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The Role of the Syk/Shp-1 Kinase-Phosphatase Equilibrium in B Cell Development and Signaling

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Signal transduction from the BCR is regulated by the equilibrium between kinases (e.g., spleen tyrosine kinase [Syk]) and phosphatases (e.g., Shp-1). Previous studies showed that Syk-deficient B cells have a developmental block at the pro/pre–B cell stage, whereas a B cell–specific Shp-1 deficiency promoted B-1a cell development and led to autoimmunity. We generated B cell–specific Shp-1 and Syk double-knockout (DKO) mice and compared them to the single-knockout mice deficient for either Syk or Shp-1. Unlike Syk-deficient mice, the DKO mice can generate mature B cells, albeit at >20-fold reduced B cell numbers. The DKO B-2 cells are all Syk-negative, whereas the peritoneal B1 cells of the DKO mice still express Syk, indicating that they require this kinase for their proper development. The DKO B-2 cells cannot be stimulated via the BCR, whereas they are efficiently activated via TLR or CD40. We also found that in DKO pre-B cells, the kinase Zap70 is associated with the pre-BCR, suggesting that Zap70 is important to promote B cell maturation in the absence of Syk and SHP-1. Together, our data show that a properly balanced kinase/phosphatase equilibrium is crucial for normal B cell development and function. ***The Journal of Immunology*****, 2014, 193: 268–276.**

The early stages of B cell development take place in the bone marrow, where the B lymphoid progenitors initiate the ordered recombination of the Ig gene loci (1, 2). A productive VHDJH recombination at the H chain gene locus leads to the synthesis of a membrane-bound μ H chain that, in association with the surrogate L chain proteins λ5 and VpreB and the signaling components Ig-α and Ig-β, form the pre-BCR (3, 4). Signaling through this receptor is an important checkpoint in B cell development; it allows the expansion of pre-B cells and initiates the recombination of the L chain genes. Subsequently, surrogate L chains are replaced by either κ or λ L chains that, together with the membrane-bound μ H chains and the Ig-α/Ig-β heterodimer, form an IgM-BCR. The BCR^+ immature B cells migrate to the spleen, where they mature into B-2 cells (2, 3). B-2 cells are the major B cell population in the spleen that also contains low numbers of B-1 cells (5). The B-1 cells ($IgM^{hi}IgD^{low/-}CD23^-CD43^+$) dominate in the peritoneal and pleural cavities. Based on CD5 expression, one distinguishes two types of B-1 cells: B-1a ($CD5^+$) and B-1b ($CD5^-$). B-1 cells are involved in T cell–independent immune responses and in the production of natural Abs (5, 6).

Signaling via the pre-BCR or the BCR is mediated by the Ig-α/Ig-β heterodimer (7, 8). Protein tyrosine kinases such as Lyn and the spleen tyrosine kinase (Syk) phosphorylate tyrosines of the ITAM within the cytoplasmic tails of Ig-α and Ig-β (9–12). The phosphorylated ITAMs serve as docking sites for the tandem Src homology 2 domains of Syk. The binding of Syk to the ITAMs activates Syk, and this results in a positive-feedback loop amplifying Syk activity (13). Once activated, Syk interacts with several adaptor proteins and phosphorylates downstream molecules that activate PI3K and phospholipase Cγ2 signaling pathways (14–16). This results in the generation of second messengers, the increase of intracellular calcium levels, and the activation of MAPKs (10, 11, 17–19). A B cell–specific deletion of *Syk* in mice results in a developmental blockage at the pre–B cell stage (20, 21). Furthermore, Syk is an essential protein for the positive selection of immature B cells into the recirculating B cell pool (22, 23).

In resting cells, the steady-state activity of protein tyrosine kinases downstream of the BCR is controlled by protein tyrosine phosphatases (24). The best-characterized tyrosine phosphatases in BCR signaling are the receptor tyrosine phosphatases CD45 and CD148, as well as the nonreceptor tyrosine phosphatases Shp-1 and Shp-2 (25). CD45 and CD148 are highly expressed in B cells (26). Together with Shp-2, CD45 and CD148 mostly play a positive regulatory role in BCR-mediated signaling (27). In contrast, Shp-1 is a key negative regulator of B lymphocyte activation (28). A loss-of-function mutation in the Shp-1 encoding gene *Ptpn6* was found in *motheaten* (*me/me*) mice and its allelic variant *viable motheaten* (me^v/me^v) (29, 30). These mutations result in immunological abnormalities such as extramedullary hematopoiesis, splenomegaly and hemorrhagic pneumonitis (31). Furthermore, these mice showed premature thymic involution, profound depletion of $B220^+$ B cell progenitors in the bone marrow, and a reduction in conventional B-2 cell numbers (32–35). A B cell–specific deletion of Shp-1 promotes development of B-1a cells and their expansion in the secondary lymphoid organs, and the mice

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; DKO, double-knockout; KI, knockin; KO, knockout; MFI, mean fluorescence intensity; PLA, proximity ligation assay; Syk, spleen tyrosine kinase; WT, wild-type.

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develop symptoms of systemic autoimmunity (36). Mature B cells that overexpress a catalytically inert form of Shp-1 (Shp-1 C453S; dominant negative) exhibit augmented calcium mobilization and homotypic adhesion in response to BCR engagement (37, 38). These studies show that Shp-1 counteracts the phosphorylation of BCR signaling molecules such as Syk, Slp-65, and Ig-α (37, 39). We have generated B cell–specific Shp-1 and Syk double-knockout (DKO) mice to study the role of the kinase/phosphatase equilibrium in the development and function of B cells in vivo.

Materials and Methods

Mice and genotyping

The DKO mice were generated by crossing floxed *Ptpn6*$^{fl/fl}$ mice (36) and *Syk*$^{fl/fl}$ mice (40) with mb1-cre-knockin (KI) mice (41). Genomic DNA isolated from sorted cells or tail biopsies was used for PCR genotyping as described previously (42). Floxed (≈550 bp), deleted (≈900 bp), and wild-type (WT; ≈350 bp) *Ptpn6* alleles were identified by PCR with primers P92 (5′-ACC-CTC-CAG-CTC-CTC-TTC-3′), P93 (5′-TGA-GGT-CCC-GGT-GAA-ACC-3′), and 124b (5′-GGG-CCC-CTA-GAC-ATA-GAC-CT-3′), respectively. Floxed (≈350 bp), deleted (≈210 bp), and WT (≈250 bp) *Syk* alleles were identified by PCR with primers SykF (5′-GCC-CGT-TCT-GTG-CCT-ACT-GG-3′), SykR (5′-GCT-GGT-TCC-CTT-TTC-CTT-CC-3′), and SykR2 (5′-TAC-CTA-ACC-AAA-CCC-ACG-GC-3′), respectively. WT *Mb1* allele (≈410 bp) was identified by PCR with primers Mb1, probe, dir (5′-CTG-CGG-GTA-GAA-GGG-GGT-C-3′) and Mb1, in2, rev (5′-CCT-TGC-GAG-GTC-AGG-GAG-CC-3′). The *hCre*-inserted (≈450 bp) allele was identified by PCR with primers hCre dir (5′-CCC-TGT-GGA-TGC-CAC-CTC-3′) and hCre rev (5′-GTC-CTG-GCA-TCT-GTC-AGA-G-3′). All experiments used 8–12 wk-old mice maintained under specific pathogen-free conditions according to institutional guidelines and animal study protocols approved by the institutional and governmental animal care and use committees.

