacity to trigger death only in those cells that expressed PKC-β.

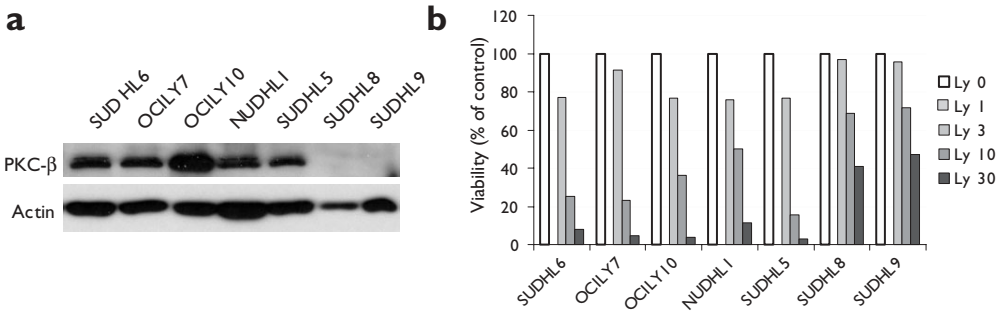

Figure 7. PKC-β expression profile and response to PKC-β inhibition in a panel of DLBCL lines. (**a**) Total cell lysates from seven B cell lymphoma lines were immunoblotted for PKC-β expression. Blots were reprobed for actin as a loading control. (**b**) Dose-response profile of B cell lymphoma lines to increasing doses of the PKC-β–specific inhibitor Ly379196 (in μM). The dose-response profile is shown with viability indicated as a percentage of the respective control (0 μM Ly379196) cells.

Discussion

We have identified here an essential role for PKC-β in BCR-dependent NF-κB survival signaling. Mice deficient in PKC-β showed a survival defect in response to BCR stimulation that correlated with an inability to induce the NF-κB–dependent anti-apoptotic protein Bcl-x_L. Consistent with this *in vitro* defect, PKC-$\beta^{-/-}$ mice also showed reduced numbers of mature recirculating BM B cells. PKC-$\beta^{-/-}$ cells failed to degrade IκB or to activate the IKK complex in response to BCR engagement, indicating that PKC-β controls the NF-κB pathway upstream of IKK activation. The role played by PKC-β in B lymphocytes is similar to the specific roles played by PKC-θ and PKC-ζ in TCR- and TNF-α–mediated NF-κB activation, respectively[20,41]. Thus, rather than having overlapping functions, specific PKC isoforms appear to regulate NF-κB in specific receptor pathways and cell types.

How PKC-β and PKC-θ regulate immunoreceptor-dependent IKK activity is not fully understood. Our work suggests that lipid raft microdomains likely play a key role in regulating IKK activity. Both PKC-β and IKK are recruited to lipid rafts upon BCR stimulation. The majority of activated IKK is localized to raft fractions and these fractions also contain the IKK substrate IκBα. Our data also show that PKC-β is required for proper formation of the raft-associated BCR signalosome and for the recruitment of IKK into this complex. Despite the reduced recruitment of PLC-γ2 and other proximal signaling molecules involved in the calcium signal, PKC-$\beta^{-/-}$ B cells show little difference in overall tyrosine phosphorylation and have intact BCR-dependent calcium flux (data not shown). This observation together with the normal cyclin D2 response and the intact follicular mature B cell pool in PKC-$\beta^{-/-}$ mice suggests that the requirement for PKC-β in NF-κB survival signaling is more stringent than for other BCR-dependent signals. The progressive recruitment and activation of IKK that we observed are also consistent with a requirement for PKC-β in sustained signalosome formation. Our data suggest that IKK recruitment into lipid rafts may be a generalized mechanism of immunoreceptor-dependent NF-κB regulation. Consistent with this view, both PKC-θ and IKK are recruited into lipid rafts upon CD3-CD28 coligation in T cells[37,42]. Our findings predict that IKK recruitment into T cell lipid rafts would similarly be abrogated in PKC-θ–deficient cells. Assessment of IKK raft recruitment in other receptor signaling pathways may facilitate a more complete understanding of NF-κB activation in a range of receptor and cell types.

