*International Immunology, Vol. 32, No. 1, pp. 17–26*
doi:10.1093/intimm/dxz055
Advance Access publication 15 August 2019



# Distinct roles of BCNP1 in B-cell development and activation

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*Received* 17 June 2019, *editorial decision* 29 July 2019; *accepted* 11 August 2019

## Abstract

**B-cell novel protein 1 (BCNP1) has recently been identified as a new B-cell receptor (BCR) signaling molecule but its physiological function remains unknown. Here, we demonstrate that mice deficient in BCNP1 exhibit impaired B-cell maturation and a reduction of B-1a cells. BCNP1-deficient spleen B cells show enhanced survival, proliferation and $Ca^{2+}$ influx in response to BCR cross-linking as compared with wild-type spleen B cells. Consistently, mutant B cells show elevated phosphorylation of SYK, B-cell linker protein (BLNK) and PLCγ2 upon BCR cross-linking. *In vivo*, BCNP1-deficient mice exhibit enhanced humoral immune responses to T-independent and T-dependent antigens. Moreover, aged mutant mice contain elevated levels of serum IgM and $IgG_3$ antibodies and exhibit polyclonal and monoclonal B-cell expansion in lymphoid organs. These results reveal distinct roles for BCNP1 in B-cell development, activation and homeostasis.**

*Keywords:* B-cell maturation, B-1a cells, BCR signaling, humoral immune response

## Introduction

B-cell receptor (BCR) signaling must be precisely regulated to allow optimal B-cell development and differentiation (1–3). Dysregulated BCR signaling can lead to impaired B-cell development and maturation, altered B-cell activation and differentiation, uncontrolled B-cell expansion and even B-cell malignancies (1–4). Cross-linking the BCR induces activation of Src family tyrosine kinases and phosphorylation of tyrosine residues in the ITAMs of Igα–Igβ complexes. Phosphorylated ITAMs recruit and activate SYK (5, 6), which then phosphorylates the B-cell linker protein (BLNK, also named SLP-65 or BASH) and activates PLCγ2, Vav and Grb2 (7), and the downstream signaling pathways (8–11). Studies thus far have identified multiple tyrosine kinases, adaptor molecules and cell surface receptors that positively or negatively regulate BCR signaling (5–13).

The B-cell novel protein 1 (BCNP1) was originally identified by Boyd *et al*. as a gene specifically expressed by B cells and up-regulated in B-cell malignancies (12). More recently, *BCNP1* gene was reported to be mutated in several types of human cancers (13). BCNP1 contains a pleckstrin homology (PH) domain, three proline-rich motifs and a potential SH2-binding motif YxxV. We have recently identified BCNP1 as a new BCR signaling molecule (14). Overexpression of BCNP1 in WEHI231 immature B cells potentiated α-IgM-induced apoptosis (14). Conversely, Disruption of the *Bcnp1* gene in WEHI231 cells by CRISPR-Cas9-mediated genome editing resulted in reduced α-IgM-induced apoptosis. Biochemical analyses further revealed that BCNP1 physically interacted with BLNK, Grb2 and PLCγ2 and regulated the phosphorylation of BLNK, SYK and PLCγ2 induced by BCR cross-linking

(14). To explore the physiological role of BCNP1, here we established and analyzed BCNP1-deficient mice. We show that BCNP1-deficient mice exhibited a partial block of B-cell maturation in the spleen and a reduction of B-1a cells in the peritoneal cavity (PC). The mutant B cells exhibited enhanced responses to BCR stimulation and consistently elevated phosphorylation of SYK, BLNK and PLCγ2. BCNP1-deficient mice produced elevated levels of antigen-specific antibodies in response to both T-independent and T-dependent antigens, and exhibited polyclonal and monoclonal B-cell expansion in lymphoid organs as they aged. Our results define a new BCR signaling molecule that has distinct roles in B-cell development, activation and homeostasis.

## Methods

### *Generation of Bcnp1$^{-/-}$ mice*

A targeting vector was constructed to replace exons 6–10 and part of exon 11 of the *Bcnp1* gene with a neomycin gene (Supplementary Figure 1). The targeting vector was linearized with PvuI and electroporated (250 V, 500 μF) into C57BL/6-derived Bruce4 embryonic stem (ES) cells. Two days after the transfection, the ES cells were cultured in the presence of 600 μg ml$^{-1}$ of G418 (G7034, Sigma) and 2 μM of ganciclovir (078-04481, WAKO Chemicals, Japan; only for the first 2 days) for positive and negative selection of the targeted cells, respectively. Correctly targeted ES cell clones were selected by long-range genomic PCR and microinjected into C57BL/6 blastocysts, and the embryos were transferred into foster mothers. Chimeric mice were generated and bred with C57BL/6 mice to obtain heterozygotes, which were further bred to obtain homozygotes. The mice were maintained under specific pathogen-free conditions in the animal facility of Fudan University. All animal experiments have been approved by the Fudan University Animal Experiment Committee.

### *Antibodies and other reagents*

Polyclonal rabbit antibodies were raised against a peptide (AQWVQEGAEEEADAQL) located in the N terminus of mouse BCNP1. The specificity of the BCNP1 antibody was verified by immunoblot of lysates derived from WEHI231 cells transfected with a BCNP1 expression vector (14). The following commercial antibody and reagents were used. FITC-B220 (RA3-6B2), APC-Cy7-B220 (RA3-6B2), PE-α-CD43 (S7), PE-α-IgM (AF6-78), APC-α-CD5 (53-7.3), FITC-α-CD21 (7G6) and α-CD8a (53-6.7) (BD Biosciences); APC-B220 (RA3-6B2), FITC-α-IgD (11-26c.2a) and PE-Cy7-α-CD23 (B3B4) (Biolegend); APC-AA4.1 (AA4.1), PE-α-CD80 (16-10A1) and 7-amino actinomycin D (eBioscience); mouse α-rabbit IgG (L27A9), α-SYK (D3Z1E) (#13198T), α-phospho-SYK (Tyr 352) (#2701s), α-BLNK (D8R3G), α-PLCγ2 (#3872), α-phospho-BLNK (Tyr 96) (#3601) and α-phospho-PLCγ2 (Tyr 1217) (#3871) (Cell Signaling Technology); biotinylated-F(ab′)$_2$-α-IgM (#ab5929; Abcam); lipopolysaccharide (LPS) (#L2630; Sigma); IL-4 (404-ML-010; R&D System); FITC-PtC (phosphatidylcholine) (kindly provided by Dr Yang Yang); 4-hydroxy-3-nitrophenylacetyl (NP)-Ficoll, NP$_{36}$-CGG (chicken γ globulin), NP$_2$-BSA and NP$_{25}$-BSA (Biosearch Technologies); N1G9, C6 and 1A86 NP-specific monoclonal antibody (kindly provided by Prof. Takachika Azuma); F(ab′)$_2$-α-IgM antibodies (#715-006-020, Jackson ImmunoResearch). The soluble CD40L-CD8a fusion protein has been described previously (15).

