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T 세포 비의존적 B 세포 반응에서 이노시톨 다인산에 의한
Bruton's tyrosine kinase 활성 조절에 관한 연구

Studies on inositol polyphosphates-mediated regulation of
Bruton's tyrosine kinase in T cell-independent humoral immunity

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Abstract

Studies on inositol polyphosphates-mediated regulation of Bruton's tyrosine kinase in T cell-independent humoral immunity

Wooseob Kim

In the early stages of microbial infection, B cells recognize immediately pathogen-derived antigens to elicit robust immune responses in T cell-independent (TI) manner. Intense and rapid TI immune response is critical for the early protection against pathogen invasion. The recognition of antigens is mediated by B cell antigen receptor (BCR) that transduces B cell-activating signals, which is essential for activating the TI humoral immunity. The regulation and activation of Bruton's tyrosine kinase (Btk) are known as a pivotal step of 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 well-established canonical BCR signaling pathway, including phosphoinositide 3-kinase (PI3K) activity, is sufficient to regulate the activity of Btk. Earlier studies have suggested that the Btk activity might be regulated through mechanisms that are independent of PtdIP$_3$ synthesis. It has been demonstrated that inositol
polyphosphates are related to the regulation of Btk in *in vitro* assays. However, its physiological meaning of them is yet to be investigated.

Here, I find that inositol polyphosphates play a critical role in TI humoral immunity. Absence of higher order inositol phosphates (InsPs), inositol polyphosphates, leads to an inability to mount immune responses against TI antigens, such as LPS or NP-Ficoll. When mice are challenged with TI antigens, the reduced cell numbers of B cells and IgM+ plasma cell are observed in the spleen of inositol polyphosphate multikinase (IPMK)-deficient mice. B cells lacking IPMK are less proliferative, and they are defective in gene expression and cytokine production upon antigenic stimulation. I have further shown that the impaired TI immune responses in IPMK-deficient mice are due to the reduced activity of Btk in B cells. The phosphorylation of Btk and its target protein, PLCγ2, upon BCR stimulation is significantly reduced in IPMK-deficient B cells.

I identify inositol hexakisphosphate (InsP_6) as a principal cofactor for modulating Btk activity within BCR signaling. The generation of higher order InsPs including InsP_5, InsP_6, and InsP_7 is induced in activated B cells, but they are absent in IPMK-deficient B cells. It appears that the lack of high order InsPs results in the signaling defects in IPMK-deficient B cells. Moreover, treatment of IPMK-deficient B cells with cell permeable InsP_6 efficiently restores the defects in TI immune responses, implying that InsP_6 acts as an upstream regulator of Btk in BCR signaling.
Interestingly, the significance of InsP₆-mediated Btk regulation is more prominent in IgM⁺ plasma cells. Although CD19-mediated activation of PI3K is essential for functional BCR complexes, CD19 expression is markedly decreased during differentiation into IgM⁺ plasma cells. However, IgM⁺ plasma cells highly express enzymes required for the generation of high order InsPs, which might compensate for the reduced PI3K activity by increasing the level of InsP₆. It seems that activation of Btk in BCR signaling is more dependent on the formation of InsP₆ in IgM⁺ plasma cells than that in B cells.

Hence, the present study identifies higher order InsPs as principal components of B cell activation upon TI antigen stimulation. These results present a novel mechanism for InsPs-mediated regulation of the BCR signaling, in which second messengers encode instruction for cellular processes. Findings on the role of IPMK and its products will provide a new strategy to treat disorders induced by malfunction of Btk, such as B cell malignancy, autoimmune disease, and immune deficiency, in the future.

**Keyword:** T cell-independent immune response, B cell antigen receptor, Bruton's tyrosine kinase, inositol phosphate, inositol polyphosphate multikinase.

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Introduction

I-1. T cell-independent humoral immunity

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 (Rawlings et al., 2012). 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 (Vos et al., 2000). Due to remarkable contribution of TI humoral immunity to elimination of microorganism infection, it is important to understand comprehensive mechanisms.

Infection of several pathogenic bacteria cause the life-threatening disease, such as pneumonia, sepsis, and meningitis (Berical et al., 2016; Pollard et al., 2009). Protective immunity to bacterial infection is mediated by cytokine secretion and antibody production of B cells against bacterial
antigens (Kelly-Scumpia et al., 2011; Rauch et al., 2012). TI B cell responses play a central role in mounting immune response to bacterial polysaccharide antigens (Taillardet et al., 2009; Weber et al., 2014). Innate-like B cells including marginal zone B cells and B-1 B cells are responsible for TI immune response (Balazs et al., 2002; Martin et al., 2001; Yang et al., 2007). These subsets of B cells directly recognize antigens through Toll-like receptors (TLR) or B cell antigen receptors (BCR). After antigen recognition, they can immediately mount rapid and intense antibody response against invading microorganisms. Because consumed time for clearing bacteria in body determines the severity and the prognosis of disease, rapid and intense TI immune response play a significant role in the early protection against pathogen invasion.

Immunization with TI antigens promotes the formation of plasma cells and memory cells to confer long-lasting defense to pathogens (Alugupalli et al., 2004; Bohannon et al., 2016; Obukhanych and Nussenzweig, 2006). Earlier studies have reported that these IgM+ plasma cells play an important role in long-term immunity against microbial infection (Blanc et al., 2016; Foote et al., 2012; Racine et al., 2011). In contrast to class-switched plasma cells, IgM+ plasma cells have a functional BCR complexes, which are signaling competent and lead to activation upon antigenic stimulation (Blanc et al., 2016; Pinto et al., 2013). Functional BCR complexes also
enable IgM+ plasma cells to maintain long-lasting humoral immunity by recognizing polysaccharide, which remain for long period due to poor biodegradability, and recurrent pathogens to immediately elicit immune response. Hence, it is important to understand how IgM+ plasma cells are generated and how signaling competency of BCR on these cells is maintained in TI humoral immunity.

I-2. T cell-dependent humoral immunity

In contrast to TI immune response, humoral immune response to antigens comprising proteins is dependent on T cell help. T cell-dependent (TD) immune response promotes the formation of germinal center (GC) in the secondary lymphoid organs, a specialized anatomical site within the lymphoid follicle. When TD antigens are transported to the secondary lymphoid organs, B cells uptake and present them on MHC class II molecules for recognition by cognate helper T cells. Then, helper T cells are activated, expressing cytokines and CD40 ligand to induce the formation of GC in the follicles. In the GC reaction, an affinity maturation of BCR, such as somatic hypermutation, clonal expansion, and affinity-based selection, occurs and memory B cells are generated. Positive selection of GC B cells expressing high-affinity BCR is a critical step in affinity maturation (Mesin
et al., 2016). B cells with high-affinity for antigens have advantages in both capturing and presenting antigens to cognate helper T cells. As BCR signaling in GC B cells is desensitized, its major role may be to process antigen for presenting to cognate helper T cell (Khalil et al., 2012). The attenuated BCR signal cooperate with cytokines and CD40 signals, which emanate from helper T cells, to support the GC reaction in TD immune response. Therefore, attenuated BCR signaling allows GC B cells to integrate both antigen-dependent signals and T cell-derived signals in TD immune response.

**I-3. B cell antigen receptor signaling**

Signal transduction via BCR is essential for TI immune response (Vos et al., 2000). 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 (Figure 1). This leads to the assembly of the signaling complex into ITAM, comprising Vav, BLNK, phosphoinositol 3-kinase (PI3K), Bruton's tyrosine kinase (Btk), and phospholipase C-γ2 (PLCγ2). This assembly form the signalosome that is an important site for the initiation of BCR signaling, including the phosphorylation cascades and the calcium mobilization. The activation of PLCγ2 by Btk generates the second messengers, such as inositol 1,4,5-
triphosphates (InsP₃) and diacylglycerol (DAG) through hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIP₂). InsP₃ opens calcium channels located in the endoplasmic reticulum to increase intracellular calcium concentration. In addition, InsP₃-mediated calcium mobilization and DAG lead the activation of PKCβ, resulting in the activation of the NF-κB pathway. These signaling complexes trigger cellular processes critical to initiate immune responses, including the regulation of gene expression, cytoskeleton reorganization, and B cell proliferation and differentiation.

**I-4. BCR-dependent TLR signaling in B cells**

B cells mediate TI antibody response through direct recognition of antigens with TLR or BCR (Rawlings et al., 2012). Interestingly, earlier reports have demonstrated that the TLR4 signaling upon LPS stimulation is dependent on BCR signaling (Otipoby et al., 2015; Pone et al., 2012; Schweighoffer et al., 2017). Treatment of B cells with LPS induces the activation of SYK tyrosine kinase, which is essential for BCR signaling. Moreover, BCR-deficient B cells are less proliferative and defective in the phosphorylation of key components in BCR signaling upon LPS stimulation. LPS consists of a lipid A moiety and a polysaccharide moiety. Therefore, LPS can trigger TLR4 and BCR signaling through the lipid A moiety and the polysaccharide moiety, respectively (Pone et al., 2012; Schweighoffer
Figure 1. B cell antigen receptor signaling pathway
et al., 2017). The polysaccharides part of LPS are capable of inducing the multivalent cross-linking of BCR, thereby bridging the two receptors. Such traits of LPS stimulation in B cells could give an explanation of how the response to LPS in B cells is dependent on BCR signaling.

