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3BP2 Deficiency Impairs the Response of B Cells, but Not T Cells, to Antigen Receptor Ligation

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The adapter protein 3BP2 is expressed in lymphocytes; binds to Syk/ZAP-70, Vav, and phospholipase C- γ (PLC- γ); and is thought to be important for interleukin-2 gene transcription in T cells. To define the role of 3BP2 in lymphocyte development and function, we generated 3BP2-deficient mice. T-cell development, proliferation, cytokine secretion, and signaling in response to T-cell receptor (TCR) ligation were all normal in 3BP2^{-/-} mice. 3BP2^{-/-} mice had increased accumulation of pre-B cells in the bone marrow and a block in the progression of transitional B cells in the spleen from the T1 to the T2 stage, but normal numbers of mature B cells. B-cell proliferation, cell cycle progression, PLC- γ 2 phosphorylation, calcium mobilization, NF-AT β dephosphorylation, and Erk and Jnk activation in response to B-cell receptor (BCR) ligation were all impaired. These results suggest that 3BP2 is important for BCR, but not for TCR signaling.

3BP2 is a protein originally characterized as an Abl SH3-interacting protein (44). It consists of an N-terminal pleckstrin homology domain, a proline-rich central region, and a C-terminal SH2 domain. 3BP2 mRNA is expressed in hematopoietic tissues. 3BP2 protein is expressed in T cells, B cells, natural killer (NK) cells, monocytic cell lines, osteoclasts, and the rat basophilic leukemia cell line RBL-2H3 (11, 37). 3BP2 associates with Syk and ZAP-70 (11, 36). Association of 3BP2 with Syk was shown to require the SH2 domain of 3BP2 and the catalytic activity of Syk. The SH2 domain of 3BP2 has also been shown to bind to the adapter protein LAT in T cells and mast cells after T-cell receptor (TCR) and Fc ϵ RI ligation, respectively (11, 46). Phospholipase C- γ (PLC- γ) and Vav were also identified as binding partners of 3BP2 (22). Other partners of 3BP2 include 14-3-3, Grb2, Cbl, and Fyn (11, 14, 15).

TCR stimulation induces a significant translocation of 3BP2 to the membrane and detergent-insoluble (cytoskeleton) fractions, suggesting a role for 3BP2 in TCR-mediated signal transduction (11). Transient overexpression of 3BP2 in Jurkat T cells induces transcriptional activation of the interleukin-2 (IL-2) gene promoter and its NF-AT and AP-1 elements and cooperates with TCR ligation and ionomycin in activating NF-AT/AP-1-driven transcription. Overexpression of 3BP2 resulted in calcineurin-dependent dephosphorylation of NF-ATc and stimulated AP-1 activity independently of NF-AT (11). The SH2 and PH domains of 3BP2, but not its proline-rich domain, are required for NF-AT activation. Furthermore, overexpression of the SH2 domain of 3BP2 inhibited TCR-mediated NF-AT activation (11). Activation of NF-AT by 3BP2 in T cells required ZAP-70, because overexpression of 3BP2 in a ZAP-70-deficient Jurkat cell clone failed to activate NF-AT (11).

Overexpression of 3BP2 enhances NK cell-mediated cytotoxicity (22). Fc ϵ RI ligation induces the phosphorylation of 3BP2 and its association with LAT in rat basophilic leukemia RBL-2H3 cells (46). Overexpression of the SH2 domain of 3BP2 in these cells suppresses Fc ϵ RI-induced signaling (46). Recently, it was shown that 3BP2 is tyrosine phosphorylated following B-cell receptor (BCR) ligation in B cells and is a substrate for Syk and Fyn, but not Btk (14). 3BP2 was shown to interact with several components of the BCR signaling pathway, including Syk, PLC- γ , and Vav, and cooperated with Vav to activate NF-AT after BCR ligation. To investigate the role of 3BP2 in lymphocyte development and function, we generated 3BP2-null mice by gene targeting.

MATERIALS AND METHODS

Generation of 3BP2-deficient mice. The *3bp2* gene was cloned from isogenic 129 genomic DNA library (Stratagene) using the full-length cDNA as a probe. The 5' 5.4-kb arm (PstI-EcoRV) and the 3' 8-kb arm (HindIII-HindIII) fragments were subcloned into pScrambler vector (Stratagene), which contains the neomycin phosphotransferase (*neo*) cDNA driven by the PGK promoter. The construct was introduced by electroporation into embryonic stem (ES) cells using standard protocols (2), and cells were then selected in medium containing 200 μ g/ml G418 and 1 μ M ganciclovir. ES clones containing the homologous recombinant event were identified by Southern blot analysis and by a 5.6-kb PCR product using forward primer a (5'-TGAGTCACTGGATCATGGGAG-3') located upstream of the 5' arm and reverse primer b (5'-CGCATGCTCCAGAC TGCCCTTG-3') located in the *neo* gene. A 0.3-kb product is amplified by PCR from the WT allele using forward primer c in exon 9 (5'-ACAGGCTGACACT GGCGA-3') and reverse primer d in exon 10 (5'-CGCAAGACTCTGTCGTG T-3'). Two ES clones (no. 3 and 7) with the recombinant allele were injected in C57BL/6 blastocysts, and 3BP2^{-/-} mice were obtained by standard methods (54). 3BP2 mRNA expression was analyzed by reverse transcription-PCR (RT-PCR) using a forward primer, e, that hybridizes to exon 2 (5'-GCTGGTACC TGCATAAG3') and a reverse primer, f, that hybridizes to the 5' half of exon 6 (5'-ATAGGTCGCTCAACTGCA3'). 3BP2 protein expression was examined by Western blotting using an antibody raised against amino acids 359 to 462 of the protein.

FACS analysis. Single-cell suspensions from spleen, bone marrow, thymus, and peritoneal cavity were isolated on a density gradient of Lympholyte-M (Cedarlane Laboratories). Cells were stained with phycoerythrin- or fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (MAbs) from PharMingen and analyzed on a FACSCalibur flow cytometer (Becton Dickinson) as previously described (2). Fluorescence-activated cell sorter (FACS) analysis was per-

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formed on cells from mice between 6 and 10 weeks of age. Annexin-FITC staining and propidium iodide (PI) staining were performed using a kit from Biovision Inc.

Antibodies. The MAbs were obtained from the following suppliers: anti-CD3 ϵ (KT3) was from Serotec, anti-CD28 was from eBioscience, antiactin was from Chemicon, anti-ZAP-70 and anti-PY 4G10 were from UpstateBiotechnology, and anti-CD40 was from R&D. The polyclonal antibodies were obtained from the following suppliers: anti-Syk was from Abcam; anti-Vav1, -PLC- γ 1, and -PLC- γ 2 were from Santa Cruz Biotechnology; anti-NF-ATp was a gift from A. Rao; anti-cyclin D2 was from Biosource; and anti-cyclin D3, -cdk4, -cdk6, -p27, and -pRb antibodies were from Cell Signaling. All antibodies to mitogen-activated protein (MAP) kinases were from Cell Signaling, except antibody to phospho-Jnk, which was from Biosource.

Cell purification, proliferation of splenic T and B cells, and Ig synthesis. Splenic T cells were purified (>95% CD3 $^{+}$ cells) by negative selection using CD3 enrichment columns (R&D) and cultured at 1×10^5 /well for 64 h in wells coated with 2 μ g/ml anti-CD3 MAb, with or without 2 μ g/ml anti-CD28 MAb or IL-2 (R&D Systems) at 40 ng/ml. Phorbol myristate acetate was used at 15 ng/ml, and ionomycin was used at 0.5 μ M. Proliferation was assessed by the incorporation of [3 H]thymidine. Concentrations of IL-2 in supernatants were measured by enzyme-linked immunosorbent assay (ELISA; R&D). B cells were purified from splenocytes labeled with biotin-conjugated MAbs to CD43 and CD11b (BD Pharmingen) and negatively sorted with streptavidin-conjugated magnetic beads (Dyna). B cells (>85% CD43-negative cells) were cultured at 1×10^5 /well in medium alone or in the presence of goat F(ab') $_2$ anti-mouse immunoglobulin M (IgM) (Jackson ImmunoResearch) without or with IL-4 (40 ng/ml; R&D Systems), anti-CD40 (2 μ g/ml; R&D), or lipopolysaccharide (LPS; 10 μ g/ml [Sigma]), and 48 h later, the cells were pulsed with 1 μ Ci [3 H]thymidine and counted. B and T cells were also incubated with 5 μ M 5,6-carboxyfluorescein succinimidyl ester (CFSE) at 37°C for 10 min and then washed in phosphate-buffered saline (PBS) before culture at 37°C with different stimuli. After 72 h, cells were analyzed by FACS and percentages of cells for each cell division were calculated. For Ig synthesis, B cells were cultured in complete medium alone or in the presence of 10 μ g/ml LPS (Sigma-Aldrich) and IL-4 without or with goat F(ab') $_2$ anti-mouse IgM as previously described (21). After 6 days, supernatants were assayed for IgM, IgG1, and IgE by ELISA.

