Differential Regulation of B Cell Development, Activation, and Death by the Src Homology 2 Domain–containing 5′ Inositol Phosphatase (SHIP)

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Abstract

Although the Src homology 2 domain–containing 5′ inositol phosphatase (SHIP) is a well-known mediator of inhibitory signals after B cell antigen receptor (BCR) coaggregation with the low affinity Fc receptor, it is not known whether SHIP functions to inhibit signals after stimulation through the BCR alone. Here, we show using gene-ablated mice that SHIP is a crucial regulator of BCR-mediated signaling, B cell activation, and B cell development. We demonstrate a critical role for SHIP in termination of phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P_3) signals that follow BCR aggregation. Consistent with enhanced PI(3,4,5)P_3 signaling, we find that splenic B cells from SHIP-deficient mice display enhanced sensitivity to BCR-mediated induction of the activation markers CD86 and CD69. We further demonstrate that SHIP regulates the rate of B cell development in the bone marrow and spleen, as B cell precursors from SHIP-deficient mice progress more rapidly through the immature and transitional developmental stages. Finally, we observe that SHIP-deficient B cells have increased resistance to BCR-mediated cell death. These results demonstrate a central role for SHIP in regulation of BCR signaling and B cell biology, from signal driven development in the bone marrow and spleen, to activation and death in the periphery.

Key words: signal transduction • phosphatidylinositol 3-kinase • antigen • BCR • phosphatidylinositol 3,4,5-triphosphate

Introduction

The B cell antigen receptor (BCR)[1] plays a central role in B cell biology, transducing signals that determine cell fate (1). BCR-mediated signals are required not only for initiation and regulation of the immune response, but also for B cell development and survival. Interestingly, a growing body of evidence indicates that the B cell continuously receives signals through the BCR, apparently independent of antigen binding (2). Evidence for the existence and importance of these signals comes from findings that genetic ablation of components of the BCR complex (3, 4), or certain BCR signal transduction intermediaries (5–11), results in developmental arrest and lack of mature B cells in the periphery. Furthermore, induced genetic ablation of the BCR, using conditional knockout mice, results in rapid depletion of the mature B cell pool (2). Thus, it seems likely that developmental progression and survival in both the bone marrow and the periphery require constitutive or "tonic" signaling through the BCR. These data support a hypothesis

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[1]*Abbreviations used in this paper:* 7AAD, 7-amino-actinomycin D; BCR, B cell antigen receptor; Btk, Bruton's tyrosine kinase; [Ca^{2+}], intracellular free calcium; HSA, heat-stable antigen; IP_3, inositol 1,4,5-triphosphate; MAP, mitogen-activated protein; mIg, membrane-bound Ig; NF, nuclear factor; PI3-K, phosphatidylinositol 3-kinase; PI(3,4)P_2, phosphatidylinositol 3,4-biphosphate; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PLC, phospholipase C; SHIP, Src homology 2 domain–containing 5′ inositol phosphatase; sIg, surface Ig.

wherein both antigen-independent and -dependent signals emanating from the BCR provide critical stimuli for the selection, development, activation, and survival of B cells.

Transduction of signals through the BCR occurs via multiple parallel, yet cross-regulating, biochemical pathways. Aggregation of the BCR triggers a rapid increase in tyrosyl phosphorylation of the Igα/β signaling components of the receptor, Src- and Syk-family kinases, and many downstream effector proteins (12, 13). One of the most rapidly phosphorylated substrates is CD19, which functions to recruit and activate phosphatidylinositol 3-kinase (PI3-K) (14). PI3-K generates phosphatidylinositol 3,4,5-triphosphate (PI[3,4,5]P_3), which activates a wide range of signaling molecules through recruitment to the plasma membrane. PI(3,4,5)P_3-mediated activation of the enzymes Bruton's tyrosine kinase (Btk) and phospholipase C (PLC)γ results in generation of inositol polyphosphates (inositol 1,4,5-triphosphate [IP_3]) leading to intracellular calcium release and extracellular calcium influx (15–19). PI(3,4,5)P_3 also regulates activation of additional signaling pathways including those involving Akt (20–22) and mitogen-activated protein (MAP) kinases (23), and regulates activation of transcription factors including nuclear factor (NF)-κB (24–26). Furthermore, loss of PI(3,4,5)P_3 generation, through genetic ablation of the regulatory subunit of PI3-K, p85α, results in an absence of mature B cells in the periphery (9, 10). Thus, signaling cascades that mediate production of PI(3,4,5)P_3 are critical not only for active BCR-mediated responses, but also for B cell development and survival.

PI(3,4,5)P_3 signals are attenuated upon BCR coaggregation with the low affinity Fc receptor, FcγRIIB1 (27–29). This inhibition occurs via recruitment of the Src homology 2 domain–containing 5′ inositol phosphatase (SHIP), which hydrolyzes the 5′ phosphate from PI(3,4,5)P_3 to produce phosphatidylinositol 3,4-biphosphate (PI[3,4]P_2) (30–32). This hydrolysis disrupts BCR-mediated activation of Btk and PLCγ, and consequently, the calcium mobilization response (32, 33). In both the chicken DT40 B cell lymphoma line and in ex vivo B cells, genetic ablation of SHIP abrogates FcγRIIB1-mediated inhibition of the biochemical processes described above (17, 21, 34).

Although these results establish SHIP as a crucial negative regulator of B cell signaling during BCR–FcγRIIB1 coaggregation, the role of SHIP during autonomous BCR signaling is not defined. However, recent data have demonstrated increased calcium mobilization and Akt activation upon aggregation of the BCR in SHIP-deficient DT40 cells (21, 35). Also important in this regard is the fact that BCR aggregation leads to tyrosine phosphorylation of SHIP and its association with linker molecules including Shc (36), suggesting a possible role for SHIP in regulation of BCR signaling. Recently, it has been reported that mice deficient in SHIP display elevated levels of serum antibody, splenomegaly, and increased mortality (34, 37).