### *Purification of spleen B cells and analysis of cell survival, proliferation and $Ca^{2+}$ influx*

Spleen B cells were isolated by negative sorting with the IMag B-cell purification kit (#557792, BD Biosciences) following the manufacturer's instruction. Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum, 5 × 10$^{-5}$ M 2-mercaptoethanol, and 100 U of penicillin and streptomycin (ThermoFisher Scientific) for 1–3 days in the presence or absence of different doses of F(ab′)$_2$-α-IgM antibodies, followed by staining with propidium iodide (PI) to analyze cell viability. For the proliferation assay, cells were cultured for 48 h and pulsed for the last 6 h with 1 μCi per well of [$^3$H] thymidine. Thymidine uptake was analyzed as described previously (16). $Ca^{2+}$ influx experiments were performed as previously described (17). In brief, purified spleen B cells were suspended at 10$^7$ cells ml$^{-1}$ and incubated with 1.2 mM of Indo 1-AM (Dojindo) at 37°C for 45 min in the dark. After washing, the cells were resuspended at 10$^6$ cells ml$^{-1}$, stimulated with 10 μg ml$^{-1}$ of F(ab′)$_2$-α-IgM antibodies and immediately analyzed with a LSR flow cytometer (BD Biosciences).

### *BCR internalization, immunization, ELISA and ELISPOT assay*

These experiments were performed as described previously (15, 18, 19).

### *Immunoblot analysis*

Cells were lysed in NP-40 buffer (P0013F, Beyotime Biotechnology) on ice for 20 min and then sonicated for 2.5 min. The lysate was resolved in a 10% sodium dodecyl sulfate (SDS) gel and transferred to an immobilon-P membrane (MILLIPORE). After blocking with block ACE (DS PHARMA BIOMEDICAL), the membrane was incubated with primary antibody and then horseradish peroxidase (HRP)-conjugated secondary antibodies, and developed with an enhanced chemiluminescence light (ECL) reagent.

### *Determination of CDR3 length*

This experiment was performed as described previously (20) with some modifications. Briefly, total RNA was isolated from splenocytes or lymph node (LN) cells and the first strand cDNA was synthesized using PrimeScript™ II (Takara). The first strand cDNA was amplified with one of the $V_H$ forward primers and the μ reverse primer under the following PCR conditions: 94°C for 3 min followed by 10 cycles of 94°C for 30 s, 64–45°C (2°C touchdown per cycle) for 30 s and 72°C for 15 s, 25 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 15 s, and an additional 72°C for 5 min. The PCR product was further subjected to a run-off elongation with one of the $J_H$ reverse primers labeled with 5′-fluorescent dye under the following conditions: 94°C for 1 min followed by 10 cycles of 94°C for 30 s, 60°C for 25 s and 72°C for 25 s, and an additional 72°C for 5 min. Finally, 2 μl of the elongation

product was loaded onto an automated DNA sequencer (Applied Biosystems). The following primers were used: $V_H$558, 5′-TCCARCACAGCCTWCATGCARCTCARC-3′; $V_H$7183, 5′-AAGAASAMCCTGTWCCTGCAAATGASC-3′; $V_H$Q52, 5′-AGACTGARCATCASCAAGGACAAYTCC-3′; μ reverse primer, 5′-GACATTTGGGAAGGACTGACTCTC-3′; $J_H$1, 5′-GACGGTGACCGTGGTCCCTGT-3′; $J_H$2, 5′-GACTGT GAGAGT GGTGCCTTG-3′; $J_H$3, 5′-GACAGTGACCAGAGTC CCTTG-3′; $J_H$4, 5′- GACGGTGACI'GAGGTTCCTTG-3′. For each sample (spleen or LN), PCR product obtained with one of the V gene primers ($V_H$558 $V_H$7183 and $V_H$Q52) and the Cμ primer was further subjected to run-off elongation with one of the 4 $J_H$ primers, resulting in 12 different CDR3 profiles. We analyzed the spleen and LNs from three wild-type (WT) and three BCNP1-deficient mice and obtained a total of 108 CDR3 profiles in each group. The proportion of CDR3 showing a single major peak among the 108 CDR3 profiles was determined.

### *Statistical analysis*

Statistical significance was assessed by an unpaired or paired *t*-test, exact probability test, χ-square test or log-rank test (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

## Results

### *Impaired B-cell maturation and reduced numbers of B-1a cells in Bcnp1$^{-/-}$ mice*

BCNP1 contains three proline-rich domains (PxxP), a PH domain and a potential tyrosine phosphorylation site (YxxV) (Supplementary Figure 1A). Q-PCR analysis confirmed that *Bcnp1* was expressed at variable levels in different B-cell subsets, but not expressed by T cells (Supplementary Figure 2). In addition, *Bcnp1* expression was up-regulated in spleen B cells after LPS, CD40L or BAFF stimulation (Supplementary Figure 2). To investigate the physiological function of BCNP1 in B cells, we generated *Bcnp1*$^{-/-}$ mice (Supplementary Figure 1B–D). BCNP1 deficiency did not affect the cellularity in the spleen, bone marrow (BM) and PC (Supplementary Figure 3). B-cell development in the BM was also normal (Supplementary Figure 4A). Analysis of the transitional B cells in the spleen revealed a significant increase of the T2 population in *Bcnp1*$^{-/-}$ mice, suggesting a partial block of T2 to T3 differentiation in the mutant mice (Fig. 1A). Consistently, there was an increase of IgM$^{high}$IgD$^{high}$ transitional B and a decrease of IgM$^{low}$IgD$^{high}$ mature B population in the spleen of *Bcnp1*$^{-/-}$ mice (Fig. 1B), indicating that B-cell maturation was partially blocked in the mutant mice. Direct comparison of the IgM and IgD levels between WT and *Bcnp1*$^{-/-}$ B cells confirmed the increased IgM but normal IgD expression in the mutant mice (Fig. 1C). The proportion of the B220$^{+}$CD21$^{high}$CD23$^{low}$ marginal zone B cells (MZB) appeared normal (Fig. 1D). Analysis of PCs revealed a significant reduction of the B-1a cells in the mutant mice (Fig. 1E). Collectively, *Bcnp1*$^{-/-}$ mice exhibited a partial block of B-cell maturation in the spleen and a decreased B-1a population in the PC.

