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RNA-binding protein Ptbp1 is essential for BCR-mediated antibody production

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Abstract

The RNA-binding protein polypyrimidine tract-binding protein-1 (Ptbp1) binds to the pyrimidine-rich sequence of target RNA and controls gene expression via post-transcriptional regulation such as alternative splicing. Although Ptbp1 is highly expressed in B lymphocytes, its role to date is largely unknown. To clarify the role of Ptbp1 in B-cell development and function, we generated B-cell-specific Ptbp1-deficient (P1BKO) mice. B-cell development in the bone marrow, spleen and peritoneal cavity of the P1BKO mice was nearly normal. However, the P1BKO mice had significantly lower levels of natural antibodies in serum compared with those of the control mice. To investigate the effect of Ptbp1 deficiency on the immune response *in vivo*, we immunized the P1BKO mice with T-cell-independent type-2 (TI-2) antigen NP-Ficoll and T-cell-dependent (TD) antigen NP-CGG. We found that B-cell-specific Ptbp1 deficiency causes an immunodeficiency phenotype due to defective production of antibody against both TI-2 and TD antigen. This immunodeficiency was accompanied by impaired B-cell receptor (BCR)-mediated B-cell activation and plasmablast generation. These findings demonstrate that Ptbp1 is essential for the humoral immune response.

Keywords: B-cell activation, immunodeficiency, RBPs

Introduction

B lymphocytes are the only cells that produce antibodies against a variety of pathogens. Post-transcriptional regulation via mechanisms such as alternative splicing (AS) is essential for antibody production by B cells (1). B cells usually express membrane-bound IgM, serving as the B-cell receptor (BCR). When activated by antigen stimulation, B cells differentiate into antibody-producing cells, and IgM mRNA is altered via AS to produce secretory instead of membrane-bound IgM (2, 3). AS also is necessary for class switching from IgM to IgD. In B cells, IgM and IgD are transcribed as one transcript containing VH, Cμ and Cδ domains (4). Subsequently, either Cμ or Cδ is selected via AS, and IgM and IgD are simultaneously produced in the same cells (5, 6). Recent genome-wide studies have shown that more than 90% of multi-exon genes are alternatively spliced upon B-cell activation (7, 8).

Additional means of post-transcriptional regulation, including alternative polyadenylation (APA), mRNA decay and altered mRNA stability, are involved in the differentiation and function of various cell types. These mechanisms are tightly regulated by RNA-binding proteins (RBPs). Polypyrimidine tract-binding protein-1 (Ptbp1/hnRNPI) is an RBP belonging to the heterogeneous nuclear ribonucleoprotein (hnRNP) family. This protein is a Ptb subfamily member, along with Ptbp2 (nPtb) and Ptbp3 (Rod1). The Ptb family proteins have four RNA recognition motifs from the N-terminus to the C-terminus, through which they bind the polypyrimidine-rich region of the target RNA. Ptbp1 is ubiquitously expressed in cells, including B cells, whereas Ptbp2 is primarily expressed in the nervous system (9). Ptbp3 is highly expressed in the hematopoietic lineage (10). Interestingly, cross-regulation among Ptbp1, Ptbp2 and Ptbp3 has been reported (11). Previous studies have reported that Ptbp1 is not only involved in AS (12) but also in mRNA 3′ end formation and APA (13–17).

For several years, we have been investigating the role of Ptbp1 in embrionic stem (ES) cells and the nervous system. We have demonstrated that Ptbp1 is essential for early mouse development and for the proliferation and differentiation of mouse ES cells (18). We also reported that Ptbp1 is necessary for the maintenance of adherens junctions in neural stem cells of the dorsal telencephalon and that loss of Ptbp1 leads to lethal hydrocephalus (19).

Upon lymphocyte activation, Ptbp1 stabilizes several gene transcripts, including Rab8A in CpG-activated B cells (20). Vavassori *et al.* reported that Ptbp1 regulates CD154 (CD40L)

transcript stability upon TCR stimulation in mouse CD4$^+$ T cells (21). In addition, Ptbp1 is reported as being required for optimal CD4$^+$ T-cell activation (22). Monzon-Casanova *et al.* recently reported that Ptbp1 is required for B-cell selection in germinal centers (GCs) (23). However, because their studies focused on the role of Ptbp1 in the early GC B-cell response, the physiological role of Ptbp1 in the development and activation of B cells is still largely unknown. This study aims to clarify the role of Ptbp1 in B-cell development and function. Using B-cell-specific Ptbp1-deficient mice, we investigated the effects of Ptbp1 deficiency on B-cell development, antibody production and the immune response *in vivo*.

Methods

Mice

Ptbp1$^{flox/flox}$ mice were generated and maintained as described previously (18, 19). Mb1-Cre mice were crossed with Ptbp1$^{flox/flox}$ mice to generate B-cell-specific Ptbp1-deficient mice. All mice were generated on a C57BL/6 genetic background. All animals were maintained under specific pathogen-free conditions in the animal facilities of the Institute of Medical Science, the University of Tokyo (Tokyo, Japan). All animal work was performed in compliance with the guidelines of the Institutional Animal Care and Use Committee of the University of Tokyo (PA15-17).

Flow cytometry

Single-cell suspensions from the indicated organs were treated with anti-CD16/32 (clone 93; Biolegend, San Diego, CA, USA) for Fc-blocking. The following antibodies were used for cell-surface staining: anti-B220 (RA3-6B2), CD19 (6D5); IgM (RMM-1); IgD (11-26c.2a); CD5 (53-7.3); CD21 (7E9); CD23 (B3B4), CD22 (OX-97); PIR-A/B (6C1); BAFFR (7H22-E16); CD3ε (145-2c11); CD4 (GK1.5); CD8α (53-6.7); CD11b (M1/70); Gr-1 (RB6-8c5); Ter-119 (TER-119); CD95 (Jo2); GL7 (GL7); CD138 (281-2); CD38 (90); IgG$_1$ (RMG1-1); and Ki67 (16A8) (all from Biolegend). Anti-phospho-Btk (pY223: N35-86) and PLCγ2 (pY759: K86-689.37) antibodies were from BD Biosciences (San Jose, CA, USA). Debris and dead cells were excluded by forward scatter, side scatter and PI staining. All samples were analyzed by FACSCalibur or FACSAria (BD Biosciences). The data were analyzed using FlowJo (TreeStar, Ashland, OR, USA).

