The Journal of Experimental Medicine

Src Homology 2–containing 5-Inositol Phosphatase (SHIP) Suppresses an Early Stage of Lymphoid Cell Development through Elevated Interleukin-6 Production by Myeloid Cells in Bone Marrow

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Abstract

The Src homology (SH)2–containing inositol 5-phosphatase (SHIP) negatively regulates a variety of immune responses through inhibitory immune receptors. In SHIP$^{-/-}$ animals, we found that the number of early lymphoid progenitors in the bone marrow was significantly reduced and accompanied by expansion of myeloid cells. We exploited an in vitro system using hematopoietic progenitors that reproduced the in vivo phenotype of SHIP$^{-/-}$ mice. Lineage-negative marrow (Lin$^-$) cells isolated from wild-type mice failed to differentiate into B cells when cocultured with those of SHIP$^{-/-}$ mice. Furthermore, culture supernatants of SHIP$^{-/-}$ Lin$^-$ cells suppressed the B lineage expansion of wild-type lineage-negative cells, suggesting the presence of a suppressive cytokine. SHIP$^{-/-}$ Lin$^-$ cells contained more IL-6 transcripts than wild-type Lin$^-$ cells, and neutralizing anti–IL-6 antibody rescued the B lineage expansion suppressed by the supernatants of SHIP$^{-/-}$ Lin$^-$ cells. Finally, we found that addition of recombinant IL-6 to cultures of wild-type Lin$^-$ bone marrow cells reproduced the phenotype of SHIP$^{-/-}$ bone marrow cultures: suppression of B cell development and expansion of myeloid cells. The results identify IL-6 as an important regulatory cytokine that can suppress B lineage differentiation and drive excessive myeloid development in bone marrow.

Key words: lymphopoiesis • inflammation • SHIP • interleukin 6

Introduction

Hematopoietic cells develop from a primitive stem cell that gives rise to two precursor populations. One population has the capacity to form myeloid and megakaryocyte lineages (1) and is marked by low expression of IgG receptors. The other population has the capacity to form all lymphoid and natural killer cell lineages (2) and is marked by expression of RAG. Sorted populations of lymphoid- or myeloid-committed progenitors, defined as above, showed minimal capacity to develop into another lineage (1, 2). In addition to expression of RAG, lymphoid-committed precursor cells can be detected in in vitro and in vivo assays using sorted populations that lack all lineage markers (Lin$^-$) and express the receptor for IL-7 and low levels of surface markers Sca-1 and c-kit (IL-7R$^+$, Sca-1[low], c-kit[low]; reference 3). Expression of CD19 occurs at the pro-B cell stage and corresponds to commitment to the B lineage (4).

Early lymphoid-committed progenitors diverge from a more primitive stem cell population found in the Lin$^-$, c-kit(high), Sca-1(high) population, a bone marrow fraction which also contains precursors for myeloid lineages (5, 6). Down-regulation of the c-kit receptor by the uncommitted stem cell corresponds to a loss of myeloid developmental potential and an increase in lymphoid potential (7). This is a critical developmental decision, representing the first commitment step from multipotent progenitors, but is the

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Abbreviations used in this paper: FcγRII, Fcγ receptor II; NOD, nonobese diabetic; PI-3K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; SH, src homology; SHIP, SH2-containing 5-inositol phosphatase.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2004/01/243/12 $8.00
Volume 199, Number 2, January 19, 2004 243–254
http://www.jem.org/cgi/doi/10.1084/jem.20031193

least understood. Information of how stem cells attenuate self-renewal and undergo lineage commitment is essential to appreciate the myeloid- and lymphoid-restricted developmental pathways.