### *Increased survival, proliferation and $Ca^{2+}$ influx in Bcnp1$^{-/-}$ spleen B cells*

While B-cell maturation was partially impaired in *Bcnp1*$^{-/-}$ mice, *Bcnp1*$^{-/-}$ spleen B cells exhibited a moderately increased survival (Fig. 2A) and proliferation (Fig. 2B) in response to different doses of F(ab′)$_2$ α-IgM antibodies. Carboxyfluorescein succinimidyl ester (CFSE) assay confirmed that *Bcnp1*$^{-/-}$ spleen B cells had increased survival and underwent more cell divisions in response to BCR stimulation (Fig. 2C). Consistently, the mutant B cells showed elevated $Ca^{2+}$ influx in response to BCR cross-linking than did WT B cells (Fig. 2D). *Bcnp1*$^{-/-}$ spleen B cells also exhibited a moderately increased response to LPS stimulation (Fig. 2C).

The expressions of various cell surface receptors were not different between WT and *Bcnp1*$^{-/-}$ B cells (Supplementary Figure 4B). Class-switch recombination (CSR) induced by CD40L+α-CD8+IL-4 was reduced in *Bcnp1*$^{-/-}$ B cells as compared with WT B cells (Supplementary Figure 4C). In contrast, CSR induced by LPS+IL-4 occurred normally (Supplementary Figure 4C). T-cell subsets in the thymus and spleen were not different between WT and *Bcnp1*$^{-/-}$ mice. *Bcnp1*$^{-/-}$ B cells exhibited normal constitutive (ligand-independent) and moderately elevated α-IgM-induced (ligand-dependent) BCR internalization compared with WT B cells (Supplementary Figure 5).

### *BCNP1 regulates the phosphorylation of BLNK and PLCγ2*

Since $Ca^{2+}$ influx induced by BCR cross-linking was elevated in *Bcnp1*$^{-/-}$ B cells, we next analyzed kinase and adaptor molecules that are involved in regulating $Ca^{2+}$ influx. BCR engagement initiates SYK activation, which phosphorylates BLNK. Phosphorylated BLNK then translocates to the cell membrane, and recruits and activates PLCγ2 (7, 21). The activated PLCγ2 hydrolyzes membrane phospholipids to produce inositol-triphosphate (IP3), which results in increases in free cytoplasmic $Ca^{2+}$ (22). In this pathway, we found that the phosphorylation of SYK, BLNK and PLCγ2 was enhanced in *Bcnp1*$^{-/-}$ spleen B cells as compared with WT B cells (Fig. 3A). Quantification of the phosphorylated and total proteins revealed a significantly enhanced phosphorylation of SYK, BLNK and PLCγ2 in *Bcnp1*$^{-/-}$ spleen B cells after α-IgM-stimulation (Fig. 3B). These results demonstrate that BCNP1 participates in BCR signaling in primary B cells by regulating the phosphorylation of SYK, BLNK and PLCγ2.

### *Enhanced humoral immune responses in Bcnp1$^{-/-}$ mice*

*Bcnp1*$^{-/-}$ mice showed a moderate increase in the steady state levels of serum IgM and IgG$_3$ and a slight decrease of serum IgG$_{2c}$ and IgA compared with WT mice (Supplementary Figure 6). Humoral immune responses to a T-independent antigen NP-Ficoll were enhanced (Fig. 4A). The production of both NP-specific IgM and IgG$_3$ was significantly elevated (Fig. 4A). These results are consistent with the elevated survival, proliferation and $Ca^{2+}$ influx in *Bcnp1*$^{-/-}$ B cells after BCR stimulation under *in vitro* conditions. Responses to a T-dependent antigen NP coupled to CGG (NP-CGG) were also significantly enhanced (Fig. 4B). The production of both total (Fig. 4B, left panel) and high-affinity (right panel)

