Inositol polyphosphates promote T cell-independent humoral immunity via the regulation of Bruton's tyrosine kinase

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T cell-independent (TI) B cell response is critical for the early protection against pathogen invasion. The regulation and activation of Bruton's tyrosine kinase (Btk) is known as a pivotal step of B cell antigen receptor (BCR) signaling in TI humoral immunity, as observed in patients with X-linked agammaglobulinemia (XLA) experiencing a high incidence of encapsulated bacterial infections. However, key questions remain as to whether a well-established canonical BCR signaling pathway is sufficient to regulate the activity of Btk. Here, we find that inositol hexakisphosphate ($InsP_6$) acts as a physiological regulator of Btk in BCR signaling. Absence of higher order inositol phosphates (InsPs), inositol polyphosphates, leads to an inability to mount immune response against TI antigens. Interestingly, the significance of $InsP_6$-mediated Btk regulation is more prominent in IgM^+ plasma cells. Hence, the present study identifies higher order InsPs as principal components of B cell activation upon TI antigen stimulation and presents a mechanism for InsP-mediated regulation of the BCR signaling.

T cell-independent immune response | B cell antigen receptor | Bruton's tyrosine kinase | inositol phosphate | inositol polyphosphate multikinase

In mammals, innate and adaptive immune systems together contribute to host defense. The rapidity of innate immunity and the specificity of adaptive immunity are critical for the protective immune response against invasive pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) is of importance in activating innate immunity. Pathogen-derived antigens can also activate adaptive immunity to elicit humoral immune response (1). Bacterial polysaccharide and repetitive epitope of viral particle mediate extensive cross-linking of antigen receptors and thereby activate B cells in a T cell-independent (TI) manner, resulting in rapid and intense antibody response against invading microorganisms (2). Therefore, TI B cell response is critical for the early protection against pathogen invasion.

Signal transduction via B cell antigen receptor (BCR) is essential for TI immune response (2). Cross-linking of BCR by multivalent antigens induces tyrosine phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) by the Src family kinases such as Lyn, thus, activating Syk. This leads to the assembly of the signaling complex into ITAM, comprising Vav, BLNK, phosphoinositide 3-kinase (PI3K), Bruton's tyrosine kinase (Btk), and phospholipase C-γ2 (PLCγ2). The activity of the signaling complex triggers cellular processes critical to initiate immune responses, including the regulation of gene expression, cytoskeleton reorganization, and B cell proliferation and differentiation.

Rapid B cell response to TI antigens is particularly important to prevent overwhelming tissue damage caused by the infection of fast-replicating bacteria. TI immune response is known to depend on functional Btk (3–6), which is a critical component of the BCR signaling cascade. High incidence of encapsulated bacterial infections was observed in a patient with X-linked agammaglobulinemia (XLA) (7–9), which was caused by loss-of-function mutations in the gene encoding Btk. Some mutations in XLA have been identified within the pleckstrin homology (PH) domain of Btk (10, 11), which is required for the association with $PtdIP_3$ in membrane and the subsequent activation (3, 12). However, it has been reported that the activity of Btk was still observed in $PtdIP_3$-deficient B cells (13, 14), suggesting that the activation of Btk is not entirely dependent on its ability to bind to $PtdIP_3$. Nevertheless, it has been poorly understood whether there is an additional mechanism for regulating Btk activity through the PH domain.

Inositol phosphates (InsPs) are known to be generated by antigen stimulation (15, 16). It is well-established that InsPs, such as $InsP_3$ (17) and $InsP_4$ (16, 18–20), act as signaling messengers in the activation of immune cells. However, little is understood about the roles of higher order InsPs in immune responses, although they can act as a structural cofactor or an allosteric regulator that modulates the stability and function of protein complex (21–23). Recently, it has been reported that a mixture of $InsP_6$ and Btk in solution might enable transient dimerization and transautophosphorylation of Btk (21). While it suggests a possibility that the higher order InsPs might be involved

Significance

T cell-independent (TI) immune response is crucial in generation of intense and rapid humoral immunity for defense against microbial infection. Bruton's tyrosine kinase (Btk) is critical for the TI immune response, and signaling components controlling its activity during B cell receptor (BCR) signaling is known. However, some mutant forms of Btk observed in X-linked agammaglobulinemia (XLA) patients suggest that there may be other mechanisms tightly regulating its activity, particularly in TI immune response. Here, we show that inositol polyphosphates are key metabolites modulating the activity of Btk in BCR signaling. Our results provide insights on the role of inositol polyphosphates in B cell immunity.

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in modulating the activity of BCR signaling via regulation of Btk activity, the physiological relevance remains to be elucidated.

Higher order InsPs, such as $InsP_5$ and $InsP_6$, are converted from $InsP_3$ through successive phosphorylation by specific inositol phosphate kinases (24). Among them, inositol polyphosphate multikinase (IPMK) is the only enzyme responsible for generating $Ins(1,3,4,5,6)P_5$ from $InsP_4$ (25, 26). In the present study, we utilized B cell-specific IPMK-deficient mice to investigate the role of higher order InsPs in B cell immunity, as IPMK is essential for the biosynthesis of higher order InsPs. The results showed that higher order InsPs play a critical role during TI immune response by modulating the Btk activity. This suggests that higher order InsPs are an important regulator of B cell immunity.