Cell preparation and flow cytometry

Single-cell suspensions of spleen and lymph nodes (cervical, axillary, brachial, and inguinal) were prepared in PBS. PBS was also used to flush femurs and for lavage of peritoneal cavities. Erythrocytes were lysed in 0.89% NH_4Cl, 0.1% $KHCO_3$, and 0.003% tetrasodium EDTA (pH 7.3). Surface staining was performed using 1×10^6 cells in PBS, 2% FCS, and 0.2% sodium azide on ice. Fluorochrome-conjugated or biotinylated B220 (RA3-6B2), IgM (11/41), IgD (11-26), CD19 (1D3), CD5 (53-7.3), CD21/CD35 (eBio8D9), CD23 (B3B4), CD93 (AA4.1), CD4 (RM4-5), CD8a (53-6.7), CD24 (30.F1), and CD43 (R2/60) mAbs were purchased from eBioscience. Fluorochrome-conjugated CD3ε (145-2C11) was purchased from BD Pharmingen, and fluorochrome-conjugated BP-1 (6C3) from BioLegend. Intracellular FACS staining was done using the "Fix and Perm Cell" permeabilization kit (ADG) and the following Abs: Zap70 PE (eBioscience; 1E7.2), purified anti-Syk (BioLegend; cloneSYK-01), and Zenon mouse IgG labeling kits (Molecular Probes, Invitrogen). Flow cytometry data were acquired on an FACSCalibur or LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star). The different stages of B cell development were isolated and ordered according to the Hardy fractionation system (43) (fraction A: pre–pro-B; B: pro-B cells; C/D: pre-B; E: immature B cells; and F: mature B cells.)

Ex vivo activation and proliferation experiments

CD19 or CD43 MACS-purified (Miltenyi Biotec) splenic B cells were cultured with or without stimuli at 2 to 3×10^5 cells/well in Iscove's DMEM cell-culture medium (Biochrome AG) supplemented with 10% FCS (PAN Biotech), 2 mM glutamine (Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 4.9×10^{-5} M 2-ME. After 24 h, cells were harvested, and the expression of CD86 was analyzed by flow cytometry using an anti-CD86 Ab (eBioscience; clone GL1). Dead cells were excluded by 7-aminoactinomycin D (7-AAD) staining. Mean fluorescence intensity (MFI) of cells stained with anti-CD86 PE was calculated. For the proliferation assay, cells were labeled with the proliferation dye (eFluor 670; eBioscience) and cultured with or without stimuli for 72 h. In these experiments, cells were stimulated with the following reagents: polyclonal anti-IgM $F(ab')_2$ (The Jackson Laboratory; 10 μg/ml), anti-RP105 (eBioscience; clone RP/14; 10 μg/ml), LEAF-purified anti-mouse CD40 (BioLegend; clone HM40-3; 10μg/ml), unmethylated CpG oligonucleotides (Invivogen; ODN 1826; 2.5 μg/ml), and anti-CD38 (eBioscience; clone 90; 10 μg/ml). LPS from *Salmonella minnesota* mutant R595 (44) was kindly provided by Dr. M. Freudenberg (10 μg/ml).

The following Abs were used for Western blotting: anti-Syk (Santa Cruz Biotechnology; N19), anti–Shp-1 (Upstate Biotechnology), and anti–Bap-31 (Cell Signaling Technology).

Western blot

B cells were flushed from the bone marrow and cultured for 4 wk in IL-7–supplemented culture medium and lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 137.5 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and protease inhibitor mixture [Sigma-Aldrich]) containing 0.1% Triton. Total cell lysates were analyzed by conventional methods for Western blot as described (13).

Calcium flux

Cells were loaded with 5 mg/ml indo-1 acetoxymethyl ester and 0.5 mg/ml pluoronic F-127 (Molecular Probes) in Iscove's DMEM cell-culture medium supplemented with 1% FCS at 37°C for 45 min. After loading, cells were washed and stained with CD19-FITC and CD5–PerCP-Cy5 Abs (eBioscience). Finally, cells were resuspended in medium containing 1% FCS. The Ca^{2+} response was induced by the addition of goat anti-mouse IgM (10 μg/ml; Southern Biotechnology Associates) at 37°C, and intracellular calcium concentrations were monitored for 8 min with an LSRII (BD Biosciences).

Serum Ig determination

Serum IgM, IgA, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 from tail-vein bleeds were quantified by standard ELISA methods (45). To detect Igs in the sera of naive mice, ELISA plates (Nunc Maxisorb; Nunc) were coated with 5μg/ml goat anti-mouse IgM, -IgA, or -IgG (Southern Biotechnology Associates) in 50 μl PBS and incubated overnight at 4°C. The sera were then applied in serial dilutions of 1:3 in dilution reagent (1% [w/v] BSA and 0.05% [w/v] sodium azide in PBS) and incubated for 2 h at 37°C. Mouse monoclonal IgM, IgA, and IgG isotypes were used as standard controls (Southern Biotechnology Associates). Alkaline phosphatase–conjugated secondary Abs were added for detection (Southern Biotechnology Associates).

Proximity ligation assay

F(ab) fragments specific for Ig-α, Zap70 (Santa Cruz Biotechnology), and Syk (SYK01; BioLegend) Abs were generated using Fab Micro Preparation Kit (Pierce) according to the manufacturer's manual. Subsequently, F(ab) fragments were conjugated with duolink probemaker oligonucleotides plus or minus (Olink Bioscience, Uppsala, Sweden) as described previously (46). B cells were negatively selected by anti-CD43 MACS beads and cultured overnight with complete Iscoves's DMEM cell-culture medium. The cells were settled in PTFE slides (Thermo Scientific) and stimulated with anti-IgM $F(ab')_2$ (10 μg/ml) for different time points. The cells were fixed with 2% paraformaldehyde and then incubated with the appropriate F(ab) fragments. The hybridization and ligation steps were performed using the Duolink II Kit (Olink Bioscience) according to the manufacturer's instructions. Proximity ligation signals were detected with a Zeiss LSM 510/780 fluorescence microscope (63 × objective; Zeiss), and images were processed with Zen (2010) software. Proteins closer than 10 nm can lead to a fluorescence signal (red dot). The signals were calculated using BlobFinder software.

Statistical analysis

All analyses were performed with the Prism 4 program (GraphPad Software). The statistical tests used are indicated in the figure legends.