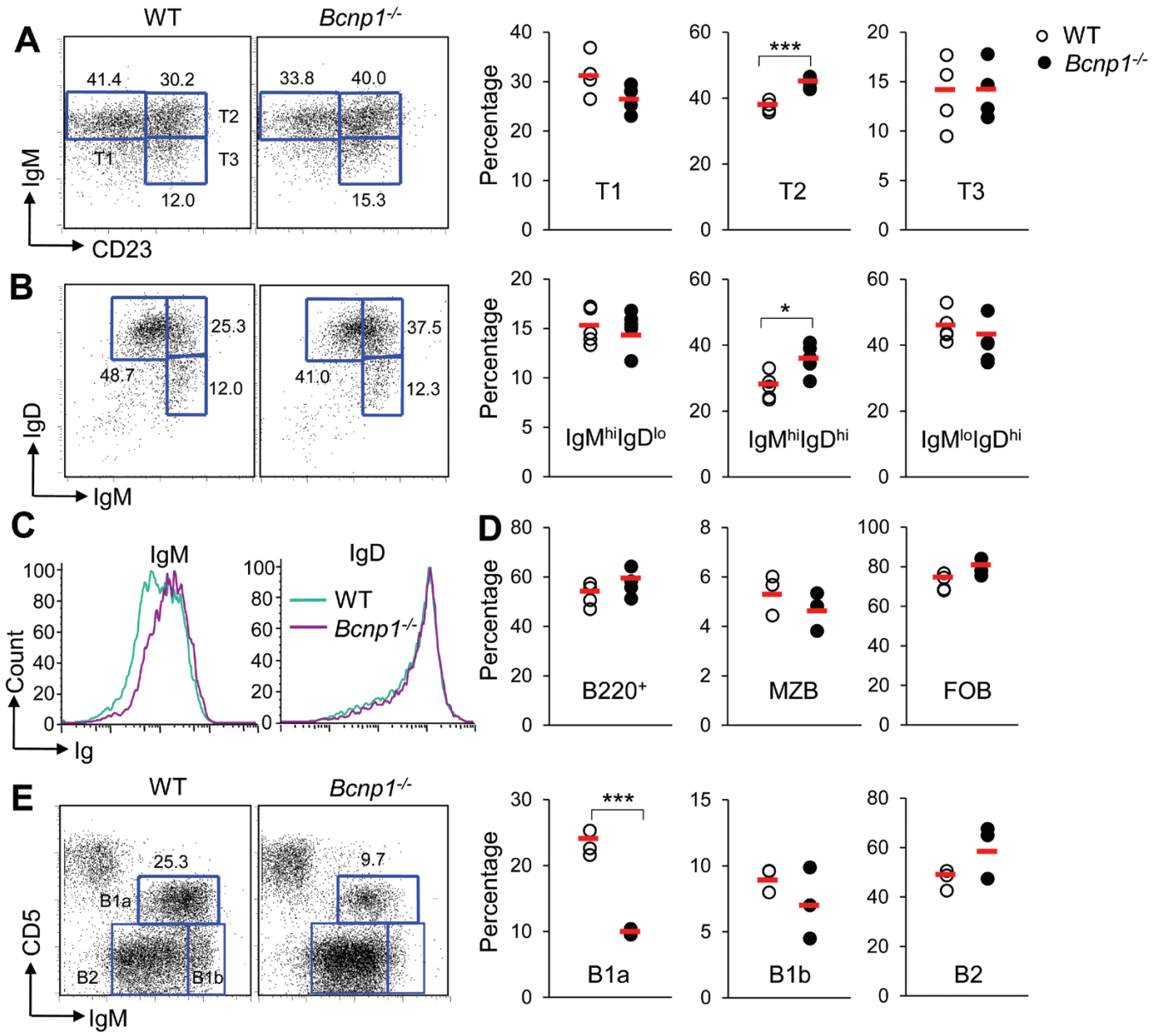


**Fig. 1.** A partial block of B-cell maturation in the spleen and a reduction of the B-1a population in the PC in *Bcnp1*$^{-/-}$ mice. (A) Spleen cells were analyzed for IgM and CD23 expression in gated B220$^{+}$AA4.1$^{+}$ cells to distinguish T1, T2, T3 populations. Left, representative FACS profiles; right, results of four pairs of WT and *Bcnp1*$^{-/-}$ mice. (B) Spleen cells were analyzed for IgM and IgD expression in gated B220$^{+}$ cells to reveal IgM$^{hi}$IgD$^{lo}$, IgM$^{hi}$IgD$^{hi}$, IgM$^{lo}$IgD$^{hi}$ populations. Left, representative FACS profiles; right, results obtained with five pairs of WT and *Bcnp1*$^{-/-}$ mice. (C) Increased IgM but normal IgD expression in *Bcnp1*$^{-/-}$ spleen B cells (gated on B220$^{+}$ cells) as compared with WT B cells. (D) Proportion of the B220$^{+}$ B cells, and MZB (CD23$^{low}$CD21$^{high}$) and FOB (CD23$^{high}$CD21$^{low}$) among the B220$^{+}$ cells in the spleen of WT and *Bcnp1*$^{-/-}$ mice. Results of four pairs of WT and *Bcnp1*$^{-/-}$ mice are shown. (E) Reduced B-1a population in gated 7-AAD$^{-}$ cells in the PC of *Bcnp1*$^{-/-}$ mice. Left panels, representative FACS profiles; right panels, summary of three pairs of WT and *Bcnp1*$^{-/-}$ mice. Open circles, WT mice; solid circles, *Bcnp1*$^{-/-}$ mice. A red bar indicates the mean value. *$P < 0.05$; ***$P < 0.005$ (two-tailed unpaired *t*-test).

NP-specific IgG$_1$ antibodies was enhanced in *Bcnp1*$^{-/-}$ mice as compared with WT mice. In agreement with the increased levels of NP-specific IgG$_1$ antibodies, ELISPOT analysis revealed a significant increase of the antibody-forming cells (AFCs) that secrete low- (as measured with NP$_{25}$-BSA) and high-affinity (measured with NP$_2$-BSA) NP-specific antibodies (Fig. 4C).

### *B-cell expansion in aged Bcnp1*$^{-/-}$ *mice*

Aged *Bcnp1*$^{-/-}$ mice exhibited a more striking increase in the serum IgM and IgG$_3$ levels and a decrease in the total serum IgG levels as compared with age-matched WT mice (Fig. 5A). Furthermore, we found an increased B-cell population in various lymphoid organs. There was a significant increase in the proportion of the B220$^{+}$, but not CD3$^{+}$, cells in