To define the role of SHIP in BCR-mediated signal transduction and in B cell biology, we examined ex vivo B cells from SHIP-deficient mice. We found that BCR ligation in SHIP-deficient B cells resulted in levels of PI(3,4,5)P_3 that were elevated over fivefold compared with wild-type littermates. Additionally, we found that SHIP regulates the biological outcome of signaling through the BCR. SHIP-deficient B cells were more sensitive to BCR-mediated induction of the activation markers CD86 and CD69. Furthermore, SHIP-deficient B cells displayed accelerated temporal development in the bone marrow, suggesting that PI(3,4,5)P_3 signals affect the rate of positive selection of immature B cells. SHIP ablation also resulted in accelerated maturation of transitional B cells in the spleen. Thus, SHIP normally limits the rate of B cell development from the immature stage onward through maturation in the periphery. Finally, we determined that SHIP functions as a positive regulator of BCR-induced cell death. These results demonstrate a central role for SHIP in regulation of BCR signaling and B cell biology, from tonic signal–driven development in the bone marrow and spleen, to activation and death in the periphery.

Materials and Methods

Reagents and Antibodies. Purified rabbit anti–mouse IgG antibody (H+L) F(ab′)$_2$ fragments were purchased from Zymed, and used for cell stimulation. Antibodies directed against the following molecules were used for flow cytometry: MHC class II (I-$A^{b/d}$, D3.137; clone was provided by S. Tonkonogy, North Carolina State University, Raleigh, NC), CD21 (7G6; PharMingen), CD23 (B3B4; PharMingen), CD24 (M1/69; PharMingen), CD45R (anti-B220, RA3-6B2; PharMingen), CD43 (Ly 48; PharMingen), CD69 (H1.2F3; PharMingen), CD86 (GL-1; PharMingen), IgD (JA12.5), IgM (polyclonal; Caltag), and mAb 493 (a gift from A. Rolink, Basel Institute for Immunology, Basel, Switzerland). 7-amino-actinomycin D (7AAD; via probe) was purchased from PharMingen, and propidium iodide and LPS (055:B5) were purchased from Sigma Chemical Co.

Animals and Cells. All animals used in this study were age-matched 6–10-wk-old $SHIP^{-/-}$ mice and control $SHIP^{+/+}$ littermates generated as the F1 progeny of $SHIP^{+/-}$ mice (37). Usually mice between 6 and 8 wk old were used to avoid the pathology associated with aged $SHIP^{-/-}$ mice (37). Splenic B cells were prepared as described previously (16). In brief, spleens were excised from mice, cells were dispersed, and RBCs were lysed using Gey's solution. For studies of induction of activation marker expression and cell survival, splenocytes were depleted of T cells by complement lysis using HO13.4 and T24 antibodies, and B cells were further purified by discontinuous Percoll density gradient centrifugation ($\rho > 1.07$). Purified B cells were cultured with 0–12 μg/ml F(ab′)$_2$ anti-IgG in 20% fetal bovine serum, with 100 IU/ml penicillin and 100 μg/ml streptomycin for 6–24 h. Bone marrow cells were prepared by flushing femurs with IMDM to dislodge cells, followed by gentle deaggregation using a 5-ml syringe. Bone marrow was depleted of RBCs using Gey's solution and washed twice in IMDM.

Phenotypic Analysis. Cells were washed, resuspended in PBS containing 1% BSA and 0.1% sodium azide, and incubated with optimal concentrations of directly conjugated antibody. Cells were incubated for 30 min at 4°C and washed twice in PBS/BSA/azide. After washing, cells were analyzed by flow cytometry. Cell viability was assessed by exclusion of 7AAD or propidium iodide.

Calcium Mobilization. For measurements of intracellular free calcium ($[Ca^{2+}]_i$), 10^6 cells/ml in IMDM cells were loaded with

Indo-AM (Molecular Probes, Inc.), and stimulated with $F(ab')_2$ anti-IgG antibody. Mean $[Ca^{2+}]_i$ was evaluated over time using a flow cytometer (model 50H; Ortho Diagnostic Systems, Inc.) with appended data acquisition system and MultiTime software (Phoenix Flow Systems) as described previously (16).

Measurement of B Cell Survival. Freshly purified splenic B cells were either left unstimulated, or were stimulated with 1–12 μg/ml of $F(ab')_2$ anti-IgG antibody. After 24 h of incubation, cell viability was assessed by exclusion of propidium iodide. Experiments were performed in triplicate wells.

Measurement of PI(3,4,5)P_3 and PI(3,4)P_2 Generation. Splenic B cells from $SHIP^{-/-}$ or $SHIP^{+/+}$ control littermates were depleted of RBCs and T cells and further purified by discontinuous Percoll density gradient centrifugation as described above. Cells were cultured for 48 h in 20% fetal bovine serum as described above with the addition of 25 μg/ml LPS (incorporation of ^{32}P label into the cellular ATP pool requires actively metabolizing cells). After 48 h in culture, cells were harvested, washed three times, and incubated for 90 min at 10^7 cells/ml in low phosphate medium with 0.5 mCi/ml ^{32}P-orthophosphate. ^{32}P-labeled cells were stimulated with $F(ab')_2$ fragments of anti-IgG for the indicated time, and immediately lysed in 2.4 N HCl/methanol/chloroform (1:0.9:1.4 vol/vol/vol). Lipids were extracted, deacylated with methanol/25% methylamine/*n*-butanol, and analyzed by HPLC on a SAX ion exchange column (Phenomenex) as described previously (38).

Measurement of IP_3 Generation. IP_3 generation was measured using a ^3H radioreceptor inhibition assay kit (DuPont-NEN) according to the manufacturer's instructions. Splenic B cells used in this study were first cultured for 48 h as described above.

Sublethal Irradiation and Autoreconstitution. Age-matched $SHIP^{-/-}$ and $SHIP^{+/+}$ littermates were treated with 500 rads of whole body irradiation 0–14 d before killing (39). For studies of autoreconstitution, single cell suspensions of spleen or bone marrow were depleted of RBCs, stained, and analyzed by flow cytometry.