Results

Additional deletion of Ptpn6 partially rescues the maturation of Syk-deficient B cells

To generate *Ptpn6* and *Syk* DKO mice, we crossed *Ptpn6*$^{fl/fl}$ mice and *Syk*$^{fl/fl}$ mice to mb1-cre mice (36, 40, 41). We investigated the presence of mature B cells in the peripheral lymphoid organs of DKO mice and compared them to WT (*Ptpn6*$^{fl/fl}$ *Syk*$^{fl/fl}$) mice and Syk or Shp-1 single-knockout (KO; *Syk*$^{fl/fl}$-mb1-cre and *Ptpn6*$^{fl/fl}$-mb1-cre, respectively) mice (Fig. 1). As previously published (23), Syk-deficient mice have hardly any or no mature B-2 cells (IgM^+ IgD^{hi}) in the spleen and peripheral blood, whereas *Ptpn6*-deficient

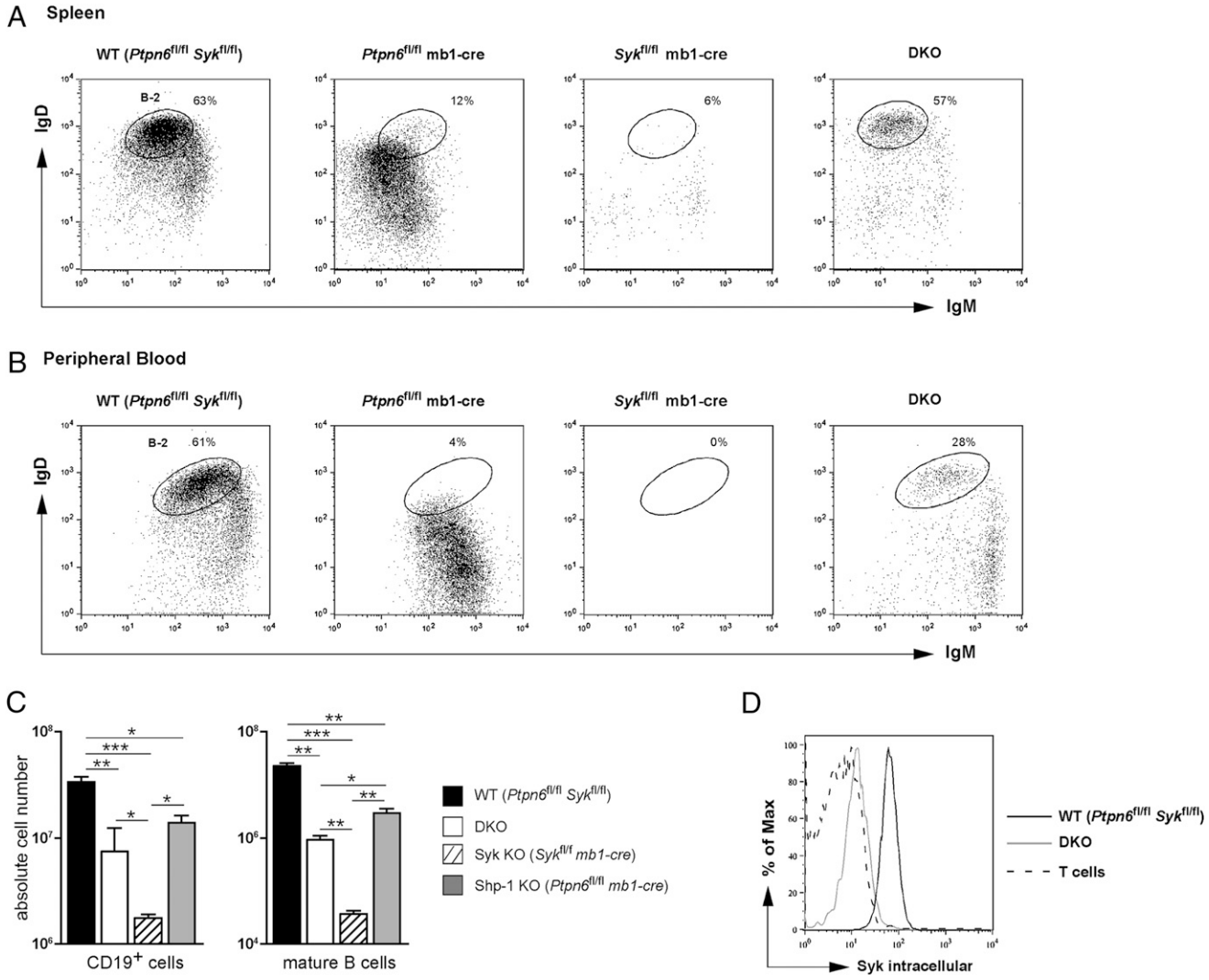

FIGURE 1. The additional Shp-1 deficiency partially rescues the maturation of Syk-deficient B cells in DKO mice. Representative analysis of lymphocytes isolated from the spleen (**A**) and peripheral blood (**B**) from WT (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$), *Ptpn6*$^{fl/fl}$ mb1-cre, *Syk*$^{fl/fl}$ mb1-cre, and DKO (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$ mb1-cre) mice. The cells are stained with anti-IgM and anti-IgD. Numbers indicate the percentages within the CD19$^+$ gate. (Data shown are representative of seven experiments with similar results.) (**C**) The absolute number of the CD19$^+$ B cells and mature IgM$^+$ IgD^{hi} splenocytes within CD19$^+$ gate from 8–12-wk-old WT (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$), DKO (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$ mb1-cre), and single KO mice. The results are shown as mean ± SEM (n = 7/group). One-way ANOVA analysis showed significant difference in cell numbers in the different mice [for CD19$^+$, $F(3,24) = 17.16$, $p < 0.0001$; for mature B cells, $F(3,24) = 38.55$, $p < 0.0001$]. Asterisks show significant differences between the indicated pairs of data groups tested with Tukey's multiple comparison test. (**D**) PBLs were stained for surface Ags, fixed, permeabilized, and stained for intracellular Syk and analyzed by flow cytometry. Histograms represent B cells within the CD19$^+$ gate. Data shown are representative of three experiments with similar results. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

mice show a relative increase in B-1a (IgM$^+$IgD$^{-/lo}$) B cells and a decrease in B-2 cells compared with WT mice (36) (Fig. 1A, 1B). Interestingly, the spleens of DKO contain a majority of mature B-2 cells (Fig. 1A, 1B), but the total number of mature B cells was reduced by >20-fold in the DKO mice in comparison with WT mice (Fig. 1C). We further analyzed the splenic B cell populations in the DKO mice and compared them to WT mice (Supplemental Fig. 1). There was a significant reduction in the total number of all subsets including mature follicular B cells and marginal zone B cells (Supplemental Fig. 1C). The DKO mice exhibited lower percentages of transitional type 1 and transitional type 3 B cells and a slight increase in transitional type 2 B cells compared with WT (Supplemental Fig. 1A), although the percentages of follicular B cells were similar in the WT and the DKO mice (Supplemental Fig. 1B).

The protein expression level of Syk in peripheral blood B cells was measured by flow cytometry. Intracellular Syk expression was not detected in the DKO B cells. Peripheral blood B cells from WT mice were used as a positive control. T cells were used as a negative control and did not show any intracellular Syk expression (Fig. 1D).

Effects of B cell–specific Ptpn6 and Syk double deletion in B cell development

To assess the effect of the double deficiency of *Ptpn6* and *Syk* in early B cell development, we performed flow cytometry analyses of bone marrow cells derived from WT and DKO as well as Syk and Shp-1 single-KO mice.