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISAs) were performed, as described previously, with some modifications (24). In brief, flat-bottom 96-well plates (NUNC AIS, Roskilde, Denmark) were coated with rat anti-mouse IgM, IgG$_1$, IgG$_3$ and IgA antibodies (Southern Biotechnology, Birmingham, AL, USA) and blocked with 3% BSA in PBS. Serially diluted standards and serum samples were then loaded into assigned wells and incubated for 1 h at room temperature. Bound antibodies were detected using HRP-conjugated goat anti-mouse isotype-specific antibodies (Southern Biotechnology), followed by the addition of SureBlue TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD, USA). Absorbance at 450 nm was measured using an iMark microplate reader (Bio-Rad, Hercules, CA, USA).

For measuring NP-specific antibodies, 96-well plates were coated with 50 μl per well of 10 μg ml^{-1} of NP$_7$-BSA [N-5050L, Biosearch Technologies, Petaluma, CA, USA; NP$_7$ plate, for higher-affinity T-cell-dependent (TD) response] or NP$_{30}$-BSA [N-5050H, Biosearch Technologies; NP$_{30}$ plate, for total TD and T-cell-independent type-2 (TI-2) response] at 4°C overnight, followed by blocking with 3% BSA in PBS. After washing with 0.05% Tween 20 in PBS, sera at various dilutions were added in triplicate and the plates were then incubated for 1 h at room temperature. The bound immunoglobulins were detected using HRP-conjugated secondary antibodies, as described above.

Immunization of mice

To elicit the TI-2 response, mice were immunized intra-peritoneally with 25 μg of NP-49-Ficoll (F1420, Biosearch Technologies). To elicit the TD response, mice were immunized intra-peritoneally with 50 μg of NP-31-CGG (N50550, Biosearch Technologies) with Imject alum (Thermo Fisher Scientific, Waltham, MA, USA), and boosted with 50 μg of NP-CGG in PBS 8 weeks later. Blood samples were collected on the indicated days after immunization from the tail.

Detection of NP-specific B cells

Spleen cells from the immunized mice were stained with lineage cocktail (Lin: anti-CD3ε, CD4, CD8α, CD11b, Gr-1 and Ter119), B220 and NP-PE (Biosearch Technologies) for 20 min at 4°C. Non-B and dead cells were excluded by gating and PI staining. The gating strategies were as follows: plasmablasts, Lin$^-$NP-PE$^+$B220^{low}CD138$^+$; GC B cells, Lin$^-$NP-PE$^+$B220$^+$IgG$_1^+$CD38^{low}; and memory B cells, Lin$^-$NP-PE$^+$B220$^+$IgG$_1^+$CD38^{high}.

In vivo B-cell proliferation

The mice were immunized intra-peritoneally with NP-Ficoll or NP-CGG, as described above. Splenocytes were collected on day 4 (for TI-2 response) or 7 (for TD response). The cells were fixed and permeabilized with the FOXP3 Fix/Perm Buffer Set from Biolegend followed by staining with APC-conjugated anti-mouse Ki-67 antibody. Lin$^-$NP-PE$^+$B220$^+$ cells were gated and the population examined for the frequency of Ki67$^+$ cells.

Western blot analysis

Purified 5 × 10^5 splenic B cells were suspended in 50 μl serum free RPMI 1640 medium. After pre-incubation for 10 min at 37°C, the cells were stimulated with F(ab′)$_2$ anti-mouse IgM (10 μg per 5 × 10^5 cells) (Jackson ImmunoResearch, West Grove, PA, USA) at 37°C. The stimulated B cells were lysed in NP-40 lysis buffer containing 1% NP-40, 50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, phosphatase inhibitor cocktail and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Cell lysates were resolved by SDS–PAGE and transferred to PVDF membranes. Then, the blots were probed with the indicated antibodies, followed by HRP-conjugated secondary antibodies and were visualized using Luminata Forte Western HRP Substrate (Merck Millipore, Darmstadt, Germany).

The following antibodies were used for immunoblotting: anti-phospho-tyrosine antibodies 4G10 (Merck Millipore, Burlington, MA, USA); anti-PLCγ2 antibodies (sc407, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-IκBα antibodies (4814); and anti-Erk1/2 antibodies (4695, Cell Signaling Technology, Danvers, MA, USA). Immunoblots were visualized using the Bio-Rad ChemiDoc MP imaging system (Bio-Rad).

Statistical analysis

Comparisons were analyzed for statistical significance with the unpaired, two-tailed Student's *t*-test using Microsoft Excel software (Microsoft, Redmond, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Ptbp1 modulates the expression levels of several markers on B cells

To clarify the role of Ptbp1 in B-cell development and function, we generated B-cell-specific Ptbp1-deficient mice by crossing $Ptbp1^{fl/fl}$ mice with Mb1-Cre mice that were known to express Cre recombinase specific for the B-cell lineage (called 'P1BKO' mice here). The development of B cells in the P1BKO mouse bone marrow was comparable with that of the control mice [Supplementary Figure 1A (left) and Supplementary Figure 1B]. In the spleen, $IgM^{high}IgD^{low}$ immature and $IgM^{+}IgD^{+}$ mature B-cell populations were equivalent in the P1BKO and control mice (Supplementary Figure 1A, middle). The number of B cells in each stage of the P1BKO mice bone marrow and spleen was comparable with that of the control mice (Supplementary Figure 1B and C). However, the expression level of IgM in the spleen slightly but significantly decreased in P1BKO mice compared with that in the control mice (Supplementary Figure 1D). The number and proportion of $CD5^{+}B1a$ cells in the peritoneal cavity were also equivalent in the P1BKO and control mice [Supplementary Figure 1A (right) and Supplementary Figure 1E].

Interestingly, in the P1BKO mouse spleens, marginal zone (MZ) B cells and follicular (Fo) B cells were observed in normal proportion (Fig. 1A); however, the expression level of complement receptor type 2 CD21 on MZ B cells was significantly decreased and that of low-affinity IgE receptor CD23 on Fo B cells was significantly elevated (Fig. 1B and C). In addition, the expression level of CD22, an inhibitory receptor expressed on B cells (25, 26), was slightly but significantly decreased in the P1BKO mice compared with the control mice (Fig. 1D and E, left panel). In Ptbp1-deficient B cells, the expression level of the inhibitory receptor PIR-B (Fig. 1D and E, right panel) and BAFFR (Fig. 1F), which is thought to promote the survival and activation of mature B cells (27–29), was comparable with that of controls. These results indicate that Ptbp1 modulates the expression of several surface receptors such as CD21, CD22 and CD23, but is dispensable for B-cell development.

