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The Adaptor Protein Shc Plays a Key Role during Early B Cell Development

Amber J. Giles, Timothy P. Bender, and Kodi S. Ravichandran

The adaptor protein Shc is phosphorylated downstream of many cell surface receptors, including Ag and cytokine receptors. However, the role of Shc in B cell development has not been addressed. Here, through conditional expression of a dominant negative Shc mutant and conditional loss of Shc protein expression, we tested a role for Shc during early B lymphopoiesis. We identified a requirement for Shc beginning at the transition from the pre-pro-B to pro-B stage, with a strong reduction in the number of pre-B cells. This developmental defect is due to increased cell death rather than impaired proliferation or commitment to the B lineage. Additional studies suggest a role for Shc in IL-7-dependent signaling in pro-B cells. Shc is phosphorylated in response to IL-7 stimulation in pro-B cells, and pro-B cells from mice with impaired Shc signaling display increased apoptosis. Together, these data demonstrate a critical role for Shc in early B lymphopoiesis with a requirement in early B cell survival. In addition, we also identify Shc as a required player in signaling downstream of the IL-7R in early B cells. The Journal of Immunology, 2009, 183: 5468–5476.

B cell development is a highly ordered process that requires cells to progress through multiple checkpoints during maturation. B cells originate from the hematopoietic stem cell pool and pass through multiple stages of development before becoming committed to the B lineage (1–3). Before B lineage commitment, potential B cells exist as common lymphoid progenitor cells (CLP). Although CLPs maintain the capacity to differentiate into other non-B lineages, they are considered to be B lineage specified and are actively undergoing D VH-J H IgHC gene rearrangement. In addition, CLPs express the IL-7R, which is required for progression along the B lineage. CLPs progress to the pre-pro-B cell stage where expression of B220 can first be detected. At this stage, D VH-J H IgHC gene rearrangement is nearly complete, and these cells are considered B lineage committed, although lineage plasticity remains (4).

Transition from the pre-pro-B to pro-B stage is accompanied by surface expression of CD19. The IgHC locus then begins to undergo V VH-D VH-J H rearrangement (4). If an in-frame coding region is produced from these recombination events, the IgHC protein is expressed on the cell surface along with components of the surrogate L chain, including the A5 and V pre B proteins and the Igα-Igβ heterodimer. This forms the pre-BCR (pre-BCR). Proper expression and intracellular signal transmission of the pre-BCR is crucial for progression from the pre-B to pro-B stage, and only one-third of pro-B cells successfully complete this process (5).

Disruption of components of the pre-BCR (such as in Rag1−/− mice) can lead to a complete block at the pre-BCR checkpoint (6–8). A functional AGr is also required for mature B cell maintenance in the periphery, because deletion of the BCR in mature splenic B cells results in rapid cell death (9). Successful signaling through the pre-BCR leads to survival, proliferation, and developmental progression. Once cells advance to the late pre-B stage, the recombination machinery initiates recombination of the L chain locus (10). The L chain pairs with IgHC, and together with the Igα-Igβ heterodimer, forms the BCR on immature B cells.

Functionally immature B cells leave the bone marrow via the bloodstream and travel to the periphery where they undergo further maturation (11). Immature B cells in the spleen are denoted transitional B cells and show a high rate of turnover in vivo with rapid cell loss (12). A significant amount of negative selection occurs at the T1-T2 transition when immature B cells from the bone marrow encounter new Ags (13). Once cells reach the T2 stage, they progress to the mature B pool after positive selection. Three distinct types of mature B cells exist: follicular, marginal zone, and B-1 cells, and there are different requirements for the development of each cell type. One current hypothesis for the formation of different mature B cell populations is the signal strength hypothesis. This hypothesis suggests that strong signaling from the BCR skews development to the follicular B developmental pathway, whereas weaker signaling through the BCR results in marginal zone B cell formation (14).