The Hardy fractionation system was used to delineate the different B cell developmental stages (43). The DKO mice had proportionally more pro–pre-B cells (fractions A–D), however, the absolute cell numbers were not significantly higher than those seen in the WT (Fig. 2B). In contrast, the total cell numbers of immature (fraction E) and recirculating B cells (fraction F) were reduced >5- and 20-fold, respectively, compared with the WT

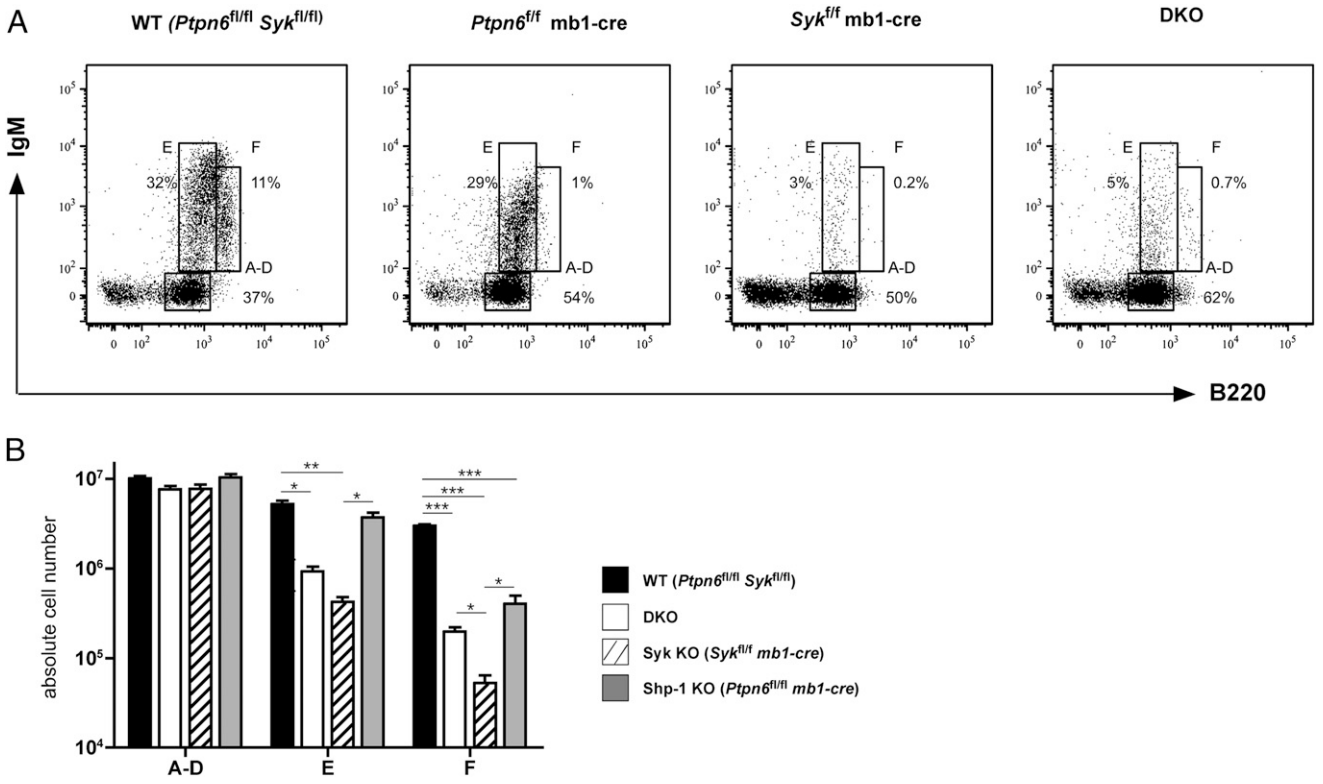

FIGURE 2. Effects of B cell–specific *Ptpn6* and *Syk* double deletion in early B cell developmental stages. (**A**) Representative data of flow cytometric analysis of lymphocytes isolated from bone marrow and stained with anti-B220 and anti-IgM to distinguish the Hardy fractions A–D, E, and F. (**B**) Absolute cell numbers of Hardy fractions A–F, plotted as mean ± SEM ($n = 7$). One-way ANOVA analysis showed significant difference between the cell numbers in the different mice in fractions E and F [fraction E, $F(3,24) = 6.818$, $p = 0.0022$; fraction F, $F(3,24) = 43.17$, $p < 0.0001$]. Asterisks show significant differences between the indicated pairs of data groups tested with Tukey's multiple comparison test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

control (Fig. 2B). DKO mice had twice as many immature B cells and 4-fold more recirculating cells as the Syk KO mice (Fig. 2B).

To rule out that mb1-cre might have an effect on its own, we compared $Ptpn6^{fl/fl}Syk^{fl/fl}$ to $Ptpn6^{fl/+}Syk^{+/+}$ mb1-cre mice at early developmental stages in the bone marrow (Supplemental Fig. 2A) and at later stages in the spleen (Supplemental Fig. 2C). The percentage and the total number of B cells in each stage were similar in $Ptpn6^{fl/fl}Syk^{fl/fl}$ and $Ptpn6^{fl/+}Syk^{+/+}$ mb1-cre mice (Supplemental Fig. 2B, 2D). The deletion of *Ptpn6* and *Syk* was confirmed by PCR analysis of genomic DNA of $CD43^-$ splenic B lymphocytes isolated from DKO ($Ptpn6^{fl/fl}Syk^{fl/fl}$ mb1-cre) B cells (Supplemental Fig. 3A). Bone marrow cells were isolated from DKO and control mice and cultured for 4 wk in IL-7–containing medium. The bone marrow cells were then lysed and immunoblotted for Shp-1 and Syk. Neither Shp-1 nor Syk could be detected in bone marrow B cells isolated from DKO mice (Supplemental Fig. 3B). The protein expression level of Syk in splenic B cells was measured by flow cytometry. Syk expression was absent in the DKO B cells (Supplemental Fig. 3C). Splenic B cells from WT mice were used as a positive control, and T cells were used as a negative control.

B cell responses in the DKO mice

To investigate whether DKO B cells can be activated, we assessed the ability of B cells purified from WT, Shp-1 single KO, and DKO spleens to upregulate the activation marker CD86 in response to various stimuli (47). Compared to WT controls and Shp-1 KO, DKO B cells were not activated upon stimulation by anti-IgM $F(ab')_2$, anti-RP105, or anti-CD38 (Fig. 3A). However, DKO B cells responded as well or better than controls upon stimulation with anti-CD40, CpG, or LPS (Fig. 3A). It was shown that Shp-1 KO cells had impaired activation to several stimuli (36). Similarly, we observed that the MFI of CD86-positive cells was reduced by >3-fold in Shp-1 KO cells compared with WT B cells after anti-IgM $F(ab')_2$ stimulation.

We also studied the proliferation of the isolated B cells triggered by the same stimuli mentioned above. Treatment with anti-IgM $F(ab')_2$ or anti-RP105 did not induce proliferation of DKO B cells. In contrast, the CpG-evoked proliferation was similar or higher in DKO B cells than in WT or Shp-1 KO but the difference was not statistically significant. Upon stimulation by anti-CD40, the DKO B cells proliferated substantially less than WT and similar to Shp-1 KO B cells (Fig. 3B). None of the DKO, WT, or Shp-1 KO B cells proliferated upon anti-CD38 stimulation. In addition, we compared $Ptpn6^{fl/+}Syk^{+/+}$ mb1-cre mice to our $Ptpn6^{fl/fl}Syk^{fl/fl}$ control mice, and we found no difference in CD86 MFI or the percentages of proliferating cells in response to any stimuli used in this experiment (Supplemental Fig. 2E, 2F).