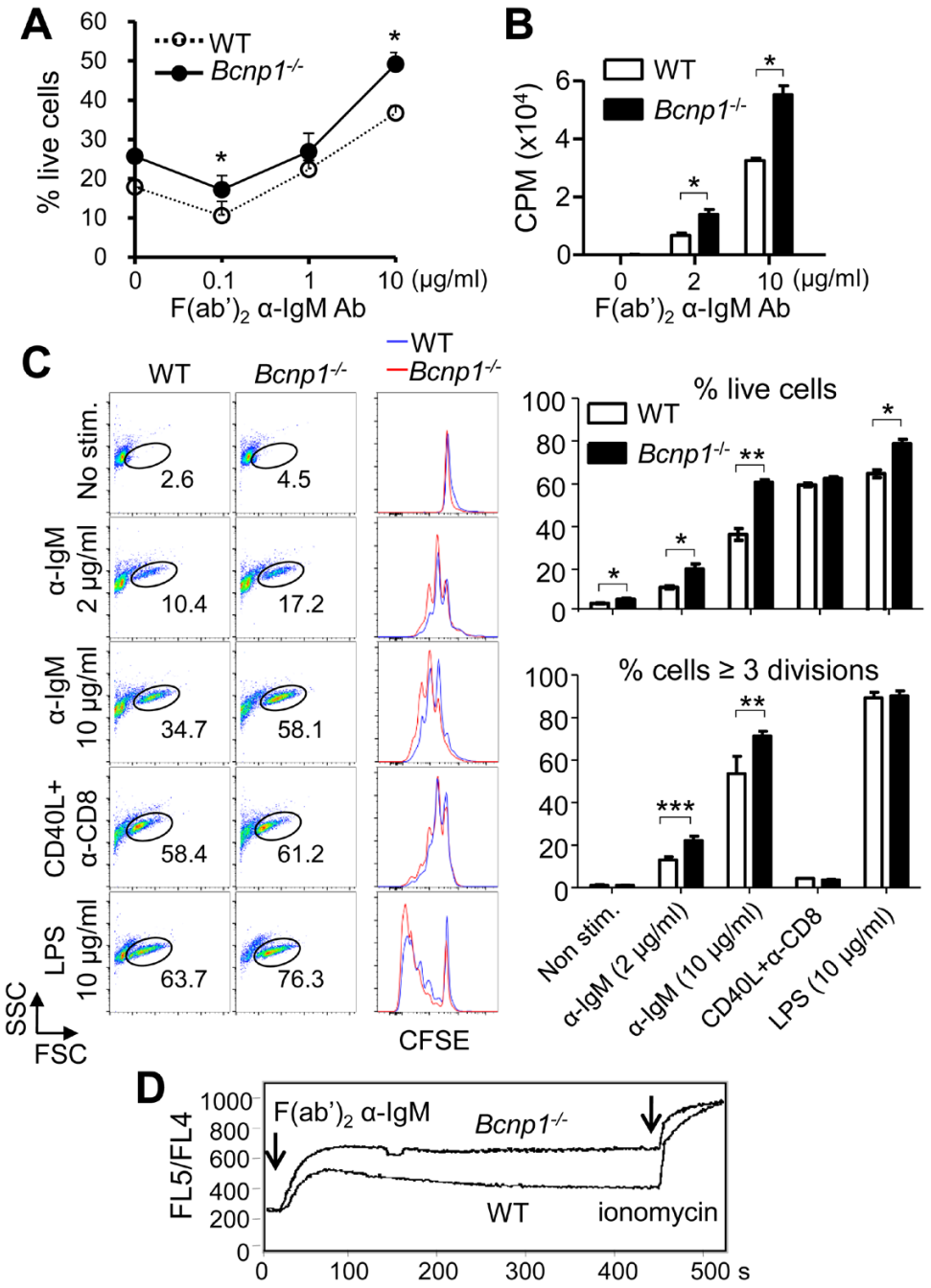


**Fig. 2.** *Bcnp1*$^{-/-}$ spleen B cells show increased survival, proliferation and $Ca^{2+}$ influx in response to BCR stimulation. (A, B) Purified WT and *Bcnp1*$^{-/-}$ spleen B cells ($5 \times 10^5$ cells ml$^{-1}$) were cultured in the presence of different doses of F(ab′)$_2$ α-IgM antibodies for 2 days and analyzed for B-cell viability by staining with PI (A) and proliferation by thymidine uptake (B). (C) Purified spleen B cells were labeled with CFSE and cultured for 3 days in medium alone or in the presence of 2 or 10 μg ml$^{-1}$ of F(ab′)$_2$ α-IgM antibodies, soluble CD40L-CD8α protein cross-linked with 2 μg ml$^{-1}$ of α-CD8 antibody (CD40L + α-CD8) or 10 μg ml$^{-1}$ of LPS. Live cells were gated based on FSC and SSC, and analyzed for CFSE intensity. Left, representative FACS profiles; upper right, % live cells; lower right, % cells divided three or more times. (D) Purified spleen B cells were stimulated with 10 μg ml$^{-1}$ of F(ab′)$_2$ α-IgM antibodies and analyzed for $Ca^{2+}$ influx. Ionomycin was used as a positive control. Representative results of three independent experiments are shown. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ (two-tailed unpaired *t*-test).

the spleen of *Bcnp1*$^{-/-}$ mice (Fig. 5B, upper panels). The majority of these B220$^+$ cells appeared to be FOB on the basis of their CD23 and CD21 expression (Fig. 5B, lower panels). The average percentages and absolute numbers of T, B, FOB and MZB in the spleen of six pairs of WT and *Bcnp1*$^{-/-}$ mice are summarized in Fig. 5C (upper and lower panels, respectively). B cells in aged *Bcnp1*$^{-/-}$ mice also contained a higher proportion of the CD80$^+$, possibly activated, cells compared with WT mice (Fig. 5D, left panel) although the difference did not reach statistical significance (Fig. 5D, right panel). There

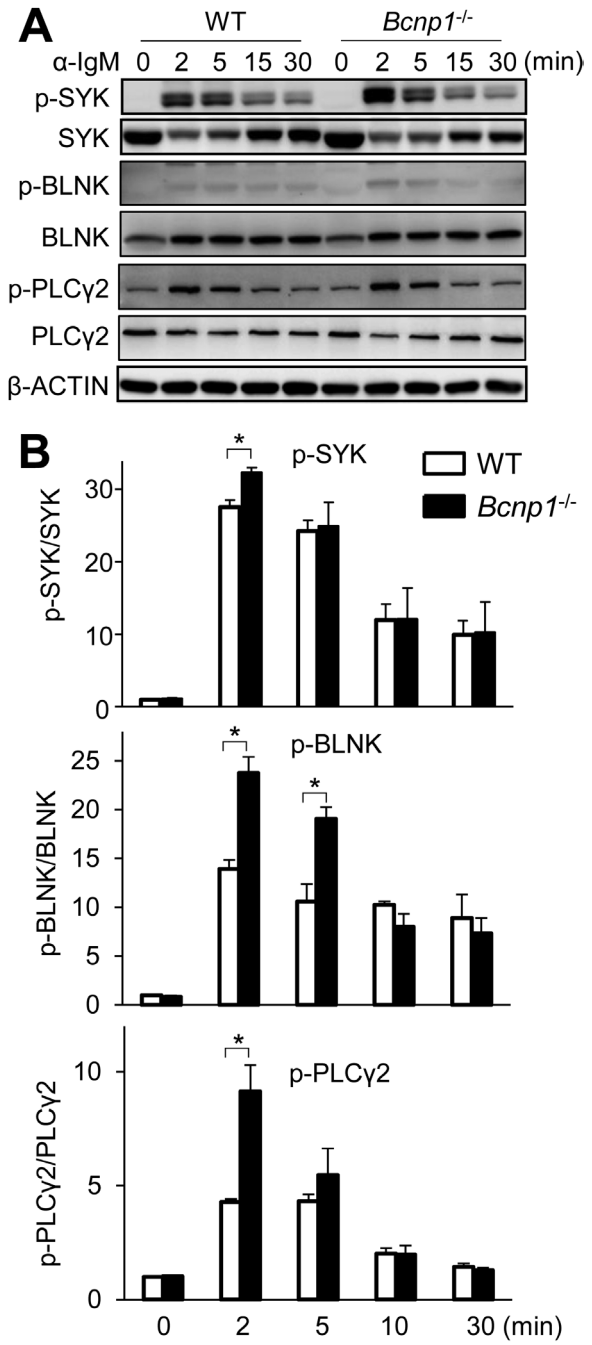


**Fig. 3.** BCNP1 regulates the phosphorylation of SYK, BLNK and PLCγ2. (A) Purified spleen B cells were stimulated with 10 μg ml$^{-1}$ of F(ab′)$_2$ α-IgM antibodies for the indicated time and the cell lysates were resolved by SDS–PAGE and blotted with the indicated antibodies. (B) Ratio of the phosphorylated SYK (p-SYK), BLNK (p-BLNK) or PLCγ2 (p-PLCγ2) to their total proteins. B cells purified from three WT and three *Bcnp1*$^{-/-}$ mice were analyzed and mean ± SEM are shown. *$P < 0.05$.