Results

$SHIP^{-/-}$ B Cells Display Elevated PI(3,4,5)P_3 Levels upon BCR Aggregation. To define the role of SHIP in modulation of BCR signaling, levels of PI(3,4,5)P_3 and its breakdown product, PI(3,4)P_2, were measured by HPLC (31, 38) after stimulation of splenic B cells derived from $SHIP^{-/-}$ mice and normal littermates. Aggregation of the BCR with $F(ab')_2$ fragments of anti–mouse Ig resulted in an increase in levels of radiolabeled PI(3,4,5)P_3 in both $SHIP^{-/-}$ and $SHIP^{+/+}$ cells (Fig. 1 A); however, the induced increase in PI(3,4,5)P_3 was significantly greater in B cells from $SHIP^{-/-}$ mice. After stimulation of $SHIP^{-/-}$ B cells, PI(3,4,5)P_3 levels increased at least fivefold at all measured time points compared with wild-type cells. Particularly striking was the more sustained nature of the PI(3,4,5)P_3 signal observed upon BCR aggregation. PI(3,4,5)P_3 levels remained highly elevated in B cells from $SHIP^{-/-}$ mice throughout the duration of the experiment. Loss of PI(3,4,5)P_3 after stimulation of wild-type cells was primarily attributable to degradation by SHIP, as levels of the SHIP breakdown product, PI(3,4)P_2, were significantly elevated in the control cells compared with $SHIP^{-/-}$ cells (Fig. 1 B). These results show that most of the PI(3,4,5)P_3 produced upon BCR ligation is rapidly hydrolyzed by SHIP, causing a reduction both in the peak levels of PI(3,4,5)P_3 and in the duration of the PI(3,4,5)P_3 response. Interestingly, a small but significant amount of PI(3,4,5)P_3 degradation and conversion to PI(3,4)P_2 was observed in $SHIP^{-/-}$ B cells, suggesting the presence of a second 5′ inositol phosphatase. This may be the recently described SHIP-2 (40). From our results, it is apparent that degradation of PI(3,4,5)P_3 that follows stimulation through the BCR is primarily mediated through SHIP, and therefore SHIP plays a significant role in regulation of BCR signaling pathways that are PI(3,4,5)P_3 dependent.

Although the rate of PI(3,4,5)P_3 degradation was increased in the $SHIP^{+/+}$ cells, the additive counts from PI(3,4)P_2 plus PI(3,4,5)P_3 (Fig. 1, A plus B) were approximately equivalent in the two cell populations, suggesting that PI3-K was equally active. BCR-mediated activation of PI3-K is primarily dependent on phosphorylation of CD19, and CD19 phosphorylation was found to be comparable between the two cell populations (data not shown). In conclusion, analysis of phosphatidylinositol levels reveals that SHIP is a primary regulator of PI(3,4,5)P_3 levels. After aggregation of the BCR, SHIP significantly attenuates both the peak and the duration of the PI(3,4,5)P_3 response.

One of the effects of BCR aggregation downstream of PI(3,4,5)P_3 is activation of PLCγ and consequent mobilization of calcium. To determine whether PI(3,4,5)P_3 generation limits BCR-mediated PLCγ activation, the BCR was aggregated and subsequent IP_3 generation and calcium mobilization were measured in $SHIP^{-/-}$ and $SHIP^{+/+}$ cells.

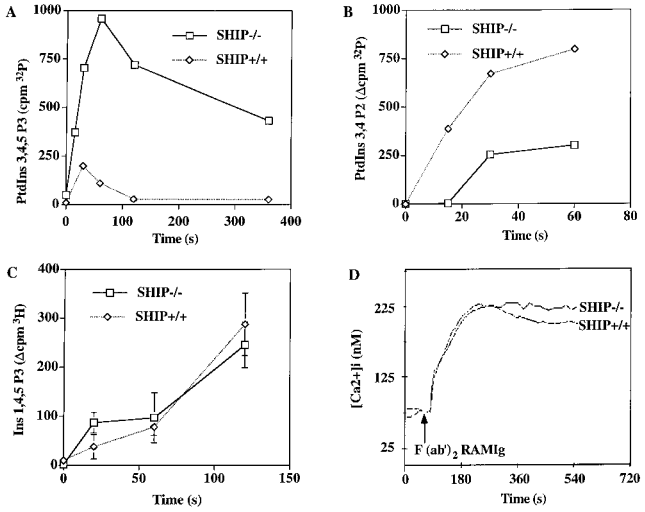

Figure 1. SHIP terminates PI(3,4,5)P_3 signals generated upon BCR aggregation. (A and B) B cells from $SHIP^{-/-}$ or control littermates were labeled with ^{32}P-orthophosphate for 1.5 h. The cells were then stimulated with $F(ab')_2$ fragments of anti-IgG (12 μg/ml) for the indicated time and immediately lysed with methanol/chloroform. Phospholipids were extracted, deacylated, and fractionated by HPLC. The fractions containing PI(3,4,5)P_3 (A) and the SHIP breakdown product, PI(3,4)P_2 (B), were quantitated by liquid scintillation. (C) IP_3 (Ins 1,4,5 P3) levels in $SHIP^{-/-}$ and control B cells after stimulation with $F(ab')_2$ anti-IgG (12 μg/ml). (D) Intracellular free calcium levels ($[Ca2+]_i$) were monitored after $F(ab')_2$ anti-IgG (10 μg/ml) stimulation of Indo-1–loaded B cells derived from $SHIP^{-/-}$ or wild-type littermates.

$SHIP^{-/-}$ B cells display equivalent IP_3 production and slightly increased calcium mobilization after $F(ab')_2$ anti-Ig stimulation (Fig. 1, C and D). These results indicate that $PI(3,4,5)P_3$ levels achieved during BCR signaling, in either $SHIP^{-/-}$ or wild-type B cells, are sufficient to support PLCγ activation. However, other $PI(3,4,5)P_3$-dependent signaling pathways, and their downstream biologic sequelae, may be affected by excessive $PI(3,4,5)P_3$ accumulation in $SHIP^{-/-}$ B cells.