Src homology (SH)2–containing 5-inositol phosphatase (SHIP) is expressed by hematopoietic cells. SHIP is a critical negative regulator, maintaining the balance of positive and negative signals in lymphocytes and myeloid cells (8). Recently, an important role of SHIP in the immune system has been manifested by establishing SHIP knockout mice. Major features of $SHIP^{-/-}$ mice include splenomegaly, caused by hyperplasia of myeloid cells, and a massive infiltration of myeloid cells into lungs, causing a shortened life span (9, 10). $SHIP^{-/-}$ mice also exhibit an alteration in B cell and myeloid numbers in the periphery and in bone marrow progenitors. The $B220^+$ B cell population is reduced by ~50% compared with wild-type littermates, whereas myeloid cells defined by expression of Mac-1^+ is elevated approximately twofold in $SHIP^{-/-}$ mice (9, 10). IL-7–responsive pre-B cell precursor cells were only ~30% of normal in bone marrow cells of $SHIP^{-/-}$ animals (10). In contrast, bone marrow cells were hyperresponsive upon stimulation with cytokines that promote myeloid development (macrophage-CSF, GM-CSF, IL-3, and Steel factor), suggesting that the increased number of granulocyte-macrophage progenitors might be explained by the hyperresponsiveness to cytokines (10). This notion is supported by reports showing that SHIP functions downstream of macrophage-CSF signaling in the FDC-P1 cell line (11) and that SHIP is involved in steel factor signaling where it negatively regulates degranulation in mast cells (12–14). Potential roles for SHIP in signal transduction by the cytokines GM-CSF and IL-3 are still unknown.

The mechanism by which SHIP affects the development of IL-7–responsive pre-B cells and the development of mature peripheral B cells was investigated in two studies. The first used a $RAG^{-/-}$ complementation system and found that the number of $B220^+CD43^+$ pro-B cells was normal, but the number of $B220^+sIgM^+$ immature and $B220^+sIgD^+$ mature cells were significantly reduced (15). The second used unirradiated nonobese diabetic (NOD)/SCID animals reconstituted with bone marrow of $SHIP^{-/-}$ animals (16). The chimeric animals exhibited a reduction of mature and immature peripheral B cells and only a slight reduction (30%) in total $B220^+$ bone marrow cells. The authors of both studies concluded that SHIP affects an intermediate stage of B cell development in the transition from pro-B to pre-B cells. The developmental defect in both cases revealed a function of SHIP intrinsic to the B lymphoid compartment, independent of the myeloproliferation defect. However, the reductions in the percentage of peripheral B cells in the $SHIP^{-/-}$ animal might be affected by the concomitant hyperexpansion of myeloid cells present in the same microenvironment. Hence, $SHIP^{-/-}$ mice may show an earlier lymphoid lineage developmental defect that was not apparent in these models, due to the absence of $SHIP^{-/-}$ myeloid cells or the absence of the myeloid hyperexpansion observed in the SHIP-deficient animal (9, 10).

Here, we report that early B cell development is impaired in the bone marrow of $SHIP^{-/-}$ mice and confirmed the developmental defect caused by SHIP deficiency using serum-free, stromal cell–free cultures that support B cell development to the pro-B cell stage. Supernatants of such cultures of $SHIP^{-/-}$ bone marrow cells when added to wild-type cultures reproduce the defects in $SHIP^{-/-}$ bone marrow: excessive myelopoiesis and defective B cell development. Thus, the impaired early B cell development by SHIP deficiency is caused by suppressive soluble factors. The soluble factor appears to be IL-6, since bone marrow–derived Mac-1^+ cells of $SHIP^{-/-}$ animals make excessive amounts of IL-6. Furthermore, we found that recombinant IL-6 in stromal-free cultures of wild-type bone marrow cells promotes myelopoiesis and suppresses B lymphoid development. Lastly, neutralizing anti–IL-6 antibodies added to cultures of normal marrow cells containing $SHIP^{-/-}$ supernatants rescues B lymphoid development and decreases myelopoiesis. Our findings reveal an important role of SHIP and the proinflammatory cytokine IL-6 in regulating the early stages of B lymphoid development.

Materials and Methods

Animals. The $SHIP^{-/-}$ mice (C57Bl/6 background) were provided by Dr. G. Krystal. C57Bl/6 mice as wild-type controls were purchased from The Jackson Laboratory. C57Bl/6 SJL mice (CD45.1 background) were purchased from The Jackson Laboratory.