was no increase in the proportion (Fig. 5E, upper panels and Fig. 5F, left panel) but a significant increase in the absolute numbers (Fig. 5F, right panel) of IgM$^+$CD5$^+$ cells in the PC of *Bcnp1*$^{-/-}$ mice. To clarify whether these IgM$^+$CD5$^+$ cells are B-1a cells, we further stained these cells with PtC. A substantial fraction of B-1a cells are known to bind PtC, which was the case for the IgM$^+$CD5$^+$ cells found in WT PCs (Fig. 5E, lower left panel). In contrast, most of the IgM$^+$CD5$^+$ cells in the PCs of *Bcnp1*$^{-/-}$ mice failed to bind PtC (Fig. 5E, lower right panel), suggesting that they are not bona fide B-1a cells. The percentage and absolute numbers of the PtC$^+$ cells are summarized in Fig. 5G (left and right panels, respectively). The B-cell population was also increased in the cervical LNs in *Bcnp1*$^{-/-}$ mice (Fig. 5H). These results collectively demonstrate that absence of BCNP1 caused aberrant B-cell expansion in lymphoid organs in aged mice.

To investigate whether the increased B-cell population in aged *Bcnp1*$^{-/-}$ mice is due to clonal expansion, we analyzed the CDR3 length of the immunoglobulin gene heavy chain genes in B cells from the spleen and LNs. We analyzed three V gene families, namely J558, 7183 and Q52, in combination with the 4 $J_H$ exons, and found a significantly higher frequency of clonal expansion of B cells in the spleen and LNs of *Bcnp1*$^{-/-}$ mice compared with WT mice (Fig. 5I). These results indicate that absence of BCNP1 resulted in an increased B-cell population in multiple lymphoid organs of aged mice, which was accompanied by clonal expansion.

## Discussion

BCNP1-deficient mice have a reduction of the B-1a population in the PC and a partial block in B-cell maturation in the spleen, which are common features observed in mice with impaired BCR signaling, including mice deficient in Bruton's tyrosine kinase (BTK), BLNK or BCAP, and in IgαFF and IgβAA mice in which the tyrosine residues in the ITAMs of Igα and Igβ were mutated to phenylalanine (F) and alanine (A), respectively (23–27). These observations implicate a role for BCNP1 in positively regulating BCR signaling during B-cell development and maturation. This conclusion is in agreement with our recent finding that BCNP1 overexpression in WEHI231 immature B cells potentiated α-IgM-induced apoptosis (14). Conversely, BCNP1-deficient WEHI231 cells, generated by CRSPR-Cas9-mediated genome editing, exhibited reduced apoptosis after BCR cross-linking (14). In contrast to immature B cells, however, *Bcnp1*$^{-/-}$ mature B cells exhibited elevated $Ca^{2+}$ influx and the phosphorylation of SYK, BLNK and PLCγ2 in response to BCR cross-linking, suggesting a role for BCNP1 in negatively regulating BCR signaling in mature B cells. Therefore, BCNP1 deficiency appeared to have opposing effects in immature versus mature B cells.

The phenotypes of *Bcnp1*$^{-/-}$ mice resemble those found in IgαFF and IgβAA mice (25, 27). Similar to IgαFF and IgβAA, BCNP1 deficiency attenuated B-1a cell development and follicular B-cell maturation but enhanced mature B-cell survival and activation. In addition, both *Bcnp1*$^{-/-}$ and IgβAA mice show increased serum IgM levels and elevated antibody production against the T-I antigen NP-Ficoll (27). However, responses to T-D antigen were different between *Bcnp1*$^{-/-}$ and IgαFF or IgβAA mice. While IgαFF and IgβAA mice show decreased antibody production against a T-dependent antigen, NP-CGG, *Bcnp1*$^{-/-}$ mice exhibit elevated antibody production against the same antigen. It was reported that both IgαFF and IgβAA B cells had impaired constitutive and antigen-induced BCR internalization (27, 28). BCR internalization is an essential