Ras signaling to Raf is required for B development as well as signaling through the BCR. Overexpression of a dominant negative Ras (H-rasN17) in early B cells blocked B cell development at the pre-pro-B to early pro-B transition (15). This block was partially rescued by expression of Raf-CAAX, which mimics Ras-induced activation of Raf. Transgenic mice that express dominant inhibitory Ras (Asn17 HA-Ras) in the late stages of pro-B development showed decreased survival of B lymphocytes although proliferation was not impaired, as assessed by BrdU incorporation in the bone marrow (16). Expression of an activated Ras (c-HA-rasV12) on a Rag-deficient background generated B lineage cells in peripheral tissues, suggesting that activated Ras was able to induce pro-B cells to differentiate beyond the pre-BCR checkpoint in the absence of the μ H chain (17). Thus, Ras plays important
roles in B cell development both before and during the pre-BCR checkpoint.

Cytokines and their cognate receptors have been shown to play critical roles in B cell development before the pre-BCR checkpoint (18–20). Deletion of cytokine receptors can inhibit B cell development at the earliest stages by blocking proliferation, differentiation, and/or survival of developing B lymphocytes. IL-7 and its cognate receptor are particularly important at the pre-pro-B to pro-B transition. Inhibiting the IL-7R by injection of blocking Abs (21) or genetic knockout (22) severely disrupts B cell development at the pre-pro-B to pro-B stage. This block has been linked to loss of commitment to the B lineage through decreased expression of the transcription factor EBF (early B factor) (23). In addition to B lineage commitment, IL-7 also functions to promote survival (24) and proliferation (25, 26) of early B subsets.

Proper intracellular transmission of receptor signals through appropriate cytoplasmic signaling intermediates is also required for successful B lymphopoiesis. The adaptor protein Shc signals downstream of multiple receptors, including Ag and cytokine receptors (27). During receptor signal transduction the p52 isoform of ShcA (hereinafter Shc) is phosphorylated on three critical tyrosine residues (Y239, Y240, and Y317), allowing the binding/recruitment of Grb2/Sos proteins leading to the activation of Ras, and in turn the MAPK cascade (28, 29). Shc protein expression and Shc tyrosine phosphorylation have been shown to be critical for thymocyte development at the β selection checkpoint. Impaired Shc-mediated signaling blocks T cell development by inhibiting the proliferative burst that accompanies successful pre-TCR signal transduction (30, 31). However, the role of Shc in B cell development remains unknown.

In this report, we attempt to address the role of Shc during B cell development by conditionally expressing a dominant negative Shc protein with mutation of its three critical tyrosine residues (hereinafter ShcFFF), or conditional loss Shc protein expression, in early B lymphocytes. Mice conditionally expressing ShcFFF had severely reduced pre-B cell numbers, suggesting a block at the pre-BCR checkpoint. However, we also noticed a surprising defect in cellularity of the pro-B population. On further analysis, we identified Shc as an important player in signaling downstream of the IL-7R and in providing survival signals to pro-B cells. In vivo and ex vivo studies indicate a critical role for Shc during early B cell development that is distinct from its role in T cell development.

Materials and Methods

Mice

The conditional ShcFFF-transgenic mouse line and conditional Shc1 knockout have been described previously (31). The MbI-Cre and Cd19-Cre mice have also been previously described (32–34). Rag1-deficient mice were obtained from The Jackson Laboratory. Mice were bred and maintained under specific pathogen-free conditions at the University of Virginia animal facility according to approved Institutional Animal Care and Use Committee protocols.

Flow cytometry

Single-cell suspensions were prepared from bone marrow and spleen of 8- to 12-wk-old mice. RBC were lysed, and cells were stained for FACS in 0.5% BSA in PBS with 0.05% NaN3. Abs specific for the following epitopes were used: B220 (RA3-6B2); CD19 (1D3); CD93 (AA4.1); BP-1 (6C3); CD24 (30-F1); TER119; CD11b (M1/70); CD3ε (145-2C11); IgM (II/41); Ly-6G (RB6-8C5); CD43 (57); CD17 (A7R34); CD117 (2B8); Sca1 (D7); and BrdU. Annexin V-FITC was used to detect surface exposure of phosphatidylserine. Abs were conjugated to FITC, Alexa 488, PE, PE-Texas Red, PE-Cy5.5, PE-Cy7, V450, allophycocyanin, Alexa 647, allophycocyanin-Alexa 750, or allophycocyanin-eFluor 780. Biotin-conjugated Abs were detected using streptavidin-FITC, PE, allophycocyanin, or Pacific Orange. Cell viability or DNA content was analyzed with 4',6-diamidino-2-phenylindole dihydrochloride, 7- aminoactinomycin D, or propidium iodide (PI). Abs and other reagents were purchased from eBioSciences, BD Pharmingen, or Sigma-Aldrich. FACS data were collected at the University of Virginia Flow Cytometry Core Facility on a CyAn ADP 9 color machine. FACS was performed using FlowJo software and gating on singlets as determined by pulse width vs forward scatter.