B cells require Ptbp1 for optimal antigen-specific antibody production

To determine whether Ptbp1 has a role in humoral immunity *in vivo*, we first analyzed standing serum immunoglobulin titers in unimmunized 8- to 12-week-old P1BKO and control mice. We found that serum immunoglobulin titers of all isotypes tested were significantly reduced in the P1BKO mice (Fig. 2A). IgM titers were 2.5-fold lower in the P1BKO mice than in the control mice and IgG_1 and IgA were 2-fold lower in the P1BKO mice than in the control mice. Most strikingly, the serum IgG_3 level was >15-fold lower in the P1BKO mice than in the control mice.

Importantly, IgG_3 is believed to be the major immunoglobulin isotype produced in response to T-cell-independent (TI) type-2 antigens (30–32). This observation may reflect the essential role of Ptbp1 in the TI-2 response. Therefore, to evaluate antibody production against TI-2 antigen, we immunized the P1BKO and control mice intra-peritoneally with TI-2 antigen NP-Ficoll. Indeed, minimal amounts of NP-specific IgM and IgG_3 were produced in the P1BKO mice (Fig. 2B).

We next examined the antibody response against a TD antigen. The P1BKO and control mice were immunized intraperitoneally with the TD antigen NP-CGG in alum. After immunization, the production of NP-specific total IgG_1 (measured with NP_{30}) was significantly lower in the P1BKO mice at 1, 3, 7 and 8 weeks than in the control mice (Fig. 2C, left panel). The production of higher-affinity antibodies (measured with NP_7) was also significantly lower in the P1BKO mice at 1, 4, 6 and 8 weeks (Fig. 2C, right panel). Boosting of mice with NP-CGG in PBS at 8 weeks resulted in a significantly lower antibody response in the P1BKO mice than in the control mice (Fig. 2C). Collectively, these data demonstrate that Ptbp1 expression in B cells is necessary for optimal antigen-specific antibody production.

Ptbp1 is essential for TI-2 antigen-specific proliferation and plasmablast differentiation

To investigate whether the loss of antibody production against TI-2 antigen in the P1BKO mice is caused by impaired B-cell activation, we challenged the P1BKO and control mice intraperitoneally with NP-Ficoll and examined the frequency and number of NP-specific proliferative B cells. Figure 3(A) shows the gating strategy for detection of NP-specific B cells (Fig. 3A, left). Four days after immunization, the proportion of $NP\text{-}PE^{+}$ cells significantly decreased in P1BKO mice (Fig. 3A, right). The proportion and number of proliferative B cells ($NP\text{-}PE^{+}B220^{+}Ki67^{+}$) in the spleen were significantly lower in the P1BKO mice than in the control mice (Fig. 3B and C, respectively). To confirm whether the defective proliferative response of Ptbp1-deficient B cells affects plasmablast differentiation, we determined the frequency and number of NP-specific plasmablasts ($NP\text{-}PE^{+}B220^{low}CD138^{+}$). The frequency of NP-specific plasmablasts was >10% in the control mice but almost zero in the P1BKO mice (Fig. 3D). Consistent with this observation, very few NP-specific plasmablasts were observed in the P1BKO mice (Fig. 3E). These findings demonstrate that Ptbpl is essential for proliferation and differentiation of plasmablasts against TI-2 antigen.

Ptbp1 is required for efficient TD-antigen-specific GC formation and memory B-cell generation

We next examined the proliferative response against TD antigen NP-CGG. As shown in Fig. 4A and B, both the proportion