A major goal of the NF-κB field remains the identification of the protein kinase(s) and/or molecular adapter(s) that directly activate the IKK complex. Our results highlight the importance of lipid raft microdomains in IKK activation and suggest that the direct activators of the IKK complex would likely be found within this specialized membrane location. Thus, a shift in focus towards lipid raft microdomains may greatly facilitate the identification of such IKK-activating kinases.

Despite the critical role for PKC-β in BCR signaling, its role appears to be relatively restricted to the NF-κB pathway. In contrast to XID mice that show a severe loss of mature splenic B cells, PKC-$\beta^{-/-}$ mice have an intact mature follicular B cell pool. Consistent with these observations, we observed distinct differences in the requirements for Btk or PKC-β in BCR-dependent gene expression and differentiation of T2 cells. Together, these observations begin to differentiate distinct pathways for BCR-dependent maintenance *versus* maturation. A Btk-PKC-β-NF-κB–dependent pathway regulates BCR-mediated survival. In contrast, a Btk-dependent, PKC-β–independent pathway (or pathways) controls BCR-induced differentiation.

Our findings help to link recent observations documenting exaggerated NF-κB activity and increased PKC-β expression in activated B cell–type and clinically refractory DLBCLs, respectively[10,24]. Our data suggest that a major feature of these lymphomas may be dysregulated activation of PKC-β–dependent NF-κB survival signaling and that PKC-β may be an attractive drug target for such disorders. Consistent with this idea, PKC-β small molecule inhibitors were effective in promoting cell death in non-Hodgkin's B lymphoma lines. This effect was similar to that obtained by blocking NF-κB activity at the level of IκB or IKKβ in identical lines to those used here[10]. Because the ubiquitous expression of NF-κB, IκB and the IKK complex pose major constraints with regard to the design of therapeutic agents, cell type–specific regulators of IKK and the NF-κB survival pathway, including PKC-β, may represent more optimal drug targets.

It remains unclear whether dysregulated NF-κB activity drives the tumorigenicity in refractory DLBCLs or whether the failure to respond to therapy is secondary to chemotherapy-induced NF-κB activation. Many standard chemotherapy agents, including vinca alkaloids and anthracyclines, activate both NF-κB and PKC[43]. Chemotherapy-induced NF-κB activity can be blocked by PKC inhibition, which suggests that a PKC-dependent NF-κB activation pathway drives chemoresistance[43,44]. In either case, our data suggest that a combination of standard chemotherapy with drugs targeting the PKC-β–dependent NF-κB survival pathway may improve the clinical outcome of selected DLBCL patients.

Based upon the B lineage–restricted phenotype of PKC-$\beta^{-/-}$ mice, the *in vivo* toxicity of PKC-β–specific inhibitors is anticipated to be relatively limited. Our results suggest that PKC-β inhibitors minimally affect cells that lack PKC-β expression. In addition, preclinical trials evaluating the oral PKC-β–specific inhibitor Ly379196, which we used here, have identified little or no toxicity *in vivo* at therapeutic doses[45,46]. The growing data on cell-specific use of PKC family members suggests that, in addition to PKC-β inhibition in B cells, inhibition of alternative PKC isoforms might also be effective in treating a range of disorders characterized by dysregulated NF-κB survival signaling.

Methods

Mouse strains. BALB/c (WT), BALB/xid (XID), PKC-$\beta^{+/-}$ and PKC-$\beta^{-/-}$ mice (on a129 background) were bred and maintained in the animal facilities of the MacDonald Research Laboratories and handled according to guidelines of the UCLA and the University of Washington Animal Research Committees. PKC-$\beta^{+/-}$ and PKC-$\beta^{-/-}$ mice were genotyped by Southern blotting, as described[17], and PKC-β protein expression was confirmed by immunoblotting. Mice used in all experiments were 6–12 weeks old.

Cells and reagents. Single-cell suspensions were prepared from splenocytes depleted of erythrocytes by lysis with ammonium chloride solution. Murine splenocytes were cultured in RPMI with 5% fetal calf serum (FCS) plus supplement (glutamine, 2-mercaptoethanol, penicillin and streptomycin in 10 mM HEPES). The SUDHL6, OCILY3, OCILY10, NUDHL1, SUDHL5, OCILY7, SUDHL8 and SUDHL9 B cell tumor cell lines were passaged as described[47,48]. The Ramos human B cell line was passaged in RPMI with 10% FCS plus supplement. Cells were stimulated at 37 °C with 10 μg/ml of polyclonal goat F(ab′)2 fragment anti–mouse IgM or anti–human IgM (Jackson Laboratory, Bar Harbor, ME), 10 μg/ml of anti–mouse CD40 (a gift of E. Clark) or 1 μg/ml each of PMA + ionomycin (CalBiochem, La Jolla, CA). The general PKC inhibitor (Ro318425) and the PKC-β–specific inhibitor (Ly379196) were used as described[19].