In vivo BrdU incorporation assay

To observe cell cycle kinetics, we used the protocol outlined by Kincade’s group (1). For bone marrow analysis, mice received an initial 0.1 mg of BrdU (Sigma-Aldrich) intraperitoneally 1 h prior to injection of 50 Ci of BrdU in water (0.8 mg/ml; changed daily). A minimum of five control and five mutant mice were sacrificed at 24, 48, and 72 h postinjection. Bone marrow from two femurs per mouse was harvested, and RBC were lysed, counted (by trypan exclusion), and stained for surface phenotype. BrdU incorporation was determined by flow cytometry using a BrdU incorporation kit (BD Biosciences). The least squares analysis was performed to obtain the renewal and production rates.

Ex vivo proliferation assays

Bone marrow B cells were sorted at the University of Virginia Flow Cytometry Core Facility with a BD Biosciences FACS Vantage SE Turbo Sorter with DIVA Option. Sorted cells were collected into PBS, rinsed in 1× PBS, and plated at 7000 cells/well in triplicate in a 96-well plate. For mice on a Rag1−/− background, pro-B cells were collected using MACS anti-Cd19 microbeads (Miltenyi Biotec) per the manufacturer’s protocol and assessed to be >95% pure by FACS of anti-B220 by anti-CD19. Pro-B cells were grown for 3 days in the indicated concentration of IL-7 (PeproTech) in 100 μl of RPMI 1640 with 5% FCS, 2 mM L-glutamine, and 50 μM 2-ME (complete RPMI) while incubating at 37°C in a humidified chamber with a 5% CO2 atmosphere. On the third day, 50 μl of fresh complete RPMI with the indicated concentration of IL-7 were added to feed the cells. During long-term cultures on OP-9 stromal cells, cultures were fed every 3 days. For [3H]thymidine uptake assays, 1 μCi of [3H]thymidine was added to each well 6–8 h before harvesting. Cells were collected on Filtermat A filters (PerkinElmer Life Sciences). For total cell counts, 100 μl of cell suspension were added to a FACS tube along with 50 μl of 5-μm-diameter counting beads (SpheroTech) and PI for viability. Live cells were gated using PI vs FSC, and total live cell number was calculated per the manufacturer’s protocol. Remaining cell suspension was used to phenotype B cell subsets by FACS.

Cell activation, immunoprecipitation, and immunoblotting

Pro-B cells from Rag1−/− bone marrow were cultured using anti-CD19 microbeads (Miltenyi Biotec) per the manufacturer’s protocol. Cells were allowed to rest in plain RPMI in a 37°C humidified chamber with a 5% CO2 atmosphere for 1 h before stimulation. Cells were then transferred to 35-mm glass bottom dishes for stimulation. Cells received 100 ng/ml IL-7, removed at the indicated times, and immediately boiled in 1× Laemmli buffer with 5% 2-ME. Cells were lysed for 10 min and assessed to be >95% pure by FACS of anti-B220 by anti-CD19. Pro-B cells were plated into 96-well plates (Eppendorf) in 100 μl of complete RPMI with the indicated concentration of IL-7. Cells were then cultured for 3 days. Cultures were subjected to immunoprecipitation by anti-FLAG agarose (Sigma-Aldrich) Immunoprecipitates were rinsed four times with 500 μl of cold lysis buffer and immunoblotted as described above. For detection of endogenous Shc protein in the conditional Shc knockout mice, total bone marrow B cells were first enriched with anti-CD19 microbeads (Miltenyi Biotec) and then sorted as described above. Total cell lysates were probed for total Shc and Erk1/2 protein as a loading control.