I-5. X-linked agammaglobulinemia

Rapid B cell response to TI antigens is particularly important to prevent overwhelming tissue damage caused by the infection of fast-replicating bacteria. T cell-independent immune response is known to depend on functional Btk (Ellmeier et al., 2000; Kerner et al., 1995; Khan et al., 1995; Mohamed et al., 2009), 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) (Hermaszewski and Webster, 1993; Lederman and Winkelstein, 1985; Ochs and Smith, 1996), which was caused by loss-of-function mutations in the gene encoding Btk. In similar with the phenotype of XLA in human, a spontaneous mutation (R28C) in the Btk gene of mice results in X-linked immunodeficiency (Xid). As Btk is essential for development, activation, and differentiation of B cells, XLA patients have reduced cellularity of circulating B cells and, thus, are unable to mount humoral immune response.

In B cells, BCR engagement lead to activation of PI3K, which induces
the formation of PtdIP\(_3\) in the membrane. Increased concentration of PtdIP\(_3\) lead to the recruitment of Btk into the inner surface of membrane via association with PH domain of Btk. This binding of Btk with PtdIP\(_3\) is prerequisite for its activation. In XLA in human and Xid in mice, some mutations are within the pleckstrin homology (PH) domain of Btk (Rawlings et al., 1993; Thomas et al., 1993; Valiaho et al., 2006), which is required for the association with PtdIP\(_3\) in membrane and the subsequent activation (Mohamed et al., 2009; Varnai et al., 1999). However, it has been reported that the activity of Btk was still observed in PtdIP\(_3\)-deficient B cells (Jou et al., 2002; Suzuki et al., 2003), 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.

I-6. Inositol polyphosphate multikinase

Inositol polyphosphate multikinase (IPMK) is a multifunctional protein including both catalytic activity and non-catalytic activity (Kim et al., 2017a) (Figure 2). Among non-catalytic activities, IPMK acts as mediator to regulate transcription and other kinase activity, such as transcriptional coactivator (Kim et al., 2013), modulation of AMPK activity (Bang et al., 2012), regulation of mTORC1 signaling, and stabilization of TRAF6 in
TLR4 (Kim et al., 2017b). Moreover, IPMK generates inositol 1,3,4,5-tetrakisphosphate (InsP₄) and inositol 1,3,4,5,6-pentakisphosphate (InsP₅) by catalytic activity. IPMK is the only one enzyme generating InsP₅, indicating that IPMK is essential for generation of higher order InsPs. IPMK is also known as physiologic PI3K, which activates Akt by converting PIP₂ to PIP₃ (Maag et al., 2011). However, little is known about a role of IPMK in immune system.

I-7. Higher order inositol phosphates

Inositol phosphates (InsPs) are known to be generated by antigen stimulation (Antony et al., 2007; Pouillon et al., 2003). It is well-established that InsPs, such as InsP₃ (Feske, 2007) and InsP₄ (Elich and Sauer, 2018; Huang et al., 2007; Marechal et al., 2007; Miller et al., 2009; Miller et al., 2007; Pouillon et al., 2003; Sauer and Cooke, 2010), act as signaling messengers in the activation of immune cells. However, little is understood about the roles of higher order InsPs in immune responses, even though they can act as a structural cofactor or an allosteric regulator that modulates the stability and function of protein complex (Dovey et al., 2018; Fu et al., 2018; Paulsen et al., 2015; Wang et al., 2015). InsP₅ play an important role in angiogenesis by regulating the stability of protein complexes. InsP₅ facilitates the interaction of HIF-α and pVHL, followed by proteasomal
Figure 2. Multifaceted function of IPMK in mammalian cells.
degradation. Depletion of InsP_5 by IPMK deletion induces an increase of HIF-α in fibroblasts, which promotes a substantial increase of vascular endothelial growth factor and leads to angiogenesis. In addition, earlier study has shown the function of higher order InsPs in necroptosis induced by cytokine-dependent death receptor activation and viral infection. Abolished production of higher order InsPs, such as InsP_5 and InsP_6, disrupts necroptosis downstream signaling. It has been demonstrated that the binding of higher order InsPs to MLKL, a critical effector of necroptosis, mediates the structural rearrangement of the N-terminal auto-inhibitory brace. Recently, it has been demonstrated that a mixture of InsP_6 and Btk in solution enables transient dimerization and trans-autophosphorylation of Btk (Wang et al., 2015). While it suggests a possibility that the higher order InsPs might be involved in modulating the activity of BCR signaling via regulation of Btk activity, the physiological relevance remains to be elucidated.

I-8. Aim of this study

T cell-independent immune response is crucial in generation of intense and rapid humoral immunity for defense against microbial infection. Bruton's tyrosine kinase is critical for the TI immune response, and signaling components controlling its activity during BCR signaling is
known. However, some mutant forms of Btk observed in XLA patients suggest that there may be other mechanisms tightly regulating its activity, particularly in TI immune response.

Higher order InsPs, such as InsP₅ and InsP₆, are converted from InsP₃ through successive phosphorylation by specific inositol phosphate kinases (Hatch and York, 2010; Irvine and Schell, 2001; Tsui and York, 2010). Among them, IPMK is the only enzyme responsible for generating InsP₅ from InsP₄ (Odom et al., 2000; Saiardi et al., 1999). In the present study, I 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.

Here, the results showed that higher order InsPs play a critical role during TI immune response by modulating the Btk activity. It was demonstrated that inositol polyphosphates are key metabolites modulating the activity of Btk in BCR signaling. These results provide novel insights on the role of inositol polyphosphates in B cell immunity.
Materials and Methods

Mice

Strategy for the generation of conditional IPMK knockout mice has been described (Kim et al., 2011). CD19-Cre transgenic mice were purchased from The Jackson Laboratories. All mice were bred and maintained in specific pathogen-free barrier facilities at Seoul National University and Institute of Molecular Biology and Genetics, and were used according to protocols approved by the Institutional Animal Care and Use Committees of Seoul National University.

Immunization

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

Data were collected using FACS Canto II (BD Bioscience) and were analyzed with FlowJo software (Tree Star). Sample preparation from bone marrow and spleen was performed as described (Choi et al., 2012). The following antibodies and chemicals were used: Anti-B220 (RA3-6B2); anti-CD4 (GK1.5); anti-CD8α (53-6.7); anti-CD19 (1D3); anti-CD23 (B3B4); anti-CD138/Syndecan-1 (281-2); anti-Fas (15A7); anti-GL7 (GL7); anti-IgM (R6-60.2) were purchased BD Bioscience. Anti-CD21/35 (eBio8D0); anti-CD5 (53-7.3) were purchased from Invitrogen. Anti-IgD (11-26C.21) was purchased from BioLegend. NP-PE (N-5070-1) was purchased from Biosearch.

Phospho flow cytometry

Level of phosphorylated proteins was measured according to manufacturer's instructions (BD Bioscience). In brief, after splenocytes were incubated for 10 min at 37°C, each samples were stimulated with anti-IgM (Jackson ImmunoResearch; AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgM, μ Chain Specific) or anti-IgD dextran (Fina Biosolutions) for indicated time in the absence or presence of cell permeable InsP₅ (Echelon Biosciences, Q-M1396) or InsP₆. Then, cells were washed with cold media
and fixed with BD Cytofix Fixation Buffer. Fixed cells were washed twice with Perm/Wash Buffer and permeabilized with BD Phosflow Perm Buffer III. After permeabilization, cells were washed twice with Perm/Wash Buffer and stained with anti-phospho antibodies for 50 min. Then, cells were washed twice with Perm/Wash Buffer and analyzed. For some experiments, splenocytes were pretreated for 1 hr at 37˚C with ibrutinib (Selleckchem, S2680) or DMSO. The following antibodies were used: Anti-pPLCγ2, Y759 (K86-689.37); anti-pSyk, Y352 (17A/P-ZAP70) were purchased BD Bioscience. Anti-pBtk, Y551 (M4G3LN) were purchased from Invitrogen. Anti-pS6, S235/236 (D57.2.2E); anti-pAkt, T308 (D25E6) was purchased from Cell Signaling Technology.

Calcium influx measurement

Intracellular calcium concentration was measured according to manufacturer's instructions (Thermo Fisher, Fluo-4 NW Calcium Assay Kit). In brief, freshly isolated splenocytes were stained with antibodies specific for B220. Total splenocytes were washed and resuspended in Ca²⁺-containing medium (HBSS, 20mM HEPES) and then were labeled for 30 min at 37˚C with dye loading solution including Flu-4 NW dye mix and probenecid. After incubation, anti-IgM or anti-IgD dextran was added as indicated concentration and fluorescence was measured using BD FACS
Measurement of inositol phosphates in primary B lymphocytes

Primary B cells were enriched from spleen using magnetic cell sorting (Miltenyi Biotec), following manufacturer's instructions. In brief, splenocytes were stained primarily with biotinylated antibodies (anti-CD3ε, anti-Gr1, anti-Mac1 and anti-Ter119 from BD Bioscience, Biotin Mouse Lineage Panel) and secondarily with streptavidin microbeads (Miltenyi Biotec). After loading stained samples onto LD column, untouched B cells that passed through column were collected. 0.7–1 × 10^8 cells were labeled for 24 hr in inositol-free RPMI 1640 medium (USBiological, R8998-03) supplemented with LPS, 10 µg/ml; 2mM L-glutamine (Gibco); 20mM HEPES (Gibco); 10% FBS (Gibco); 100 U/ml penicillin and streptomycin; 50 µg/ml 2-mercaptoethanol (Gibco); 50 µl/ml [³H]myo-inositol (PerkinElmer, NET114A001MC, 1 mCi). After washing labeled cells with PBS, inositol phosphates were extracted and analyzed as described (Azevedo and Saiardi, 2006). 300 µl of extraction buffer was added to each sample, which were incubated on ice for 10 min. Then, samples were neutralized with 90 µl neutralization buffer and leaved on ice for 1.5 hr. After centrifuged at maximum speed for 10 min, supernatant was injected into HPLC and collect 1ml fractions. 4 ml of Ultima-Flo AP liquid scintillation
cocktail (Perkin-Elmer, 6013599) was added to each fraction, then the radioactivity of the eluted fractions was quantified in the scintillation counter. Inositol incorporated into lipid was measured by extracting cell pellet with solution (0.1M NaOH, 0.1% Triton X-100) overnight and solubilized material are quantified in the scintillation counter. Data were normalized against lipid count for each sample. I performed all these procedures with Seyun Kim and his colleagues (Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Korea).