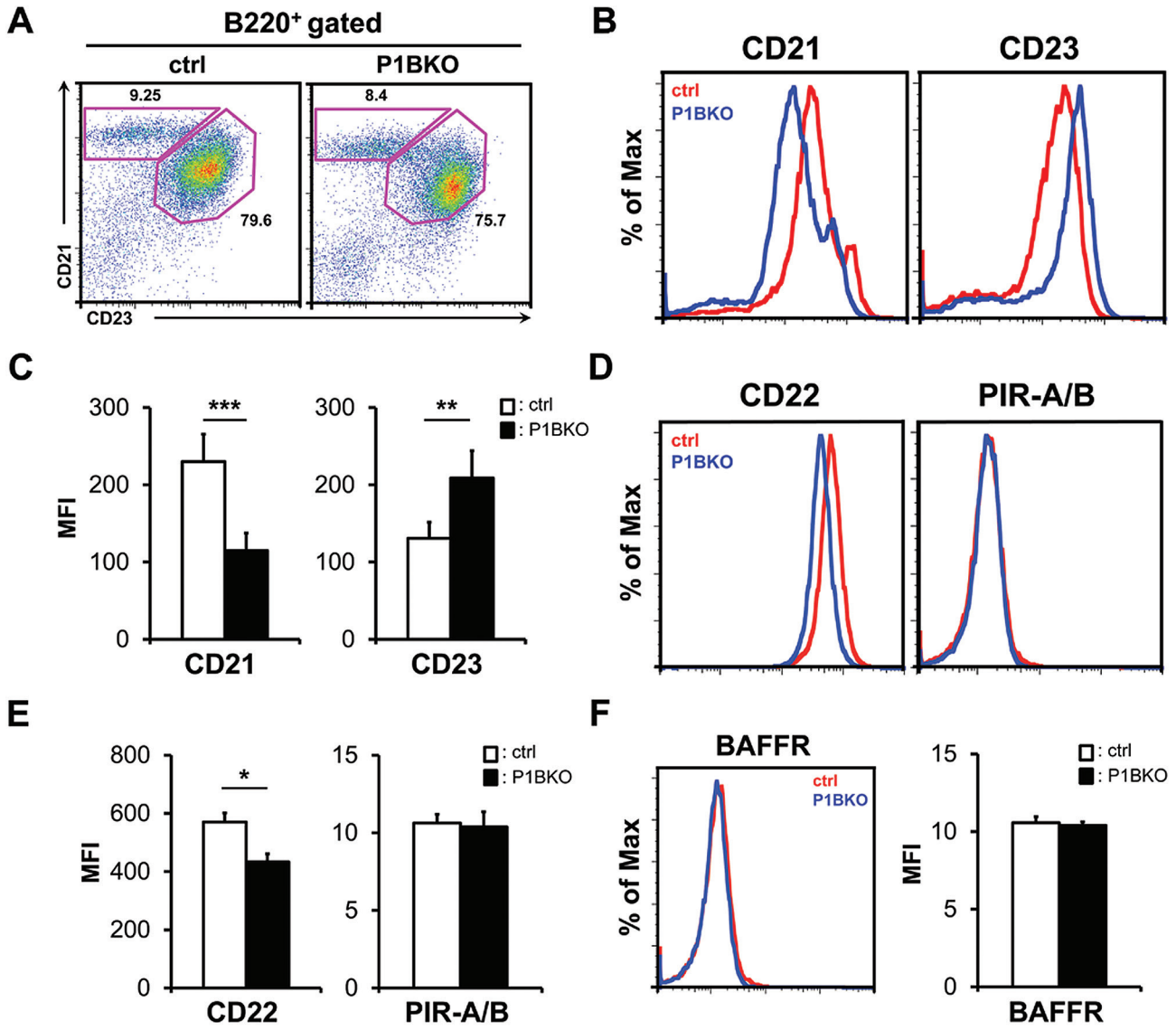

Fig. 1. Ptbp1 deficiency affects the expression of several surface markers on B cells. (A) Typical FACS profiles of B220^{+}/CD21^{high}/CD23^{low} MZ B cells and B220^{+}/CD21^{low}/CD23^{high} Fo B cells in the spleens of control (left) and P1BKO mice (right). The numbers in the plots indicate the percentages of each B-cell subset. (B) Histogram depicts the expression levels of CD21 on MZ B cells and CD23 on Fo B cells. Red solid line, control B cells; blue solid line, Ptbp1-deficient B cells. (C) Mean fluorescence intensity (MFI) of CD21 (left) and CD23 (right). The mean values of seven independent experiments are shown. Error bars indicate the SD for each sample. $^{**}P < 0.01$; $^{***}P < 0.001$, two-tailed unpaired Student's *t*-test. (D) Histogram depicts the expression levels of CD22 (left) and PIR-A/B (right) on splenic B cells. (E) MFI of CD22 (left) and PIR-A/B (right). The mean values of seven independent experiments are shown. Error bars indicate the SD for each sample. $^{*}P < 0.05$, two-tailed unpaired Student's *t*-test. (F) Histogram (left) depicts the expression level of BAFFR; bar graph (right) shows the MFI of BAFFR. The mean values of seven independent experiments are shown. Error bars indicate the SD for each sample.

and number of NP-specific Ki67^{+} B cells (NP-PE^{+} B220^{+}Ki67^{+}) were significantly lower in the P1BKO mice than in the control mice. Furthermore, both the frequency and number of NP-specific plasmablasts (NP-PE^{+}B220^{low}CD138^{+}) were significantly lower in the P1BKO mice than in the control mice (Fig. 4C and D, respectively).

As shown in Fig. 2(C), the P1BKO mice showed a markedly lower secondary response than primary response as compared with the control mice. We next examined the NP-specific GC formation and memory B-cell differentiation in the P1BKO mice. As expected, the proportion and number of NP-specific GC B cells (NP-PE^{+}B220^{+}IgG1^{+}CD38^{low}) were lower in the P1BKO mice than in the control mice 2 weeks after immunization (Fig. 4E and F). Although the proportion of NP-specific memory B cells (NP-PE^{+}B220^{+}IgG1^{+}CD38^{high}) between the P1BKO and control mice was comparable (Fig. 4G, left panel), the absolute number of memory B cells was significantly lower in P1BKO mice than that in control mice (Fig. 4G, right panel) perhaps due to a reduction in GC B cells. In addition, Ptbp1-deficient memory B cells had higher IgG_1 expression levels than did controls (Fig. 4H).