Results

IPMK cKO Mice Display No Significant Defects in B Cell Development. To gain insight on the physiological role of higher order InsPs in B cell immunity, IPMK-floxed mice, in which one allele was floxed and the other was deleted, were crossed with CD19-Cre mice to ablate IPMK, specifically in B cells. To confirm IPMK deletion in B cells, protein levels were evaluated in splenic B cells of $CD19^{Cre}$; $IPMK^{flox/-}$ (IPMK cKO) and $CD19^{Cre}$; $IPMK^{+/-}$ or $IPMK^{+/-}$ (control) mice. The results confirmed that IPMK was efficiently depleted in B cells of the IPMK cKO mice (*SI Appendix*, Fig. S1). The analysis of B cell development in the bone marrow revealed that IPMK cKO mice displayed no significant alteration (*SI Appendix*, Fig. S2). This is probably because $CD19^{Cre}$-mediated deletion is inefficient in early stages of B cell development (27), thereby limiting the assessment of IPMK function early in the B cell lineage. The frequency and cellularity of B cells in the spleen were also similar between IPMK cKO and control mice (*SI Appendix*, Fig. S3), suggesting that the peripheral development of immature B cells into mature cells is normal in the IPMK cKO mice. No significant change was observed in mature B cell populations, such as follicular (Fo) B cells and marginal zone (MZ) B cells, in the spleen of IPMK cKO mice (Fig. 1*A* and *SI Appendix*, Fig. S4*A*). CD19-Cre also induces IPMK deletion in B-1 B cells, mainly located in the peritoneal cavity. However, we did not observe any defects in B-1 B cell population in IPMK cKO mice (Fig. 1*B* and *SI Appendix*, Fig. S4*B*). Thus, IPMK appears to be dispensable for B cell development in the periphery and the bone marrow.

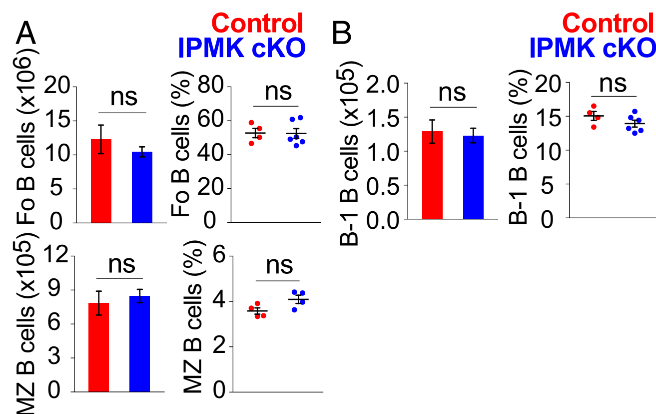

Fig. 1. The development of B cells is normal in IPMK cKO mice. (*A*) Percentages and cell numbers of follicular B cells [n = 4 mice (control) or n = 6 mice (IPMK cKO)] and marginal zone B cells (n = 4 mice per genotype) in spleen. (*B*) Percentages and cell numbers [n = 4 mice (control) or n = 6 mice (IPMK cKO)] of B-1 B cells in peritoneal cavity. All data are presented as mean ± SEM. Student's t test was used to calculate P values. ns, not significant ($P > 0.05$).

IPMK cKO Mice Display Defects in TI Immune Responses. Given that InsPs including $InsP_3$ and $InsP_4$ are robustly synthesized in activated B cells (15) and that IPMK is the only enzyme forming $Ins(1,3,4,5,6)P_5$ and, thus, $InsP_6$ from $InsP_4$, we investigated the functional consequences of IPMK deletion in B cell immunity by challenging mice with specific antigens. Mice were first challenged with lipopolysaccharide (LPS), a TI type I antigen, which causes vigorous proliferation of B cells and differentiation into plasma cells. Two days after LPS challenge, splenic B cell responses were assessed. We noticed that both B cell frequency (74% vs. 66.9% in control and IPMK cKO, respectively) and cell number (5.5×10^7 vs. 3.6×10^7) in the spleen were significantly reduced in IPMK cKO mice compared with those in the control mice (Fig. 2*A*). In addition, IPMK cKO mice failed to produce IgM^+ plasma cells in response to LPS immunization (Fig. 2*B*). Both the frequency (2% vs. 0.36%) and number (1.2×10^6 vs. 0.18×10^6) of IgM^+ plasma cells were greatly reduced in IPMK cKO mice compared with those in the control mice.