**Isolation and quantification of RNA**

Total RNA was extracted from cells with TRI Reagent (Molecular Research Center, Inc.), following the manufacturer's instructions. Equivalent quantities of total RNA were reverse-transcribed with QuantiTect Reverse Transcription Kit (QAIGEN) and cDNA was analyzed by real-time PCR (Applied Biosystem, StepOnePlus). The expression of genes is normalized to Actb expression. The primer sets used for amplification of cDNA are listed in Figure 3.

**Proliferation assay**

A BrdU incorporation assay was performed using BrdU Flow Kits (BD
<table>
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<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>c-myc</td>
<td>5'-ATGCCCTCAAACGTGAATCTTC-3'</td>
<td>5'-CGCAACATAGGATGGGAGGCA-3'</td>
</tr>
<tr>
<td>Irf4</td>
<td>5'-GCTGCATATCTGGCTTGATTACC-3'</td>
<td>5'-GTGGATAACGTGTTCAAGGTACTGTAAGAG-3'</td>
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<tr>
<td>Tnfa</td>
<td>5'-CCCTCAACACTCAAGTACTTCTTCT-3'</td>
<td>5'-GCTACGACGTGGTGCTACAG-3'</td>
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<tr>
<td>Il-6</td>
<td>5'-TAGTCCTCTACCCCCTATTTCC-3'</td>
<td>5'-TTGGTCCTTAGCCACTCTCCCT-3'</td>
</tr>
<tr>
<td>Il-10</td>
<td>5'-GCTCTTACTGACTGCGCATGAG-3'</td>
<td>5'-CGCAGCTCTAGGACATGTG-3'</td>
</tr>
<tr>
<td>Actb</td>
<td>5'-GTGGGACATCGATGAAACTACA-3'</td>
<td>5'-CTCATCGTACTCCTGCTTG-3'</td>
</tr>
<tr>
<td>Ipmk</td>
<td>5'-TGAAGATTGGCGGGAAGAGC-3'</td>
<td>5'-GCCATTTGTGAAAACCTTGG-3'</td>
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<td>5'-CGCACTTCCCCTGTCTACA-3'</td>
<td>5'-CCGAGCTTGTGCAAGGAG-3'</td>
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<td>5'-ACCTGCGAGTCTCCATGAT-3'</td>
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**Figure 3.** Primers for real-time PCR
Bioscience, 559619) according to manufacturer's protocol. For CFSE assay, B cells were purified from spleen using SH800 Cell Sorter (Sony Biotechnology Inc.) at the Institute of Molecular Biology and Genetics at Seoul National University. Purified B cells were labeled with CellTrace™ CFSE Proliferation Kit (Invitrogen), then cultured for indicated times with LPS (5 µg/ml), anti-IgM (10 µg/ml) or IL-4 (20 ng/ml).

**Western blot**

Cell lysates were extracted using RIPA buffer (150mM NaCl, 1mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 25mM Tris-HCl pH 7.6) supplemented with protease inhibitor cocktail (Roche). Cell lysates were loaded and separated within SDS-PAGE gels, and transferred to Immobilon PVDF transfer membranes (Merck). Immunoblotting was performed with indicated antibodies: anti-TFAF6 (abcam, ab33915), anti-IPMK (kindly gifted by S. K.).

**Statistical analyses**

The statistical significance was assessed by two-tailed Student's t test using Prism 7 (GraphPad software). The P-values are represented in individual figure legends by ns (non-significant, P > 0.05) or asterisks (*P
Synthesis of cell permeable InsP6

The synthesis and the verification of cell permeable InsP6 was conducted by Henning J. Jessen and his colleagues (Institute of Organic Chemistry, University of Freiburg, Germany).

1. General information

Reactions were carried out using oven-dried glassware under an atmosphere of dry N2 and magnetically stirred, unless noted otherwise. Air- and moisture-sensitive liquids and solutions were transferred via syringe or stainless steel canula.

Reagents were purchased from commercial suppliers (Acros, Aldrich, Fluka, TCI) and used without further purification, unless noted otherwise.

Solvents (methylene chloride, diethyl ether, tetrahydrofuran, acetonitrile, toluene) for reactions were purified by filtration and dried by passage over activated anhydrous neutral A-2 alumina (MBraun solvent purification system) under an atmosphere of dry nitrogen. Analytical grade solvents were used as received for extractions and chromatographic purifications.

Deuterated solvents were obtained from Armar Chemicals, Switzerland, in the indicated purity grade.

Thin Layer Chromatography were used for monitoring reactions and
carried out using Merck silica gel 60 F254 plates, visualized with UV light or developed either with phosphormolybdic acid solution or with potassium permanganate solution followed by heating.

**Flash Chromatography** was performed using Fluka silica gel 60 (230-400 Mesh) at a pressure of ca. 0.3 bar. Eluents and Rf are indicated.

**Lyophilizations** were performed on a Christ Freeze Dryer Alpha 1-2 LD+.

**1H-NMR** spectra were recorded on Bruker 400 MHz spectrometers or Bruker 500 MHz spectrometers (equipped with a cryo platform) at 298K in the indicated deuterated solvent. Data are reported as follow: chemical shift (δ, ppm), multiplicity (s, singulet; d, doublet; t, triplet; q, quartet; m, multiplet or not resolved signal; br, broad signal), coupling constant(s) (J, Hz), integration. All signals were referenced to the internal solvent signal as standard (CDCl₃, δ 7.26; CD₃OD, δ 3.31; DMSO-d₆, δ 2.50).

**13C-NMR** spectra were recorded with ¹H-decoupling on Bruker 101 MHz or Bruker 125 MHz spectrometers (equipped with a cryo platform) at 298K in the indicated deuterated solvent. All signals were referenced to the internal solvent signal as standard (CDCl₃, δ 77.0; CD₃OD, δ 49.0; DMSO-d₆, δ 39.5).

**31P-NMR** spectra were recorded with proton coupling and 1H-decoupling on Bruker 162 MHz or Bruker 202 MHz spectrometers (equipped with a cryo platform) at 298K in the indicated deuterated solvent.
All signals were referenced to an internal standard (PPP).

**IR** spectra were recorded on a JASCO FT-IR-4100 spectrometer and data are reported in terms of frequency of absorption (cm⁻¹).

**Mass spectra** were recorded by the Mass spectroscopy Service of UZH on Finnigan MAT95 MS, Bruker EsquireLC MS, Bruker maXis QToF HR MS and Finnigan TSQ700 MS machines.

2. Experimental procedures (Figure 4)

Synthesis of **1** (Figure 5). The compound was synthesized as described before in three steps starting from *myo*-inositol. Analytical data were identical with the values reported in the literature (Capolicchio et al., 2013).

Synthesis of **2** (Figure 6). The compound was synthesized as described before in three steps. Analytical data were identical with the values reported in the literature (Jessen et al., 2008).

Synthesis of **4** (Figure 7). A) 500 mg (0.78 mmol, 1.0 eq.) **1** and 477 mg (1.03 mmol, 1.3 eq) acyloxybenzyl phosphoramidite **2** were dissolved in dry MeCN (5 mL). 131 mg (1.11 mmol, 1.4 eq) of DCI were added and the mixture was stirred for 15 minutes at room temperature. Progress of the reaction was monitored by $^{31}$P-NMR. Oxidation was achieved by addition of 0.353 mL (3.90 mmol, 5.0 eq) $t$-BuOOH. The solvent was evaporated and the obtained oil **3** was directly used in the next step.

B) The intermediate was dissolved in DCM (25 mL) and 5% of TFA (0.75
ml) and stirred for 3 h at room temperature. After completion of the reaction, the solvent was concentrated in vacuo and the residue was crystalized from Et₂O and MeOH multiple times to obtain the pure product 4 as colorless crystals (90 mg, 22%).

**¹H-NMR** (Figure 8) (500 MHz, 298K, DMSO-d₆, δ/ppm): 7.38 (d, J = 7.0 Hz, 4H), 7.07 (d, J = 5.0 Hz, 4H), 5.17- 4.99 (m, 4H), 4.97 (s, 1H), 4.59 (s, 2H), 4.03- 3.89 (m, 1H), 3.75- 3.66 (m, 2H), 3.59 (s, 5H), 2.22 (s, 6H); **¹³C-NMR** (Figure 9) (126 MHz, 298K, DMSO-d₆, δ/ppm) 169.58, 150.70, 134.74, 129.46, 122.16, 83.91, 72.88, 71.86, 68.03, 40.57, 40.40, 40.24, 40.07, 39.90, 39.73, 39.57, 21.29; **³¹P{¹H}NMR** (Figure 10) (203 MHz, 298K, DMSO-d₆, δ/ppm): -1.53; **³¹P-NMR** (Figure 11) (203 MHz, 298K, DMSO-d₆, δ/ppm): -1.52 (d, J = 11.7 Hz); **IR** (neat, cm⁻¹): 3442.3, 3326.6, 2361.4, 1747.2, 1508.1, 1212.0, 1010.15, 918.0, 705.7 **Rf** (DCM: MeOH; 1:1) 0.35; **HRMS** (ESI) calcd 579.1238 for C₂₄H₂₉NaO₁₃P [(M+Na)⁺], found 579.1237 (Figure 12).