For in vitro survival, purified B cells were cultured at 5×10^5 cells/ml in the presence or absence of 200 and 500 ng/ml BAFF (Biosource). At various time points, viable cells were counted in a light microscope using a Neubauer hemocytometer and the trypan blue exclusion test. B cells were also washed, incubated with 10 μ g/ml merocyanine 540 (Sigma-Aldrich) in PBS-0.1% bovine serum albumin, and analyzed by FACS.

Immunoprecipitation and Western blotting. Cells were lysed in ice-cold lysis buffer containing 1% Triton X-100, and lysates were centrifuged for 15 min at 4°C and precleared for 1 h at 4°C with protein G-Sepharose (Amersham Pharmacia Biotech). Immunoprecipitation was performed overnight at 4°C with antibody preadsorbed onto protein G-Sepharose. Beads were washed five times with modified lysis buffer containing 0.2% Triton X-100. Bound proteins were eluted, run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by Western blotting with the indicated antibodies followed by goat anti-mouse or rabbit antibodies conjugated to horseradish peroxidase (Amersham) and enhanced chemiluminescent (ECL) detection (Perkin-Elmer). As indicated, Western blot images were scanned and quantified using NIH Image (version 1.63) software.

Calcium mobilization. Measurement of calcium flux was done as described previously (30). Briefly, 2×10^6 purified T or B cells/ml were incubated with 4 μ g/ml Fluo-4-AM (Molecular Probes). T cells were preloaded with 2 μ g/ml anti-CD3 antibody and then stimulated with 10 μ g/ml goat F(ab') $_2$ anti-rat IgG. B cells were stimulated with 5 to 20 μ g/ml goat F(ab') $_2$ anti-mouse IgM. Cells were analyzed by FACS. Intracellular Ca $^{2+}$ concentration was measured by FACS as Fluo-4AM fluorescence intensity using the FL1 channel.

Serum Ig levels and antibody responses. Serum Ig levels were quantified by ELISA. Immunizations with keyhole limpet hemocyanin (KLH), 2,4,6-trinitrophenyl (TNP)-LPS, and TNP-Ficolin were performed, and the serum antibody responses to KLH and TNP were determined by ELISA as previously described (54).

Statistical analysis. Statistical analysis of the data using Student's *t* test was performed with Prism software.

RESULTS

Generation of 3BP2 $^{-/-}$ mice. A targeting construct, in which the 3' half of exon 6 plus all of exons 7 through 13 of murine

3bp2 were replaced with a neomycin-resistant gene, was introduced into ES cells (Fig. 1A). The deletion includes the proline-rich region and the SH2 domain of 3BP2. ES clones with targeted disruption of *3bp2* were identified by Southern blotting and genomic PCR (data not shown). Of 250 ES clones analyzed, 5 were found to have a disrupted allele and 2 were used to generate 3BP2 $^{-/-}$ mice. Mice with a disrupted *3bp2* gene were identified by PCR on tail genomic DNA, as evidenced by the amplification of a novel 5.6-kb fragment derived from the targeted allele compared to the 0.3-kb fragment derived from the WT allele (Fig. 1B). Lysates and immunoprecipitates from thymus and spleen probed by Western blotting using an antibody raised against an amino acid sequence immediately upstream of the SH2 domain of 3BP2 (amino acids 359 to 462) revealed no detectable 3BP2 protein in 3BP2 $^{-/-}$ mice (Fig. 1C) (data not shown). Because there are no available antibodies directed against the N-terminal region of 3BP2, we looked for the presence of a transcript encoded by the N-terminal exons preserved in the targeted allele. RT-PCR analysis of mRNA from splenocytes with primers that hybridize to sequences in exon 2 and exon 6, which are preserved in the targeted allele, amplified a 392-bp product in WT mice, but no detectable product in 3BP2 $^{-/-}$ mice (Fig. 1D). 3BP2 $^{-/-}$ mice did not display apparent differences from WT littermates in growth, weight, or health. Results of all analyses presented below were similar in the two independently derived 3BP2 $^{-/-}$ mouse strains.

Lymphoid development in 3BP2 $^{-/-}$ mice. Cellularity of the thymus, bone marrow, spleen, and peritoneum was normal in 3BP2 $^{-/-}$ mice. Detailed FACS analysis of thymocytes from 3BP2 $^{-/-}$ mice and WT littermates at 6 to 10 weeks of age revealed no obvious difference in the percentages of CD4 $^{+}$ and CD8 $^{+}$ cells or of CD3 $^{+}$, TCR- $\alpha\beta$ $^{+}$, TCR- $\gamma\delta$ $^{+}$, and CD2 $^{+}$ cells (data not shown). Bone marrow cells from 3BP2 $^{-/-}$ mice had a normal percentage of the B220 $^{+}$ CD43 $^{+}$ pro-B-cell population (Fig. 2A, upper panel), but an increased percentage of B220 lo IgM $^{-}$ population (Fig. 2A, lower panel) that includes pro-B cells and pre-B cells, indicating that pre-B cells were increased (approximately twofold). The percentages of B220 lo IgM $^{+}$ immature B cells (Fig. 2A, lower panel) and B220 hi IgM $^{+}$ /B220 hi CD43 $^{-}$ mature recirculating B cells (Fig. 2A, upper and lower panels) were normal. The absolute numbers of pro-B, pre-B, immature, and recirculating B cells in bone marrow from femurs of 3BP2 $^{-/-}$ mice and WT controls were determined using triple staining for IgM, B220, and CD43. The results revealed a significant increase in the numbers of pre-B cells in 3BP2 $^{-/-}$ mice and no significant difference in the numbers of the other B-cell populations (Table 1). There were no obvious differences in the numbers and percentages of Thy1 $^{+}$ CD4 $^{+}$, CD8 $^{+}$, B220 $^{+}$ IgM $^{+}$, and IgD $^{+}$ cells in spleens and lymph nodes of 3BP2 $^{-/-}$ mice and WT littermates (Fig. 2B) (data not shown).

Immature IgM hi HSA/CD24 $^{+}$ bone marrow B cells are transported into the spleen, where they start to express the complement receptor CD21, a part of the CD19-CD21-CD81 coreceptor complex, and the low-affinity IgE receptor CD23. Splenic transitional B cells have been divided into IgM $^{+}$ CD24 hi CD21 $^{-}$ CD23 $^{-}$ T1 cells, which mature into IgM hi CD21 $^{+}$ CD23 $^{+}$ T2 cells (8). T2 cells mature into IgM lo CD21 $^{+}$ CD23 $^{+}$ follicular B cells, which recirculate. Marginal zone (MZ) B cells are a