As the Toll-like receptor 4 (TLR4) signaling on B cells is dependent on the BCR signaling pathway (28, 29), we evaluated TI type II immune responses to investigate whether the impaired response to LPS is due to a defect in BCR signaling in IPMK cKO mice. We immunized mice with 4-hydroxy-3-nitrophenyl-acetyl conjugated to Ficoll (NP-Ficoll), which is recognized by BCR to stimulate B cell. Three days after immunization, we analyzed splenic B cells responding specifically to NP-Ficoll. The frequency (0.18% vs. 0.1%) and cellularity (10.2×10^4 vs. 4.43×10^4) of NP^+ B cells in the spleen were considerably reduced in IPMK cKO mice compared with those in the control mice (Fig. 2*C*). IPMK cKO mice also showed decreased frequency (0.078% vs. 0.034%) and cellularity (4.4×10^4 vs. 1.77×10^4) of plasma cells expressing NP-specific immunoglobulins (Fig. 2*D*). Overall, these results clearly show that IPMK cKO mice are defective in B cell responses against TI antigens, in which BCR signaling is involved.

However, IPMK deficiency did not affect a response to TD antigens significantly. When IPMK cKO mice were immunized mice with 4-hydroxy-3-nitrophenyl-acetyl conjugated to keyhole limpet hemocyanin (NP-KLH), germinal center (GC) were formed and short-lived plasma cells were generated within 5–7 d in the spleen (*SI Appendix*, Fig. S5 *A* and *B*). The frequency and cellularity of NP-specific plasma cells (IgD^{low} NP^+ $B220^{low}$ $CD138^+$) and GC B cells (IgD^{low} NP^+ $B220^{high}$ $CD138^-$ $GL7^+$) were similar between IPMK cKO and control mice.

IPMK Deficiency Impairs B Cell Proliferation During a Response to TI Antigens. To verify whether IPMK deletion affects the proliferation of B cells in response to TI antigens, we carried out the BrdU incorporation assay in vivo. Two days after injection with LPS and BrdU, B cells were isolated from the spleen and BrdU incorporation was measured. The frequency of $BrdU^+$ B cells was greatly reduced in IPMK cKO mice (6.9%) compared with that in the control mice (17.7%) (Fig. 3*A*). It is notable that $IgM^{high}IgD^{low}$ B cells comprising mostly MZ B cells are labeled by BrdU more efficiently than Fo B cell-enriched $IgM^{low}IgD^{high}$ cells (Fig. 3*B*). The effect of IPMK deficiency on the decrease in $BrdU^+$ cells was more severe in $IgM^{high}IgD^{low}$ cells (6.6-fold decrease) than in $IgM^{low}IgD^{high}$ cells (3.5-fold decrease). LPS challenge significantly increased the number of $IgM^{high}IgD^{low}$ cells and $IgM^{low}IgD^{high}$ cells in the control mice (Fig. 3*C*). However, this LPS-stimulated elevation in the number of $IgM^{high}IgD^{low}$ B cells was markedly blunted in IPMK cKO mice (Fig. 3 *C*, *Left*). Similarly, the number of $IgM^{low}IgD^{high}$ B cells in response to LPS was not increased in IPMK cKO mice, compared with that in the control mice (Fig. 3 *C*, *Right*).

Further, we assessed the defects in proliferation against TI antigens directly, by staining purified splenic B cells with CFSE and culturing them in vitro with LPS or anti-IgM. B cells from IPMK cKO mice displayed significantly reduced proliferation in