Synthesis of 5 (Figure 13). 30 mg (54.9 μmol, 1.0 eq.) of inositol monophosphate 4 and 249 mg (0.55 mmol, 10 eq.) acyloxybenzyl phosphoramidite 2 were coevaporated with dry acetonitrile (2 mL). The residue was dissolved in dry MeCN (2 mL). To this solution, 64 mg (0.549 mmol, 10 eq.) DCI were added. Progress of the reaction was monitored by **³¹P-NMR.** After completion of the reaction (30 min), oxidation was achieved by slow (!) addition of 172 mg (0.55 mmol, 10 eq.) mCPBA (70%
moistened with water) at 0°C. The reaction mixture was concentrated in vacuo. The product was purified by FC twice (gradient: EtOAc to EtOAc/MeOH 20:1) yielding 70 mg of 5 as a colorless oil (28.7 μmol, 53 %).

1H-NMR (Figure 14) (400 MHz, 298K, CDCl₃, δ/ppm): 7.35- 7.13 (m, 26H), 7.03- 6.92 (m, 22H), 5.70 (d, J = 9.0 Hz, 1H), 5.13- 4.88 (m, 24H), 4.41 (t, J = 9.3 Hz, 2H), 4.25 (q, J = 9.8 Hz, 1H), 4.16 (q, J = 7.1 Hz, 2H), 2.36- 2.25 (m, 36H); 13C NMR (Figure 15) (101 MHz, 298K, CDCl₃, δ/ppm): 169.31- 169.16 (m), 150.91- 150.76 (m), 140.47, 132.88, 132.81, 129.59- 129.14 (m), 121.88- 121.76 (m), 77.35, 77.23, 77.03, 76.71, 74.73, 73.13, 69.45, 69.34, 69.29, 60.42, 53.49, 50.81, 21.09, 21.06, 14.20; 31P{1H}NMR (Figure 16) (162 MHz, 298K, CDCl₃, δ/ppm): 0.22 , 0.02 , -0.68 , -1.57; 31P NMR (Figure 17) (162 MHz, 298K, CDCl₃, δ/ppm): 0.49-0.21 (m), -0.68 (h, J = 8.2 Hz), -1.57 (d, J = 9.2 Hz); IR (neat, cm⁻¹): 2956.3, 2331.5, 1757.8, 1509.2, 1370.2, 1216.9, 1194.7, 1006.7; Rf (SiO₂, EtOAc): 0.2; HRMS (ESI) calcd for 1241.7362 C₁₁₄H₁₁₄Na₂O₄₈P₆ [(M+2Na)²⁺], found 1241.7360 (Figure 18).
Figure 4. Experimental procedures for synthesis of cell permeable InsP₆.
Figure 5. Structural formula of compound 1.
Figure 6. Structural formula of compound 2.
Figure 7. Structural formula of compound 4.
Figure 8. $^1$H-NMR spectra of compound 4.
Figure 9. $^{13}$C-NMR spectra of compound 4.
Figure 10. $^{31}P\{^1H\}$NMR spectra of compound 4.
Figure 11. $^{31}$P-NMR spectra of compound 4.
Figure 12. Mass spectra of compound 4.
Figure 13. Structural formula of compound 5.
Figure 14. $^1$H-NMR spectra of compound 5.
Figure 15. $^{13}$C NMR spectra of compound 5.
Figure 16. $^{31}$P{¹H} NMR spectra of compound 5.
Figure 17. $^{31}$P NMR spectra of compound 5.
Figure 18. Mass spectra of compound 5.
3. AB cleavage and cellular uptake of cell permeable InsP\textsubscript{6} in HeLa cells.

To identify the conversion of (AB)\textsubscript{12}-InsP\textsubscript{6} into InsP\textsubscript{6} by enzymatic hydrolysis, cell permeable InsP\textsubscript{6} was incubated in freshly prepared HeLa cell extracts. Then, \textit{in vitro} reactions were resolved by polyacrylamide gel electrophoresis (Losito et al., 2009) (Figure 19A). In addition, the ability of cell permeable InsP\textsubscript{6} to enter intact HeLa cells and to release InsP\textsubscript{6} was assessed (Wilson et al., 2015) (Figure 19B). All experiments were conducted according to a previous study with InsP\textsubscript{7} prometabolites (Hauke et al., 2019; Pavlovic et al., 2015). Areas under the curve of each samples (A\textsubscript{S}) and corresponding Orange G dye (A\textsubscript{OGD}) were calculated by Image J. The normalized ratios of areas (A\textsubscript{S}/A\textsubscript{OGD}) were plotted against the applied concentrations of cell permeable InsP\textsubscript{6} (Figure 20).
Figure 19. AB cleavage and cellular uptake of cell permeable InsP₆.

(A) Experiment 1 (Lane 1–5) and experiment 2 (Lane 6–10) for AB cleavage. Lane 1, 10: InsP₆ (standard). Lane 2, 6: InsP₆ (standard) in cell extract. Lane 3, 7: cell extract only. Lane 4, 8: incubation of 0.33 mM cell permeable InsP₆ with cell extract for 15 min. Lane 5, 9: incubation of 0.66 mM cell permeable InsP₆ with cell extract for 15 min. (B) Experiment 3 (Lane 1–4) and experiment 4 (Lane 5–8) for cellular uptake. Lane 4, 8: InsP₆ (standard). Lane 3, 5: extract from intact cells treated with DMSO for 24 hr. Lane 1, 6: extract from intact cells treated with 30 μM cell permeable InsP₆ for 24 hr. Lane 2, 7: extract from intact cells treated with 60 μM cell permeable InsP₆ for 24 hr.
Figure 20. Plots of the normalized $A_S/A_{OGD}$ for AB cleavage and cellular uptake against the applied concentrations of $(AB)_{12}$-InsP$_6$.

Areas under the curve of each samples ($A_S$) and corresponding Orange G dye ($A_{OGD}$) were calculated. All data are presented as mean ± S.E.M.
Results

III-1. 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$^{\text{Cre}}$; IPMK$^{\text{flox/}}$ (IPMK cKO) and CD19$^{\text{Cre}}$; IPMK$^{+/}$ or IPMK$^{+/}$ (control) mice. The results confirmed that IPMK was efficiently depleted in B cells of the IPMK cKO mice (Figure 21). The analysis of B cell development in the bone marrow revealed that IPMK cKO mice displayed no significant alteration, although CD19$^{\text{Cre}}$-mediated ablation of IPMK occurs during early stages of B cell development (Figure 22). The frequency and cellularity of B cells in the spleen were also similar between IPMK cKO and control mice (Figure 23), 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 (Figure 24). CD19-Cre also induces
IPMK deletion in B-1 B cells, mainly located in the peritoneal cavity. However, I did not observe any defects in B-1 B cell population in IPMK cKO mice (Figure 25). Thus, IPMK appears to be dispensable for B cell development in the periphery and the bone marrow.
Figure 21. Efficient deletion of IPMK is observed in B cells of IPMK cKO mice

Western blot for analyzing the IPMK protein levels in splenic B cells purified from control and IPMK cKO mice.
Figure 22. The B cell development in the bone marrow is normal in IPMK cKO mice

Flow cytometry staining for the pro/pre-B cells (IgM⁻ B220⁺), immature B cells (IgM⁺ B220⁺) and mature recirculating B cells (IgM⁺ B220^{high}) in bone marrow.
Figure 23. The B cell development in the spleen is normal in IPMK cKO mice

Flow cytometry staining of B cells (B220⁺) in spleen (left). Cell numbers and percentages (n=5 mice (control) or n=8 mice (IPMK cKO)) of B220⁺ cells in spleen (right). All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05).
Cells

Control

IPMK cKO

B220

53.1

50.6

Control

IPMK cKO

B220+ cells (x10⁷)

ns

B220+ cells (%)

ns
Figure 24. The generation of peripheral mature B cells is normal in IPMK cKO mice

(A) Flow cytometry staining of splenic B cells for follicular B cells (CD23$^{+}$ CD21$^{\text{low}}$) and marginal zone B cells (CD23$^{-}$ CD21$^{\text{high}}$) among B220$^{+}$ cells. 
(B) 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. All data are presented as mean ± S.E.M. Student's $t$ test was used to calculate $P$ values. ns, not significant ($P > 0.05$).
A

Gated on B220^+

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IPMK cKO</th>
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<tbody>
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<td>CD21</td>
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<td>3.86</td>
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B

<table>
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<th>Follicular B cells (%)</th>
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<td>IPMK cKO</td>
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</table>
Figure 25. The development of B-1 B cells in peritoneal cavity is normal in IPMK cKO mice

(A) Flow cytometry staining of B-1 B cells (CD19+ CD5+) in peritoneal cavity. (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 ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05).
A