Detection of FLAG-tagged ShcFFF transgenic protein. B cells from bone marrow or spleen were positively selected using anti-CD19 microbeads (Miltenyi Biotec). Cells were rinsed with PBS and lysed. Total protein from cleared lysates was determined with Bradford reagent. Equal protein levels between control (Cre ShcFFF−/) and mutant (Cre+ ShcFFF+) were subjected to immunoprecipitation by anti-FLAG agarose (Sigma-Aldrich). Immunoprecipitates were rinsed four times with 500 μl of cold lysis buffer and immunoblotted as described above. For detection of endogenous Shc protein in the conditional Shc knockout mice, total bone marrow B cells were first enriched with anti-CD19 microbeads (Miltenyi Biotec) and then sorted as described above. Total cell lysates were probed for total Shc and Erk1/2 protein as a loading control.

Results

Targeted disruption of Shc during B cell development

To determine the role of Shc during B cell development (Fig. 1A), we took two genetic approaches to impair Shc-mediated signaling via the Cre/loxP approach: first, we targeted expression of a transgene encoding a dominant negative Shc protein (Shc Y239F/Y240F/Y317F, hereinafter ShcFFF); and second, we...
selectively deleted Shc1 at different stages of B cell development. To target the early B cell developmental stages, we used the Cre-transgenic lines Mbi1-Cre and Cd19-Cre. Mbi1-Cre expression begins at the CLP stage and continues throughout B cell development, allowing strong expression of Cre recombinase during early B development (32, 33). In Mbi1-Cre/ShcFFF mice, the transgenic ShcFFF protein was detected by anti-FLAG immunoprecipitation (IP) with anti-FLAG, and immunoblotted (IB) with anti-Shc Ab to detect ShcFFF transgenic protein. ShcFFF is visible in both bone marrow and spleen of Mbi1-Cre/ShcFFF mice, but only in the spleen of Cd19-Cre/ShcFFF mice.

Disruption of Shc results in a block at the pre-pro-B to pro-B transition

Conditional expression of ShcFFF or deletion of Shc1 with Mbi1-Cre resulted in a severe block in B cell development that was first apparent in the pro-B cell compartment before the pro-B stage. Importantly, we saw no defect in the hematopoietic stem cell (HSC) compartment, which is before Mbi1-Cre expression, suggesting that the fidelity of Cre (and in turn ShcFFF) expression is retained in Mbi1-Cre/ShcFFF mice. There was also no detectable difference in the numbers of CLPs, the stage when Mbi1-Cre-mediated recombination begins (Fig. 2A). This places the defect in Mbi1-Cre/ShcFFF mice during early B cell development after the CLP stage but before the pro-B stage.

To control for expression of a transgenic protein, we crossed the Mbi1-Cre mice to transgenic mice that conditionally express wild-type Shc (ShcWT), based on the same Cre/IoxP approach as the ShcFFF mutant. Mbi1-Cre/ShcWT mice had no detectable defect in B cell development (Fig. 2B). Instead, these mice displayed a reproducible increase in B cell numbers beginning at the pre-B stage.

Although this increase was not statistically significant, it could be explained by overexpression of the wild-type Shc protein, which has previously been shown to be beneficial to cell proliferation (35).

In contrast to Mbi1-Cre/ShcFFF mice, conditional expression of ShcFFF under Cd19-Cre resulted in no detectable defect in marrow B cell subsets (Fig. 2C). Although this result does not rule out a role for Shc in peripheral B cells, it suggests that Shc plays a nonredundant role during early stages of B cell development and may not have a similar requirement during later stages of development.

In addition to conditional expression of transgenic Shc proteins, we also examined the requirement for Shc during B cell development with conditional Shc1 knockout mice (Shc1f/f) (supplemental Fig. 2). Although Shc protein was efficiently removed in sorted bone marrow B cells beginning at the pre-B stage, we noticed residual protein in the pro-B subset of Mbi1-Cre/Shc1f/f mice (supplemental Fig. 2A). In addition, there was no reproducible, statistically significant defect in B cell subsets in the bone marrow or spleen (supplemental Fig. 2B). Because residual Shc protein was present in pro-B cells, we examined mice that were allowed to age to 9 mo. Senescence can allow subtle defects in B cell development to become more apparent, because CLPs, pre-pro-B cell, and pro-B cell numbers and proliferative capacity are reduced in aged mice (36). Aged Mbi1-Cre/Shc1f/f mice showed developmental defects in the bone marrow beginning at the pro-B stage (supplemental Fig. 2C), consistent with Mbi1-Cre/ShcFFF mice. This demonstrates that both Shc protein and its ability to be tyrosine phosphorylated on the three critical tyrosine residues are required for early B cell development.