We also assessed the ability of splenic and lymph node B cells in WT and DKO mice to mobilize calcium upon anti-IgM cross-linking. Splenic and lymph node DKO B cells did not show any calcium responses when stimulated with anti-IgM $F(ab')_2$ (Fig. 3C). However, their intracellular Ca^{2+} content was not different, as they gave similar signals upon ionomycin treatment (data not shown).

Effects of B cell–specific Ptpn6 and Syk double deletion on B cell populations of the peritoneal cavity

It has been shown that *Syk*-deficient mice do not generate peritoneal B cells (21), whereas Shp-1 KO mice display an expansion of the B-1a cells (36). To analyze the peritoneal B cell populations in the DKO mice, we stained B cells for CD5, a marker of murine B-1a cells (6). Similar to the Shp-1 KO mice, there was a 10-fold

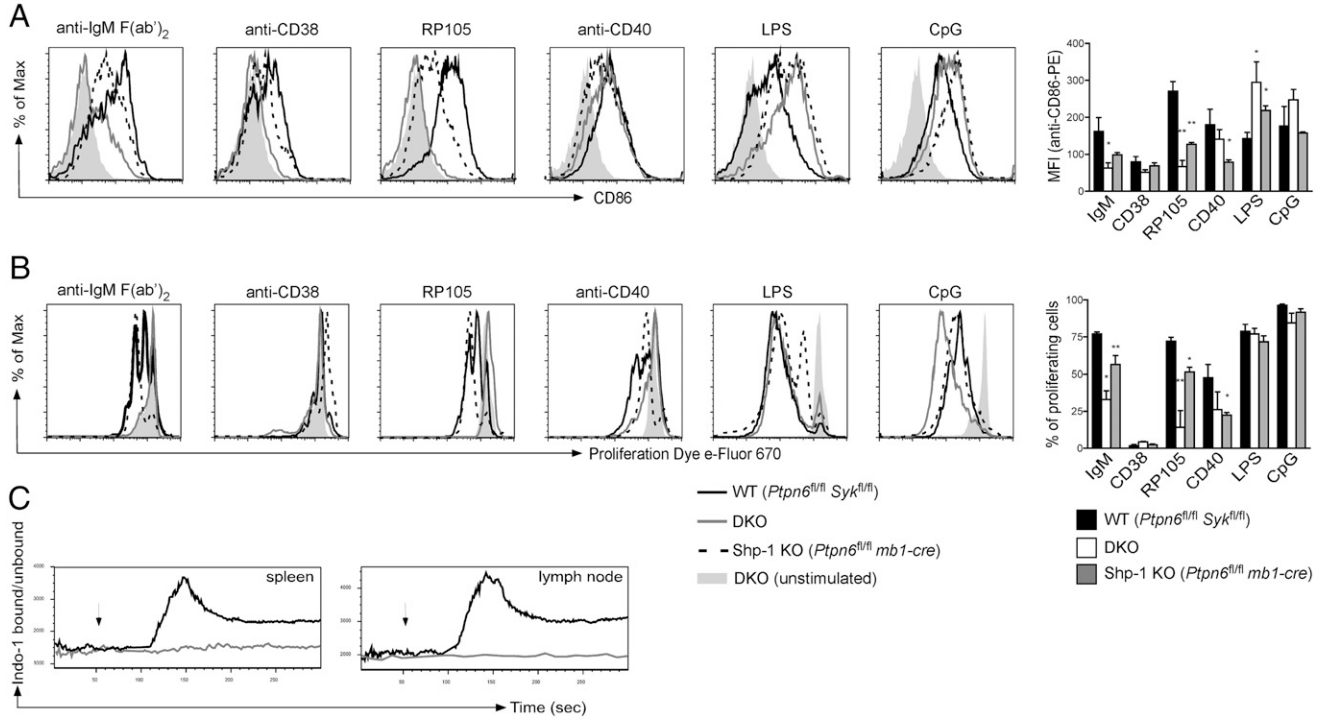

FIGURE 3. B cell responses in the DKO mice. (**A**) Ex vivo activation assay. Splenocytes from 9- to 10-wk-old WT, Shp-1 KO, or DKO mice were MACS sorted for $CD19^+$ cells and cultured ex vivo without stimulation or with stimulation: anti-IgM $F(ab')_2$ (10 μg/ml), anti-CD38 (10 μg/ml), anti-RP105 (10 μg/ml), anti-CD40 (10 μg/ml), LPS (10 μg/ml), and CpG (2.5 μg/ml) for 24 h. 7-AAD^- cells were analyzed by flow cytometry for the surface expression of the activation marker CD86 (*left panel*). Results for one of four independent experiments are shown (black curve: WT; gray curve: DKO; and shaded histogram: unstimulated DKO). The MFI of CD86 PE was calculated (*right panel*). (**B**) Ex vivo proliferation assay. Splenocytes from 9- to 10-wk-old WT, DKO, or Shp-1 KO mice were sorted by MACS. $CD43^-$ B cells were labeled with proliferation dye (eFluor 670). The cells were then cultured without stimulus or with stimulus: anti-IgM $F(ab')_2$ (10 μg/ml), anti-CD38 (10 μg/ml), anti-RP105 (10 μg/ml), anti-CD40 (10 μg/ml), LPS (10 μg/ml), and CpG (2.5 μg/ml) for 72 h. Living 7-AAD^- cells were analyzed by flow cytometry (*left panel*). Histograms shown are representative of one of four experiments with similar results ($n = 4$). The percentage of proliferating cells was calculated (*right panel*). Histograms shown are representative of one of four experiments with similar results ($n = 4$). (**C**) B cells from spleen (*right panel*) and lymph nodes (*left panel*) were stimulated with anti-IgM Ab (10 μg/ml) at the indicated time. Intracellular calcium concentrations of $CD19^+$ B cells are presented as the ratio of bound to unbound Indo-1. Data shown are representative of one of three experiments with similar results. $^*p < 0.05$, $^{**}p < 0.01$ by Mann–Whitney *U* test; $n = 4$.

reduction in the percentages of B-2 and B-1b cells in the peritoneal cavity of DKO mice compared with WT animals. In contrast, the percentages of B-1a B cells were similar in the DKO and WT (Fig. 4A); however, there was a reduction in the absolute numbers of all B cell subsets in the DKO mice (Fig. 4B). As expected, Syk KO mice hardly showed any B cells (<1%) in the peritoneal cavity, whereas Shp-1 KO had increased B-1a cell numbers (Fig. 4A, 4B). Interestingly, the peritoneal DKO B-1a cells mobilized calcium upon anti-IgM stimulation (Fig. 4C). This is in line with the intracellular staining analysis showing that Syk was not deleted in the peritoneal DKO B cells, suggesting a strong selection for the peritoneal B-1a cells that did not lose their Syk expression (Fig. 4D).