Control

IPMK cKO

CD5

CD19

40

42.1

B

B-1 B cells (x10^5)

ns

B-1 B cells (%)

ns

Control

IPMK cKO

Control

IPMK cKO
III-2. IPMK cKO mice display defects in TI immune responses

Given that InsPs including InsP₃ and InsP₄ are robustly synthesized in activated B cells (Antony et al., 2007) and that IPMK is the only enzyme forming Ins(1,3,4,5,6)P₅ and, thus, InsP₆ from InsP₄, I 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. I noticed that both B cell frequency (74% vs. 66.9% in control and IPMK cKO, respectively) and cell number (5.5 × 10⁷ vs. 3.6 × 10⁷) in the spleen were significantly reduced in IPMK cKO mice compared with those in the control mice (Figure 26). In addition, IPMK cKO mice failed to produce IgM⁺ plasma cells in response to LPS immunization (Figure 27). Both the frequency (2% vs. 0.36%) and number (1.2 × 10⁶ vs. 0.18 × 10⁶) of IgM⁺ plasma cells were greatly reduced in IPMK cKO mice when 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 (Otipoby et al., 2015; Pone et al., 2012; Schweighoffer et al., 2017), I 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. I 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, I analyzed splenic B cells responding specifically to NP-Ficoll. The frequency (0.18% vs. 0.1%) and cellularity (10.2 × 10⁴ vs. 4.43 × 10⁴) of NP⁺ B cells in the spleen were considerably reduced in IPMK cKO mice when compared with those in the control mice (Figure 28). IPMK cKO mice also showed decreased frequency (0.078% vs. 0.034%) and cellularity (4.4 × 10⁴ vs. 1.77 × 10⁴) of plasma cells expressing NP-specific immunoglobulins (Figure 29). Overall, these results clearly show that IPMK cKO mice are defective in B cell responses against TI antigens, in which BCR signaling is involved.
Figure 26. IPMK cKO mice show the reduced numbers of B cells in immune responses against TI type I antigens

Flow cytometry staining of splenic B cells (B220+) 2 days 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). All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ***P < 0.001.
LPS immunization, Day 3

Control

IPMK cKO

B220+

Cells

75.7

63.6

B220

Cells

B220+ cells (x10^7)

Control

IPMK cKO

B220+ cells (%)

Control

IPMK cKO

***

***
Figure 27. IPMK cKO mice show defects in the generation of IgM$^+$ plasma cells against TI type I antigens

Flow cytometry staining of plasma cells (IgM$^+$ CD138$^+$) as in A (upper). Cell numbers and percentages (n=7 mice (control) or n=8 mice (IPMK cKO)) of IgM$^+$ CD138$^+$ cells in spleen (lower). All data are presented as mean $\pm$ S.E.M. Student's $t$ test was used to calculate $P$ values. $***P < 0.001$. 
LPS immunization, Day 3

Control

IPMK cKO

**2.49**

**0.35**

IgM^+ CD138^+ cells (x10^6)

IgM^+ CD138^+ cells (%)

---

Control

IPMK cKO

***

***
Figure 28. IPMK cKO mice show the reduced numbers of NP-specific B cells in immune responses against TI type II antigens

Flow cytometry staining of NP-reactive B cells (B220\text{low} NP^+) in spleen 3 days after NP-Ficoll immunization (upper). Cell numbers and percentages (n=3 mice (control) or n=4 mice (IPMK cKO)) of B220\text{low} NP^+ in spleen (lower). All data are presented as mean ± S.E.M. Student's $t$ test was used to calculate $P$ values. **$P < 0.01$. 
NP-Ficoll immunization, Day 4

Control

IPMK cKO

B220^low NP^+ cells (%)

Control

IPMK cKO

B220^low NP^+ cells (x10^4)

Control

IPMK cKO

**
Figure 29. IPMK cKO mice show defects in the generation of NP-specific plasma cells against TI type II antigens

Flow cytometry staining of NP-reactive plasma cells (CD138$^+$ NP$^+$) as in C (upper). Cell numbers and percentages (n=3 mice per genotype) of CD138$^+$ NP$^+$ cells in spleen (lower). All data are presented as mean ± S.E.M. Student's $t$ test was used to calculate $P$ values. **$P < 0.01$. 


NP-Ficoll immunization, Day 4

Control | IPMK cKO
--- | ---
0.083 | 0.043

CD138

NP

CD138+ NP+ cells (x10^4)

Control | IPMK cKO
--- | ---

CD138+ NP+ cells (%)

Control | IPMK cKO
--- | ---

**
III-3. 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, I carried out the BrdU incorporation assay \textit{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%) (Figure 30). It is notable that IgM$^{\text{high}}$IgD$^{\text{low}}$ B cells comprising mostly MZ B cells are labeled by BrdU more efficiently than Fo B cell-enriched IgM$^{\text{low}}$IgD$^{\text{high}}$ cells (Figure 31). The effect of IPMK deficiency on the decrease in BrdU$^+$ cells was more severe in IgM$^{\text{high}}$IgD$^{\text{low}}$ cells (6.6-fold decrease) than in IgM$^{\text{low}}$IgD$^{\text{high}}$ cells (3.5-fold decrease). Lipopolysaccharide challenge significantly increased the number of IgM$^{\text{high}}$IgD$^{\text{low}}$ cells and IgM$^{\text{low}}$IgD$^{\text{high}}$ cells in the control mice (Figure 32). However, this LPS-stimulated elevation in the number of IgM$^{\text{high}}$IgD$^{\text{low}}$ B cells was markedly blunted in IPMK cKO mice (Figure 32 left). Similarly, the number of IgM$^{\text{low}}$IgD$^{\text{high}}$ B cells in response to LPS was not increased in IPMK cKO mice, compared with that in the control mice (Figure 32 right).

Further, I assessed the defects in proliferation against TI antigens directly, by staining purified splenic B cells with CFSE and culturing them \textit{in vitro} with LPS or anti-IgM. B cells from IPMK cKO mice displayed significantly
reduced proliferation in response to both stimuli when compared with that of B cells from control mice (Figure 33). These results collectively suggest that IPMK is essential for B cell proliferation during the response to TI antigens.
Figure 30. IPMK-deficient B cells show a decrease in proliferation induced by TI antigens

Flow cytometry analyzing the proportion of BrdU$^+$ cells gated on B220$^+$ in spleen 2 days after daily injection with LPS and BrdU (n=4 mice (control) or n=3 mice (IPMK cKO)). All data are presented as mean ± S.E.M. Student's $t$ test was used to calculate $P$ values. **$P < 0.01$; ***$P < 0.001$. 
Figure 31. B cell subsets of IPMK cKO mice show a decrease in proliferation induced by TI antigens

Flow cytometry analyzing the proportion of BrdU-incorporated cells in IgM$^\text{high}$ IgD$^\text{low}$ B cells and IgM$^\text{low}$ IgD$^\text{high}$ B cells gated on B220$^+$ as in A. All data are presented as mean ± S.E.M. Student's $t$ test was used to calculate $P$ values. **$P < 0.01$; ***$P < 0.001$. 

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>IPMK cKO</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM$^\text{high}$ IgD$^\text{low}$</td>
<td>39.8</td>
<td>6.03</td>
</tr>
<tr>
<td>IgM$^\text{low}$ IgD$^\text{high}$</td>
<td>5.46</td>
<td>1.57</td>
</tr>
</tbody>
</table>
Figure 32. Cell numbers of B cell subsets in IPMK cKO mice are unaltered in response to LPS

Cell numbers of IgM$^{\text{high}}$ IgD$^{\text{low}}$ B cells (left) and IgM$^{\text{low}}$ IgD$^{\text{high}}$ B cells (right) in spleen at steady state or 2 days 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). All data are presented as mean ± S.E.M. Student's $t$ test was used to calculate $P$ values. **$P < 0.01$; ***$P < 0.001$. 
Figure 33. IPMK-deficient B cells show a decrease in *in vitro* proliferation upon stimulation with TI antigens

Flow cytometry analyzing CFSE-labeled B cells left unstimulated (shaded histograms) or stimulated with LPS (5 µg/ml) for 2 days or anti-IgM (10 µg/ml) for 3 days in the presence of IL-4 (20 ng/ml). All data are presented as mean ± S.E.M. Student's *t* test was used to calculate *P* values. **P < 0.01; ***P < 0.001.
III-4. IPMK cKO mice display a normal response to TD antigens

To evaluate whether IPMK mediates TD immune response, I immunized mice with 4-hydroxy-3-nitrophenyl-acetyl conjugated to keyhole limpet hemocyanin (NP-KLH). Immunization with TD antigens results in the formation of GC and generation of short-lived plasma cells within 5–7 d in the spleen. I observed that the production of GC B cells (B220\(^+\) GL7\(^+\) Fas\(^+\)) and plasma cells (IgD\(^{low}\) CD138\(^+\)) was similar in the spleen of IPMK cKO and control mice (Figure 34). In addition, 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 also similar between IPMK cKO and control mice (Figure 35). Thus, B cell-specific ablation of IPMK do not seem to cause any significant defects in immune responses against the TD antigens.
Figure 34. The formation of germinal center and the differentiation of plasma cells are unaltered in spleen 6 days after immunization with NP-KLH.