We then sought to narrow the window at which the developmental defect occurred in Mbi1-Cre/ShcFFF mice. Using flow cytometry, we first gated out non-B lineage and IgM+ B cells (CD3ε−, Gr1−, Ter119−, NK1.1−, Ly6C−, IgM+), then gated on B220+CD43−AA4.1+ cells and displayed early B fractions as CD19+BP1+ (pre-pro-B), CD19+BP1− (early pro-B), and CD19+BP1− (late pro-B). This revealed a 67% decrease in the early pro-B stage of Mbi1-Cre/ShcFFF mice compared with littermate controls. This was not seen when the wild-type Shc transgene was expressed in Mbi1-Cre/ShcWT mice (Fig. 2D). Because a consensus in defining the pre-pro-B compartment is lacking, we used multiple gating strategies with flow cytometry but observed no defect in the pre-pro-B cell compartment of Mbi1-Cre/ShcFFF mice (supplemental Fig. 3). This places the most proximal defect in B cell development due to impaired Shc-mediated signaling at the pre-pro-B to early pro-B transition.

Diminished B cell compartment in the spleen of Mbi1-Cre/ShcFFF mice

Immature B cells from the bone marrow populate the spleen as transitional B cells. Transitional B cells are nonproliferative and undergo measurable cell loss as they mature (11, 12, 37). Analysis of splenic B cell subsets from Mbi1-Cre/ShcFFF mice revealed decreased immature, AA4.1+ transitional B cells (T1, T2, and T3 based on IgM vs CD23 expression) and AA4.1− mature follicular B cells, but no apparent defect in marginal zone B cells or T cells (Fig. 3A).

Importantly, B cell development was not impaired in mice conditionally expressing the wild-type Shc transgene (Fig. 3B). Moreover, when we assessed the spleen of mice expressing ShcFFF under Cd19-Cre, we saw no defect in splenic B cell subsets (Fig. 3C). Taken together, these studies point to a critical role for Shc-mediated signaling specifically during early B cell development rather than later maturational events.
Pro-B cells from Mb1-Cre/ShcFFF mice fail to accumulate in response to IL-7

Both the cytokine IL-7 and its receptor (composed of the IL-7Rα and common γ chain) have been shown to be critically required for development of early B cell subsets (18, 22, 38). Expression of IL-7Rα is increased from pre-pro-B to early pro-B, maintained through the late pro-B, and begins to decline by the pre-B stage (Fig. 4A, left; Ref. 39). IL-7Rα expression in Mb1-Cre/ShcFFF mice is comparable with expression in littermate controls during early B cell development (Fig. 4A, right). To determine whether Mb1-Cre/ShcFFF bone marrow B lymphocytes could respond to IL-7 stimulation, we measured [3H]thymidine incorporation of pro-B cells after culture with varying concentrations of IL-7 (Fig. 4B). Mb1-Cre/ShcFFF pro-B cells had significantly lower [3H]thymidine incorporation than those of littermate controls. This suggests that although Mb1-Cre/ShcFFF mice express normal levels of the IL-7R, bone marrow B cells are defective in their ability to respond to IL-7 when Shc-mediated signaling is impaired.

It has been reported that IL-7Rα signaling can be influenced by the pre-BCR (40, 41). Because we were specifically interested in early pro-B cells, which do not express a pre-BCR, and to rule out any contributions from the pre-BCR, we used Rag1−/− mice. To test the phosphorylation of Shc in response to IL-7 stimulation, pro-B cells from Rag1−/− bone marrow were stimulated with IL-7 for the indicated times (Fig. 5A). Phosphorylated Shc was detected as early as 2 min after IL-7 stimulation, increased at 5 min, and began to decrease by 10 min. This demonstrated that Shc is phosphorylated in freshly isolated pro-B cells in response to IL-7 stimulation.