Serum Ig production in the DKO mice

Serum Ig levels from unimmunized 8–10-wk-old mice were measured by ELISA. Although DKO mice had similar IgM concentrations as the WT mice, they had reduced serum titers of all IgG isotypes (IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3) (Fig 5). This differs from Shp-1 KO mice, which show increased serum IgM, IgG_1, and IgG_3 and normal IgG2a and IgG_{2b} concentrations. In contrast, IgA concentrations were not altered significantly in the DKO mice compared with WT. Syk KO mice show lower Ig titers than the DKO mice; however, these differences do not reach statistical significance. To study if mb1-cre itself has an effect on the serum Ig titers, we measured serum Ig levels of $Ptpn6^{fl/+}Syk^{+/+}$ mb1-cre mice as well (Supplemental Fig. 2G). $Ptpn6^{fl/fl}Syk^{fl/fl}$ and $Ptpn6^{fl/+}Syk^{+/+}$ mb1-cre mice had similar serum Ig titers.

The colocalization of Ig-α and Zap70 upon BCR stimulation

It has been reported that Zap70, a member of the Syk kinase family, is expressed in all B cell developmental stages (48), albeit at low levels in mature B cells. To test whether Zap70 can take over the function of Syk in the DKO B cells, we used the proximity ligation assay (PLA) to monitor the colocalization of these two kinases with the BCR in WT and DKO B cells. For this, we used oligo-coupled F(ab) fragments from anti–Ig-α, anti-Zap70, and anti-Syk Abs, allowing us to test the proximity of these proteins in the range of 10–20 nm. In resting WT B cells, there is already some Syk localized at or near the BCR as indicated by the PLA signal (red dots, Fig. 6A). As found previously (49), more Syk is recruited to the BCR as early as 1 min after B cell stimulation with anti-IgM $F(ab')_2$. A more extended time course and quantification shows that the BCR/Syk interaction is only transient and declines after 5 min of stimulation (Fig. 6E). Interestingly, Zap70 behaves quite differently from Syk in WT B cells. It colocalizes in larger amounts with the BCR only at later time points, 15–30 min after B cell stimulation (Fig. 6C, 6G). As expected, in DKO B cells, no BCR/Syk interaction can be found (Fig. 6B, 6F). However, in these B cells, a BCR/Zap70 colocalization is found in the resting state and early (1 min) after B cell stimulation, whereas Zap70 no longer is colocalized with the BCR at later time points of B cell activation (Fig. 6D, 6H). Thus, Zap70 behaves quite differently in DKO B cells than in WT B cells. The localization of Zap70 in DKO B cells is similar to that of Syk in WT B cells (compare Fig. 6E with 6H). This suggests that Zap70 partially sub-

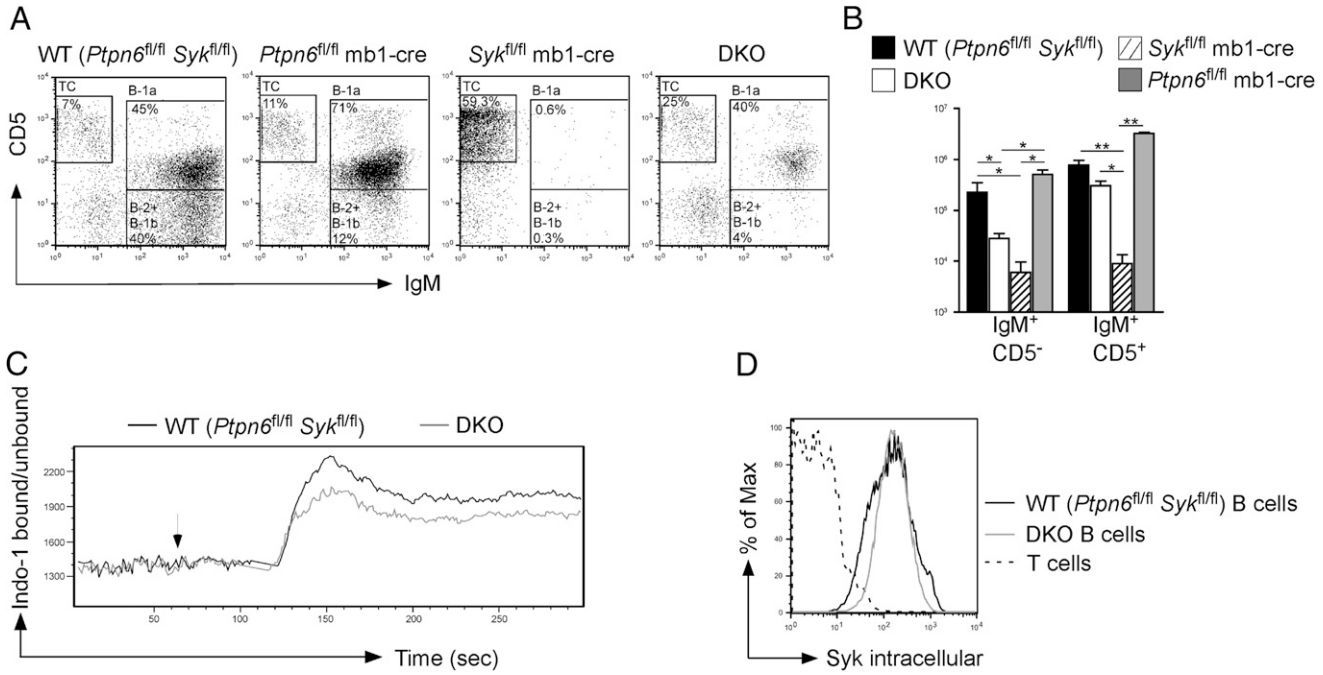

FIGURE 4. Effects of B cell–specific *Ptpn6* and *Syk* double deletion in the peritoneal cavity B cell populations. (**A**) Peritoneal lavage cells of WT (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$), *Ptpn6*$^{fl/fl}$ mb1-cre, *Syk*$^{fl/fl}$ mb1-cre, and DKO (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$ mb1-cre) mice were stained with IgM versus CD5 to study B-1a (IgM$^+$CD5$^+$), B-2 + B-1b (IgM$^+$CD5$^-$), and T cells (TC; IgM$^-$CD5$^+$). (**B**) Absolute numbers of B-1a and B-2 + B-1b cells are plotted as mean ± SEM. Kruskal–Wallis analysis showed significant difference of the cell numbers in the different mice (for B-2 + B-1b cells, $p = 0.0062$; for B-1a cells, $p = 0.0044$; $n = 5$). Asterisks show significant differences between the indicated pairs of data groups tested with Dunn's multiple comparison test. (**C**) The B cells from the peritoneal cavity of WT or DKO mice were stimulated with anti-IgM Ab (10 μg/ml) at the indicated time. Intracellular calcium concentrations of CD19$^+$ B cells are presented as the ratio of bound to unbound Indo-1. Results shown are representative of three experiments with similar results ($n = 3$). (**D**) Peritoneal cavity lymphocytes were stained for surface Ags, fixed, permeabilized, and stained for intracellular Syk and analyzed by flow cytometry. Histograms represent B cells within the CD19$^+$ gate. CD3ε$^+$ T cells were used as a negative control. *$p < 0.05$, **$p < 0.01$.

stitutes for the missing Syk in DKO B cells, although it cannot promote a calcium response in these cells (Fig. 3C). Zap70 expression in DKO mature B cells was not different from in WT B cells (Supplemental Fig. 3C).