(A) Flow cytometry staining (left), and percentages and cell numbers (right) of GC B cells (GL7⁺ Fas⁺) gated on B220⁺. (B) Flow cytometry staining (left), and percentages and cell numbers (right) of plasma cells (IgD⁻ CD138⁺) in spleen. All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05).
Figure 35. The immune response to TD antigens remains unchanged in IPMK cKO mice

Flow cytometry analysis of spleen 6 days after immunization with NP-KLH. (A) Flow cytometry staining of NP-reactive B cells in spleen. Analysis of plasma cells (B220^{low} CD138^{+}) and GC B cells (B220^{+} CD138^{-} GL7^{+}) gated on CD4^{-} CD8^{α^{-}} IgD^{-} NP^{+}. (B) Cell numbers and percentages of NP-specific plasma cells (n=3 mice per genotype) and GC B cells (n=4 mice per genotype) as in A. All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05).
A

<table>
<thead>
<tr>
<th></th>
<th>Unimmunized</th>
<th>Immunized with NP-KLH, Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; CD8α&lt;sup&gt;+&lt;/sup&gt;</td>
<td>NP&lt;sup&gt;+&lt;/sup&gt; IgD&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.021</td>
<td>0.48</td>
</tr>
<tr>
<td>IPMK cKO</td>
<td>0.32</td>
<td>21.7</td>
</tr>
</tbody>
</table>

|                  | CD138<sup>-</sup> B220<sup>+</sup> |
|                  |                                     |
| Control          |                                       |
| IPMK cKO         | 62.4                                   |

B

- **Unimmunized**
  - NP<sup>+</sup> plasma cells (x10<sup>4</sup>): Control, 2.0 ± 0.3; IPMK cKO, 1.8 ± 0.2
  - NP<sup>+</sup> GC B cells (x10<sup>4</sup>): Control, 6.5 ± 0.5; IPMK cKO, 7.0 ± 0.6

- **Immunized with NP-KLH, Day 7**
  - NP<sup>+</sup> plasma cells (%): Control, 0.0 ± 0.1; IPMK cKO, 0.0 ± 0.1
  - NP<sup>+</sup> GC B cells (%): Control, 60.0 ± 5.0; IPMK cKO, 65.0 ± 5.0

*ns* indicates no significant difference.
III-5. 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 (Figure 36). Both BCR and TLR4 signaling activate the NF-κB pathway, which induces the expression of genes such as *c-myc* (Grumont et al., 2002; Meyer-Bahlburg et al., 2009) and *Irf4* (Grumont and Gerondakis, 2000; Shaffer et al., 2008) (Figure 37). 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 (Schweighoffer et al., 2017), I evaluated whether LPS-induced cytokine production is also affected by deficiency of IPMK in splenic B cells. I found that IPMK-deficient B cells showed substantially decreased production of these cytokines (Figure 38 and 39). It is also known that BCR signaling enhances IL-10 production in LPS-stimulated B cells (Matsumoto et al., 2011; Schweighoffer et al., 2017); however, it was considerably less in IPMK-deficient B cells than that in the control (Figure 39). It seems that the impaired responses to TI antigens is due to hypo-responsiveness to TLR4 and BCR signaling in IPMK-deficient B cells.
Figure 36. The expression of Ipmk is induced in activated B cells

Level of mRNA for Ipmk (n=3 per each time point) in purified B cells stimulated for indicated times with LPS (10 µg/ml) or anti-IgM (10 µg/ml).
Figure 37. Gene expression induced by TLR and BCR stimuli is diminished in IPMK-deficient B cells

Quantitative RT-PCR analysis of indicated genes in purified B cells cultured for 4 hr with anti-IgM (n=4 mice per genotype) and LPS (n=3 mice per genotype). All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. *P < 0.05; **P < 0.01; ***P < 0.001. AU, arbitrary unit.
Figure 38. Expression of proinflammatory cytokines induced by TLR stimuli is diminished in IPMK-deficient B cells

Quantitative RT-PCR analysis of proinflammatory cytokines (n=4 mice per genotype) in purified B cells cultured for 2 days in the presence of LPS (10 µg/ml). All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. *P < 0.05; **P < 0.01; ***P < 0.001. AU, arbitrary unit.
**Figure 39. Production of *Il-10* induced by TLR and BCR stimuli is diminished in IPMK-deficient B cells**

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 hr after stimulation with anti-IgM. All data are presented as mean ± S.E.M. Student's *t* test was used to calculate *P* values.

*P* < 0.05; **P* < 0.01; ***P* < 0.001. AU, arbitrary unit.
III-6. 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 (Jumaa et al., 1999; Wang et al., 2000; Yamazaki et al., 2002). To investigate whether IPMK is required for BCR signal transduction, I assessed the phosphorylation patterns of various components in BCR signaling after stimulating splenic Fo B cells and MZ B cells with anti-IgM. I could not detect any abnormality in the phosphorylation of Syk, Akt, and S6, which are involved in the early stage of BCR signaling (Figure 40). I also examined the TRAF6 protein levels, which were found to be downregulated in IPMK-deficient macrophages (Kim et al., 2017b), but found no changes when IPMK was deleted in B cells (Figure 41).

As it has been reported that the Btk activity can be regulated by InsP_{6} (Wang et al., 2015), and the defects in B cell response to TI antigens observed in IPMK cKO mice were similar to the phenotypes of Btk-deficient mice (Ellmeier et al., 2000), I postulated that the activity of Btk in the BCR signaling pathway might be defective in IPMK-deficient B cells. Thus, I 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 (Figure 42). The phosphorylation of PLCγ2, a Btk
target protein, was reduced significantly in both subsets from IPMK cKO mice when compared with that from the control mice (Figure 43). As BCR-mediated activation of PLCγ2 triggers calcium mobilization to increase cytoplasmic calcium concentration, I accordingly confirmed that calcium influx was conspicuously diminished in IPMK-deficient B cells after stimulation with anti-IgM (Figure 44). As TLR4 signal is dependent on BCR signaling, it is likely that the phosphorylation of Btk and PLCγ2 induced by LPS is defective in IPMK-deficient B cells. To analyze the response to polysaccharide in B cells, I utilized dextran-conjugated anti-immunoglobulin antibodies (anti-IgD dextran), an analogue of polysaccharide (Pone et al., 2012; Vos et al., 2000). As expected, the calcium mobilization induced by anti-IgD dextran was significantly reduced in IPMK-deficient B cells, compared with that of normal B cells (Figure 45). Meanwhile, when increases in intracellular 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 (Figure 46). These results reveal that the deficiency of IPMK in B cells results in abnormal BCR signaling, most likely due to impaired activation of Btk.
Figure 40. The phosphorylation of signaling molecules involved in TLR and BCR signaling is unaffected in IPMK-deficient B cells

(A) Following stimulation with anti-IgM (10 µg/ml) for 3 min, flow cytometry staining for phospho-Syk (Y352) (left) and phosphor-Akt (T308) (right) in Fo B cells (B220⁺ IgD<sup>high</sup> CD21<sup>low</sup>) and MZ B cells (B220⁺ IgD<sup>low</sup> CD21<sup>high</sup>) in spleen. (B) Following stimulation with LPS (10 µg/ml) or anti-IgM (10 µg/ml) for indicated time, flow cytometry staining for phospho-S6 (S235/S236) in Fo B cells (B220⁺ CD23⁺ CD21<sup>low</sup>) and MZ B cells (B220⁺ CD23⁻ CD21<sup>high</sup>) in spleen.
A

MZ

F0

pAkt (T308)

Cells

MZ

F0

pSyk (Y352)

Cells

B

MZ

F0

anti-IgM, 10 min

MZ

F0

LPS, 1 hr

MZ

F0

ps6 (S235/S236)

Cells
Figure 41. TRAF6 protein levels are unchanged in splenic B cells form IPMK cKO

Western blot for analyzing the TRAF6 protein levels in splenic B cells purified from control and IPMK cKO mice.
Figure 42. The activation of Btk is compromised in IPMK-deficient B cells

(A) Following stimulation with anti-IgM (10 µg/ml) for 3 min, flow cytometry staining for phospho-Btk (Y551) in Fo B Cells (B220^+ IgD^{high} CD21^{low}) and MZ B cells (B220^+ IgD^{low} CD21^{high}) in spleen. Numbers are MFI of indicated populations. (B) Relative mean fluorescence intensity (MFI) of phospho-Btk (Y551) in Fo B cells and MZ B cells at indicated time (n=4 mice per genotype). All data are presented as mean ± S.E.M. Student’s t test was used to calculate P values. ns, not significant (P > 0.05); **P < 0.01.
A

Fo

MZ

Cells

pBtk (Y551)

Unstim.
Control
IPMK cKO

B

Fo

MZ

Relative MFI of pBtk

Time (min)

ns
ns
ns

ns
ns
ns

ns
ns

ns

**

Control
IPMK cKO
Figure 43. The phosphorylation of PLCγ2 is decreased in IPMK-deficient B cells

(A) Following stimulation with anti-IgM (10 µg/ml) for 3 min, flow cytometry staining for phospho-PLCγ2 (Y759) in Fo B Cells (B220+ IgD^{high} CD21^{low}) and MZ B cells (B220+ IgD^{low} CD21^{high}) in spleen. Numbers are MFI of indicated populations. (B) Relative mean fluorescence intensity (MFI) of phospho-PLCγ2 (Y759) in Fo B cells and MZ B cells at indicated time (n=4 mice per genotype). Numbers are MFI of indicated populations. All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05); **P < 0.01; ***P < 0.001.
A

Fo

MZ

Cells

pPLCγ2 (Y759)

Unstim.
Control
IPMK cKO

B

Fo

MZ

Relative MFI of pPLCγ2

Time (min)

Control
IPMK cKO

ns

**

***

ns
Figure 44. Calcium mobilization in IPMK-deficient B cells is compromised upon stimulation with anti-IgM

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). Numbers are MFI of indicated populations. All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05); **P < 0.01; ***P < 0.001.
Figure 45. Calcium mobilization in IPMK-deficient B cells is compromised upon stimulation with anti-IgD dextran, an analogue of polysaccharide

Profile of Ca$^{2+}$ mobilization of purified splenic B cells (B220$^+$) induced by stimulation with anti-IgD dextran (500 ng/ml).
Figure 46. Gene expression of IPMK-deficient B cells in response to LPS and anti-IgM were fully restored by treatment with calcium ionophore (A, B) Quantitative RT-PCR analysis of indicated genes in purified B cells cultured with or without ionomycin (50 ng/ml) for 4 hr in the presence of anti-IgM (10 μg/ml) and LPS (10 μg/ml). (C) Quantitative RT-PCR analysis of proinflammatory cytokines in purified B cells cultured with or without ionomycin (50 ng/ml) for 2 days in the presence of LPS (10 μg/ml). (D) Purified B cells were cultured with LPS (10 μg/ml) for 2 days, followed by stimulation with anti-IgM (10 μg/ml). Quantification of Il-10 mRNA at 3 hr after stimulation with or without ionomycin (500 ng/ml) in the presence of anti-IgM. All data are presented as mean ± S.E.M. Student's t test was used to calculate P values. ns, not significant (P > 0.05); *P < 0.05; **P < 0.01; ***P < 0.001. AU, arbitrary unit.
A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)

D

![Graph D](image)
III-7. Inositol hexakisphosphate is required for the Btk activity

I 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 $[^3H]$myo-inositol, and the 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 (Figure 47). In IPMK-deficient B cells, higher order InsPs comprising InsP$_5$, InsP$_6$, and InsP$_7$ were greatly reduced (Figure 48), which was expected because IPMK regulates the synthesis of InsP$_5$ from InsP$_4$.