Pro-B cells cultured in IL-7 ex vivo can differentiate over time to yield a mixed population of early B cells. To avoid this caveat, along with any possible influence of impaired pre-BCR signaling in ShcFFF-expressing mice, we crossed the Mb1-Cre/ShcFFF mice onto a Rag1−/− background, blocking B cell development at the pro-B stage. Once again we observed a defect in development to the early pro-B cell stage in mice conditionally expressing ShcFFF (Fig. 5B). These pro-B cells expressed the IL-7Rα-chain at levels comparable with those of littermate controls (Fig. 5C), yet the pro-B cells from Mb1-Cre/ShcFFF/Rag1−/− mice failed to respond to varying concentrations of IL-7 as shown by diminished [3H]thymidine incorporation as well as decreased live cell numbers compared with littermate controls (Fig. 5D).
Although the previous experiments demonstrated that ShcFFF-expressing pro-B cells were defective in their ability to respond to IL-7, we wanted to determine whether placing cells under a more robust ex vivo culture system could overcome this defect. To this end, we plated pro-B cells from *Mb1-Cre/ShcFFF* mice on OP-9 stromal cells in the presence of 5 ng/ml IL-7. Even in the presence of OP-9 stromal cells, ShcFFF-expressing pro-B cells were reduced in cellularity compared with those of littermate controls (Fig. 5E). By placing *Mb1-Cre/ShcFFF* mice onto a *Rag1*-*H11002* background, we were able to test the effects of impaired Shc signaling on IL-7 response specifically in pro-B cells. ShcFFF-expressing pro-B cells displayed an impaired response to IL-7 even in the presence of OP-9 stromal cells. Furthermore, this defect is independent of the pre-BCR.

In addition to IL-7, we tested the ability of ShcFFF-expressing bone marrow B cells to migrate to SDF-1, which has been shown to be critical for B cell development by directing developing lymphocytes to distinct areas in the bone marrow niche (19, 20, 42). We did not observe any defect in ShcFFF-expressing B cells to migrate to SDF-1 over a range of concentrations (data not shown). Moreover, we did not observe an accumulation of immature B cells in the spleen, which is observed in *Cxcr4* conditional knockout mice (43). Normal migration to SDF-1 in addition to defective response of pro-B cells to IL-7 when cultured on OP-9 stromal cells allowed us to rule out defective

![Figure 4](http://www.jimmunol.org/) ShcFFF expression impairs response of pro-B cells to IL-7, although IL-7R is present. **A**, Left, Normal expression pattern of the IL-7R during early B cell development. Right, Comparable expression of IL-7R in *Mb1-Cre* and *Mb1-Cre/ShcFFF* littermates in the early B cell subsets. **B**, Sorted pro-B cells were cultured with the indicated concentration of IL-7. On the fourth day after plating, cells were pulsed with 1 μCi of [3H]thymidine for 8 h, and thymidine incorporation was determined. *, p < 0.01; **, p < 0.001; ***, p < 0.0001.

![Figure 5](http://www.jimmunol.org/) Pre-pro to pro-B block in *Mb1-Cre/ShcFFF* mice and Shc tyrosine phosphorylation in response to IL-7 occur independently of the pre-BCR. **A**, CD19+ cells from Rag1−/− bone marrow were allowed to rest for 1 h in plain RPMI before incubation with either no IL-7 or 100 ng/ml IL-7 for the indicated times. Total lysates were probed with Abs against phospho-Shc (pY239/pY240) and then total Shc. **B**, Total bone marrow cell numbers. Block in pre-pro-B to early pro-B in *Mb1-Cre/ShcFFF* mice on a *Rag1*-*H11002* background. **C**, Pro-B cells from *Mb1-Cre/ShcFFF/Rag1*−/− mice express IL-7R at levels comparable with that of littermate controls. **D**, CD19+ bone marrow pro-B cells from mice on a Rag1−/− background were grown in varying concentrations of IL-7, and proliferation was measured after 4 days. Cell growth was assessed with [3H] incorporation (left) or by total cell counts (right). **E**, CD19+ pro-B cells from mice on a Rag1−/− background were grown on OP-9 stromal cells in the presence of 5 ng/ml IL-7 for the indicated times. Total cell counts were quantified with a flow cytometry-based bead assay. IB, Immunoblot; HSC, hematopoietic stem cell.
production rates, respectively. The slope provides the renewal rate of the population. Similarly, control and ShcFFF-expressing mice exhibited comparable labeling of the pre-B compartment, but pre-B cells from ShcFFF-expressing mice had a production rate of only 14% relative to littermate controls (54 × 10^3 cells/day vs 389 × 10^3 in controls). This suggests that ShcFFF affects the number of pro-B and pre-B cells generated under steady-state conditions, rather than the renewal rate of the respective pools.