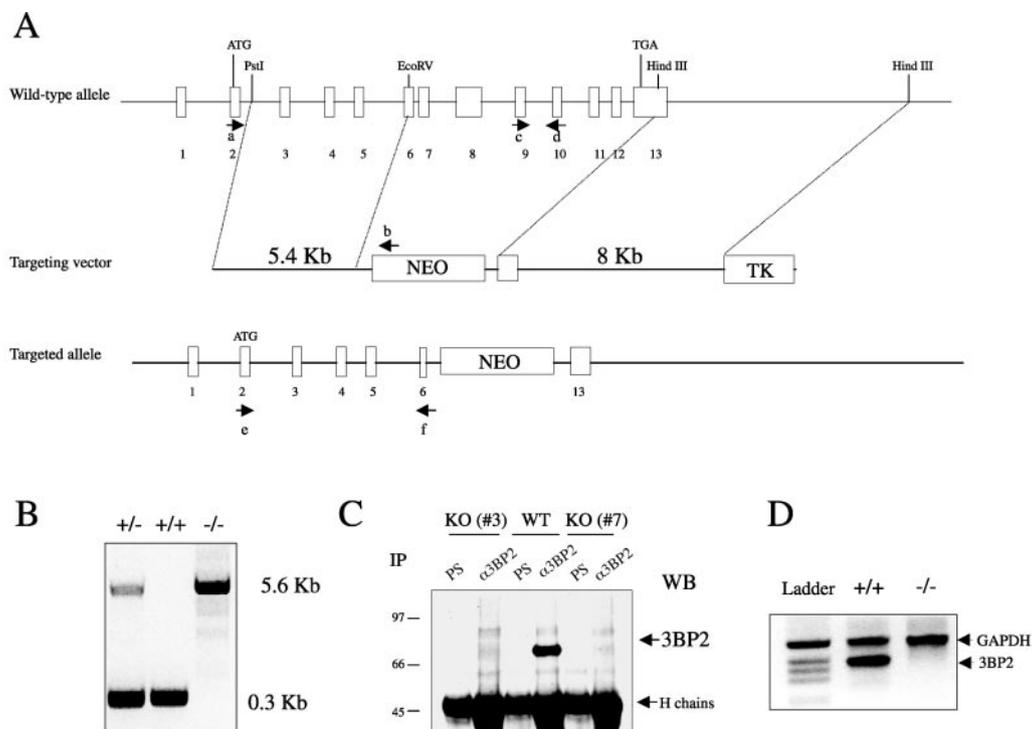


FIG. 1. Generation of 3BP2-deficient mice (A). Genomic structure of the murine *3bp2* gene, targeting vector, and predicted structure of the targeted allele after homologous recombination. Exons are represented by boxes. Neo, neomycin resistance gene; TK, thymidine kinase gene. (B) PCR analysis of tail genomic DNA from 3BP2^{+/+}, 3BP2^{+/-}, and 3BP2^{-/-} littermates. Two sets of primers were used in the same reaction and are shown in Fig. 1A. Primers a and b amplify a 5.6-kb band in the rearranged allele. Primers c and d amplify a 0.3-kb band in the WT allele. (C) Western blot (WB) analysis of 3BP2 immunoprecipitates from thymocytes of two 3BP2^{-/-} mice (knockout [KO] mice 3 and 7) derived from different founders and a WT control (PS, preimmune rabbit serum). (D) RT-PCR analysis of mRNA from splenocytes of WT and 3BP2^{-/-} mice using primers e and f, which hybridize to exon 2 and the 5' region of exon 6 that is preserved in the targeted allele. These two primers amplify a 392-bp product from cDNA. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is used as a positive control.

sessile population of IgM⁺ CD21⁺ CD23⁻ cells generated from T2 cells and possibly from follicular B cells and are located close to the marginal sinus. MZ B cells play an important role in the response to TI antigens (1, 7). Detailed analysis of spleen B cells was performed by simultaneously staining for IgM, CD21, and CD24 or IgM, CD21, and CD23 (Fig. 2C). 3BP2^{-/-} mice had an increased percentage of IgM⁺ CD24^{hi} CD23⁻ transitional T1 cells (17.2% ± 3.2% of splenic B cells in 3BP2^{-/-} mice versus 11.4% ± 1.3% in controls; $P < 0.05$, $n = 3$), a decreased percentage of IgM^{hi} CD21⁺ CD23⁺ T2 cells (5.3% ± 2.0% of splenic B cells in 3BP2^{-/-} mice versus 12.4% ± 3.3% in controls; $P < 0.05$, $n = 3$), and a normal percentage of IgM^{lo} CD21⁺ CD23⁺ follicular B (FO) cells (66.5% ± 10.7% of splenic B cells in 3BP2^{-/-} mice versus 68.7% ± 8.4% in controls; $n = 3$). There was no significant difference in the percentage of IgM⁺ CD21⁺ CD23⁻ MZ B cells (3.7% ± 2% in 3BP2^{-/-} mice versus 4.2% ± 1.3% in controls; $n = 5$). There was a significant decrease of CD23 expression on IgM⁺ splenic B cells from 3BP2^{-/-} mice (mean fluorescence intensity [MFI], 222 ± 76.4 in 3BP2^{-/-} mice versus 773 ± 268.4 in controls; $P < 0.0001$, $n = 9$). A similar decrease in CD23 expression was observed on peripheral blood B cells (data not shown). The percentage of IgM⁺ CD5⁺ B1 cells in the peritoneum of 3BP2^{-/-} mice was normal (Fig. 2D). These results suggest that 3BP2 plays a role in the progression from pre-B cells to immature B cells and from T1 to T2 B cells.

Normal T-cell function in 3BP2^{-/-} mice. The proliferation of T cells from 3BP2^{-/-} mice in response to plates coated with anti-CD3 was similar to that of T cells from WT littermates over the range of anti-CD3 concentrations tested (0.3 to 10 μg/ml) (Fig. 3A). Similar proliferation of 3BP2^{-/-} and WT T cells was also observed when cells were stimulated with anti-CD3 plus anti-CD28 or anti-CD3 plus IL-2 (Fig. 3B). Consistent with the results from [³H]thymidine incorporation, similar percentages of T cells from 3BP2^{-/-} mice and WT controls underwent a comparable number of rounds of cell division in response to anti-CD3, with or without anti-CD28, as determined by CFSE dilution assay (Fig. 3C). 3BP2^{-/-} T cells normally upregulated CD69 and CD25 surface expression following TCR ligation (data not shown) and secreted normal amounts of IL-2 following stimulation with anti-CD3 plus anti-CD28 (Fig. 3D). These results suggest that 3BP2 is not essential for T-cell activation in response to TCR ligation.

TCR signaling is normal in 3BP2^{-/-} mice. TCR ligation results in protein tyrosine phosphorylation that includes phosphorylation of ZAP-70, Vav, and PLC-γ1. Total protein tyrosine phosphorylation and phosphorylation of ZAP-70, Vav, and PLC-γ1 were normal in 3BP2^{-/-} T cells (Fig. 4A and B). PLC-γ1 activation results in IP3 generation and calcium mobilization. Calcium mobilization after TCR ligation was normal in T cells from 3BP2^{-/-} mice (Fig. 4C). Phosphorylation of the