To confirm that BCR signaling requires higher order InsPs, I 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$_6$ (Chen et al., 2015; Hauke et al., 2019; Pavlovic et al., 2015), and then the phosphorylation of Btk and PLC$\gamma_2$ was measured. The treatment with cell permeable InsP$_6$ only resulted in substantial restoration of phosphorylation of both Btk and PLC$\gamma_2$ after stimulation with anti-IgM (Figure 49 and Figure 50) or anti-IgD dextran (Figure 51), indicating that Btk is efficiently activated. However, InsP$_6$-mediated increments in the phosphorylation of Btk and PLC$\gamma_2$ were inhibited by Btk inhibitor, ibrutinib (Figure 52). Thus, our results clearly indicate that InsP$_6$ plays an essential role in BCR signaling by modulating the Btk activity.
Figure 47. Level of higher-order InsPs increases in LPS-stimulated B cells

HPLC elution profiles of soluble InsPs extracted from B cells radiolabeled in the absence or presence of LPS (10 μg/ml). D.P.M., disintegrations per minute.
Figure 48. Production of higher-order InsPs are reduced in IPMK-deficient B cells

Figure 49. Cell permeable InsP₆ restores the activity of Btk and PLCγ2 upon anti-IgM stimulation

Following stimulation with anti-IgM (10 µg/ml) in the presence of DMSO or cell permeable InsP₆ (80 µM), flow cytometry staining of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in Fo B cells (B220⁺ IgDhigh CD21low) and MZ B cells (B220⁺ IgDlow CD21high). Numbers are MFI of indicated populations.
**Figure 50. the phosphorylation of Btk and PLCγ2 is unaltered in InsP5-treated B cells**

Following stimulation with anti-IgM (10 μg/ml) in the presence of DMSO or cell permeable InsP5 (100 μM), flow cytometry staining of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in follicular B cells (B220+ IgD^{high} CD21^{low}) and marginal zone B cells (B220+ IgD^{low} CD21^{high}). Numbers are MFI of indicated populations.
Figure 51. The lack of InsP₆ in IPMK-deficient B cells leads to the defects in the activation of Btk and PLCγ2 upon anti-IgD dextran stimulation

Following stimulation with anti-IgM (10 μg/ml) or anti-IgD dextran (10 μg/ml) for 5 min in the presence of DMSO or cell permeable InsP₆ (80 μM), flow cytometry staining for phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in purified splenic B cells. Numbers are MFI of indicated populations.
Figure 52. InsP₆-mediated restoration is diminished by the treatment with Btk inhibitor, ibrutinib

Following stimulation with anti-IgM (10 μg/ml) for 3 min in the presence of DMSO or cell permeable InsP₆ (80 μM), flow cytometry staining for phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in marginal zone B cells (B220⁺ IgDlow CD21high) pretreated with DMSO or ibrutinib (75 μM) for 1h. Numbers are MFI of indicated populations.
III-8. 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 (Blanc et al., 2016; Martin et al., 2001). 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 (Blanc et al., 2016; Pinto et al., 2013). As I 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 (Figure 53). These defects in BCR signaling displayed by IPMK-deficient IgM+ plasma cells can be rescued by supplying exogenous cell permeable InsP6 during ex vivo stimulation (Figure 54). Interestingly, LPS immunization induced upregulation of key enzymes responsible for the production of InsP6, such as Ipmk (6.2-fold higher level) and inositol pentakisphosphate 2-kinase (Ippk) (2.7-fold higher level), in IgM+ plasma cells when compared with that in MZ B cells (Figure 55). However, there was no obvious change in other InsP or PtdIP3 regulatory genes (Figure 56). These results suggest that
InsP₆ 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.
Figure 53. IPMK is required for BCR signaling in IgM+ plasma cells

(A) Following stimulation with anti-IgM (10 µg/ml) for 3 min, flow
cytometry staining of phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in
plasma cells (IgD⁻ B220low CD138⁺) obtained from immunized mice with
LPS. Numbers are MFI of indicated populations. (B) Relative MFI of
phospho-Btk (Y551) and phospho-PLCγ2 (Y759) in plasma cells at
indicated time (n=4 mice (control) or n=5 mice (IPMK cKO)). All data are
presented as mean ± S.E.M. Student's t test was used to calculate P values.

**P < 0.01; ***P < 0.001. AU, arbitrary unit.
A

B

** ns

*** ns
Figure 54. Cell permeable InsP₆ restores the activity of Btk and PLCγ₂ in IgM⁺ plasma cells

Following stimulation with anti-IgM (10 µg/ml) in the presence of DMSO or cell permeable InsP₆ (60 µM), flow cytometry staining of phospho-Btk (Y551) and phospho-PLCγ₂ (Y759) in plasma cells (IgD⁻ B220low CD138⁺) as in A. Numbers are MFI of indicated populations.
Figure 55. The expression of *Ipmk* and *Ippk* is increased in IgM⁺ plasma cells

Quantitative RT-PCR of indicated genes (n=3 per each subset) in Fo B cells (B220⁺ CD23⁺ CD21low), MZ B cells (B220⁺ CD23⁻ CD21high) and plasma cells (PC, IgM⁺ CD138⁺). All data are presented as mean ± S.E.M.
Figure 56. The expression of InsP and PtdIP regulator genes in B cell subsets

Quantitative RT-PCR of indicated genes (n=3 per each subset) in follicular B cells (B220$^+$ CD23$^+$ CD21$^{low}$), marginal zone B cells (B220$^+$ CD23$^-$ CD21$^{high}$) and plasma cells (IgM$^+$ CD138$^+$). All data presented as mean ± S.E.M. Numbers are fold change of indicated genes relative to them in follicular B cells.
Discussion

IV-1. Inositol hexakisphosphate is identified as a novel regulator of Btk in B cells

Inositol polyphosphate multikinase exhibits complex catalytic activities that eventually yield water soluble InsPs, such as InsP₄ and InsP₅ (Odom et al., 2000; Saiardi et al., 1999), and lipid bound PtdIP₃ (Maag et al., 2011). In the present study, it has been 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. Inositol polyphosphate multikinase-deficient B cells exhibited reduced activation of Btk and its target protein, PLCγ₂, and diminished calcium influx after stimulation. Higher order InsPs comprising InsP₅, InsP₆, and InsP₇ were greatly reduced in these cells. As InsP₆ is produced by IPPK from InsP₅, which is produced solely by IPMK from InsP₄, the deficiency of IPMK leads to the substantial reduction in InsP₆ level.

In this study, it has shown that cell permeable InsP₆ can successfully restore the signaling defects in IPMK-deficient B cells. Treatment with cell permeable InsP₆ led to an increased phosphorylation of Btk and PLCγ₂, but did not induce any change in the presence of ibrutinib. Thus, these results
strongly indicate that higher order InsPs produced by IPMK are required for Btk activity during B cell responses. It has been shown that constitutively active mutant of Btk binds to InsP₆ with high affinity compared with that of wild type Btk (Fukuda et al., 1996), suggesting a possibility that Btk activity is correlated with InsP₆ binding capacity. It has also been reported that InsP₆ acts as an allosteric regulator to stimulate the dimerization of Btk, which contributes to autophosphorylation (Wang et al., 2015). These results, together with the findings of previous studies, indicate that InsP₆ generated by IPMK modulates Btk activity during B cell immunity \textit{in vivo}.

IV-2. I PMK plays a major role in the production of higher order InsPs in B cells

Inositol metabolic-labeling experiment demonstrated that the appreciable levels of InsP₅ and InsP₆ was detected in IPMK-deficient B cells. It is important to note that in addition to the IPMK-mediated synthesis of InsP₅, an alternative minor pathway is also reported to be involved in the production of InsP₅ (Saiardi et al., 2018; Verbsky et al., 2002). Moreover, earlier studies have also reported low levels of InsP₅ and InsP₆ in the IPMK-null embryonic stem cell (Frederick et al., 2005) and mouse embryonic fibroblast (Kim et al., 2011). In this study, there was an approximately 85 percent reduction in the level of InsP₆ in the IPMK-deficient B cells,
compared with that of the normal B cells. Thus, although there is an alternative pathway for the synthesis of InsP$_5$ in the mammalian cells, IPMK appears to play a major role in the synthesis of InsP$_5$ and InsP$_6$ in the B cells. The residual activity of Btk in the IPMK-deficient splenic B cells and IgM$^+$ plasma cells may be due to the appreciable level of InsP$_6$ observed in these cells.