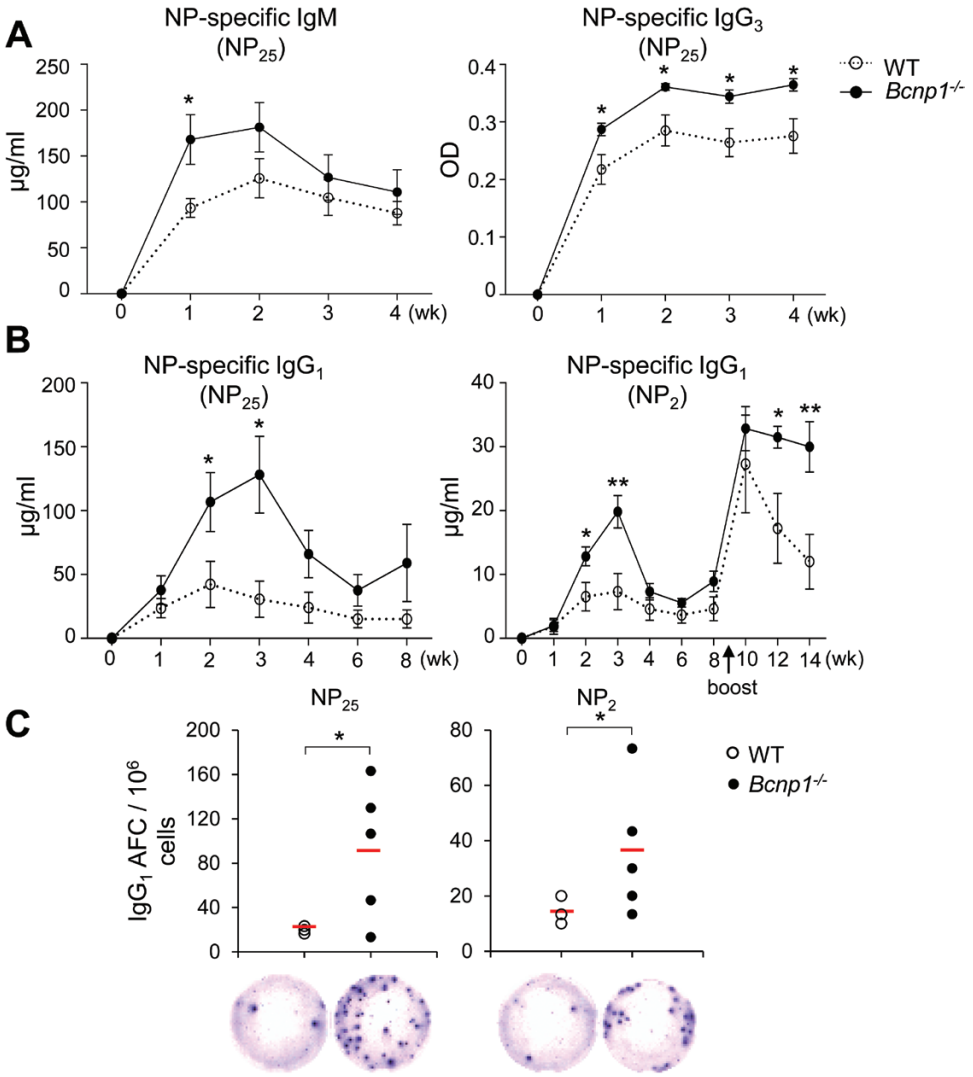


**Fig. 4.** Elevated antibody production against both T-I and T-D antigen in *Bcnp1*$^{-/-}$ mice. (A) Five pairs of WT and *Bcnp1*$^{-/-}$ mice were immunized i.p. with 10 µg of NP-FICOLL and analyzed for the NP-specific IgM (left panel) and $IgG_3$ (right panel) in the sera by ELISA. (B) Seven pairs of WT and *Bcnp1*$^{-/-}$ were immunized with 10 µg of NP-CGG precipitated in alum. The mice were boosted with the same amount of NP-CGG in phosphate-buffered saline (PBS) 9 weeks later. Total (left panel) and high affinity (right panel) NP-specific $IgG_1$ antibody in the sera were analyzed by ELISA. For A and B, mean ± SEM are shown. (C) AFC in the BM of five pairs of WT and *Bcnp1*$^{-/-}$ mice at 20 weeks after immunization with NP-CGG in alum. AFCs were measured by ELISPOT. Open circles, WT mice; solid circles, *Bcnp1*$^{-/-}$ mice. $^{*}P < 0.05$; $^{**}P < 0.01$ (two-tailed unpaired *t*-test).

step for antigen endocytosis and subsequent presentation to T cells. The decreased antibody production against T-D antigen in IgαFF and IgβAA mice could in part be caused by the impaired B-cell antigen presentation to T-follicular helper cells. In contrast to IgαFF and IgβAA B cells, *Bcnp1*$^{-/-}$ B cells have normal constitutive and moderately enhanced α-IgM-induced BCR internalization (Supplementary Figure 5). *Bcnp1*$^{-/-}$ B cells also show a moderately increased response to LPS, a TLR4 ligand. Recently, it was reported that TLR4 signaling in B cells requires BCR and SYK (29). The elevated LPS response in *Bcnp1*$^{-/-}$ spleen B cells may be attributable to the enhanced BCR signaling although a role for BCNP1 in directly regulating TLR4 signaling cannot be excluded.

Several mutually nonexclusive possibilities could be considered for the seemingly opposing roles for BCNP1 during B-cell development versus mature B-cell activation. B-cell development proceeds in an antigen-independent manner and the BCR signaling in these cells may be substantially different from that in mature B cells. In fact, the expression levels of various cell surface and intracellular molecules involved in BCR signal transduction are quite different between immature and mature B cells. For instance, mature B cells, but not immature B cells, express IgD, which is known to affect the tonic and antigen-triggered BCR signaling (30–37). The differential roles of BCNP1 in immature and mature B cells may be attributable to the differences in the composition or context of BCR signaling molecules. Alternatively, BCNP1 positively regulates BCR signaling but its deficiency has caused the selection of B cells with elevated BCR levels and stronger BCR signaling, leading to enhanced BCR responses in mature