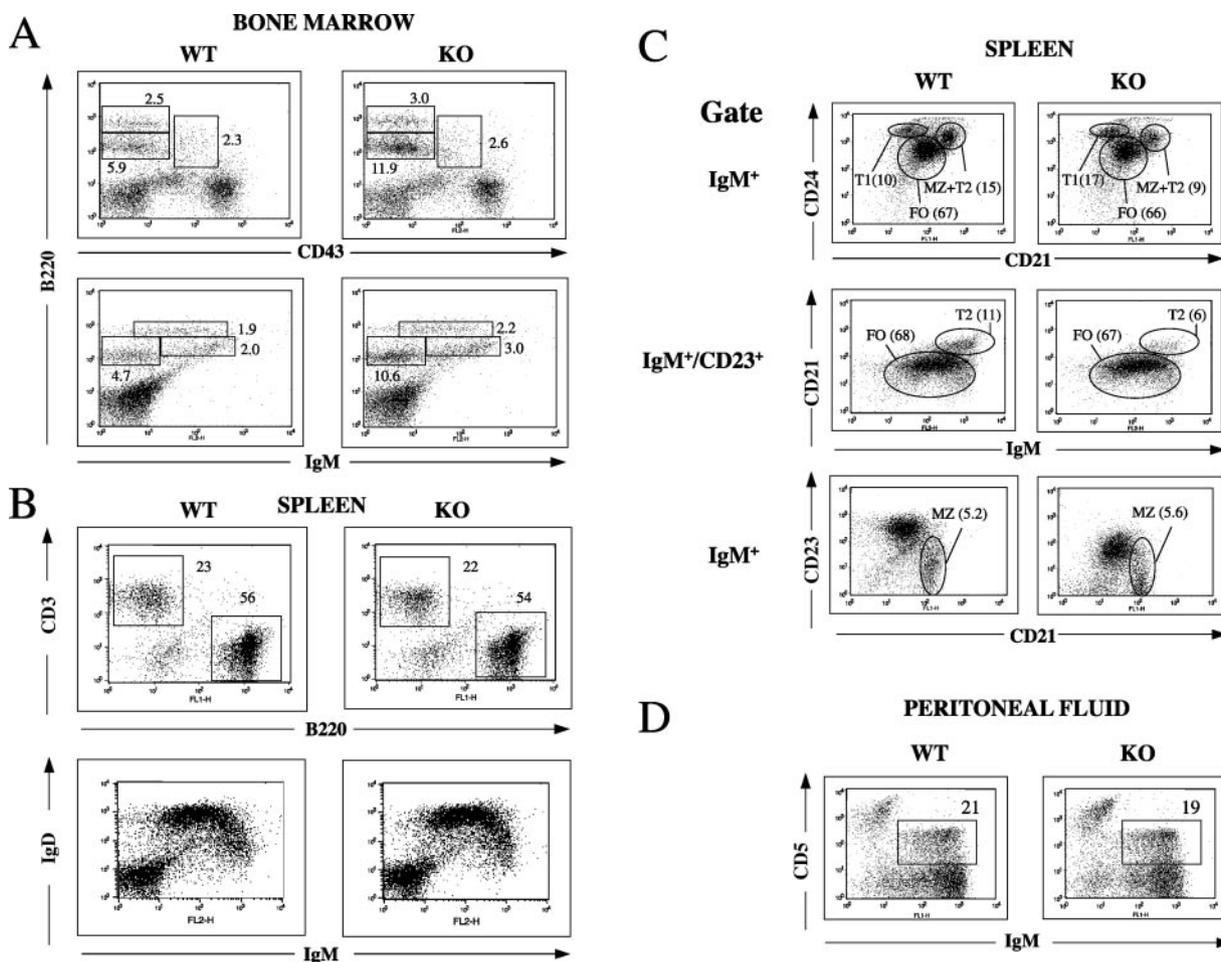


FIG. 2. Detailed FACS analysis of B cells in bone marrow (A), spleen (B and C), and peritoneum (D). In panel C, T1 cells were identified as CD24^{hi} CD21⁻ cells in the IgM⁺ gate (top panel), T2 cells as IgM^{hi} CD21⁺ cells in the IgM⁺/CD23⁺ gate (middle panel), and follicular B cells as CD24⁺ CD21⁺ cells in the IgM⁺ gate (top panel) and as IgM^{lo} CD21⁺ in the CD23⁺ gate (middle panel). MZ B cells were identified as CD21⁺ CD23⁻ cells in the IgM⁺ gate (lower panel). The percentages shown in the middle panel were adjusted to depict the percentage of total IgM⁺ cells. KO, knockout.

MAP kinases Erk, Jnk, and p38 was also normal in these cells (Fig. 4D).

Impaired B-cell proliferation in response to BCR ligation in 3BP2^{-/-} mice. Primary mouse splenic B cells express 3BP2 (data not shown). Highly purified B cells (>90% B220⁺) from 3BP2^{-/-} mice showed a significant defect in proliferation in response to BCR cross-linking with anti-IgM over the range of concentrations tested (0.8 to 12 μg/ml), as assessed by [³H]thy-

midine incorporation ($P < 0.05$, $n = 6$) (Fig. 5A). This defect was partially corrected by IL-4, although the proliferation of B cells from 3BP2^{-/-} mice to anti-IgM plus IL-4 remained significantly lower than that of WT B cells (Fig. 5B). Consistent with results from [³H]thymidine incorporation, CFSE dilution assays showed that after anti IgM treatment a lower percentage of B cells from 3BP2^{-/-} mice entered cell division compared to WT B cells (Fig. 5C, middle panel). Cell division was also delayed in 3BP2^{-/-} B cells treated with anti-IgM plus IL-4 (Fig. 5C, right panel). 3BP2^{-/-} B cells proliferated normally in response to anti-CD40 ± IL-4 and LPS ± IL-4, as determined by [³H]thymidine incorporation (Fig. 5D) and CFSE dilution assay (data not shown). 3BP2^{-/-} B cells normally upregulated surface expression of the major histocompatibility complex class II and CD86 in response to all stimuli tested, including anti-IgM (data not shown).

Normal B-cell survival in response to BCR ligation, but impaired expression of cell cycle regulatory proteins in B cells in 3BP2^{-/-} mice. To determine whether the impaired B-cell

TABLE 1. Absolute numbers of B lineage cells in bone marrow

Mouse genotype	No. of B lineage cells (10 ⁶) ^a				
	Total	B220 ^{lo} CD43 ⁺ (pro-B)	B220 ^{lo} CD43 ⁻ IgM ⁻ (pre-B)	B220 ^{lo} CD43 ⁻ IgM ⁺ (immature)	B220 ^{hi} IgM ⁺ (recirculating)
WT	15.51 ± 1.21	0.37 ± 0.03	0.63 ± 0.08	0.44 ± 0.07	0.30 ± 0.02
3BP2 ^{-/-}	17.62 ± 1.81	0.49 ± 0.05	1.56 ± 0.11*	0.38 ± 0.06	0.35 ± 0.04

^a The means ± standard deviation of the absolute numbers are shown for the indicated populations in one femur each from WT and 3BP2^{-/-} mice ($n = 6$ each). *, $P \leq 0.05$.

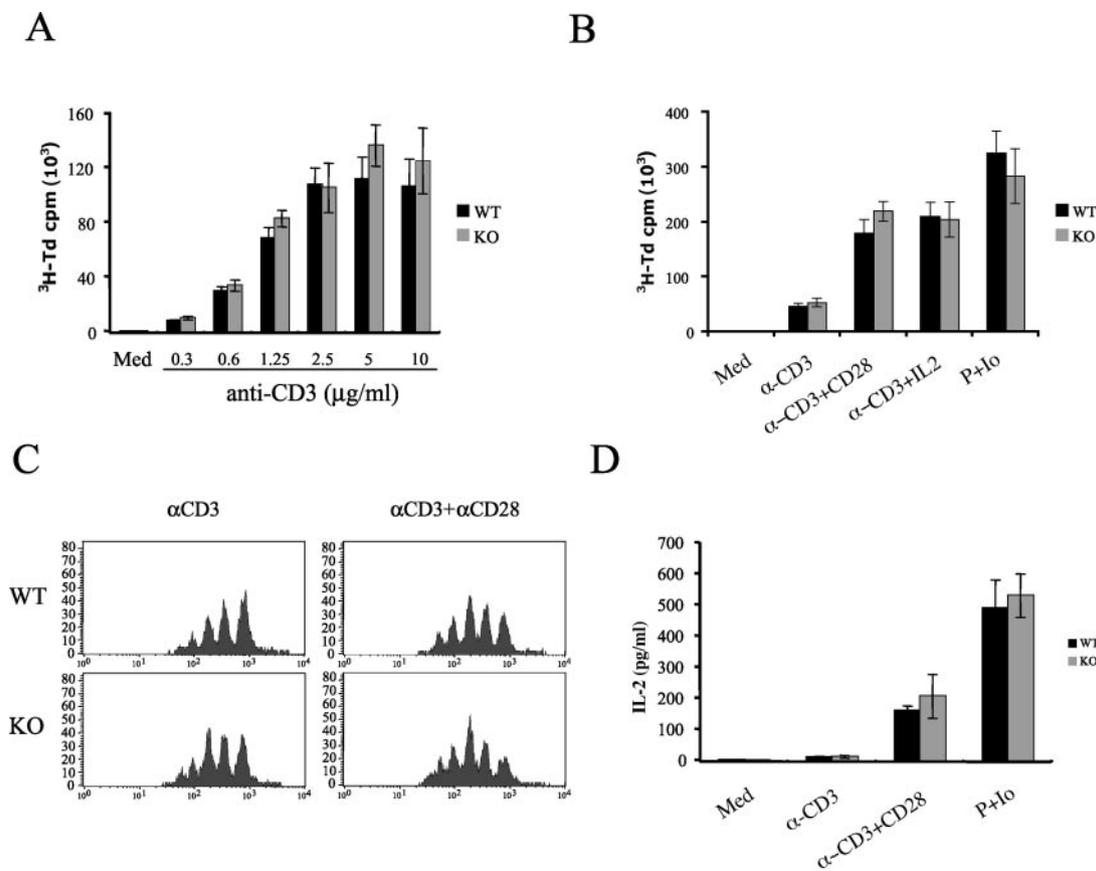


FIG. 3. T-cell proliferation and cytokine secretion in $3BP2^{-/-}$ mice. (A and B) Proliferation of purified T cells from 6- to 10-week-old WT mice and $3BP2^{-/-}$ littermates (knockout [KO] mice) to increasing concentrations of anti-CD3 (α -CD3) used to coat wells (A) and to anti-CD3 (2 μ g/ml) with or without soluble anti-CD28 (α -CD28; 2 μ g/ml) or IL-2 (40 ng/ml), and to phorbol myristate acetate and ionomycin (P+Io) (B), as assessed by [3 H]thymidine incorporation. Med, medium. (C) Proliferation of purified T cells to anti-CD3 \pm anti-CD28 as assessed by CFSE dilution assay. (D) Secretion of IL-2 in supernatants. Results shown are representative of three experiments.

proliferation in response to anti-IgM was due to impaired survival, we performed dual staining with annexin V and PI. We found no difference in the percentage of annexin V⁺ apoptotic cells and annexin V⁺/PI⁺ necrotic cells between B cells from $3BP2^{-/-}$ mice and WT littermates at 24 h and 48 h poststimulation with anti-IgM \pm IL-4 or LPS (Fig. 6A). These results suggest that $3BP2$ is not essential for the survival of B cells following BCR ligation.