Reduced Numbers of Immature and/or Transitional B Cells and Increased Numbers of Mature B Cells in the Periphery of SHIP-Deficient Mice. The biological consequences of SHIP modulation of BCR signaling are poorly defined. It has been reported that mice deficient in SHIP have elevated levels of serum antibody, splenomegaly, and increased mortality (34, 37). B cells from $SHIP^{-/-}$ mice are reduced in number by 50% in the bone marrow, and yet surprisingly, increased by ≤50% in the spleen (34, 37). As these observations imply a role for SHIP in regulation of B cell development, we compared splenic B cell development in $SHIP^{-/-}$ and wild-type mice. The majority of $IgM^{hi}IgD^{low}$ cells are transitional (T1) cells (39, 41, 42), which are recent immigrants from the bone marrow. These cells require constitutive BCR-derived signals to progress to the $IgM^{hi}IgD^{hi}$ (T2) stage (42). Additional BCR-mediated signals then allow the cell to progress to the $IgM^{lo}IgD^{hi}$ (M) mature stage (42). In spleens of $SHIP^{-/-}$ mice, there is a two- to threefold reduction in the total number of the most immature (T1) cells, as well as a 1.5-fold reduction in the T2 population (Fig. 2 A, and Table I). This conclusion is based on analyses in which contaminating marginal zone cells, distinguished as $IgM^{hi}IgD^{low}CD21^{hi}$, were excluded from the T1 population (Fig. 2 B, and Table I). Curiously, marginal zone cells are also underrepresented in the spleens of SHIP-deficient mice. Finally, the mature $IgM^{lo}IgD^{hi}$ population of cells appears to be overrepresented. It is also of interest that surface Ig (sIg)M levels on mature, $SHIP^{-/-}$ B cells are reduced by two- to fivefold compared with control B cells. The reduction in numbers of transitional B cells in the spleen of $SHIP^{-/-}$ mice, as well as the downregulation of sIgM, suggests a role for SHIP in regulation of maturation, possibly due to overamplification of BCR-generated signals.

To confirm that the unusual splenic B cell phenotype represents increased numbers of mature and reduced numbers of transitional cells, we examined levels of heat-stable antigen (HSA) and B220 on splenic B cells (39). B cells from the $SHIP^{-/-}$ mice showed reduced numbers of HSA^{hi} cells (Fig. 2 C), which further demonstrates that the B cell population has a more mature phenotype. Two additional surface markers expressed on mature cells, CD23 and MHC class II, were also significantly elevated on $SHIP^{-/-}$ cells (data not shown). In conclusion, SHIP deficiency causes

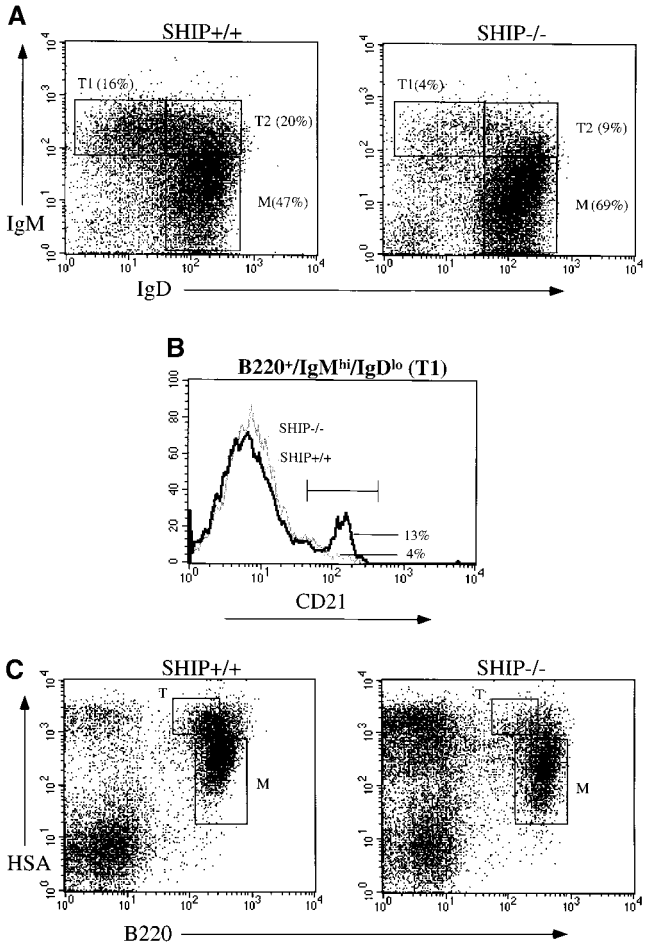

Figure 2. Reduced numbers of immature/transitional, but increased numbers of mature B cells in the spleens of $SHIP^{-/-}$ mice. Spleen cells from 7-wk-old $SHIP^{-/-}$ and $SHIP^{+/+}$ littermates were RBC-lysed and directly stained (A) for B220, IgM, 7AAD (via probe), and IgD. Cells were analyzed by flow cytometry, and gated for viable/$B220^+$ cells. Early transitional cells in region T1 ($mIgM^{hi}mIgD^{lo}$) decreased from 16 (control) to 4% of the B cell population in SHIP deficiency, late transitional B cells in region T2 are reduced from 20 to 9%, while the percentage of mature cells in region M ($IgM^{lo}IgD^{hi}$) are increased 1.5-fold by SHIP deficiency. To eliminate marginal zone cells from the presumptive T1 population (T1), gated cells ($IgM^{hi}IgD^{lo/-}$) were stained for CD21 (B) to determine the population of marginal zone B cells. To further analyze B cell maturation, cells were stained for B220, and HSA (C). A decrease in the transitional HSA^{hi} population is apparent in $SHIP^{-/-}$ B cells.