Discussion

We have shown in this study that Syk/Shp-1 DKO mice generate mature B cells, allowing us to study the function of B cells in the absence of these central BCR signaling components. These mice also shed a new light on the phenotype of the Syk and Shp-1 single-deficient mice. Syk-deficient mice exhibit a (partial) developmental block at the pre–B cell stage (22, 23), and our data suggest that, in the absence of Shp-1, this block can be overcome presumably by Zap70.

However, the development of the DKO B cells is partially impaired because the total numbers of immature and mature B cells are reduced. This indicates that Zap70 can promote early B cell development but cannot replace Syk at later B cell developmental

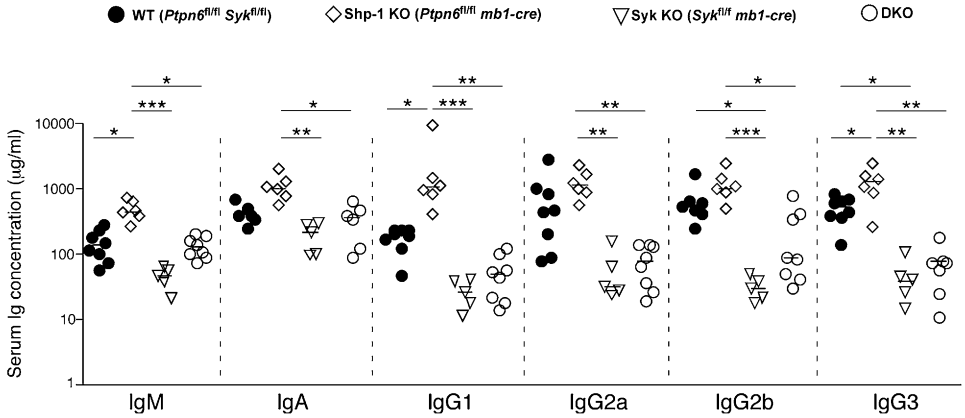

FIGURE 5. Serum Ig production in the DKO mice. Serum Ig levels of 8–10-wk-old WT (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$), *Ptpn6*$^{fl/fl}$ mb1-cre, *Syk*$^{fl/fl}$ mb1-cre, and DKO (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$ mb1-cre) mice were measured by ELISA. Each symbol represents an individual mouse. The bar indicates the median titer. Kruskal–Wallis analysis showed significant difference of the Ig titers of the different mice (for IgM, $p = 0.0002$; for IgA, $p = 0.0018$; for IgG1, $p < 0.0001$; IgG2a, $p = 0.0006$; IgG2b, $p = 0.0003$; IgG3, $p = 0.0002$). Asterisks show significant differences between the indicated pairs of data groups tested with Dunn's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

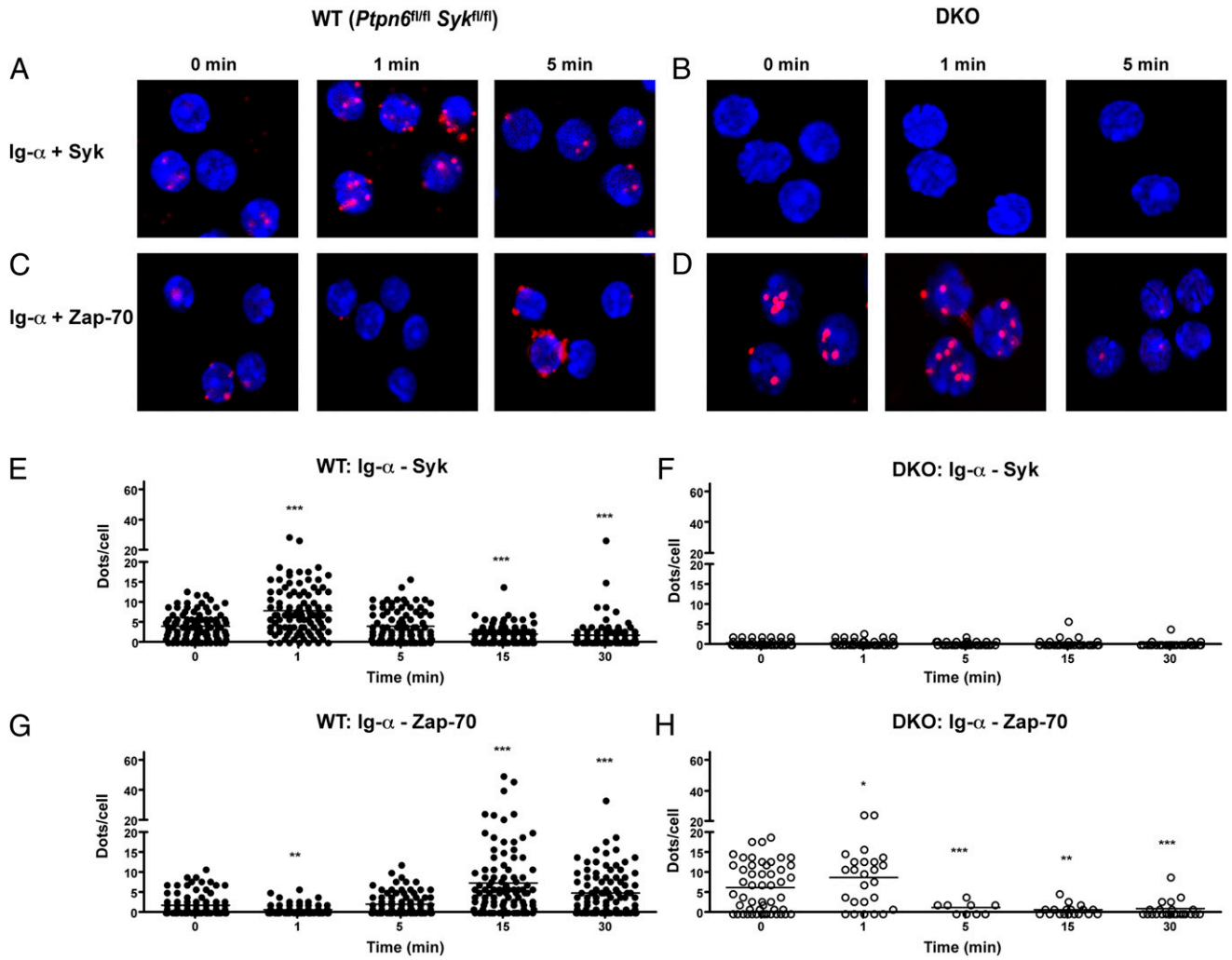

FIGURE 6. Zap70 is colocalized with Ig-α upon BCR stimulation. Splenic cells were isolated from WT (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$) or DKO (*Ptpn6*$^{fl/fl}$*Syk*$^{fl/fl}$ mb1-cre) mice, and after depleting CD43^{+} cells by MACS beads, the CD43^{-} B cells were cultured ex vivo overnight to rest. PLA was performed to study colocalization of Ig-α and Zap70 or Ig-α and Syk upon anti-IgM F(ab')$_2$ stimulation at different time points (0, 1, 5, 10, 15, 30, and 60 min). PLA using anti-Ig-α F(ab) fragment and anti-Syk Fab fragment in WT B cells (**A**) and DKO B cells (**B**) after 0, 1, and 5 min of BCR stimulation. Nuclei were stained with DAPI (blue), PLA signals are shown in red (red dots), and overlay shows the merged image of DAPI staining and PLA signals. PLA using anti–Ig-α F(ab) fragment and anti-Zap70 F(ab) fragment in WT B cells (**C**) and DKO B cells (**D**). Quantification of the PLA signals of Ig-α and Syk in WT (**E**) or DKO (**F**) cells. PLA of Ig-α and Zap70 in WT (**G**) or DKO (**H**) cells. Each circle represents one cell. The bar indicates the mean. Data shown are representative of three independent experiments with similar results. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ by Mann–Whitney *U* test.