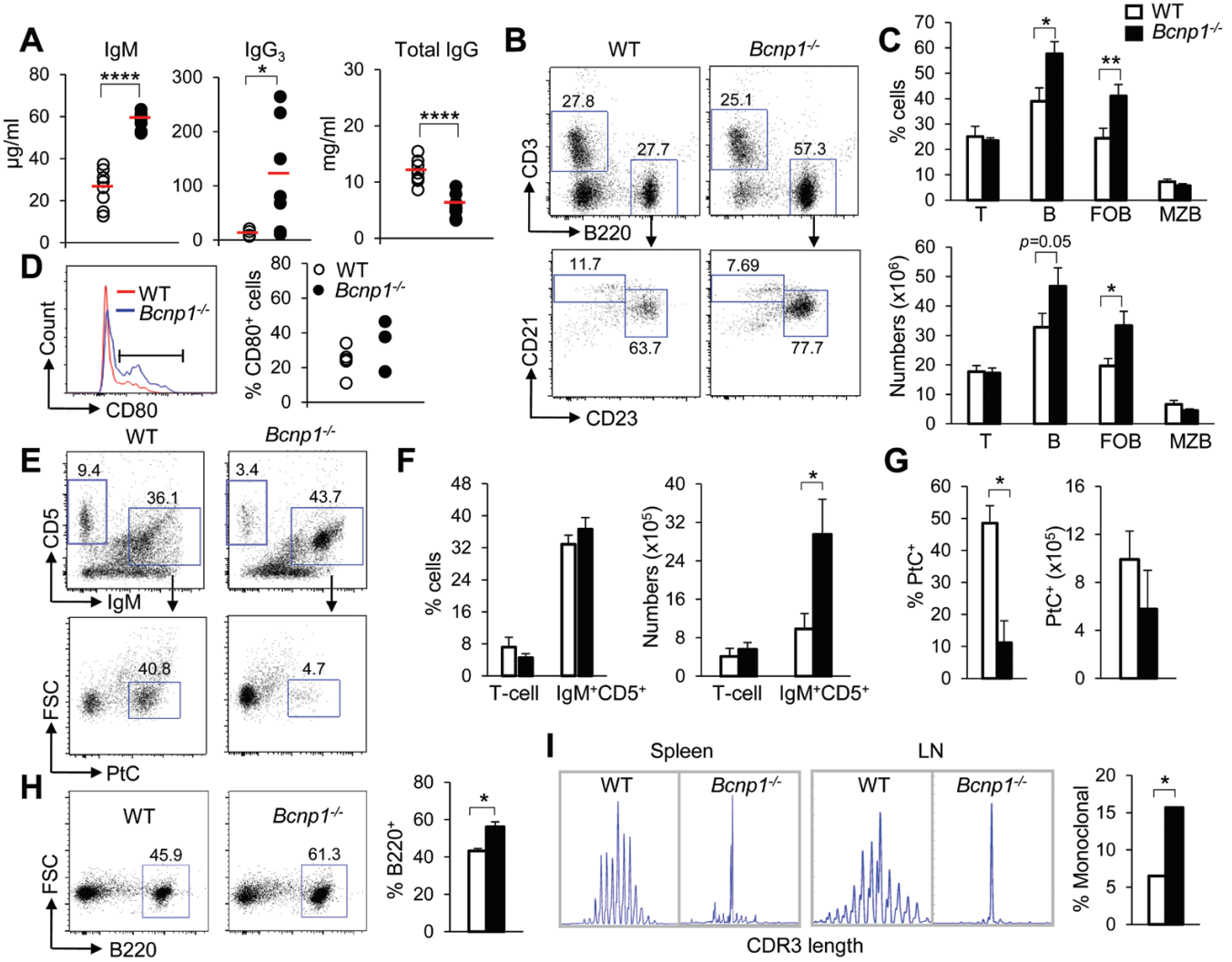


**Fig. 5.** Chronic B-cell expansion in aged (14 months old) *Bcnp1*$^{-/-}$ mice. (A) Increased IgM and $IgG_3$ but decreased total IgG levels in aged *Bcnp1*$^{-/-}$ mice compared with aged WT mice. Mice were bled and analyzed for serum IgM (nine pairs of WT and *Bcnp1*$^{-/-}$ mice), $IgG_3$ (six WT and seven *Bcnp1*$^{-/-}$ mice) and total IgG (nine pairs of WT and *Bcnp1*$^{-/-}$ mice) levels by ELISA. (B) Increased proportion of B220$^+$ but normal proportion of CD3$^+$ cells (upper panels) in the spleen of aged mutant mice (gated on 7-AAD$^-$ cells). The B220$^+$ cells were mostly CD23$^+$CD21$^-$ FOB cells (lower panels). (C) Percentages (upper panel) and absolute numbers (lower panel) of T cells, B cells, FOB and MZB in seven WT and six *Bcnp1*$^{-/-}$ mice. (D) Aged *Bcnp1*$^{-/-}$ mice contained an increased proportion of CD80$^+$ B cells in the spleen than did WT mice. Left panel, representative FACS profile (gated on B220$^+$7-AAD$^-$ cells); right panel, results of four WT and three *Bcnp1*$^{-/-}$ mice. (E) Analysis of the IgM$^+$CD5$^+$ (upper panels) and the PtC-binding (lower panels) population in the PC of aged WT and *Bcnp1*$^{-/-}$ mice. (F) Summary of the proportion (left) and absolute numbers (right) of T cells and the IgM$^+$CD5$^+$ population in the PC in seven pairs of aged WT and *Bcnp1*$^{-/-}$ mice. (G) The proportion (left panel) and the absolute numbers (right panel) of the PtC-binding cells among the IgM$^+$CD5$^+$ population in the PC of three pairs of aged WT and *Bcnp1*$^{-/-}$ mice. (H) Increased B-cell proportion in the cervical LN in aged *Bcnp1*$^{-/-}$ mice. Left two panels, representative FACS profiles; right panel, summary of three pairs of WT and *Bcnp1*$^{-/-}$ mice. (I) Polyclonal and monoclonal expansion of B cells in the spleen and LN in aged *Bcnp1*$^{-/-}$ mice. Left panels, representative profiles of CDR3 length distribution; right panel, frequency of monoclonal B-cell expansion in the spleen and LN of three WT (open column) and three *Bcnp1*$^{-/-}$ (solid column) mice. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ (two-tailed unpaired *t*-test and chi-square test).

B cells. Since Igα or Igβ mutated spleen B cells show increased BCR signaling even though these mutations disrupt their interaction with SYK and the subsequent activation of downstream signaling molecules, we think that the latter possibility is more likely and that BCNP1 is primarily a positive regulator of BCR signaling. Further studies are required to completely understand the role of BCNP1 in BCR signaling at different stages during B-cell development and at different B-cell subsets.

Serum IgM and $IgG_3$ levels were significantly elevated in aged *Bcnp1*$^{-/-}$ mice. The enhanced BCR responses in *Bcnp1*$^{-/-}$ mature B cells may have resulted in T-cell-independent chronic B-cell activation and subsequent differentiation into plasma cells that secrete IgM and $IgG_3$. Aged *Bcnp1*$^{-/-}$ mice also have increased numbers of B cells in various lymphoid organs, which could be due to the enhanced B-cell survival that resulted in their gradual accumulation. Notably, the IgM$^+$CD5$^+$ cells accumulated in the

PC of *Bcnp1*$^{-/-}$ mice are not bona fide B-1a cells as shown by their poor binding to PtC. These IgM$^{+}$CD5$^{+}$ cells might be activated B cells that aberrantly express CD5 (38, 39). Although aged BCNP1-deficient mice show polyclonal and monoclonal B-cell expansion in the spleen and LNs, we have not observed B-cell lymphomas in these mice. The lack of B lymphomas could be due to the relatively short life span of mice. It is possible that BCNP1 deficiency may accelerate B lymphoma development in tumor-prone mouse lines such as Myc-transgenic or p53-deficient mice. In conclusion, the present study revealed distinct functions of BCNP1 in B-cell development, activation and homeostasis. Our results provide new insights into the mechanisms that fine-tune BCR signaling to allow optimal B-cell responses yet prevent uncontrolled B-cell expansion.

## Funding

This work was supported by the National Basic Research Program of China (2015CB943300 to J.-Y.W.), the National Natural Science Foundation of China (81571529, 81811540035 and 31870898 to J.-Y.W) and a grant-in-aid for scientific research (C) from Japan Society for the Promotion of Science (17K08878 to J.-Y.W).

## Acknowledgements

We thank Lumin Zhang for excellent technical assistance and Fudan University Animal Facility for maintaining and breeding the mice. R.H. and N.L. performed most of the experiments; R.H. drafted the manuscript; R.O. generated *Bcnp1*$^{-/-}$ mice; E.X. analyzed CDR3 length of B cells; Y.T., J.S. and Y.Z. analyzed the signaling molecules; M.H., T.T., M.T. and Y.W. supervised the study; J.-Y.W. designed the study, conceived the experiments and corrected the manuscript.

*Conflicts of interest statement:* the authors declared no conflicts of interest.

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