BMMC cultures. BMMCs were generated from PKC-$\beta^{+/-}$ and PKC-$\beta^{-/-}$ mice as described[19]. Mast cells were sensitized overnight with 1 μg/ml of monoclonal anti–DNP IgE and then stimulated with antigen—DNP–human serum albumin conjugates (100 ng/ml)—for various times.

Flow cytometry. For cell-surface staining, 5×10^5 cells per sample were incubated with various antibodies. Data was collected on a FACSCalibur flow cytometer and analyzed with CELLQuest software (both from BD Biosciences, Mountain View, CA). Anti-CD21 (7G6), anti-CD24 (M1/69), and anti-IgM (R6-60.2) were from BD PharMingen (San Diego, CA) and anti-IgD (11-26) was from Southern Biotechnology Associates (Birmingham, AL). For cell sorting, 5×10^7 cells per sample were incubated in 500 μl of staining media (RPMI with

2.5% FCS plus supplement) with various antibodies. Cells were sorted on a FACSVantage cell sorter (BD Biosciences) into 1 ml of collection media (RPMI with 20% FCS plus supplement). Post-sort analysis revealed sort purities of 82–86%. The *in vitro* T2 cell differentiation assay was done as described[27].

Immunoblotting. Total cell lysates were prepared by boiling in SDS-containing sample buffer for 10 min. Samples were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was done with standard procedures. Immunoblotting antibodies used included: anti–cyclin D2 (M-20), anti–Bcl-x_L (S-18), anti–IKKα-IKKβ (H-470), anti-IκBα (C-21), anti-Shc (H-108), anti–PLC-γ2, anti-Syk, anti-Lyn (H-6) and anti–PKC-β2 (C-18) (all from Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti–mouse IgM (The Jackson Laboratory); anti–phospho-ser^{180}–IKKα-ser^{181}–IKKβ (Cell Signaling Technologies, Beverly, MA); and anti-actin (A-2066; Sigma-Aldrich, St. Louis, MO).

IKK assay. Cells were solubilized in lysis buffer and cleared at 14,000 rpm for 15 min. Cell extracts were subjected to immunoprecipitation with anti–IKKα-IKKβ (Santa Cruz Biotechnology) and protein A Sepharose beads for 2 h at 4 °C with rocking. Immunocomplexes were washed twice in lysis buffer, followed by one wash in kinase buffer (20 mM HEPES at pH 7.5, 20 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 mM p-nitrophenyl phosphate, 50 mM NaCl, 1 mM Na_3VO_4, 1 mM dithiothreitol and protease inhibitors). The immunocomplexes were resuspended in 30 μl of kinase buffer containing 10 μM ATP, 1.0 μCi [^{32}P]ATP and 1 μg of glutathione S-transferase–IκB substrate. The reaction was left for 30 min at 30 °C and terminated by the addition of 4× SDS sample buffer. The samples were then resolved by 10% SDS-PAGE and visualized by autoradiography.

Sucrose-gradient raft fractionation. Sucrose-gradient raft fractions were prepared as described[35]. In brief, splenocytes were stimulated with anti-IgM (10 μg/ml) at 37 °C for 2 min and lysed in 1 ml of ice-cold MBS (25 mM MES at pH 6.5, 150 mM NaCl at pH 6.5, 0.5% Triton X-100, 1 mM Na_3VO_4 and protease inhibitors). Lysates were mixed with an equal volume of 85% sucrose (w/v) in MBS and overlaid with 6 ml of 35% sucrose and 3 ml of 5% sucrose in MBS. Ultracentrifugation was done at 200,000*g* for 16 h at 4 °C and 1 ml fractions were collected. All fractions (1–11) were immunoblotted for Lyn and GM1 to confirm the presence of lipid rafts in fractions 3–5.

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Competing interests statement

The authors declare that they have no competing financial interests.

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