stages. That Zap70 can only partially replace Syk in promoting B cell development is also shown by the analysis of Syk-deficient mice overexpressing Zap70 (50). In this study, *Zap70* cDNA was inserted into the *Syk* gene locus to generate Zap70 KI mice. However, in comparison with these Zap70 KI animals, our DKO mice display a greater expansion of large pre–B cells, indicating that Shp-1 limits pre–B cell proliferation (Fig. 2B). Earlier studies showed that Zap70 is expressed in all stages of B cell development (48). The absence of both Syk and Zap70 results in a complete developmental block at the transition from pro–B to pre–B cell stage, whereas mice deficient for only Syk show a partial block at this stage. This block is not due to a defect in synthesis or assembly of the pre-BCR but rather results from defective pre-BCR signaling (48). In addition, we crossed the DKO mice with *Zap70*$^{-/-}$ mice to generate triple-knockout mice. Our preliminary data suggest that in contrast to the DKO mice, that have both immature and mature B cells in the bone marrow, the triple-knockout mice indeed exhibit a complete block at the transition from pro–B to pre–B cell stage, and no mature B cells were detected in the spleen in these animals (data not shown).

Pro/pre–B cells show an increased Zap70 mRNA expression (51) and also contain LAT and Slp-76 that are associated with TCR signaling (52, 53). It is thus feasible that Zap70 employs either the TCR or BCR signaling components to promote the expansion and differentiation of pro/pre–B cells. Interestingly, the pre–B cell differentiation is also partially affected in Zap70 single KO mice, as these mice have a decrease in the absolute numbers of pre–B cells and immature B cells (54). In the DKO mice, the percentages and the total number of pre–B cells (fraction C) are higher than in Syk-deficient mice, indicating that Shp-1 acts as a gatekeeper downstream the pre-BCR (Fig. 2). Previous studies showed that Shp-1 could negatively regulate Zap70 and Lck/Fyn activation in TCR signaling (55, 56) and may bind directly to Zap70 and catalyze its dephosphorylation (57, 58). In addition, Shp-1 interacts with several other signaling molecules, such as Vav, Grb2, Slp-76, and PI3K (59).

Why Zap70 cannot efficiently support mature B cell development even in the absence of Shp-1 is less clear. In comparison with the WT control, the expression of Zap70 is not altered in mature DKO B cells (Supplemental Fig. 3). In contrast, long-term cultured

pre–B cells we generated from the bone marrow of DKO mice highly overexpressed Zap70 (data not shown). It is thus possible that the low Zap70 expression in mature B cells hampers the proper function of DKO B cells. At the same time, it is also feasible that the low responsiveness of the DKO B cells is due to a deregulated (pre)BCR/Zap70 signaling module caused by the Shp-1 deficiency. In our PLA analysis, we found that substantial amounts of Zap70 are colocalized with the BCR already in resting DKO B cells. It is thus possible that a deregulated and continuously signaling (pre) BCR/Zap70 complex in DKO B cells may interfere with B cell maturation. Alternatively, Syk and Zap70 might have some main differences in substrate specificity that prevent Zap70 from acting more efficiently downstream of the BCR.

Although the DKO splenic B cells are defective in BCR signaling, other receptors on these B cells, such as CD40, TLR4, and TLR9, show a normal or increased signaling output (Fig. 3). This is in line with the phenotype of CD22-defective B cells, which also show an augmented response after CD40 or TLR4 engagement. This may indicate that a CD22/Shp-1 complex is not only a negative regulator of the BCR, but also of CD40 and of innate immune receptors (60, 61). The normal or even increased signaling of these receptors may promote the differentiation of DKO splenic B cells to plasma cells. Indeed, the serum titer of IgM and IgA Abs was the same in WT and DKO mice (Fig. 5). Previous studies showed the importance of CD40 and TLR signaling for IgA production during a humoral immune response (62–64). The reduced IgG titers indicate, however, that the germinal center reaction and/or the class switch are partially defective in DKO mice and thus dependent on the proper function of the BCR/Syk signal transduction module.

Shp-1–deficient mice have increased numbers of B-1 B cells in all lymphoid organs, suggesting that Shp-1 controls the proliferation of these cells in the periphery (36). Similarly, mice deficient for the two ITIM-containing inhibitory receptors CD22 and Siglec-G have more B-1a cells with enhanced calcium signaling (65, 66). The B-1a B cell pool is not increased in the DKO mice (Figs. 1, 4). As a recent study showed that Syk is not necessary for the survival of B-1 cells (67), our data thus indicate that the development of B-1a B cells is strictly dependent on Syk even in the absence of Shp-1. The intracellular staining for Syk showed that all B-1a B cells in the peritoneum of DKO mice expressed Syk, and these cells showed calcium response upon BCR engagement. As mb1-cre is very efficient in deleting the floxed *Syk* gene, the peritoneal B-1a B cells in DKO mice display a remarkable selection for a functional *Syk* gene. These cells must have managed to silence the mb1-cre allele without interfering with the expression of the second WT mb1 allele, as all of these cells still produce Ig-α and express a BCR on their surface. Alternatively, these cells may have blocked the accessibility to one or both loxP sites of the floxed *Syk* gene, again without interfering with Syk expression. Still, the silencing or blockage of the Cre recombinase must be a rare event, as the total number of B-1a B cells in peritoneal cavity is >8-fold reduced in DKO mice. Altogether, this reflects a major difference in B-2 and B-1 cell development in DKO mice. For the development of B-1 B cells, the proper functioning of the BCR/Syk signaling module is essential even in the absence of Shp-1, whereas for B-2 cells it is not strictly required.

A surprising finding was that in activated WT B cells, Zap70 is recruited to the BCR at a late time point (15–30 min) when Syk is no longer found close to Ig-α. This suggests that Zap70 plays a yet unknown signaling role in the late phase of B cell activation. As Zap70 is under the stringent control of Shp-1, it is not clear how Zap70 can then be activated. One possibility is that an increase in reactive oxygen species production in activated B cells oxidizes and inhibits Shp-1, thus allowing Zap70 to become active. Indeed, the oxidant concentration reach high levels only 10–15 min after BCR activation (68). In DKO B cells, we do not detect the late recruitment of Zap70 to the BCR complex, and this may be due to the signaling defect in these cells. It is known that a defective calcium signal can prevent reactive oxygen species production in B cells (69).

Taken together, our data show that Syk and Zap70 have a partially redundant role in B cell development and that Shp-1 is an essential regulator for the activities of Syk and Zap70 in B cells. In addition, we show that the equilibrium between these enzymes is essential to maintain normal B cell development and function.

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Disclosures

The authors have no financial conflicts of interest.

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