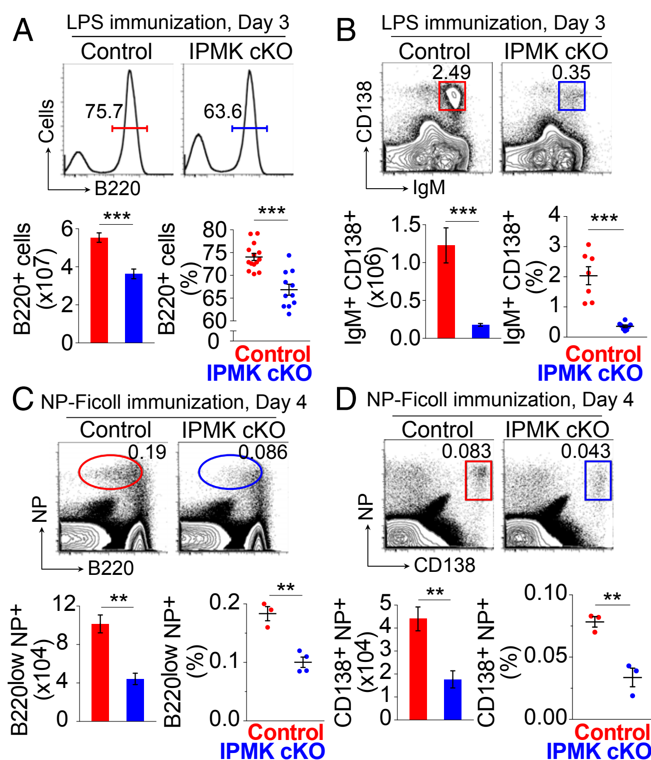

Fig. 2. IPMK cKO mice show the defects in immune responses against TI antigens. (*A*) Flow cytometry staining of splenic B cells ($B220^+$) 2 d after daily LPS injection (*Upper*). Cell numbers and percentages [$n = 14$ mice (control) or $n = 11$ mice (IPMK cKO)] of $B220^+$ cells in spleen (*Lower*). (*B*) Flow cytometry staining of plasma cells (IgM^+ $CD138^+$) as in *A* (*Upper*). Shown are cell numbers and percentages [$n = 7$ mice (control) or $n = 8$ mice (IPMK cKO)] of IgM^+ $CD138^+$ cells in spleen (*Lower*). (*C*) Flow cytometry staining of NP-reactive B cells ($B220^{low}$ NP^+) in spleen 3 d after NP-Ficoll immunization (*Upper*). Shown are cell numbers and percentages [$n = 3$ mice (control) or $n = 4$ mice (IPMK cKO)] of $B220^{low}$ NP^+ in spleen (*Lower*). (*D*) Flow cytometry staining of NP-reactive plasma cells ($CD138^+$ NP^+) as in *C* (*Upper*). Shown are cell numbers and percentages ($n = 3$ mice per genotype) of $CD138^+$ NP^+ cells in spleen (*Lower*). All data are presented as mean ± SEM. Student's *t* test was used to calculate *P* values. $^{**}P < 0.01$; $^{***}P < 0.001$.

response to both stimuli compared with that of B cells from control mice (Fig. 3*D*). These results collectively suggest that IPMK is essential for B cell proliferation during the response to TI antigens.

IPMK Is Required for the Expression of Genes Induced by TI Antigens. When splenic B cells were activated with LPS or anti-IgM, the expression of *Ipmk* mRNA was increased significantly (*SI Appendix*, Fig. S6). Both BCR and TLR4 signaling activate the NF-κB pathway, which induces the expression of genes such as *c-myc* (30, 31) and *Irf4* (32, 33) (Fig. 4 *A* and *B*). However, the induction of *c-myc* and *Irf4* mRNAs by TLR4 or BCR signaling was reduced in IPMK-deficient B cells. In addition, as it has been reported that the stimulation of B cells with LPS induced the secretion of cytokines, such as TNFα, IL-6, and IL-10, in a BCR-dependent manner (28), we evaluated whether LPS-induced cytokine production is also affected by deficiency of IPMK in splenic B cells. We found that IPMK-deficient B cells showed substantially decreased production of these cytokines (Fig. 4 *C* and *D*). It is also known that BCR signaling enhances IL-10 production in LPS-stimulated B cells (28, 34); however, it was considerably less in IPMK-deficient B cells than that in the control (Fig. 4*D*). It seems that the impaired responses to TI antigens is due to hyporesponsiveness to TLR4 and BCR signaling in IPMK-deficient B cells.

Btk Activation Is Defective in IPMK-Deficient B Cells. IPMK cKO mice failed to mount immune responses against TI antigens, similar to that in mice lacking key signaling molecules involved in BCR signaling (35–37). To investigate whether IPMK is required for BCR signal transduction, we assessed the phosphorylation patterns of various components in BCR signaling after stimulating splenic Fo B cells and MZ B cells with anti-IgM. We could not detect any abnormality in the phosphorylation of Syk, Akt, and S6, which are involved in the early stage of BCR signaling (*SI Appendix*, Fig. S7). We also examined the TRAF6 protein levels, which were found to be down-regulated in IPMK-deficient macrophages (38), but found no changes when IPMK was deleted in B cells (*SI Appendix*, Fig. S8).

As it has been reported that the Btk activity can be regulated by $InsP_6$ (21), and the defects in B cell response to TI antigens observed in IPMK cKO mice were similar to the phenotypes of Btk-deficient mice (6), we postulated that the activity of Btk in the BCR signaling pathway might be defective in IPMK-deficient B cells. Thus, we analyzed phosphorylation patterns of Btk and PLCγ2 after BCR stimulation. The phosphorylation of Btk in Fo B cells was marginally less in IPMK-deficient cells than that in the control, and the difference was more obvious in MZ B cells (Fig. 5*A* and *SI Appendix*, Fig. S9*A*). The phosphorylation of PLCγ2, a Btk target protein, was reduced significantly in both subsets from IPMK cKO mice compared with that from the control mice (Fig. 5*B* and *SI Appendix*, Fig. S9*B*). As BCR-mediated activation of PLCγ2 triggers calcium mobilization to increase cytoplasmic calcium concentration, we accordingly confirmed that calcium influx was conspicuously diminished in IPMK-deficient B cells after stimulation with anti-IgM or anti-IgD dextran, an analog of polysaccharide (Fig. 5*C* and *SI Appendix*, Fig. S10). Meanwhile, when increases in intracellular