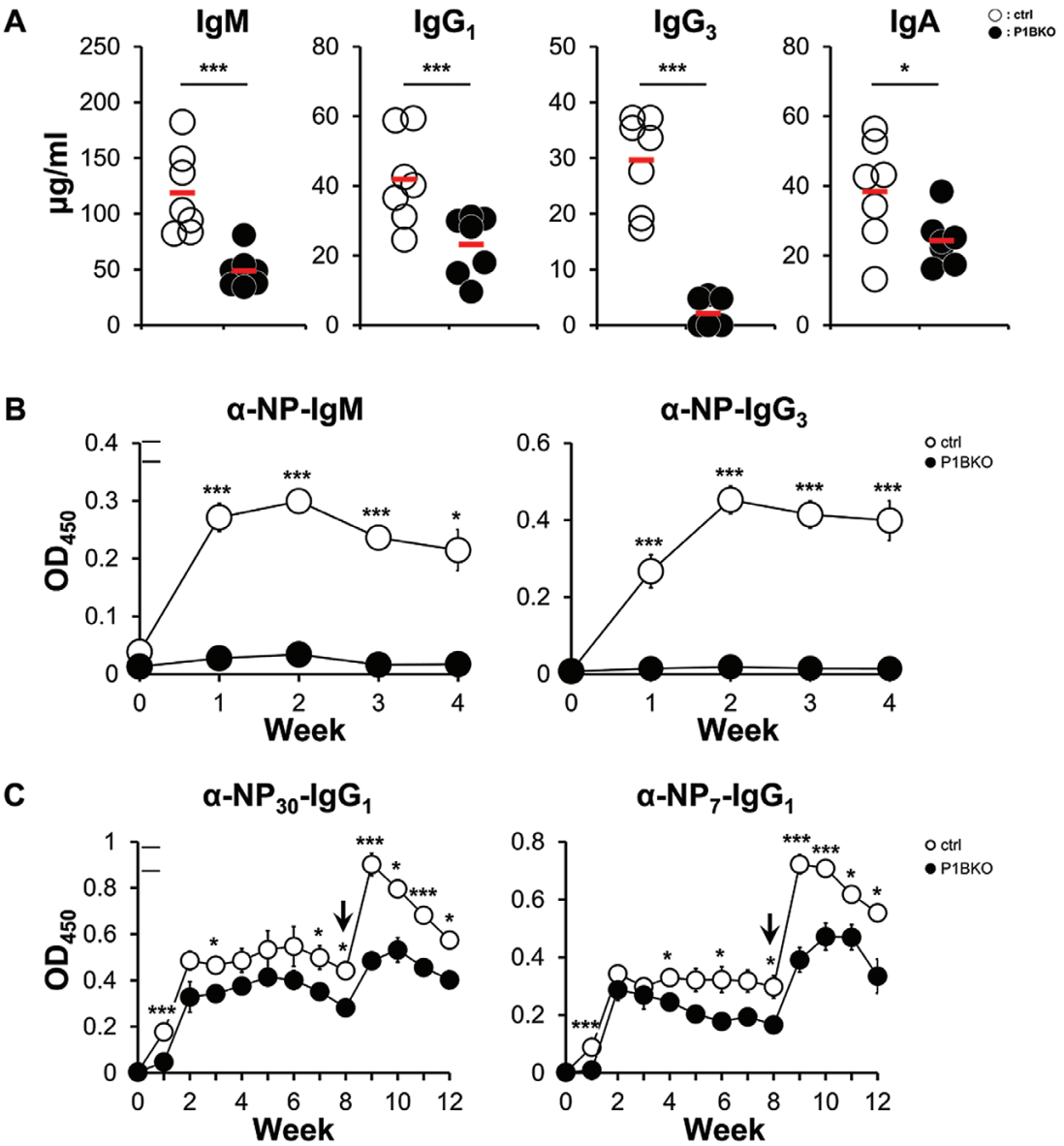

Fig. 2. B cells require Ptbp1 for optimal antibody response. (A) Serum immunoglobulin (Ig) levels of unimmunized P1BKO mice. Sera from 8- to 12-week-old control and P1BKO mice were collected and immunoglobulin concentrations were determined by ELISA. The mean values of each isotype are indicated by the red bars. Seven pairs of ctrl and P1BKO mice were analyzed. *$P < 0.05$; ***$P < 0.001$, two-tailed unpaired Student's *t*-test. (B) Response to TI-2 antigen, NP-Ficoll. NP-specific IgM (left) and IgG_3 (right) serum antibody levels at 1–4 weeks after NP-Ficoll immunization. The mean values of five pairs of ctrl and P1BKO mice are shown. Error bars indicate the SD for each sample. *$P < 0.05$; ***$P < 0.001$, two-tailed unpaired Student's *t*-test. (C) Response to TD antigen, NP-CGG. Five pairs of ctrl and P1BKO mice were immunized with 25 μg of NP-CGG in alum at week 0 and boosted with the same amount of NP-CGG in PBS 8 weeks later (indicated arrows). Serum levels of NP-specific IgG_1 antibody were measured with NP_{30}-BSA (for total, left) and NP_7-BSA (for higher-affinity, right) at the indicated times. The mean values of five pairs are shown. Error bars indicate the SD for each sample. *$P < 0.05$; ***$P < 0.001$, two-tailed unpaired Student's *t*-test.

These results indicate that Ptbp1 is required not only for TD antigen-specific proliferation and plasmablast differentiation but also for efficient GC formation.

BCR signaling is impaired in Ptbp1-deficient B cells

Since TI-2 and TD antigens are recognized by BCR and the expression level of IgM was significantly decreased in Ptbp1-deficient B cells (Supplementary Figure 1D), we examined whether the observed dysfunction of Ptbp1-deficient B cells results from impaired BCR signaling. To address this question, we first examined the immediate early signaling events of Ptbp1-deficient B cells upon activation by BCR ligation *in vitro*. The overall tyrosine phosphorylation profile of Ptbp1-deficient B cells upon BCR engagement was distinct from that of control B cells (Fig. 5A). For example, the level of tyrosine phosphorylation of proteins of about 150 and 100 kDa was lower in Ptbp1-deficient B cells, while that of 60 kDa protein was higher than in controls.

We next investigated BCR-induced tyrosine phosphorylation of Btk, a protein kinase important in BCR signaling, as