Table I. *Representation of Splenic B Cell Subpopulations from $SHIP^{+/+}$ and $SHIP^{-/-}$ Mice*

	$SHIP^{+/+}$	$SHIP^{-/-}$
	$\times 10^6$	$\times 10^6$
Total ($B220^+$)	37.9 ± 3.0	58.1 ± 3.5
Mature (IgD^+IgM^+)	24.2 ± 1.9	50.4 ± 3.2
T2 (IgD^+IgM^{hi})	7.6 ± 0.6	5.2 ± 0.4
T1 ($IgD^-IgM^hCD21^-$)	5.3 ± 0.5	2.2 ± 0.2
MZ ($IgD^-IgM^{hi}CD21^{hi}$)	0.8 ± 0.1	0.2 ± 0.1

Single cell suspensions were prepared from the spleens of 7-wk-old mice, labeled with the indicated antibodies, and analyzed by FACS®. The data are the mean of the number of cells (in millions) ± SD of three individual mice analyzed. MZ, marginal zone.

developmental abnormalities in B cells leading to a decrease in the number of transitional cells and increased numbers of mature splenic B cells.

Accelerated Development of Transitional B Cells in SHIP-deficient Mice. Data described above suggest that SHIP regulates B cell development. Specifically, levels of $PI(3,4,5)P_3$, determined by SHIP expression and function, may affect the rate of B cell maturation. As a consequence, B cells may traverse development more rapidly in $SHIP^{-/-}$ mice. To address this possibility, we used the autoreconstitution system described by Cancro and colleagues (39, 41) to analyze B cell development. In this system, the B cell compartments of adult mice are depleted by sublethal irradiation, allowing newly generated bone marrow–derived B cells to repopulate the spleen. In normal mice, peripheral lymphoid organs are devoid of lymphocytes 9 d after irradiation, with significant reconstitution appearing at 11–13 d. Virtually all newly formed B cells detected at this time point bear the transitional phenotype, HSA^{hi}, $B220^{lo}$, IgM^{hi}, and $sIgD^{lo}$.

B cell development in irradiated mice was evaluated based on quantitation of total numbers of B cells in the spleen, as well as their surface expression of IgM, IgD, CD24 (HSA), and B220. The kinetics of regeneration of B cells in the spleen was compared between $SHIP^{-/-}$ and control littermates (Fig. 3 A). 5 d after irradiation, >99% of the B cells were depleted from the spleens of both $SHIP^{-/-}$ and $SHIP^{+/+}$ mice, indicating that B cells in both mice were radiation sensitive. Although no production of cells was seen at day 7, by day 10 there were 5–10-fold more B cells in the spleens of the $SHIP^{-/-}$ mice than in wild-type littermates. In $SHIP^{-/-}$ mice, B cells repopulated the spleen 9–11 d after irradiation, while in wild-type littermates, reconstitution occurred after 11–13 d. Through the subsequent period of the experiment, increased numbers of B cells accumulated in the spleens of $SHIP^{-/-}$ mice compared with $SHIP^{+/+}$ mice.

We were interested in determining whether progression of transitional B cells to the mature stage was also accelerated in irradiated $SHIP^{-/-}$ mice. Typically, this phenotypic transition occurs over a 2–4-d period (39). The kinetics of appearance of the mature phenotype, as determined by decreasing expression of membrane-bound Ig (mIg)M and HSA (Fig. 3 B), revealed changes consistent with accelerated conversion of immature to mature splenic B cells in the SHIP-deficient mice. By day 12 after irradiation, only the SHIP-deficient B cells displayed the lower levels of mIgM and HSA typical of mature B cells. The trend for increased rate of maturation in the SHIP-deficient B cells continued 14 d after irradiation. Furthermore, significant numbers of $SHIP^{-/-}$ B cells rapidly downmodulate mIgM to very low levels. In summary, deletion of SHIP apparently leads to increased entry of immature B cells into the spleen and increased rate of maturation of transitional cells as reflected by downmodulation of IgM and HSA.

SHIP Deficiency Causes Accelerated Development of Immature Cells in the Bone Marrow. The results presented above indicate that SHIP deficiency leads to prolonged elevation of $PI(3,4,5)P_3$ and altered developmental progression from the transitional to mature stage in B cells, supporting a hypothesis that BCR-derived signals regulate developmental progression in the periphery. As tonic, or constitutive, signals also appear to be required for B cell development in the bone marrow, and because emigration of B cells into the spleen of $SHIP^{-/-}$ mice is accelerated, it seemed plausible that bone marrow development also could be accelerated. To explore this possibility, we examined bone marrow populations during autoreconstitution in $SHIP^{-/-}$ and $SHIP^{+/+}$ mice. Although total numbers of $B220^+$ cells in the bone marrow were similar between the autoreconstituted B cell populations of $SHIP^{-/-}$ and control littermates, phenotypic analysis revealed differences in the rates of development of early B cell subpopulations. To characterize these populations, we used the 493 mAb, which has been recently reported by Rolink et al. as a marker of early B cell development (43, 44). This mAb specifically recognizes pro-, pre-, and immature cells of the B cell lineage, while mature cells are not recognized. Furthermore, its expression is significantly diminished on normal transitional