**ShcFFF-expressing pro-B cells abnormally undergo apoptosis**

Pro-B cell response to IL-7 is manifested as proliferation, survival, and commitment to the B lineage. Although pro-B cells from *Mb1-Cre/ShcFFF/Rag1<−/−> mice maintained B lineage even after 9 days ex vivo culture on OP-9 stromal cells (Fig. 7A), they displayed a 65% increase in the fraction of PI-positive cells (indicative of late-stage apoptosis) after 3 days (Fig. 7B). We pulsed pro-B cells cultured ex vivo in IL-7-containing medium with BrdU to examine proliferation and observed a similar percentage of BrdU+ pro-B cells between *Mb1-Cre/ShcFFF* and littermate controls (Fig. 7C), suggesting that proliferation in response to IL-7 was not impaired. However, in these experiments, we noticed an increased percentage of cells in the sub-2n gate with 7-AAD, suggesting that ShcFFF-expressing cells stimulated with IL-7 ex vivo were abnormally induced to undergo apoptosis.

We next asked whether the increase in cell death observed in the ex vivo culture of *Mb1-Cre/ShcFFF* could be observed in B cells freshly isolated from bone marrow. ShcFFF-expressing mice showed an increase in the percentage of cells that stained positive for annexin V, beginning at the pro-B stage (Fig. 7D). This annexin V staining is likely underrepresented for multiple reasons: first, apoptotic cells are generally cleared quite rapidly in vivo; second, the cells isolated from the bone marrow were washed and stained for surface immunophenotyping (potentially losing the more fragile apoptotic cells in the washing/centrifugation steps); yet, the ShcFFF-expressing bone marrow B cells clearly demonstrated increased annexin V staining compared with those of littermate controls. In addition, early bone marrow B cell subsets displayed an increased percentage of cells in the sub-2n gate beginning at the pro-B stage and continuing through the immature B stage (Fig. 7E). Together with the data under ex vivo culture conditions in the presence of IL-7, these in vivo data provide evidence that ShcFFF-expressing pro-B cells undergo apoptosis in the bone marrow, and this likely contributes to the developmental defects seen in *Mb1-Cre/ShcFFF* mice.

**Discussion**

The adaptor protein Shc was initially shown to become phosphorylated in B cell lines in response to BCR and FcR ligation (44–46). However, due to conflicting results seen in B cell lines (47–52), the role of Shc in primary B cells during development has remained unknown. By using the Cre/loxP system, we demonstrate a novel requirement for Shc during B cell development. Disruption of Shc by conditional deletion of *Shc1* or transgenic expression of ShcFFF blocked B cell development before the pro-B stage (supplemental Figs. 1 and 2 and Fig. 2). The developmental block was further defined to lie at the pre-pro-B to pro-B transition (Fig. 2). Although critical roles for cell surface receptors have been demonstrated during this stage of development, the requirements for intracellular signaling components responsible for the pleiotropic effects of these receptors remains incomplete. Previous studies have demonstrated requirements of other adaptor proteins during multiple stages of B cell development, yet their functional significance is found to lie beyond to pre-B stage (18, 22). To our knowledge, the data presented here are the first demonstrations of a requirement for an adaptor protein in the developmental transition from the pre-pro-B to pro-B stage.

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**Table 1. Renewal and production rates of bone marrow B cell subsets**

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<td>Pro</td>
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<td><em>Mb1-Cre/ShcFFF</em></td>
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*Percentages of BrdU+ pro-B and pre-B cells for each mouse were determined by flow cytometry and multiplied by total bone marrow cell counts (trypan) from two femurs of each mouse to obtain total BrdU cell numbers. The regression coefficients of percent and absolute BrdU labeling vs time provide an estimate of renewal and production rates, respectively.*