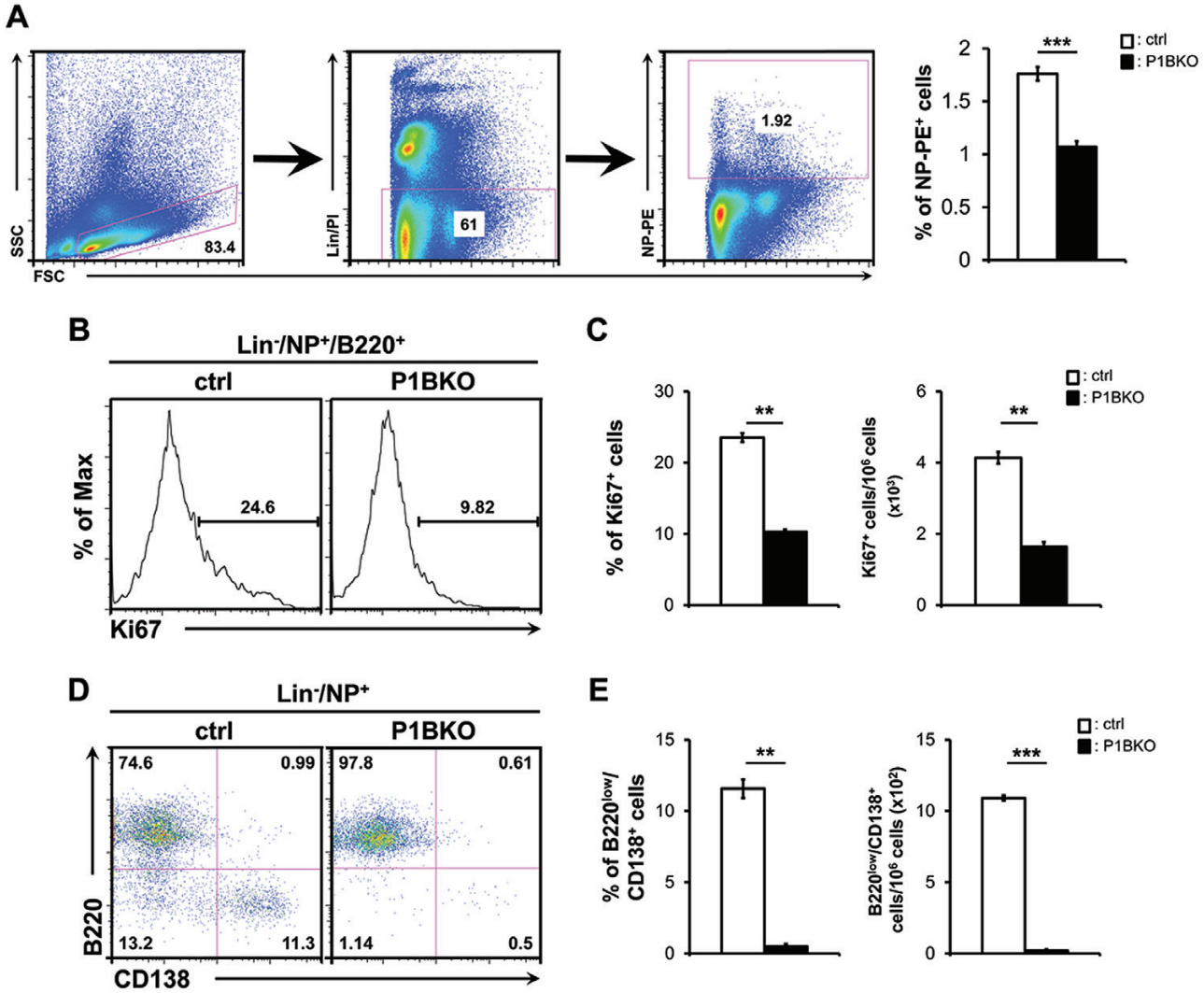

Fig. 3. TI-2 antigen-specific proliferation and plasmablast formation are impaired in P1BKO mice. (A) Gating strategy for the analysis of NP-specific B cells (left) and percentage of the NP-PE$^+$ cells (right). The Lin- (CD3/CD4/CD8/CD11b/Gr-1/Ter-119)/PI$^-$/NP-PE$^+$ cells were analyzed for Ki67$^+$ cells or plasmablasts (B220^{low}CD138$^+$). (B) Histogram depicts the frequency of NP-PE$^+$B220$^+$Ki67$^+$ proliferative B cells in mice 4 days after immunization with NP-Ficoll. (C) Percentages (left) and number (right) of NP-PE$^+$Ki67$^+$ cells. (D) Representative FACS profiles of NP-specific B220^{low}CD138$^+$ plasmablasts from mice 7 days after immunization with NP-Ficoll. (E) Percentages (left) and number (right) of NP-specific plasmablasts. Five pairs of ctrl and P1BKO mice were analyzed. Error bars indicate the SD for each sample. $^{**}P < 0.01$; $^{***}P < 0.001$, two-tailed unpaired Student's *t*-test.

evaluated by intracellular phospho-Btk staining. As a result, the tyrosine phosphorylation level of Btk after BCR cross-linking was significantly lower in Ptbp1-deficient B cells than it was in control B cells (Fig. 5B and C). We also examined the tyrosine phosphorylation level of PLCγ2, a major downstream target of Btk. As expected from the results of phospho-Btk staining, BCR-induced PLCγ2 phosphorylation was also significantly lower in Ptbp1-deficient B cells (Fig. 5D and E). Interestingly, the tyrosine phosphorylation level of Btk and PLCγ2 was also significantly lower in non-stimulated Ptbp1-deficient B cells than in control cells (Fig. 5C and E, right panel, respectively). These results appear to reflect the decrease in the expression level of surface IgM in P1BKO mice.

Signal transduction via BCR eventually activates transcription factors such as NF-κB. Therefore, we investigated the activation of NF-κB in Ptbp1-deficient B cells after BCR stimulation, using the degradation of IκBα as an index. Ptbp1-deficient and control B cells were stimulated with anti-IgM antibody for the indicated times, and lysates were prepared, and then were western blotted for IκBα. In control B cells, a progressive, time-dependent IκBα degradation was observed upon BCR stimulation (Fig. 5F, ctrl). In contrast, BCR-induced IκBα degradation was attenuated in Ptbp1-deficient B cells (Fig. 5F, P1BKO). These results suggest that the decreased expression level of IgM on Ptbp1-deficient B cells could affect BCR-mediated signaling, at least in the Btk-PLCγ2-NF-κB axis.

Discussion

In this study, we observed that B-cell-specific Ptbp1-deficient mice exhibit an immune deficiency phenotype caused by defective antibody production; in particular, antibody