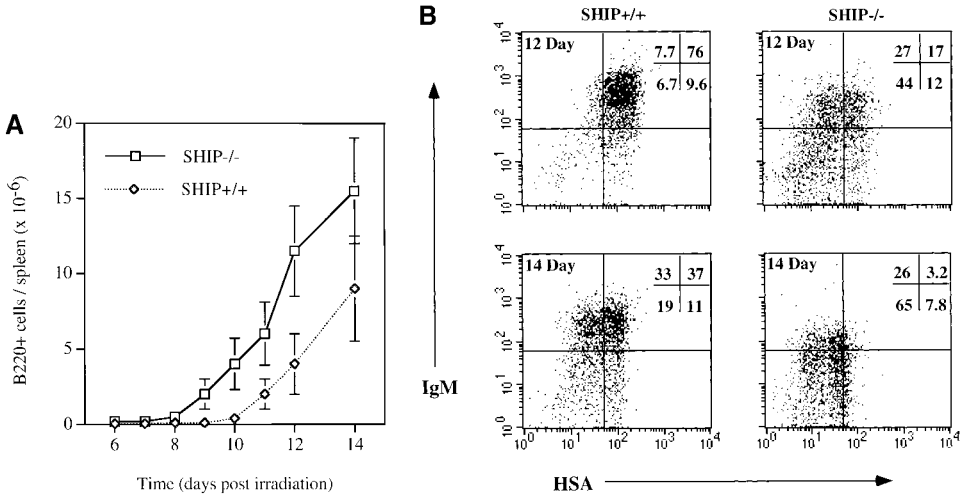

Figure 3. B cell development in $SHIP^{-/-}$ mice is accelerated. 6–10-wk-old $SHIP^{-/-}$ mice and wild-type littermates were treated with 500 rads of radiation, and the kinetics of regeneration of B cells in the spleen was monitored. (A) Time course of autoreconstitution of $B220^+$ cells in the spleens of $SHIP^{+/+}$ and $SHIP^{-/-}$ mice. (B) 12 and 14 d after sublethal irradiation, splenocytes from 8-wk-old $SHIP^{+/+}$ and $SHIP^{-/-}$ littermates were harvested and stained for the maturation markers IgM and HSA (live/$B220^+$ gate). Data shown represent the mean of three independent experiments.

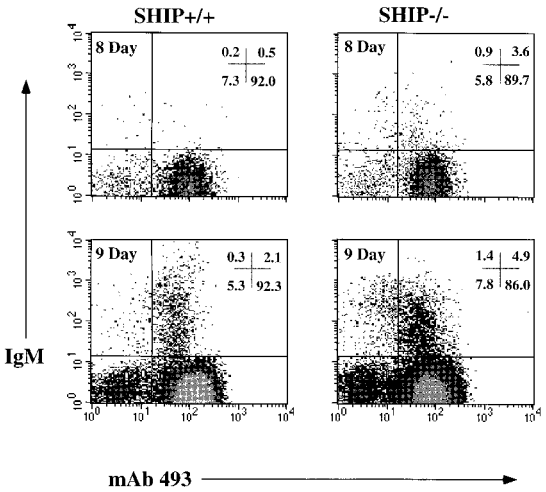

Figure 4. Increased rate of development of immature (IgM^+) cells in the bone marrow. 7-wk-old $SHIP^{-/-}$ mice and wild-type littermates were treated with sublethal irradiation (500 rads) to follow the kinetics of regeneration of bone marrow B cells. Bone marrow was harvested from two femurs at the indicated time points (8 and 9 d) after irradiation. Cells were counted and stained for B220, IgM, mAb 493, and 7AAD (via probe). Data show flow cytometric analysis of viable/$B220^+$ cells from the bone marrow of $SHIP^{+/+}$ and $SHIP^{-/-}$ mice 8 d (top) and 9 d (bottom) after sublethal irradiation. 250,000 total cells were counted in each case. 8 d after irradiation, the number of $B220^+$ cells in the bone marrow of $SHIP^{+/+}$ mice was 5.0×10^5, while $SHIP^{-/-}$ bone marrow had 4.7×10^5 $B220^+$ cells. 9 d after irradiation, the number of $B220^+$ cells in the bone marrow of $SHIP^{+/+}$ mice was 22×10^5, while $SHIP^{-/-}$ bone marrow had 26×10^5 $B220^+$ cells. Data shown are representative of three replicate experiments

cells (data not shown, and reference 44). Therefore, progression to the transitional/mature phenotype can be followed by decreased mAb 493 binding, and allows a simple way to distinguish between immature and mature B cells in the bone marrow. 493/anti-IgM analysis of normal bone marrow during autoreconstitution reveals that B cells progress through the $493^{hi}IgM^-$ pool (pro/pre), and the $493^{hi}IgM^+$ (immature) to the $493^{lo}IgM^+$ (transitional) stages. From the data in Fig. 4 (top), mAb 493^+ (pro/pre) cells appear with similar kinetics in both the SHIP-deficient and wild-type mice. However, $493^{hi}IgM^+$ (immature) B cells are increased twofold, and $493^{lo}IgM^+$ (transitional) B cells are increased fourfold in $SHIP^{-/-}$ mice by day 9 (Fig. 4, bottom). In the autoreconstitution model, downmodulation of 493 correlates well with emigration from the bone marrow to the periphery, as repopulation of B cells into the spleen also starts to occur at day 9 in the SHIP-deficient but not the control mice (Fig. 3 A). In control mice, IgM^+493^{lo} (transitional) cells appeared ~1–2 d later. It should be noted that at later time points the percentage of IgM^+ cells in the bone marrow of $SHIP^{-/-}$ mice was reduced compared with the $SHIP^{+/+}$ mice (data not shown), possibly reflecting the accelerated maturation and emigration of IgM^+ cells to the spleen. In view of previous studies implicating BCR-mediated signaling in development, these results suggest that increased levels of signaling drive accelerated maturation of the $SHIP^{-/-}$ bone marrow B cells through the immature, transitional, and mature stages of development.

SHIP Attenuates BCR-mediated Activation of CD69 and CD86 Expression. We next assessed the role of SHIP in modulating active BCR-mediated responses. Aggregation of the BCR has been shown to induce increased expression of CD69 and CD86 on resting mature B cells (45), the latter preparing them to present antigen to T cells. However, the impact of specific signaling pathways on BCR-mediated induction of activation marker expression is not well defined. As BCR-mediated signals are amplified in SHIP-deficient B cells, it seemed plausible that BCR-mediated induction of activation marker expression could be similarly amplified.