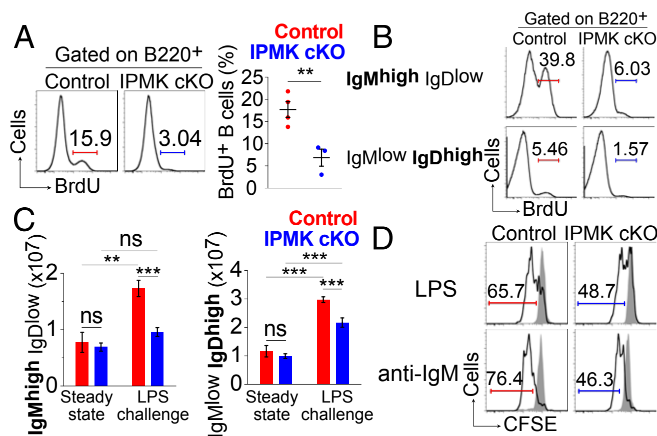

Fig. 3. IPMK-deficient B cells show a decrease in proliferation induced by TI antigens. (*A*) Flow cytometry analyzing the proportion of $BrdU^+$ cells gated on $B220^+$ in spleen 2 d after daily injection with LPS and BrdU [$n = 4$ mice (control) or $n = 3$ mice (IPMK cKO)]. (*B*) Flow cytometry analyzing the proportion of BrdU-incorporated cells in IgM^{high} IgD^{low} B cells and IgM^{low} IgD^{high} B cells gated on $B220^+$ as in *A*. (*C*) Cell numbers of IgM^{high} IgD^{low} B cells (*Left*) and IgM^{low} IgD^{high} B cells (*Right*) in spleen at steady state or 2 d after daily LPS injection. [$n = 3$ mice (control) or $n = 5$ mice (IPMK cKO) at steady state; $n = 13$ mice (control) or $n = 10$ mice (IPMK cKO) upon LPS challenge]. (*D*) Flow cytometry analyzing CFSE-labeled B cells left unstimulated (shaded histograms) or stimulated with LPS (5 μg/mL) for 2 d or anti-IgM (10 μg/mL) for 3 d in the presence of IL-4 (20 ng/mL). All data are presented as mean ± SEM. Student's *t* test was used to calculate *P* values. ns, not significant ($P > 0.05$); $^{**}P < 0.01$; $^{***}P < 0.001$.

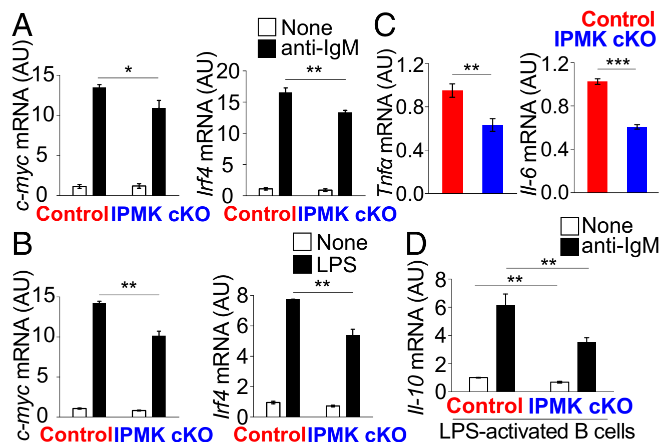

Fig. 4. Gene expression induced by TLR and BCR stimuli is diminished in IPMK-deficient B cells. (*A* and *B*) Quantitative RT-PCR analysis of indicated genes in purified B cells cultured for 4 h with anti-IgM ($n = 4$ mice per genotype) and LPS ($n = 3$ mice per genotype). (*C*) Quantitative RT-PCR analysis of proinflammatory cytokines ($n = 4$ mice per genotype) in purified B cells cultured for 2 d in the presence of LPS (10 μg/mL). (*D*) Purified B cells were cultured as in *D*, followed by stimulation with anti-IgM (10 μg/mL). Quantification of *Il-10* mRNA [$n = 6$ mice (control) or $n = 7$ mice (IPMK cKO)] at 3 h after stimulation with anti-IgM. All data are presented as mean ± SEM. Student's *t* test was used to calculate *P* values. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. AU, arbitrary unit.

calcium concentration were induced by treatment with calcium ionophore, the defects of gene expression in response to LPS and anti-IgM were fully restored in IPMK-deficient B cells (*SI Appendix*, Fig. S11). These results reveal that the deficiency of IPMK in B cells results in abnormal BCR signaling, most likely due to impaired activation of Btk.

Inositol Hexakisphosphate Is Required for the Btk Activity. We evaluated the higher order InsPs that are present in B cells and are affected by IPMK ablation. B cells purified from the spleen were labeled with [^{3}H]myo-inositol, and the high-performance liquid chromatography (HPLC) elution profile of soluble InsPs was obtained from cell extracts. Higher order InsPs up to $InsP_7$ were detected, and, interestingly, $InsP_5$ and $InsP_6$ constituted the major components in LPS-stimulated B cells (*SI Appendix*, Fig. S12). In IPMK-deficient B cells, higher order InsPs comprising $InsP_5$, $InsP_6$, and $InsP_7$ were greatly reduced (Fig. 5*D*), which was expected because IPMK regulates the synthesis of $InsP_5$ from $InsP_4$.