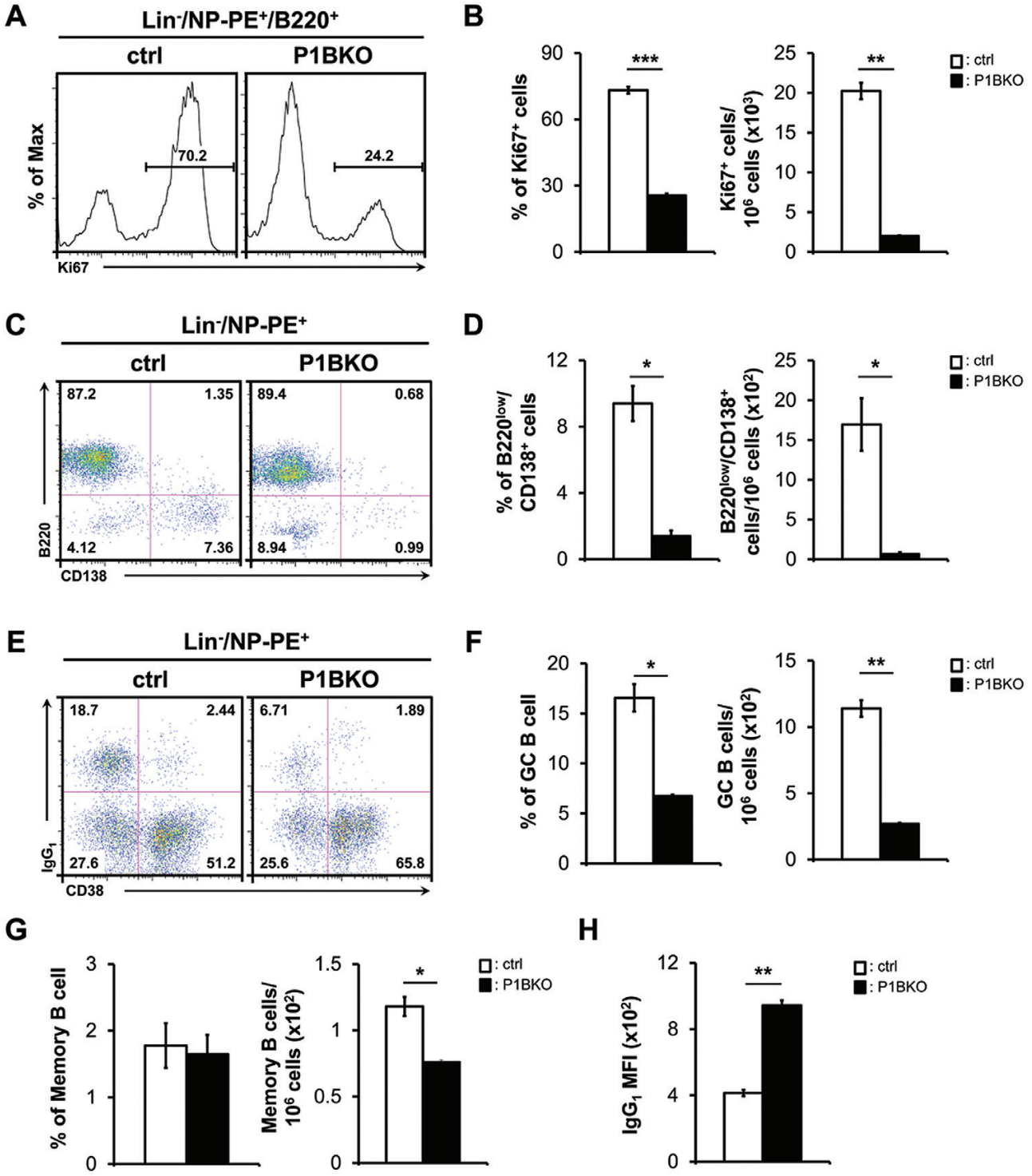

Fig. 4. Impaired TD responses in P1BKO mice. (A) Histogram depicts the frequency of NP-PE$^+$B220$^+$Ki67$^+$ proliferative B cells in mice 1 week after immunization with NP-CGG. Left panel, control mice (ctrl); right panel, P1BKO mice. (B) Percentage (left) and number (right) of NP-PE$^+$Ki67$^+$ cells. (C) Representative FACS profiles of NP-specific B220^{low}CD138$^+$ plasmablasts from mice 1 week after immunization with NP-CGG. (D) Percentages (left) and number (right) of plasmablasts. (E) Typical FACS profiles of NP-specific GC B cells (IgG$_1^+$/CD38^{low}; upper left) and memory B cells (IgG$_1^+$/CD38^{high}; upper right) from mice 2 weeks after immunization with NP-CGG. (F) Percentages (left) and number (right) of GC B cells. (G) Percentages (left) and number (right) of memory B cells. (H) IgG$_1$ mean fluorescence intensity (MFI) of memory B cells. The mean values of five pairs are shown. Error bars indicate the SD for each sample. *P < 0.05; **P < 0.01; ***P < 0.001, two-tailed unpaired Student's *t*-test.