Cyclins and cyclin-dependent kinases (Cdks) as well as Cdk inhibitors control cycle progression. With entry into the cell cycle, the levels of cyclins D2 and D3 and of their partners Cdk4 and Cdk6 are upregulated, the level of the Cdk inhibitor p27 decreases, and the Cdk target retinoblastoma tumor suppressor protein Rb is phosphorylated on Ser807 and Ser811 (5, 32, 51). We examined these events in WT and $3BP2^{-/-}$ B cells stimulated with anti-IgM. Stimulation of WT B cells with anti-IgM resulted in upregulation of the levels of cyclins D2 and D3 and of their partners Cdk4 and Cdk6, decreased p27 levels, and Rb phosphorylation (Fig. 6B). $3BP2^{-/-}$ B cells exhibited defective upregulation of cyclins D2 and D3, Cdk4, and, to a lesser extent, Cdk6; no detectable decrease in p27 levels; and no detectable Rb phosphorylation after stimulation with anti-IgM (Fig. 6B). These defects were partially rescued by IL-4. $3BP2^{-/-}$ B cells responded nor-

mally to LPS. Taken together, these results suggest that $3BP2$ is important for the progression of B cells through the cell cycle following BCR ligation.

BAFF is important for B-cell survival (45). We examined the survival response of B cells from $3BP2^{-/-}$ mice to BAFF (200 ng/ml and 500 ng/ml). Cells were analyzed every 24 h by trypan blue exclusion for 5 days. No significant difference in the percentages of viable cells was found between WT and $3BP2^{-/-}$ mice ($n = 4$ each) at any time point. At 5 days, the viability of WT B cells was $4\% \pm 2\%$ in medium versus $41\% \pm 5\%$ in 200 ng/ml BAFF. At that time point, the viability of $3BP2^{-/-}$ B cells was $6\% \pm 2\%$ in medium versus $38\% \pm 6\%$ in BAFF (data not shown). Similar results were obtained when the cells were analyzed by FACS for exclusion of merocyanine 540 (data not shown).

Impaired BCR signaling in $3BP2^{-/-}$ mice. $3BP2$ has been shown to associate with Syk, Vav, and PLC- γ 2, which are phosphorylated and activated following BCR ligation (11, 14). There was no detectable decrease in the tyrosine phosphorylation of Syk and Vav1 after BCR ligation in $3BP2^{-/-}$ B cells (Fig. 7A). In contrast, PLC- γ 2 phosphorylation was decreased (Fig. 7A). Calcium mobilization after BCR ligation was impaired in B cells from $3BP2^{-/-}$ mice over several concentrations of anti-IgM (5, 10, 15, and 20 μ g/ml) tested (Fig. 7B)

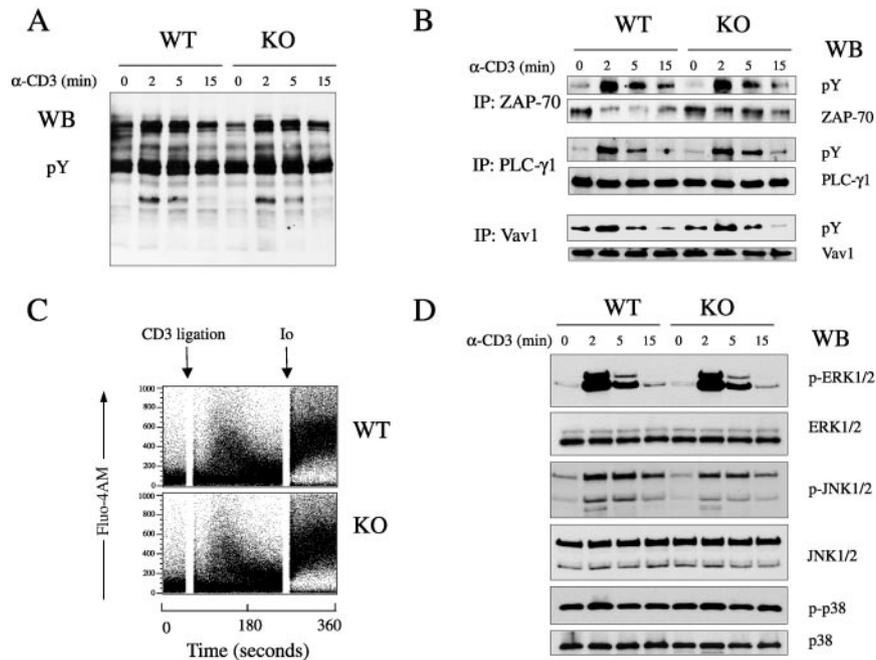


FIG. 4. TCR signaling in $3BP2^{-/-}$ mice. Purified splenic T cells were stimulated with anti-CD3 (α -CD3; $3 \mu\text{g/ml}$) and then cross-linked with $10 \mu\text{g/ml}$ goat $\text{F}(\text{ab}')_2$ anti-rat Ig. (A) Protein tyrosine phosphorylation, pY, phosphotyrosine. (B) Phosphorylation of ZAP-70, Vav1, and PLC- γ 1. IP, immunoprecipitation. (C) Calcium flux measured as intensity of Fluo-4-AM. Io, ionomycin. (D) MAP kinase phosphorylation.

(data not shown), suggesting impaired activation of PLC- γ 2. B cells from $3BP2^{-/-}$ mice responded with normal calcium influx to ionomycin.

Calcium mobilization plays a critical role in calcineurin-dependent dephosphorylation of the transcription factor NF-ATp, which allows it to translocate to the nucleus (35). NF-ATp dephosphorylation after BCR ligation, as indicated by a downward shift in molecular weight, was readily apparent in WT cells but barely detectable in $3BP2^{-/-}$ B cells (Fig. 7C). $3BP2^{-/-}$ B cells responded normally to ionomycin with complete dephosphorylation of NF-ATp. BCR stimulation of splenic B cells induces NF-AT-dependent upregulation of surface CD5 expression (4, 53). B cells from $3BP2^{-/-}$ mice were impaired in their ability to upregulate CD5 in response to BCR ligation (Fig. 7D).

BCR ligation results in the phosphorylation and activation of the MAP kinases Erk, Jnk, and p38, which are implicated in BCR-mediated proliferation (23, 39). Phosphorylation of ERK1/2 and JNK after BCR ligation was reduced in $3BP2^{-/-}$ cells, whereas phosphorylation of p38 was unaffected (Fig. 7E). Phosphorylation of MEK1/2, which is upstream of ERK, was also decreased after BCR ligation in $3BP2^{-/-}$ B cells (Fig. 8).

BCR ligation also results in phosphatidylinositol 3-kinase activation and subsequent phosphorylation and activation of Akt. Akt contributes to NF- κ B activation, which is important for B-cell survival (25, 41, 43). Phosphorylation of Akt and of the NF- κ B inhibitor I κ B following BCR ligation was normal in $3BP2^{-/-}$ B cells (Fig. 8).

Serum immunoglobulin levels and serum antibody responses in $3BP2^{-/-}$ mice. Serum levels of IgM, IgG subclasses, and IgA were not significantly different between 6- and 8-week-

old $3BP2^{-/-}$ mice and WT littermates (data not shown). To determine the role of 3BP2 in the antibody response, we immunized $3BP2^{-/-}$ mice and WT littermates with the T-dependent (TD) antigen TNP-KLH, the type I T-independent (TI) antigen TNP-LPS, and the type II TI antigen TNP-Ficoll. IgM and IgG anti-TNP antibody responses of $3BP2^{-/-}$ mice to all three antigens were comparable to those of WT controls (Fig. 9A). In addition, $3BP2^{-/-}$ mice made a normal response to KLH (data not shown). Furthermore, the size and number of germinal centers in the spleen, as determined by staining for bcl-6, were comparable in $3BP2^{-/-}$ mice and WT controls (data not shown).

Activation of B cells by TI antigens, and particularly type II TI antigens, is dependent on BCR cross-linking and activation (38). Given their defective BCR signaling, the normal antibody response of $3BP2^{-/-}$ mice to TI antigens was unexpected. BCR cross-linking is known to inhibit IgM synthesis and isotype switching (26). This raised the possibility that decreased sensitivity to BCR inhibition of Ig synthesis may compensate for impaired BCR activation in the antibody response of $3BP2^{-/-}$ mice. To test this hypothesis, we examined the effect of BCR cross-linking on IgM secretion and isotype switching to IgG1 and IgE in $3BP2^{-/-}$ B cells. Increasing concentrations of anti-IgM resulted in a dose-dependent inhibition of IgM secretion and of isotype switching to IgG1 and IgE in LPS/IL-4-stimulated WT B cells (Fig. 9B). B cells from $3BP2^{-/-}$ mice secreted significantly more IgM, IgG1, and IgE than B cells from WT controls in the presence of anti-IgM. At all the concentrations used, anti-IgM exerted no inhibitory effect on the proliferation of WT or $3BP2^{-/-}$ B cells to LPS plus IL-4 (data not shown).