To confirm that BCR signaling requires higher order InsPs, we tested if cell permeable InsP could rescue the impairment in activation of IPMK-deficient B cells. Fo B cells and MZ B cells isolated from IPMK cKO mice were activated in the presence of cell permeable $InsP_5$ or $InsP_6$ (39–41), and then the phosphorylation of Btk and PLCγ2 was measured. The treatment with cell permeable $InsP_6$ only resulted in substantial restoration of phosphorylation of both Btk and PLCγ2 after stimulation with anti-IgM (Fig. 5*E* and *SI Appendix*, Fig. S13) or anti-IgD dextran (*SI Appendix*, Fig. S14), indicating that Btk is efficiently activated. However, $InsP_6$-mediated increments in the phosphorylation of Btk and PLCγ2 were inhibited by Btk inhibitor, ibrutinib (*SI Appendix*, Fig. S15). Thus, our results clearly indicate that $InsP_6$ plays an essential role in BCR signaling by modulating the Btk activity.

IPMK Is Essential for Functional BCR Complex in IgM$^+$ Plasma Cells. Recently, it has been shown that IgM-expressing plasma cells can be generated by immunization with TI antigens (42, 43). These IgM-expressing mature plasma cells appear to be critical for response against polysaccharide antigens, and they have the functional BCR complexes that promote cytokine production by recognizing antigens (43). As we have shown that higher order InsPs are critical for TI immune responses via the regulation of Btk activity, it is likely that IgM$^+$ plasma cells of IPMK cKO mice display defects in the activation of Btk and PLCγ2. As expected, the phosphorylation of Btk and PLCγ2 was significantly reduced in IgM$^+$ plasma cells from IMPK cKO mice than that in IgM$^+$ plasma cells from control mice (Fig. 6*A* and *SI Appendix*, Fig. S16). These defects in BCR signaling displayed by IPMK-deficient IgM$^+$ plasma cells can be rescued by supplying exogenous cell permeable $InsP_6$ during ex vivo stimulation (Fig. 6*B*). Interestingly, LPS immunization induced up-regulation of key enzymes responsible for the production of $InsP_6$, such as *Ipmk* (6.2-fold higher level) and *inositol pentakisphosphate 2-kinase*