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**FIGURE 6.** Early B subsets expressing ShcFFF show normal turnover, but decreased production of lymphocyte populations. Mice were given an initial i.p. injection of BrdU and then continuous BrdU administration in water. Mice were then sacrificed at the indicated times. Bone marrow was collected, stained for surface Ags, and then fixed and stained for BrdU incorporation. A. Relative fraction of dividing BrdU+ labeling of the indicated subsets over time. The slope provides the renewal rate of the population. B. Absolute numbers of BrdU+ pro-B and pre-B cells over the time course of the experiment. Slope provides the renewal rate of the subsets. A minimum of five mice were used for each genotype at each time point. Solid and dashed lines are linear regressions for *Mb1-Cre* control and *Mb1-Cre/ShcFFF* mice, respectively. *, *p < 0.01; **, *p < 0.001.
We also present here evidence that Shc may function down-stream of the IL-7R. The IL-7R promotes proliferation, differentiation, survival, and commitment to the B lineage. Deficiency of the cytokine IL-7 or the IL-7R severely disrupts early B cell development (53). Defective survival is associated with decreased Bcl-2 protein, although expression of Bcl-2 is not sufficient to rescue B lymphopoiesis in IL-7R-deficient mice (54). However, deletion of the pro-apoptotic Bcl-2 family member Bim was able to partially rescue B lymphopoiesis in IL-7R-deficient mice (54). However, deletion of the pro-apoptotic Bcl-2 family member Bim was able to partially rescue B lymphopoiesis in the absence of IL-7 (27, 30, 31). It appears that the IL-7R mediates a balance of pro-apoptotic and anti-apoptotic proteins to allow survival during early B lymphopoiesis, although the requirements for this balance remain elusive. In our initial tests, we did not observe a defect in Bcl-2 mRNA in Mb1-Cre/ShcFFF bone marrow subsets (pro, pre, immature, or mature recirculating; data not shown). Nevertheless, the contribution of other Bcl-2 family members must be considered as possible causes for the observed increase in apoptotic cells.

Shc is linked to proliferation in response to cytokine and AgR signaling (55). However, it also appears to play a role in cell survival. Expression of a phosphorylation-defective Shc mutant (Y239F/Y240F) in the IL-3-dependent Ba/F3 cell line caused these cells to become sensitive to apoptosis in response to IL-3 withdrawal and serum starvation (29). This was linked to reduced expression of c-myc mRNA. Likewise, the Y239F/Y240F Shc mutant abolished c-myc mRNA induction in response to TCR/CD3 cross-linking in Jurkat T cells (15, 16). This was correlated with an increase in activation-induced cell death. Thus, Shc can affect multiple cellular responses. In developing thymocytes, conditional expression of ShcFFF blocked proliferation whereas in early B cells, conditional expression of ShcFFF appears to impair cell survival and lead to apoptosis.

To date, the role of Shc is best characterized as a signaling component of the Ras/MAPK pathway. Expression of a dominant negative Ras mutant during early B cell development severely disrupted B lymphopoiesis at the pre-pro-B to pro-B transition. Pro-B and pre-B cells from the dominant negative Ras transgenic mice incorporated BrdU at a rate equivalent to that of littermate controls, suggesting no defect in the renewal rates of these subsets (16). This is quite similar to the phenotype observed in B cells expressing ShcFFF. Disruption of Ras has also been shown to affect survival of pre-B cells, although upstream players have remained elusive. Given the known involvement of Shc in the Ras signaling pathway, it is intriguing to consider the possibility that...
ShcFFF may disrupt Ras signaling at the pre-pro-B to pro-B transition and induce cells to undergo apoptosis. The intracellular signaling components of early B lymphocytes before the pre-BCR checkpoint remain largely unresolved, although these early B progenitors must pass multiple checkpoints before advancing along the developmental pathway. For the first time, we show here that Shc plays a key role during B cell development. Intriguingly, we identify a novel requirement for Shc in IL-7R signaling that is independent of pre-BCR signaling. Expression of ShcFFF impairs B cell development at the pre-pro-B to pro-B transition with a reduction in the production rate of early B subsets, although the turnover rate is unaffected. We have shown that Shc is phosphorylated in response to IL-7 in pro-B cells. Furthermore, although pro-B cells from Mbl-Cre/ShcFFF mice proliferate normally in response to IL-7 when cultured ex vivo, as demonstrated with BrdU incorporation, they fail to accumulate and proliferate normally in response to IL-7 when cultured ex vivo, as demonstrated with BrdU incorporation, they fail to accumulate and show a substantial increase in the percentage of cells undergoing apoptosis. This represents a novel role for Shc during B lymphopoiesis before expression of the pre-BCR that is linked to survival. These data also reveal a new player in the IL-7R-mediated survival pathway during B lymphopoiesis.

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