As shown in Fig. 5, A and B, ex vivo $SHIP^{-/-}$ B cells expressed slightly elevated levels of CD86 and CD69 and were more sensitive to BCR-mediated induction of these markers than were their wild-type counterparts. In addition, because both molecules were induced at significantly lower Ig concentrations in $SHIP^{-/-}$ cells, SHIP increased signaling thresholds for activation marker expression. These results suggest that both BCR-linked signaling pathways and modulation of gene expression are specifically regulated by SHIP.

Splenic B Cells from $SHIP^{-/-}$ Mice Are Less Susceptible to BCR-mediated Cell Death. Under certain circumstances, aggregation of the BCR leads to activation-induced cell death via induction of apoptotic signaling pathways (46). As $PI(3,4,5)P_3$ has been shown to be a survival signal in

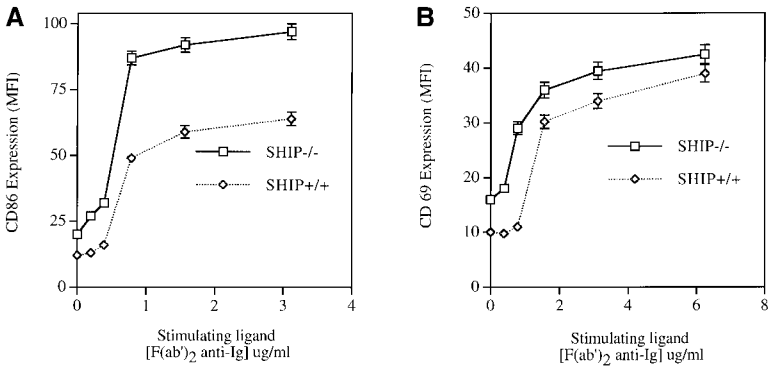

Figure 5. $SHIP^{-/-}$ B cells display increased sensitivity to BCR-mediated induction of activation marker expression. $SHIP^{-/-}$ or wild-type B cells were stimulated with the indicated concentration of $F(ab')_2$ fragments of anti-IgG. (A) After stimulation for 20 h, cells were analyzed. The mean fluorescence intensity of CD86 expression was measured on live/$B220^+$ cells. (B) After a 6-h stimulation, the mean fluorescence intensity of CD69 expression was measured. Data shown represent the mean of triplicate determinants.

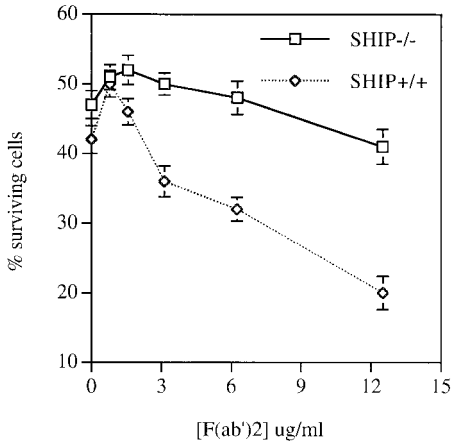

Figure 6. $SHIP^{-/-}$ splenic B cells are resistant to BCR-mediated induction of cell death. Freshly isolated splenic B cells from $SHIP^{-/-}$ mice or control littermates were incubated at 2.0×10^6 cells/ml for 24 h with the indicated concentrations of $F(ab')_2$ fragments of anti-IgG. The percentage of surviving cells was determined 24 h later based on recovery of viable (propidium iodide excluding) cells. Data shown represent the mean of triplicate determinants.

many cell models, SHIP-mediated degradation of $PI(3,4,5)P_3$ could thus promote cell death in response to BCR ligation. To test this possibility, resting splenic B cells were cultured with increasing concentrations of $F(ab')_2$ anti-Ig, and the percentage of surviving cells was determined 24 h later by propidium iodide exclusion. As shown in Fig. 6, the $SHIP^{-/-}$ cells were more resistant to BCR-mediated cell death than their $SHIP^{+/+}$ counterparts, especially at elevated concentrations of stimulus. Therefore, SHIP enhances the BCR-mediated apoptotic response of B cells, presumably through elimination of $PI(3,4,5)P_3$.

Discussion

Results reported here demonstrate an important role for SHIP in regulation of signal transduction through the BCR. Ligation of the BCR alone in $SHIP^{-/-}$ B cells resulted in dramatic and sustained elevations in $PI(3,4,5)P_3$ compared with $SHIP^{+/+}$ B cells. These results demonstrate that, at least in part, SHIP regulatory function is mediated through its enzymatic activity. Despite the overproduction of $PI(3,4,5)P_3$, SHIP-deficient B cells displayed no detectable increase in IP_3 production, and only very modest increases in calcium mobilization upon aggregation of the BCR. Thus, the large increases in $PI(3,4,5)P_3$ do not translate into proportionally large increases in activation of PLCγ. These findings indicate that a distinct signaling component must be limiting for BCR-mediated PLCγ activation. This conclusion is consistent with reports that levels of Btk, an effector necessary for PLCγ activation, has been shown to limit BCR-mediated calcium mobilization in the A20 B cell line (19, 32). However, these observations are discordant with studies showing that SHIP ablation in the DT40 chicken lymphoma line significantly enhances BCR-mediated calcium mobilization responses (35). It is possible that in the DT40 system, Btk is expressed at higher levels, making $PI(3,4,5)P_3$ the limiting element in the PLC activation cascade.

Mutations in components of the BCR signaling complex can severely influence B cell population dynamics and function. SHIP ablation is clearly associated with an altered biological phenotype. Characterization of the B cells from SHIP-deficient mice revealed a 50% reduction in the numbers of pre- and immature B cells in the bone marrow, and yet increased numbers of B cells in the spleen (34, 37). We found that immature and transitional B cells are significantly reduced in the splenic B cell compartment. Two possible mechanisms could account for this phenotype, and be a consequence of the amplified BCR signaling seen in $SHIP^{-/-}$ B cells: (a) an unusually large proportion of immature B cells may be deleted, with survivors expanding to fill available space in the periphery; and (b) the rate of B cell development may be accelerated, depleting the pre-, immature, and transitional B cell pools. While the former possibility was not formally excluded, we found that SHIP-deficient bone marrow cells were fully competent to reconstitute the mature splenic B cell compartment. $SHIP^{-/-}$ and $SHIP^{+/+}$ littermates differed primarily with respect to the rapidity with which they traversed the immature and transitional stages. In our studies of autoreconstitution, we found that in $SHIP^{-/-}$ mice B cells more rapidly repopulated the splenic compartment. Although these studies support a hypothesis in which deletion of SHIP results in accelerated maturation, the increased production of B cells could additionally be a result of enhanced survival of transitional B cells as they traverse into the mature B cell pool.