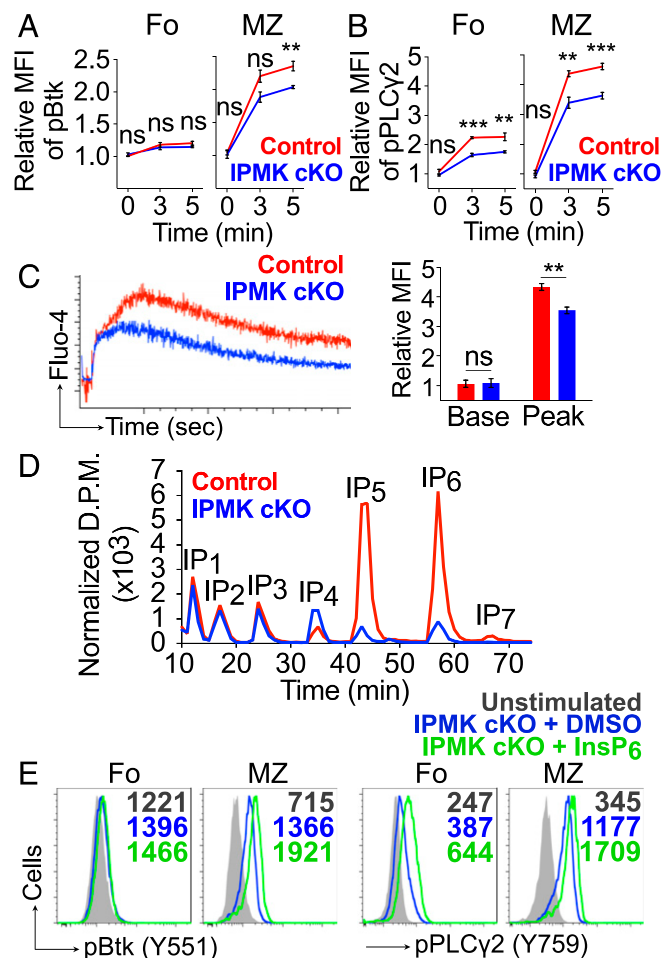

Fig. 5. IPMK is essential for Btk-mediated BCR signaling. (*A* and *B*) Following stimulation with anti-IgM (10 μg/mL) for indicated time, relative mean fluorescence intensity (MFI) of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in Fo B cells (B220$^+$ IgD^{high} CD21^{low}) and MZ B cells (B220$^+$ IgD^{low} CD21^{high}) in spleen ($n = 4$ mice per genotype). (*C*) Profiles of Ca^{2+} mobilization of splenic B cells (B220$^+$) induced by stimulation with anti-IgM (*Left*). Relative MFI of baselines and peaks ($n = 3$ mice per genotype) of calcium influx in response to anti-IgM (*Right*). (*D*) HPLC elution profiles of soluble InsPs extracted from B cells radiolabeled with [^{3}H]myo-inositol in the presence of LPS (10 μg/mL). (*E*) Following stimulation with anti-IgM (10 μg/mL) in the presence of DMSO or cell permeable $InsP_6$ (80 μM), flow cytometry staining of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in Fo B cells (B220$^+$ IgD^{high} CD21^{low}) and MZ B cells (B220$^+$ IgD^{low} CD21^{high}). Numbers are MFI of indicated populations. All data are presented as mean ± SEM. Student's *t* test was used to calculate *P* values. ns, not significant ($P > 0.05$); **$P < 0.01$; ***$P < 0.001$. D.P.M., disintegrations per minute.

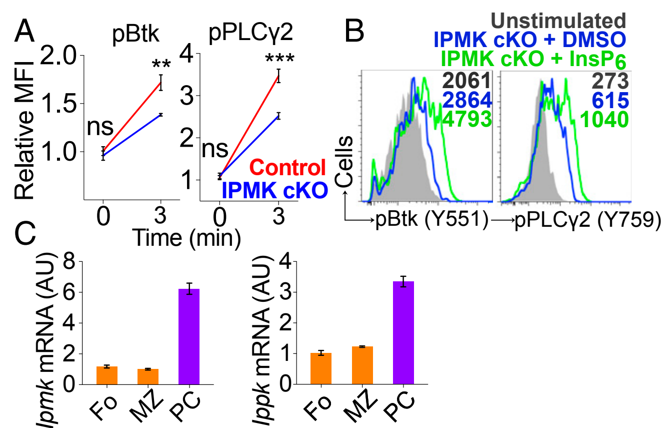

Fig. 6. IPMK is required for BCR signaling in IgM^+ plasma cells. (*A*) Following stimulation with anti-IgM (10 μg/mL) for indicated time, relative MFI of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in plasma cells (IgD^- $B220^{low}$ $CD138^+$) obtained from immunized mice with LPS [n = 4 mice (control) or n = 5 mice (IPMK cKO)]. (*B*) Following stimulation with anti-IgM (10 μg/mL) in the presence of DMSO or cell permeable $InsP_6$ (60 μM), flow cytometry staining of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in plasma cells (IgD^- $B220^{low}$ $CD138^+$) as in *A*. Numbers are MFI of indicated populations. (*C*) Quantitative RT-PCR of indicated genes (n = 3 per each subset) in Fo B cells ($B220^+$ $CD23^+$ $CD21^{low}$), MZ B cells ($B220^+$ $CD23^-$ $CD21^{high}$), and plasma cells (PC, IgM^+ $CD138^+$). All data are presented as mean ± SEM. Student's t test was used to calculate P values. ns, not significant ($P > 0.05$); $^{**}P < 0.01$; $^{***}P < 0.001$. AU, arbitrary unit.

(*Ippk*) (2.7-fold higher level), in IgM^+ plasma cells compared with that in MZ B cells (Fig. 6*C*). However, there was no obvious change in other InsP or $PtdIP_3$ regulatory genes (*SI Appendix*, Fig. S17). These results suggest that $InsP_6$ is a core component involved in the regulation of BCR signaling in IgM^+ plasma cells, and its generation seems to be accelerated by increasing the expression of IPMK and IPPK in these cells.

Discussion

IPMK exhibits complex catalytic activities that eventually yield water soluble InsPs, such as $InsP_4$ and $InsP_5$ (25, 26), and lipid-bound $PtdIP_3$ (44). In the present study, we showed that IPMK modulates B cell immunity via generation of higher order InsPs regulating Btk activity and that it plays a critical role in the B cell responses against TI antigens. IPMK-deficient B cells exhibited reduced activation of Btk and its target protein, PLCγ2, and diminished calcium influx after stimulation. Higher order InsPs comprising $InsP_5$, $InsP_6$, and $InsP_7$ were greatly reduced in these cells. As $InsP_6$ is produced by IPPK from $InsP_5$, which is produced solely by IPMK from $InsP_4$, the deficiency of IPMK leads to the substantial reduction in $InsP_6$ level. We have also shown that cell permeable $InsP_6$ can successfully restore the signaling defects in IPMK-deficient B cells. Thus, our results strongly indicate that higher order InsPs produced by IPMK are required for Btk activity during B cell responses. It has been shown that a constitutively active mutant of Btk binds to $InsP_6$ with high affinity compared with that of wild-type Btk (45), suggesting a possibility that Btk activity is correlated with $InsP_6$ binding capacity. It has also been reported that $InsP_6$ acts as an allosteric regulator to stimulate the dimerization of Btk, which contributes to autophosphorylation (21). Our results, together with the findings of previous studies, indicate that $InsP_6$ generated by IPMK modulates Btk activity during B cell immunity in vivo.