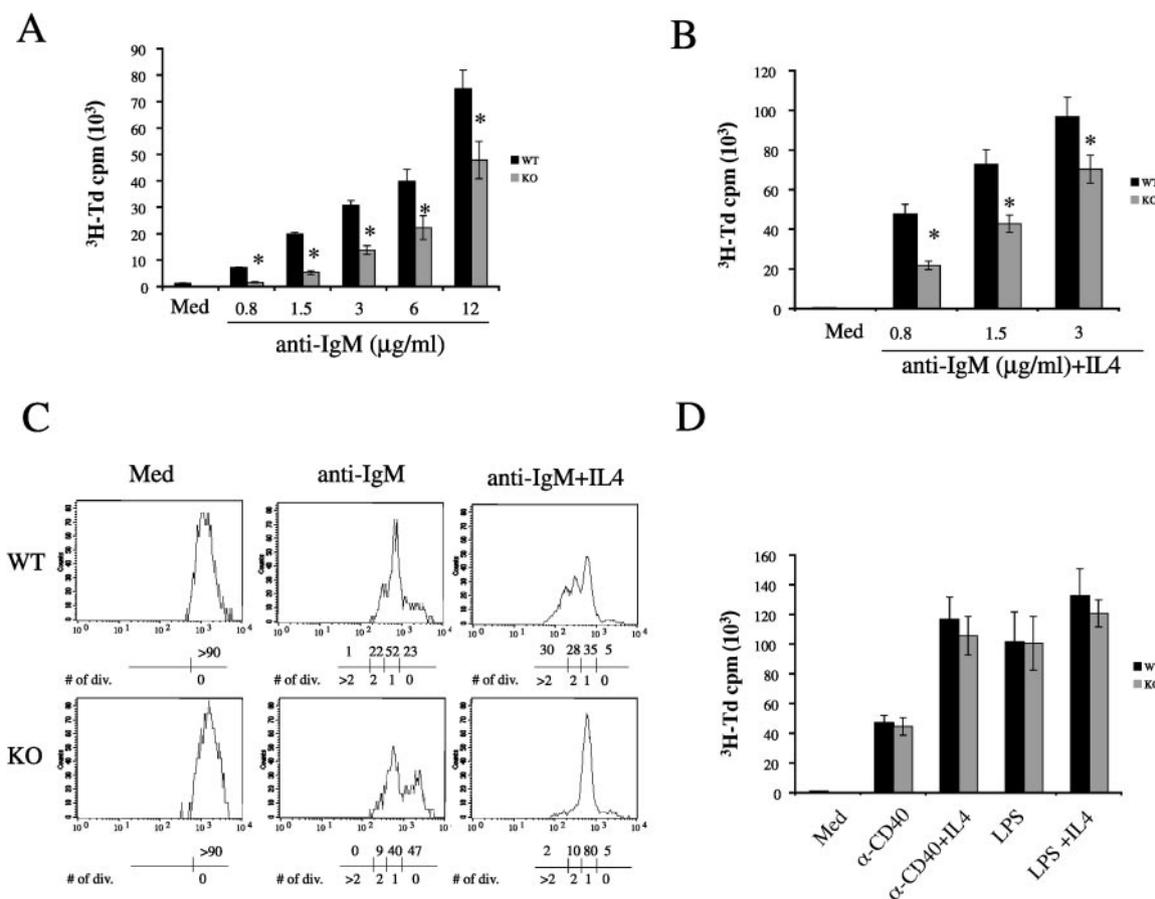


FIG. 5. B-cell proliferation in 3BP2^{-/-} mice. (A to C) Purified splenic B cells from WT mice and 3BP2^{-/-} littermates (knockout [KO] mice) were stimulated with anti-IgM without or with or IL-4 and examined for proliferation as assessed by [³H]thymidine incorporation (A and B) or CFSE dilution (C). div., divisions. (D) Proliferation of B cells to anti-CD40 and LPS without or with IL-4. Bars represent standard deviation. *, *P* < 0.05.

DISCUSSION

This study demonstrates that the adapter protein 3BP2 is important for BCR, but not TCR, signaling.

3BP2-deficient mice were viable and exhibited no apparent anatomical defects. Hemizygous point mutations of 3BP2 in humans have been associated with cherubism, which is characterized by multiple symmetric cysts in the jaw bones filled with multinucleated osteoclasts, stromal cells, and mast cells (20, 33, 55). All mutations in cherubism are located in exon 9 between the central proline-rich region and C-terminal SH2 domain and affect 3 amino acids within a 6-amino-acid sequence. The mutation in cherubism affects only one of the two 3BP2 alleles, and overexpression of the mutant in RBL-2H3 cells inhibits FcεRI-induced mast cell activation (37). Thus, the mutant gene product may function as a dominant-negative or gain-of-function mutant, rather than exerting its effect due to haploinsufficiency. Our observation that the gross and radiographic appearance of the jaws in 3BP2^{+/-} and 3BP2^{-/-} mice is normal (our unpublished observations) is consistent with this hypothesis.

Based on studies using overexpression of 3BP2 and 3BP2 mutants in Jurkat cells, it has been suggested that 3BP2 plays an important role in TCR-mediated activation (11). Unexpectedly,

our studies in 3BP2^{-/-} mice indicate that 3BP2 is not essential for either T-cell development or activation via TCR. T cells from 3BP2^{-/-} mice proliferated normally and secreted normal amounts of IL-2 in response to TCR ligation in vitro (Fig. 3A to D). Biochemical studies revealed that ZAP-70, PLC-γ1, Vav1, and MAP kinases are normally phosphorylated in 3BP2^{-/-} T cells (Fig. 4). Thus, although these three proteins interact with 3BP2, their phosphorylation after TCR ligation proceeds in the absence of 3BP2.

3BP2^{-/-} mice had a twofold increase in pre-B cells in the bone marrow (Fig. 2A and Table 1). Signaling via the pre-BCR is critical for the transition of pre-B cells to immature B cells and involves signaling pathways similar to those activated by the BCR (18). Defective signaling via the pre-BCR may have resulted in the accumulation of pre-B cells in 3BP2^{-/-} mice. In one study, pro-B cells were increased in mice with a disrupted λ5 gene, suggesting that pre-BCR signaling also influences pro-B-cell-to-pre-B-cell transition (29). However, pro-B cells were normal in 3BP2^{-/-} mice.

3BP2^{-/-} mice had a twofold increase in splenic T1 B cells and a twofold decrease in splenic T2 B cells (Fig. 2C), consistent with a role for BCR signaling in the development of T1 cells into T2 cells, as suggested by Syk- and Igα-deficient mice

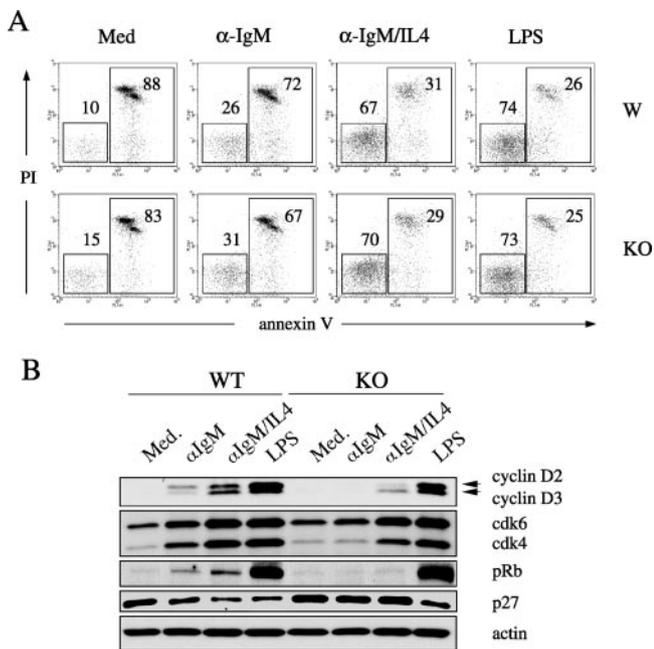


FIG. 6. B-cell survival and progression through the cell cycle. Purified B cells were stimulated with anti-IgM (α -IgM) without or with IL-4 or LPS for 24 h. (A) Annexin V and PI staining. Similar results were obtained at 48 h. (B) Lysates at 36 h were Western blotted for cyclins D2 and D3, cdk4, cdk6, p27, pRb, and actin as a control. Results in panels A and B are representative of three experiments. KO, knockout.