Antibodies and Reagents. Anti-CD19 mAb (1D3), anti-CD45RA mAb (14.8), anti–Mac-1/CD11b mAb (M1/70), and anti–IL-7R mAb (SB199) were used as described earlier (5). Anti-SHIP mAb (P1C1) was purchased from Santa Cruz Biotechnology and biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce Chemical Co.). Anti–IL-6 mAb (MP520F3) was purchased from R&D Systems and used for neutralization. Anti-TdT antibody was purchased from Supertechs. FITC-labeled $F(ab')_2$ fraction of goat anti–rabbit IgG antibody was purchased from Zymed Laboratories. All other antibodies were purchased from BD Biosciences. Recombinant mouse IL-7, IL-6, and Flk-2 ligand were purchased from R&D Systems. Recombinant stem cell factor was purchased from Peprotech.

Flow Cytometry. For staining of surface antigens, cells were incubated with combinations of labeled antibodies in PBS containing 3% FCS and 5 μg/ml of 2.4G2 mAb (BD Biosciences). The cells were washed and incubated with streptavidin-RED613 (Caltag) to detect biotinylated primary antibodies. For intracellular staining for TdT, cells were first stained with antibodies to surface antigens and then fixed, permeabilized, and stained using anti-TdT antibody FITC-labeled $F(ab')_2$ fraction of goat anti–rabbit IgG antibody as described (5). For intracellular staining for SHIP, cells were first stained with antibodies to surface antigens and then permeabilized in PBS with 2% BSA, 2% FCS, 1 mM EDTA, 0.2% Tween 20, and 0.01% SDS. After washing, the cells were stained in the permeabilization buffer using anti-SHIP mAb for 30 min at 4°C followed by streptavidin-RED613 for 30 min at 4°C. The stained cells were analyzed by FACSCalibur and CELLQUEST software (Becton Dickinson) and FloJo software (Treestar).

Isolation of Lineage-negative (Lin^-) Cells and Cell Sorting. Bone marrow cells were isolated by flushing femurs and tibias and incu-

bated with unconjugated antibodies to lineage markers (Gr-1, Mac-1, CD19, CD45R, Ter119, CD3). Bone marrow cells were washed, incubated with goat anti–rat IgG microbeads (Miltenyi Biotec), and applied to a magnetic separator to remove lineage-positive cells. For further purification of Lin^-, c-kit(high), $Sca\text{-}1^+$ cells, the Lin^- cells were incubated with FITC-conjugated antibodies to lineage markers (CD3, CD8, Gr-1, Mac-1, and CD45R), APC-conjugated anti–c-kit antibody, and PE-conjugated anti–Sca-1 antibody. The stained cells were subjected to cell sorting on MoFlo (Cytomation). For purification of $Mac\text{-}1^+$ cells in 1-wk cultures of Lin^- cells, the cultured cells were stained with FITC-conjugated anti–Mac-1 antibody and PE-conjugated anti-CD19 antibody and sorted as described above.

Cell Culture. The Lin^- cells (2×10^4) or Lin^-, c-kit(high), $Sca\text{-}1^+$ cells (2.5×10^3) were placed into 24-well plates in 1 ml of X-VIVO15 medium (Biowhittaker) containing 1% detoxified BSA (Stem Cell Technologies), 50 μM β-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 ng/ml IL-7, 100 ng/ml Flk-2 ligand, and 20 ng/ml stem cell factor as described (5).

RNase Protection Assay. Total RNA was isolated from sorted $Mac\text{-}1^+CD19^-$ cells of 1-wk cultures of Lin^- cells with MicroPoly(A) Pure (Ambion) according to the manufacturer's instructions. 5 μg of polyA-containing RNA was hybridized with ^{32}P-labeled riboprobes provided by Multi-Probe RNase Protection Assay System mCK-3b (BD Biosciences). The hybrid mRNA were digested with RNase A + T1 mix and separated on acrylamide gel electrophoresis according to the manufacturer's instruction. The dried acrylamide gel was exposed to x-ray film or applied to a Molecular Dynamics Storm system.

RT-PCR. Total RNAs were reverse transcribed to cDNAs using SuperScript II reverse transcriptase (Invitrogen). Primer pairs for mouse TNFα, IL-6, IL-1β, β-actin were obtained from Biosource. Primers for mouse LTβ was designed as described in Weih et al. (17). The PCR was performed at 25–30 cycles as follows: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. PCR products were not saturated under these conditions. PCR products were visualized and quantified by LumiImager (Roche).