IPMK did not significantly affect the development of B cells in the bone marrow and in the periphery. However, Btk-deficient mice displayed a 30–50% decrease in the number of peripheral B cell with notable loss in mature B cell population (4, 6). IPMK-deficient mice showed normal TD immune response when they were immunized with NP-KLH, in contrast to Btk-deficient mice displaying weakened TD immune responses with reduced serum level of IgM and IgG_1 (6). The differences in TD immune responses between IPMK cKO and Btk-deficient mice might be, at least in part, due to their difference in B cell development. These differences in TD immune responses between the two mice is in line with the results that inducible Btk deletion in mature B cells, in which B cell development is normal, results in a milder phenotype in immune responses than that by Btk deletion in the whole body (46).

Mice lacking IPMK were defective in TI immune responses; cellularity of B cells responding to LPS or NP-Ficoll was significantly decreased and plasma cells were inefficiently generated. We have further shown that the impaired TI immune responses in IPMK cKO mice are mainly due to a defect in the Btk activity, which was accompanied by defective proliferation of B cells. It is also noteworthy that the effect of IPMK deficiency on the decrease of proliferation is considerably more severe in MZ B ($IgM^{high}IgD^{low}$) cells than in FO ($IgM^{low}IgD^{high}$) cells. This is consistent with the findings of previous studies that MZ B cells respond to LPS more efficiently than FO B cells (42, 47). The activation of PLCγ2 by Btk induces the generation of second messengers, such as $InsP_3$ and DAG, by hydrolysis of $PtdIP_2$. $InsP_3$-mediated calcium influx and DAG mediate the activation of PKCβ, enabling the activation of the NF-κB pathway. Upregulation of c-Myc (30, 31) and IRF4 (32, 33), which are the targets of the NF-κB pathway, promotes cell cycle entry and proliferation in response to antigen stimulation. IPMK-deficient B cells displayed impaired induction of c-Myc and IRF4 by TLR4 and BCR signaling. These results are consistent with the proliferative defects of IPMK-deficient B cells, implying that they are defective in both the signaling pathways. Recently, IPMK has been shown to promote TLR4 signaling by stabilizing TRAF6 in macrophages (38). IPMK-deficient macrophages showed increased proteasomal degradation of TRAF6, thereby, reducing TLR4-dependent proinflammatory cytokine production. IPMK-deficient B cells were also defective in the production of TNFα, IL-6, and IL-10. However, protein level of TRAF6 was unaltered in IPMK-deficient B cells, suggesting that IPMK participates in responses to LPS in B cells via a pathway different from the one in macrophages.

TI immune responses promote the generation of plasma cells and memory B cells to confer long-lasting defense against pathogens (48, 49). IgM^+ plasma cells play a key role in defense against bacterial infection by rapidly secreting antibodies. In contrast to class-switched plasma cells, IgM^+ plasma cells have functional BCR complexes that are signaling competent and enable the production of cytokines upon recall antigen challenge (43). However, transcriptional regulation during plasma cell differentiation results in the loss of CD19 expression (43, 50). To initiate the BCR signaling cascade in activated B cells, CD19-mediated activation of PI3K is essential for recruitment and subsequent activation of PH domain-containing kinases, such as Btk and Akt, by producing $PtdIP_3$ at the membrane (12, 51, 52). Interestingly, the structural analysis of $InsP_6$/PH domain complex showed that the PH domain of Btk has two $InsP_6$ binding sites (21). The occupation of both sites in PH domain is important for the activation of Btk by dimerization (21, 53). One of them is a canonical binding site that is essential for binding to $PtdIP_3$ in the membrane. It suggests that $InsP_6$ might compensate the loss of CD19 expression in IgM^+ plasma cells. The results of the present study showed defects in the phosphorylation of Btk and PLCγ2 in IgM^+ plasma cells from IMPK cKO mice, which were efficiently restored by the treatment with cell permeable $IsnP_6$. Moreover, the expression of enzymes required for the generation of $InsP_6$, such as IPMK (from $InsP_3$ and $InsP_4$ to $InsP_5$) and IPPK (from $InsP_5$ to $InsP_6$), was strongly induced in IgM^+ plasma cells, probably complementing the requirement of

$InsP_6$ for a functional BCR. Thus, despite CD19 deficiency in IgM^+ plasma cells, $InsP_6$ generated by IPMK seems to provide an alternative mechanism for the regulation of Btk in BCR signaling.

In the present study, we showed that IPMK is a regulator of TI immune responses and that $InsP_6$ confers a mechanism to regulate the activity of Btk in BCR signaling. Thus, we believe that our findings on the role of IPMK and its products will offer ways to treat abnormal Btk-mediated disorders in the future.

Materials and Methods

Immunization. To mount NP-specific antibody responses, mice were injected intraperitoneally (i.p.) with 40 μg of NP-Ficoll (Bioresearch Technologies) or 100 μg of NP-KLH (Bioresearch Technologies) precipitated in Imject Alum (Thermo Scientific). For induction of immune response to LPS, 10 μg of LPS (Sigma) was administered daily by i.p. injections over the course of 2 d.

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