(34). B-cell survival in response to BAFF, which is important for the transition from T1 cells (48), was normal. Although BCR signaling also plays a role in the maturation of T2 cells into follicular cells (34), the percentage of follicular cells was normal in 3BP2^{-/-} mice, possibly because recirculating mature B cells in these mice proliferate normally in response to CD40 and Toll receptor engagement and accumulate (Fig. 5D).

An inverse relationship between the strength of BCR signaling and the development of MZ B cells has been suggested (9). We did not detect a difference in the percentage of IgM⁺ CD21⁺ CD23⁻ MZ B cells in the spleens of 3BP2^{-/-} mice. Low numbers of T2 precursors in 3BP2^{-/-} mice may negate the potentially enhancing effect of weak BCR signaling on the development of MZ B cells (6). BCR signaling is important for the generation and maintenance of peritoneal CD5⁺ B1 cells, as the numbers of these cells are decreased in several gene knockouts that interfere with BCR signaling (10, 12, 17, 24, 28, 47, 52, 58, 59). Residual BCR signaling in 3BP2^{-/-} mice may be sufficient to sustain the development and maintenance of normal numbers of CD5⁺ B1 cells in these mice.

B cells from 3BP2^{-/-} mice exhibited an activation defect restricted to BCR ligation. 3BP2-deficient B cells had impaired proliferation in response to BCR ligation as determined by [³H]thymidine incorporation and CFSE dilution assay (Fig. 5A to C). B cells from 3BP2^{-/-} mice stimulated via the BCR failed to upregulate cyclins D2 and D3 and the Cdk6, Cdk4, and Cdk6, to degrade the Cdk inhibitor p27, and to phosphorylate Rb protein (Fig. 6B), all of which are necessary for progression

through the cell cycle. However, phosphorylation of Akt and I κ B, which results in activation of NF- κ B and is important for B-cell survival (25, 41, 43), was normal in 3BP2^{-/-} mice (Fig. 8), consistent with the normal survival of these cells in response to BCR ligation (Fig. 6A). Tyrosine phosphorylation of Syk and its substrate Vav1 following BCR ligation was normal in 3BP2^{-/-} B cells (Fig. 7A). This suggests that activation of upstream src kinases that phosphorylate Syk is independent of 3BP2. These kinases include Lyn and Fyn, which have been shown to interact with 3BP2 (11). Tyrosine phosphorylation of PLC- γ 2, the major isoform of PLC- γ in B cells, was impaired in 3BP2^{-/-} B cells following BCR ligation (Fig. 7A). PLC- γ 2 phosphorylation following BCR ligation depends on src kinases, Syk, and the Tec kinase Btk (31, 50). It has been shown that PLC- γ 2 interacts via its SH2 domain with pY183 of 3BP2 (14, 22). The interaction of Syk and 3BP2 involves a phosphotyrosine residue in the bridge region of Syk and the SH2 domain of 3BP2 (11), while interaction of the src kinase Lyn with 3BP2 involves the SH2 and SH3 domains of Lyn with pY486 and the proline-rich region of 3BP2, respectively (36). Furthermore, the SH3 domain of the Tec kinase Itk is a candidate for interaction with the proline-rich region of 3BP2 (49). This raises the possibility that 3BP2 may serve as a scaffold to promote the phosphorylation of PLC- γ 2 by Syk, src kinases, and Tec kinases.

Calcium mobilization after BCR ligation was impaired in B cells from 3BP2^{-/-} mice (Fig. 7B). This probably reflects decreased activation of PLC- γ 2. More importantly, Ca²⁺/calmodulin-dependent dephosphorylation of the transcription factor NF-ATp after BCR ligation was impaired in B cells from 3BP2^{-/-} mice (Fig. 7C). Furthermore, NF-AT-dependent induction of CD5 expression after BCR ligation was deficient in these B cells (Fig. 7D). This suggests that 3BP2 is important for the calcium-dependent activation of NF-AT transcriptional activity after BCR ligation. Since NF-AT activation is important for B-cell proliferation following BCR ligation (3, 56), defective NF-AT activation probably contributes to impaired proliferation of 3BP2^{-/-} B cells in response to anti-IgM.

Activation of the MAP kinase Erk and of its upstream kinase MEK1/2 after BCR ligation was impaired in B cells from 3BP2^{-/-} mice (Fig. 7E and Fig. 8). This is possibly a consequence of impaired PLC- γ 2 activation and decreased generation of diacylglycerol, which binds to RasGRP and activates the Ras pathway (13, 40). Since the MEK1/2-Erk pathway is important for cyclin D2 expression, progression into the cell cycle and proliferation in response to BCR ligation (42), decreased Erk activation following BCR ligation may contribute to the proliferative defect in 3BP2^{-/-} B cells. Decreased phosphorylation of Jnk after BCR ligation in 3BP2^{-/-} B cells is compatible with the observations that overexpression of 3BP2 results in the stimulation of AP-1 activity, independently of NF-AT (11), and that a GCSF-R mutant that fails to bind 3BP2 and Grb2 is impaired in its ability to activate Jnk (27).

In a number of knockouts that selectively disrupt BCR signaling, the antibody response to TD antigens is preserved, while the antibody response to TI type II antigens is impaired (16, 19, 24, 28, 57, 59). In some of these knockouts, the total serum IgG3 level is decreased (28, 59), while in others only the IgG but not the IgM response to type II TI antigens is affected (19). Serum Igs were normal in 3BP2^{-/-} mice, and these mice