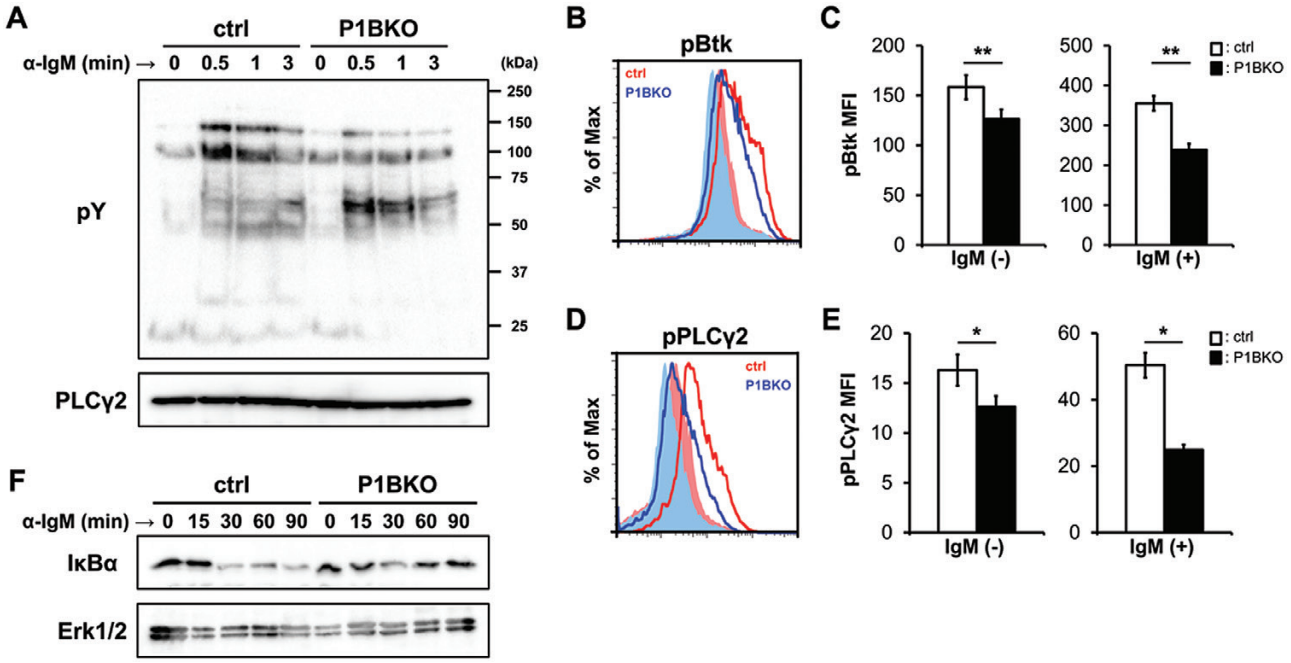

Fig. 5. Impaired BCR-mediated NF-κB activation in Ptbp1-deficient B cells. (A) Overall tyrosine phosphorylation upon anti-IgM stimulation. MACS-purified splenic B cells from control and P1BKO mice were stimulated with 10 μg ml^{-1} of F(ab′)$_2$ anti-IgM for the indicated times. Whole-cell lysates were subjected to immunoblotting with the anti-phosphotyrosine antibody 4G10. (B) Splenic B cells stimulated for 2 min with 10 μg ml^{-1} of F(ab′)$_2$ anti-IgM and stained with anti-phospho-Btk (Y223) antibody. Red solid line, control B cells; blue solid line, Ptbp1-deficient B cells. Shaded histograms indicate unstimulated cells. (C) Phospho-Btk mean fluorescence intensity (MFI) of anti-IgM stimulated splenic B cells. Left, unstimulated cells; right, anti-IgM stimulated cells. The mean values of three independent experiments are shown. Error bars indicate the SD for each sample. $^{**}P < 0.01$, two-tailed unpaired Student's *t*-test. (D) Splenic B cells stimulated for 2 min with 10 μg ml^{-1} of F(ab′)$_2$ anti-IgM and stained with anti-phospho-PLCγ2 (Y759) antibody. Red solid line, control B cells; blue solid line, Ptbp1-deficient B cells. Shaded histograms indicate unstimulated cells. (E) Phospho-PLCγ2 MFI of anti-IgM stimulated splenic B cells. Left, unstimulated cells; right, anti-IgM stimulated cells. The mean values of three independent experiments are shown. Error bars indicate the SD for each sample. $^{*}P < 0.05$, two-tailed unpaired Student's *t*-test. (F) Control and Ptbp1-deficient B cells were stimulated with 10 μg ml^{-1} of F(ab′)$_2$ anti-IgM for the indicated times. Whole-cell lysates were subjected to immunoblotting with anti-IκBα antibody.

production against TI-2 antigen was drastically deficient. This immunodeficiency was associated with impaired BCR-mediated B-cell activation. Indeed, BCR-mediated signaling in Ptbp1-deficient B cells was attenuated in the Btk-PLCγ2-NF-κB pathway *in vitro*.

Monzon-Casanova *et al*. recently reported that Ptbp1 is necessary for B-cell proliferation and affinity maturation in GCs (23). Here, we observed that GC formation also is impaired in the P1BKO mice after TD antigen immunization (Fig. 4E and F; Supplementary Figure 2). In addition, antigen-specific plasmablast generation is severely impaired in the P1BKO mice (Figs. 3D, E, and 4C, D), suggesting that Ptbp1 is essential not only for GC formation but also for plasmablast generation. Furthermore, deficient antibody production in the P1BKO mice was observed more prominently in the TI-2 response than in the TD response (Fig. 2B). Since TI-2 antigen mainly promotes activation of MZ B cells and peritoneal B1 B cells, the effect of Ptbp1 deficiency may be more pronounced in these cells than in GC B cells. A complete understanding of the differing roles of Ptbp1 between MZ/B1 B and GC B cells requires further investigation.

Our results have showed that IgG$_1$ production against TD antigen is significantly lower in the P1BKO mice than in controls at several time points during the primary response (Fig. 2C). This observation may be related to the absence of GC B cells in the P1BKO mice 1 week after immunization [Supplementary Figure 2A (upper) and Supplementary Figure 2B (left)]. The loss of this initial GC formation may affect the threshold of subsequent antibody production. In the TD immune response, the amount of antigen captured and presented by GC B cells regulates their proliferation (33, 34). Thus, given that the proportion of GC B cells in the P1BKO mice is elevated at 2 weeks after immunization [Supplementary Figure 2A (lower) and Supplementary Figure 2B (middle and right)], the impaired initial GC formation may result from the attenuation of antigen capture and/or presentation ability to T cells by Ptbp1-deficient B cells, in addition to their low proliferation ability.

The number of memory B cells was significantly reduced in P1BKO mice. However, their proportion compared with that of the control mice was not reduced; this was probably due to a greater reduction in GC B cells and other B-cell subsets in the NP-specific B-cell population. Therefore, the lower antibody recall response in P1BKO mice appears to be due to reduced number of memory B cells. On the other hand, the expression level of IgG$_1$-BCR in memory B cells was significantly higher in Ptbp1-deficient B cells than that in control B cells (Fig. 4H). Published studies indicate that IgG$_1$-type memory B cells differentiate into antibody-producing cells with high efficiency in the secondary immune response (35–37). Therefore,

Ptbp1-deficient memory B cells might amplify the BCR signal by increasing the expression level of IgG_1-type BCR, thus compensating for the diminished number of memory B cells by increasing the Ptbp1-deficient memory B-cell response. Furthermore, balanced expression of regulatory factors such as Bach2 is reported to be important for rapid differentiation of memory B cells into antibody-producing cells (38). Thus, it is also conceivable that Ptbp1 controls the expression of these key molecules in memory B cells. Additional work is needed to elucidate the detailed mechanism by which Ptbp1 regulates memory B-cell generation and activation.

We observed that BCR-mediated signaling was attenuated in the Btk-PLCγ2-NF-κB axis in Ptbp1-deficient B cells after anti-IgM antibody stimulation *in vitro* (Fig. 5). These observations appear to be related to a decrease in surface IgM levels in Ptbp1-deficient B cells. Both Btk and PLCγ2 are known to be essential for B-cell differentiation and function (39–42). In addition, Petro *et al*. reported that Btk is required for degradation of IκBα in response to BCR cross-linking (43). Moreover, Bell *et al*. reported that PLCγ2 is required for MZ and Fo B-cell survival (44). Furthermore, Hikida *et al*. reported that PLCγ2 is essential for efficient GC formation and differentiation of memory B cells (45). Thus, the observed insufficient activation of these signal molecules confirms that Ptbp1-deficient B cells are dysfunctional with respect to intracellular signal transduction. More recently, La Porta *et al*. have reported that Ptbp1 knockdown in human $CD4^+$ T cells attenuates the activation of PLCγ1, ERK1/2 and NF-κB (22). Although differences between TCR signaling and BCR signaling need to be considered, this report by La Porta *et al*. seems to support our results. Interestingly, they also pointed out that some of the Ptbp1-mediated defects in human $CD4^+$ T cells potentially result from loss of Ptbp1-regulated microRNA function. Therefore, the function or expression of Ptbp1-regulated microRNA also may be impaired in Ptbp1-deficient B cells.

The difference in expression patterns between several surface antigens (Fig. 1) might affect BCR signaling and activation via BCR in Ptbp1-deficient B cells. In particular, Barrington *et al*. reported that CD21 is a co-receptor for the BCR and amplifies BCR signaling and B-cell proliferation/survival by cooperating with CD19 and CD81 (46). Furthermore, Haas *et al*. reported that antigen-specific antibody production is impaired in CD21-deficient mice (47). In contrast to CD21, CD23 was reported to negatively regulate BCR-mediated signaling in a study using CD23-deficient mice (48–50). Therefore, in addition to the decreased expression level of IgM, the decreased expression level of CD21 and increased expression level of CD23 in Ptbp1-deficient B cells might attenuate the BCR signal, decreasing BCR-mediated activation.

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