Development from the transitional to the mature B cell stage is an active process that is reportedly dependent on the strength of the constitutive BCR-mediated signal. For example, loss of constitutive signal in CD45-deficient mice (42, 47) results in a block at the late transitional stage, and loss of mature cells in the periphery. Conversely, loss of CD22 (48, 49), a negative regulator of B cell signaling, results in increased numbers of mature splenic B cells. SHIP deficiency appears to amplify the constitutive BCR signal, resulting in a rapid conversion from the transitional to the mature stage, based on downmodulation of sIgM and HSA. Therefore, modifications of the BCR complex that enhance constitutive signaling function also appear to enhance B cell development.

SHIP-deficient B cells also emigrated from the bone marrow at an accelerated rate, thus partially accounting for the reduction in numbers of immature cells in SHIP-deficient bone marrow. It is possible that B cell development and survival could be promoted through increased accumulation of $PI(3,4,5)P_3$ in SHIP-deficient B cells. Although other signaling pathways may be necessary for signaling of development, levels of $PI(3,4,5)P_3$ appear to limit progression.

Previous reports have proposed that elevations in tyrosine phosphorylation, as a consequence of loss of tyrosine phosphatases, can also change the signaling thresholds that effect development and selection (50). Combined, these studies suggest that levels of PI3-K activity and tyrosine ki-

nase activity are central in regulation of signaling and development mediated through the BCR. However, tyrosine kinase activity is linked to PI3-K activity (51). For example, efficient PI(3,4,5)P_3 generation, during BCR signaling, requires tyrosine phosphorylation of CD19, which subsequently recruits and activates PI3-K. Consistent with findings reported here, B cells derived from CD19-deficient mice are both deficient in PI3-K activation (15) and unusually immature, expressing the elevated levels of sIgM characteristic of the transitional phenotype (52). Conversely, overexpression of CD19 renders B cells hyperresponsive to transmembrane signals. CD19 transgenic mice have increased levels of autoantibodies, and the B cell population is shifted toward the mature $sIgM^{lo}IgD^{hi}$ phenotype (53). Deficiency in the regulatory subunit of PI3-K, p85α, also leads to impaired B cell development. Thus, evidence from several models supports a central role for PI(3,4,5)P_3 in driving development.

Although the signaling events that trigger various stages of development are not well defined, at least three signaling molecules downstream of SHIP and PI3-K have recently been implicated as potential regulators of B cell development. Dominant active ras, as well as raf and MAP kinases (42, 54, 55), can provide signals required for developmental maturation in B cells. Intriguingly, the activation of ras/raf and MAP kinase–mediated signaling events has been linked positively to PI(3,4,5)P_3 and negatively to SHIP. For example, the PI3-K inhibitor, wortmannin, inhibits ras/raf-mediated downstream activation of the MAP kinase pathway (56), as does SHIP expression (34). Conversely, generation of PI(3,4,5)P_3 can activate downstream signaling pathways, including the induction of gene expression through NF-κB (24). Interestingly, NF-κB is essential for B cell development, as mice deficient in both NF-κB p50 and p52 (57) demonstrate a loss of peripheral B cells. Thus, there are multiple parallel downstream pathways linked to PI(3,4,5)P_3 that are likely candidates for inhibition by SHIP during B cell development. It is important to note that, in addition to its enzymatic activity, SHIP may also function as a linker protein. In some systems, SHIP has been shown to bind with Shc and/or p62 dok (57a), both of which are implicated in ras activation (58).

We also observe that some active BCR-mediated responses are negatively regulated through SHIP, as SHIP-deficient B cells exhibit increased sensitivity to $F(ab')_2$ anti-Ig stimulation of CD86 and CD69 expression. As upregulation of CD86 is required for efficient T cell–B cell collaboration, this provides an additional mechanism through which SHIP could negatively regulate immune responses and prevent aberrant stimulation. Although previous studies using pharmacological agents indicate that CD86 and CD69 expression can be induced by calcium-activated pathways (45), physiologic activation of these responses appears to be limited by SHIP. SHIP may further mediate its inhibitory effects through a block in ras signaling, as previous studies in T cells have demonstrated inhibition of CD69 induction upon overexpression of dominant negative ras (59).

Finally, we show the positive role of SHIP in regulation of BCR-mediated cell death. Previous studies have shown that in vitro ligation of the BCR results in apoptotic death of B cells (46). Although SHIP negatively influences CD86 and CD69 expression, it is a positive mediator of the apoptotic response (60), suggesting that degradation of PI(3,4,5)P_3 promotes cell death. The mechanism of PI(3,4,5)P_3-mediated protection is unknown; however, Akt is activated through PI(3,4,5)P_3 and provides an antiapoptotic signal in some tissues. Akt is believed to promote cell survival by phosphorylation of Bad (61) and caspase-9 (62), and promote cell cycle progression (63). Thus, SHIP may contribute to the apoptotic signal through its degradation of PI(3,4,5)P_3 and resulting failed activation of Akt (21, 22).

Previous studies have demonstrated a critical role for SHIP in mediating inhibitory signaling through FcγRIIB1 in B cells and mast cells (64). In this report, we demonstrate an active role for SHIP as a critical regulator of B cell development and antigen receptor–mediated activation. Our results indicate that B cell development in both the bone marrow and spleen is regulated by SHIP. We hypothesize that SHIP deficiency enhances both tonic- and antigen-mediated BCR signals driving B cell development, maturation, and activation.

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