**IV-3. IPMK is dispensable for the B cell development and TD immune response**

Inositol polyphosphate multikinase 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 (Ellmeier et al., 2000; Khan et al., 1995). Because CD19$^{cre}$-mediated deletion is inefficient in the immature B cells (Hobeika et al., 2006), it might be possible that the residual Btk activity is sufficient for B cell development in IPMK cKO mice. Inositol polyphosphate multikinase-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$_1$ (Ellmeier et al., 2000). 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 (Nyhoff et al., 2018). Additionally, it has been reported that Tec, another member of the same kinase family, has a redundant role with Btk during TD immune response (Ellmeier et al., 2000), and it is possible that Tec activity provided compensatory activation signal for reduced Btk activity to mount TD immune response in IPMK cKO mice.

IV-4. IPMK is essential for TI immune responses, in which BCR signaling is engaged

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. I 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\textsuperscript{high}IgD\textsuperscript{low}) cells than in FO (IgM\textsuperscript{low}IgD\textsuperscript{high}) cells. This is consistent with
The findings of previous studies that MZ B cells respond to LPS more efficiently than FO B cells (Martin et al., 2001; Oliver et al., 1999). The activation of PLCγ2 by Btk induces the generation of second messengers, such as InsP3 and DAG, by hydrolysis of PtdIP2. InsP3-mediated calcium influx and DAG mediate the activation of PKCβ, enabling the activation of the NF-κB pathway. Upregulation of c-Myc (Grumont et al., 2002; Meyer-Bahlburg et al., 2009) and IRF4 (Grumont and Gerondakis, 2000; Shaffer et al., 2008), 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 (Kim et al., 2017b). 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.
IV-5. Inositol hexakisphosphate confers an alternative mechanism for regulation of Btk in IgM+ plasma cells

T cell-independent immune responses promote the generation of IgM+ plasma cells, which contributes to long-lasting defense against bacterial infection by rapidly secreting antibodies (Foote et al., 2012; Obukhanych and Nussenzweig, 2006). Especially, IgM+ plasma cells have functional BCR complexes that are signaling competent and enable the production of cytokines upon recall antigen challenge (Blanc et al., 2016; Pinto et al., 2013). However, transcriptional regulation during plasma cell differentiation results in the loss of CD19 expression (Blanc et al., 2016; McManus et al., 2011). 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 PtdIP₃ at the membrane (Otero et al., 2001; Tuveson et al., 1993; Varnai et al., 1999). In a previous study using primary murine splenic B cells, it was shown that the activation of Btk in BCR signaling was not significantly affected by the deletion of PI3K gene in the B cells (Suzuki et al., 2003) (Jou et al., 2002). However, the phosphorylation of Akt and S6K, the target of Akt-mTOR signaling, was strongly compromised in the PtdIP₃-deficient B cells (Bilancio et al., 2006; Suzuki et al., 2003). These results suggested that the Btk activity may also be regulated through a
mechanism that is independent of PtdIP₃ synthesis.

Interestingly, the structural analysis of InsP₆/PH domain complex showed that the PH domain of Btk has two InsP₆ binding sites (Wang et al., 2015). The occupation of both sites in PH domain is important for the activation of Btk by dimerization (Chung et al., 2018; Wang et al., 2015). One of them is a canonical binding site that is essential for binding to PtdIP₃ in the membrane. It suggests that InsP₆ 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 IsnP₆. Moreover, the expression of enzymes required for the generation of InsP₆, such as IPMK (from InsP₃ and InsP₄ to InsP₅) and IPPK (from InsP₅ to InsP₆), was strongly induced in IgM⁺ plasma cells, probably complementing the requirement of InsP₆ for a functional BCR. Thus, despite CD19 deficiency in IgM⁺ plasma cells, InsP₆ generated by IPMK seems to provide an alternative mechanism for the regulation of Btk in BCR signaling.

IV-6. The significance of InsP₆-mediated Btk regulation

Btk plays the role of a central signal transducer in the BCR signaling pathway, which is essential for B cell biology and humoral immunity. It has been found that irregular functions of Btk are associated with the
development of B cell malignancy (Hendriks et al., 2014), autoimmune diseases (Di Paolo et al., 2011; Kil et al., 2012), and XLA in humans. As Btk is considered an attractive therapeutic target, it is important to understand the intricate and fine-tuned network of mechanisms involved in tightly regulating Btk activity in B cell-activating signal. Overall, in the present study, I showed that IPMK is a new regulator of TI immune responses and that InsP$_6$ confers a novel mechanism to regulate the activity of Btk in BCR signaling. Moreover, its significance is more apparent in IgM$^+$ plasma cells that highly express key enzymes required for producing InsP$_6$. Thus, I believe that our findings on the role of IPMK and its products will offer new ways to treat abnormal Btk-mediated disorders in the future.
Figure 57. The role of inositol hexakisphosphates in BCR signaling of B cell.
Figure 58: The role of inositol hexakisphosphates in BCR signaling of plasma cell.
References


an analysis of 96 patients. Medicine 64, 145-156.


Pax5 regulates its target genes by recruiting chromatin-modifying proteins in committed B cells. EMBO J 30, 2388-2404.


Oliver, A.M., Martin, F., and Kearney, J.F. (1999). IgM\text{high}CD21\text{high} lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. J Immunol 162, 7198-7207.


국문초록

T 세포 비의존적 B 세포 반응에서 이노시톨 다인산에 의한

Bruton's tyrosine kinase 활성 조절에 관한 연구

김 우섭

미생물 감염의 초기 단계에서 B 세포는 즉각적으로 항원을 인식하여
T 세포 비의존적(T cell-independent) 방식으로 면역 반응을 일으킨다.
즉각적이며 강렬한 T 세포 비의존적 B 세포 면역 반응은 병원체의 감염에
대응하는 초기 방어 기제에서 핵심적인 역할을 한다. 이러한 T 세포
비의존적 B 세포 반응에서, 브루튼 티로신 키나제(Brunon's tyrosine kinase,
Btk)의 조절과 활성은 B 세포 항원 수용체(B cell antigen receptor) 신호
전달에서 중추적인 단계이다. T 세포 비의존적 B 세포 반응 과정에서
Btk의 중요성은 박테리아 감염 질병이 번번하게 발생하는 X 연관성
무감마글로불린혈증(X-linked agammaglobulinemia) 환자의 연구를
통해서 밝혀졌다. 하지만 기존에 잘 정립된 B 세포 항원 수용체 조절
메커니즘으로 Btk의 활성 조절을 이해하기에는 부족한 부분이 남아 있다.


본 연구에서, 이노시톨 핵사키스인산(inositol hexakisphosphate)을 B 세포 항원 수용체 신호 전달에서 Btk의 생리적 핵심 조절 인자로 규명하였다. 이노시톨 다인산의 결핍은 T 세포 비의존적 B 세포 반응 반응의 저하를 유도하였다. T 세포 비의존적 항원을 생쥐에게 접종하였을 때, B 세포와 형질 세포의 수가 이노시톨 다인산 멀티키나제(inositol polyphosphate multikinase, IPMK) 결핍 생쥐에서 감소하였다. IPMK의 발현이 억제된 B 세포는 항원 자극에 의한 유전자 발현 및 사이토카인 생성에서 문제가 발생하였다. 이러한 IPMK 결핍 생쥐의 약화된 T 세포 비의존적 면역 반응은 Btk 활성의 저하로 인하여 유발됨을 확인 하였다. 특히, BCR 신호에 의한 Btk와 PLCγ2의 인산화가 현저히 줄어 들었다.
본 연구를 통해, 이노시톨 핵사키스인산이 B 세포 항원 수용체의 신호전달에서 Btk 활성을 유도하는 핵심 인자로 역할을 하는 것을 증명하였다. 활성화된 B 세포에서 이노시톨 다인산이 형성되지만, IPMK 결핍 B 세포에서는 형성이 억제되어 있는 것을 확인하였다. 이노시톨 다인산의 결핍이 T 세포 비의존적 면역 반응의 약화를 초래하는 것으로 추정이 된다. 뿐만 아니라, 세포막을 통과 가능한 이노시톨 핵사키스인산에 의해 IPMK 결핍 B 세포에서의 결합들이 효과적으로 회복되는 것을 확인하였다. 이는 이노시톨 핵사키스인산이 BCR 신호에서 Btk의 상위 조절 인자로 역할을 하는 것을 의미한다.

홍미롭게도, 이노시톨 다인산을 통한 Btk 활성 조절은 IgM을 발현하는 형질 세포에서 더욱 중요한 역할을 수행한다는 것을 확인하였다. 비록 CD19에 의한 PI3K 활성은 정상 BCR 복합체의 작용에 필수적이지만 불구하고, CD19의 발현은 IgM⁺ 형질 세포에서 두드러지게 감소하였다. 그러나 IgM⁺ 형질 세포는 이노시톨 다인산의 형성에 필수적인 효소를 높게 발현하였다. 이로 인하여, 세포내 이노시톨 핵사키스인산의 증가가
줄어든 PI3K 활성을 보완할 수 있으며, 형질 세포에서 이노시톨 핵사키스인산에 의한 Btk 활성 조절의 중요성은 더욱 부각된다.

따라서, 본 연구는 T 세포 비의존적 항원에 대한 면역 반응에서 이노시톨 다인산의 역할을 규명하였다. 이러한 결과는 2 차 신호 전달 물질이 세포 작용의 방향성을 결정하는 일례로써, 이노시톨 다인산에 의한 B 세포 항원 수용체 신호 전달 조절의 새로운 메커니즘을 제시한다. IPMK와 이의 생산물의 역할에 대한 본 연구는 B 세포 종양, 자가 면역 질환, 면역 결핍과 같이 비정상적인 Btk에 의해서 발생하는 질병 치료에 대한 새로운 접근법을 제안한다.

주요어: T 세포 비의존적 면역 반응, B 세포 항원 수용체, 브루튼 타로신 키나제, 이노시톨 다인산, 이노시톨 다인산 멀티키나제
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