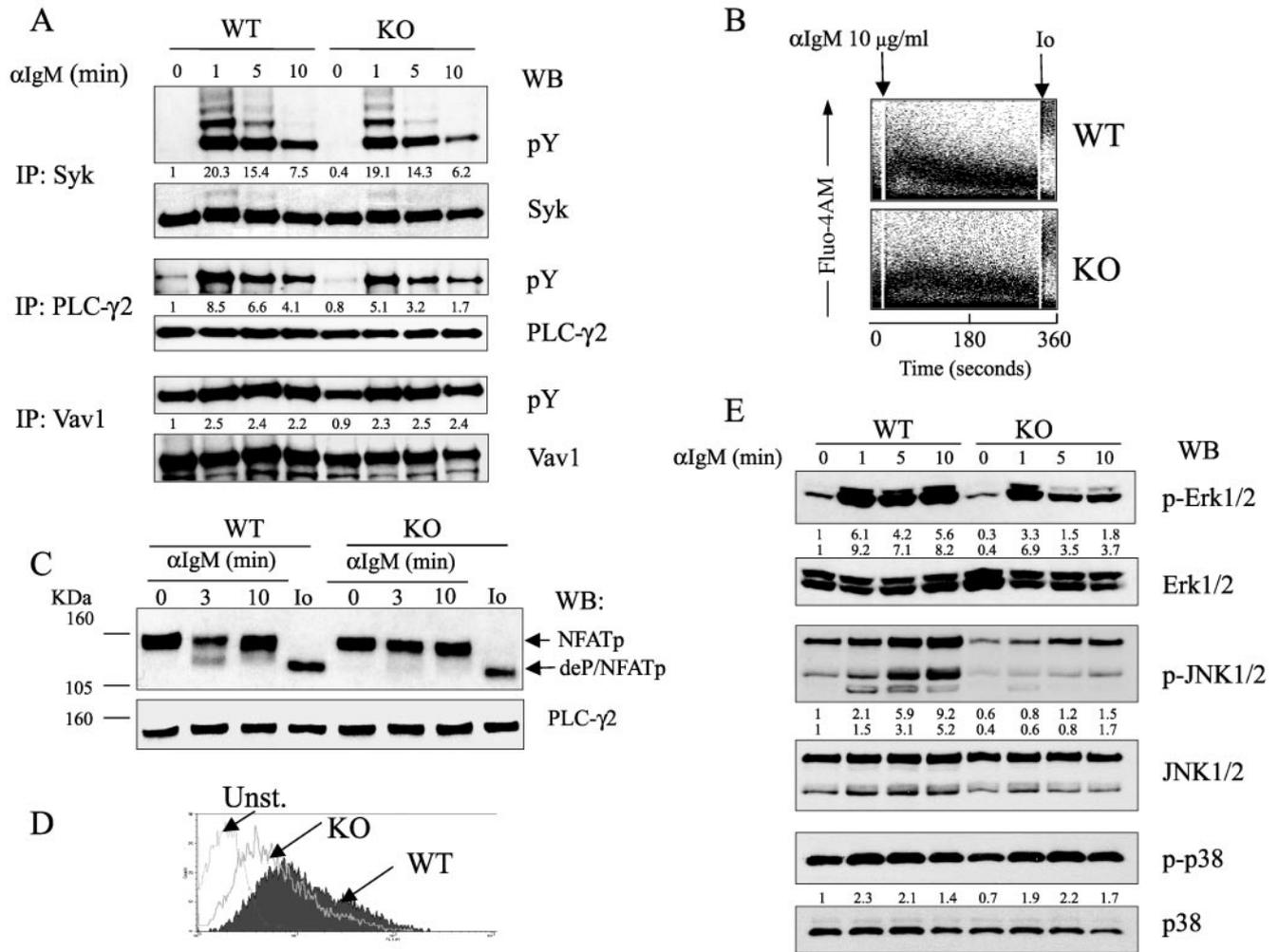


FIG. 7. BCR signaling in $3BP2^{-/-}$ mice. Purified B cells were stimulated with anti-IgM (α -IgM). (A) Lysates were immunoprecipitated with anti-Syk, anti-PLC- γ 2, or anti-Vav1 antibodies and probed with antiphosphotyrosine (pY) MAb 4G10 and reprobed with anti-PLC- γ 2, anti-Syk, or anti-Vav1 antibodies as loading controls. WB, Western blotting. (B) Calcium mobilization measured as Fluo-4-AM intensity. Io, ionomycin. (C) NFATp dephosphorylation. deP, dephosphorylation. (D) CD5 surface expression at 72 h. Unst., unstimulated. (E) MAP kinase phosphorylation. Lysates were probed with phospho-specific antibodies to ERK1/2, Jnk, and p38. Blots were reprobed with antibodies to Erk1/2, Jnk, and p38 as loading controls. Results in panels A to E are representative of three experiments. Numbers in panels A and E reflect the relative intensity of bands representing phosphorylated protein in relation to total protein.

mounted a normal antibody response and germinal center formation in response to TD antigens (Fig. 9A) (data not shown). The normal response of these mice to TI antigens (Fig. 9A), particularly to type II TI antigens, which activate B cells by cross-linking the BCR, was unexpected. This may be simply explained by the fact that the BCR signaling defect is incomplete in $3BP2^{-/-}$ mice. However, the magnitude of this defect is comparable to that seen in $BLNK^{-/-}$ and $Bam32^{-/-}$ mice, which have impaired antibody response to type II TI antigens (19, 58). BCR ligation inhibits IgM secretion and isotype switching in normal B cells (26). We demonstrated that $3BP2^{-/-}$ B cells are less susceptible to the inhibitory effect of BCR ligation on IgM secretion and isotype switching (Fig. 9B). The effects of impaired BCR activation and decreased BCR-mediated inhibition of immunoglobulin production may cancel out each other in $3BP2^{-/-}$ mice, resulting in a normal antibody response to TI antigens.

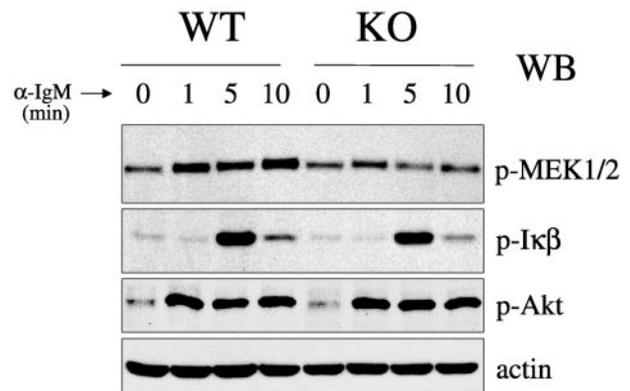


FIG. 8. Phosphorylation of MEK1/2, Akt, and $I\kappa B$ in anti-IgM-stimulated B cells. Purified B cells were stimulated with anti-IgM (α -IgM) at different time points. Lysates were probed with phospho-specific antibodies to MEK1/2, $I\kappa B$, and Akt, and with antiactin MAb as a loading control. WB, Western blotting.

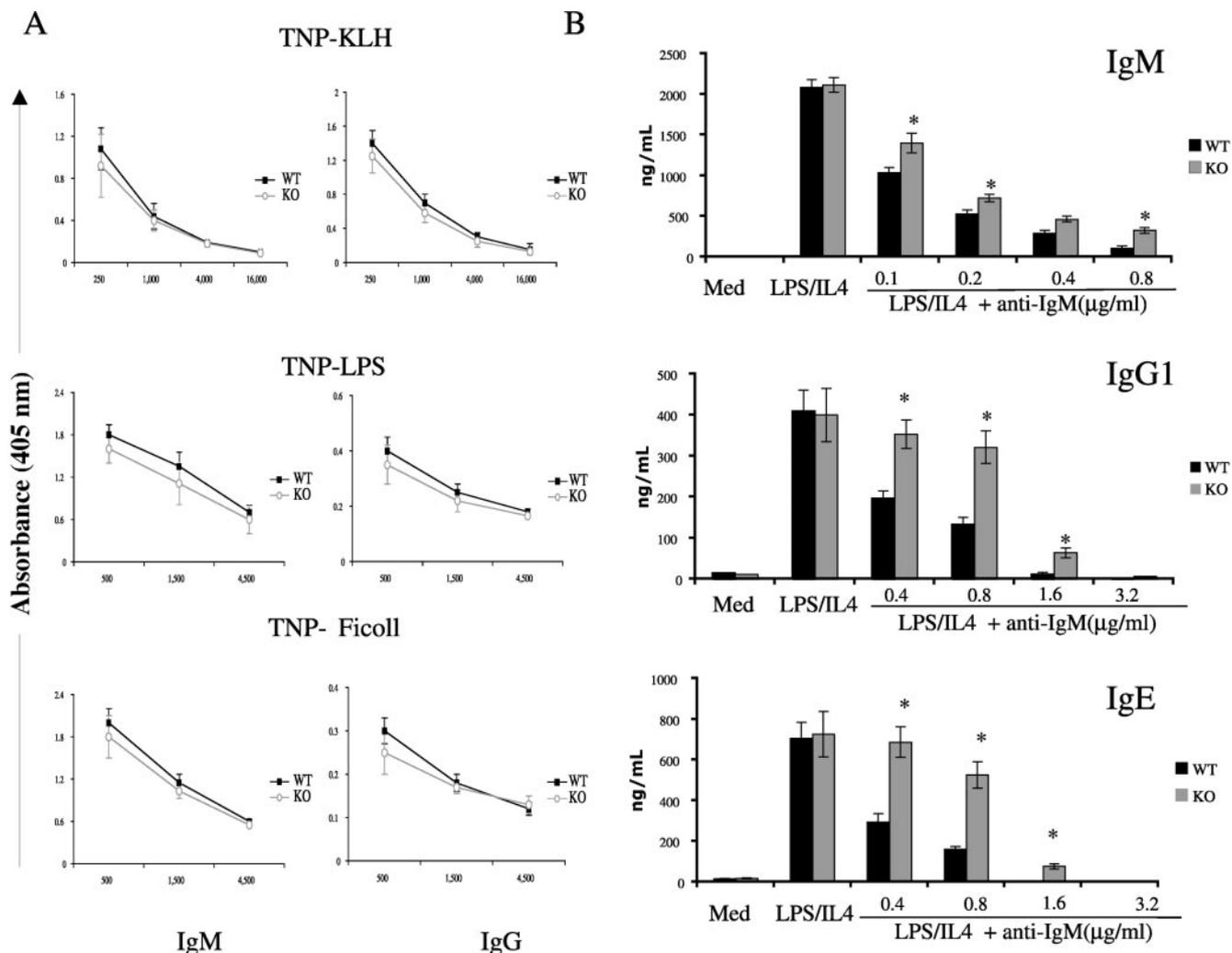


FIG. 9. Antibody production in 3BP2^{-/-} mice. (A) TNP-specific IgM and IgG antibody responses of WT and 3BP2^{-/-} (knockout [KO]) mice (*n* = 6 each) to TNP-KLH, TNP-LPS, and TNP-Ficoll as determined by ELISA. (B) Effect of BCR cross-linking on IgM, IgG1, and IgE secretion in LPS/IL-4-stimulated B cells. Because of the higher sensitivity of IgM secretion to inhibition by BCR cross-linking, lower concentrations of anti-IgM were required to reveal the dose dependence of its inhibition. Cultures were done in triplicate. Bars represent standard deviation. *, *P* < 0.05. Results are representative of three experiments.

Our results clearly show that 3BP2 is important for BCR signaling, but redundant for TCR signaling. 3BP2 may be redundant in T cells because of the expression of a functional homologue. This homologue may not be expressed or may be poorly expressed in B cells. The observation that TCR signaling is inhibited by overexpression of the isolated SH2 domain of 3BP2 in Jurkat cells (11) can be explained by inhibition of the function of both 3BP2 and its homologue. The presence of a 3BP2 homologue would be consistent with the observation that 3BP2 sequence was normal in 3 out of 12 families with cherubism (55).

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