Results

Early Stage of B Cell Development Is Impaired in the Bone Marrow of $SHIP^{-/-}$ Mice. To investigate the role of SHIP at early stages of B lymphoid development, we examined lymphoid progenitors and immature B cells in the bone marrow of $SHIP^{-/-}$ mice compared with the wild-type littermates by flow cytometry. The results of FACS® profiles are shown in Fig. 1. Total cell numbers for the populations in the bone marrow examined are also shown in Table I. As reported previously (16), the total B cell population, defined by the surface expression of CD19, was reduced in total bone marrow cells in $SHIP^{-/-}$ mice, whereas the population of myeloid cells, defined by the surface expression of Mac-1 was increased (Fig. 1 A and Table I). We found that the number of $CD19^+$ cells declines with increasing age (Fig. 1 A and unpublished data). In $SHIP^{-/-}$ mice, we observed that pro-B cell population defined earlier (18) as $CD43^+B220^+$ was reduced to ~50% relative to wild-type mice, although it was reported that pro-B cell number is normal in $SHIP^{-/-}$ bone marrow (16). Further investigation of the earlier stage of B lymphoid development demonstrated that the number of Lin^-, c-kit(low), IL-7Rα$^+$ cells in $SHIP^{-/-}$ mice was also decreased to 30% of the wild-type level. Additionally, prolymphocytes defined previously (19) as Lin^-, c-kit(low), IL-7Rα$^+$, $Flk\text{-}2^+$ cells were also reduced to 30% of normal in $SHIP^{-/-}$ mice. This prolymphocyte population overlaps to a large extent with common lymphoid progenitors defined by others on the basis of slightly different characteristics (3, 20).

Table I. *Populations on Early Lymphocyte Precursors, B Lymphocytes, and Myeloid Cells in $SHIP^{-/-}$ Bone Marrow Cells*

	Percentage		Cell number	
$SHIP^{-/-}$ bone marrow cells	$SHIP^{+/+}$	$SHIP^{-/-}$	$SHIP^{+/+}$	$SHIP^{-/-}$
$CD19^+$	28 ± 11	7.5 ± 5.3[a]	16 ± 8 (×10^6)	3.4 ± 2.7 (×10^6)[a]
$Mac\text{-}1^+$	30 ± 6	54 ± 12[a]	18 ± 7 (×10^6)	24 ± 10 (×10^6)[b]
$CD43^+B220^+$	6.2 ± 1.6	4.2 ± 1.1[b]	3.8 ± 0.8 (×10^6)	1.8 ± 0.7 (×10^6)[a]
$CD43^-$B220(low)	24 ± 7	5.2 ± 4.4[a]	16 ± 6 (×10^6)	0.6 ± 0.2 (×10^6)[a]
$CD43^-$B220(high)	6.0 ± 0.8	2.4 ± 2.0[a]	3.8 ± 0.8 (×10^6)	0.7 ± 0.6 (×10^6)[a]
Lin^-	1.4 ± 0.6	2.2 ± 1.3[b]	8.1 ± 2.3 (×10^5)	9.1 ± 4.0 (×10^5)
Lin^-, c-kit(low), IL-7Rα$^+$	0.13 ± 0.09	0.06 ± 0.04[b]	8.7 ± 6.3 (×10^4)	2.5 ± 1.9 (×10^4)[b]
Lin^-, c-kit(low), IL-7Rα$^+$, $Flk\text{-}2^+$	0.09 ± 0.05	0.04 ± 0.03[b]	5.8 ± 3.3 (×10^4)	1.8 ± 1.4 (×10^4)[b]
Lin^-, c-kit(high), $Sca\text{-}1^+$	0.08 ± 0.03	0.14 ± 0.08[b]	4.9 ± 2.5 (×10^4)	7.7 ± 5.7 (×10^4)[b]
Lin^-, c-kit(high), $Sca\text{-}1^+$, $CD27^+$, TdT^+	0.024 ± 0.002	0.022 ± 0.012	1.55 ± 0.10 (×10^4)	1.29 ± 0.30 (×10^4)

Total bone marrow cells or Lin^- cells were labeled with the indicated antibodies and analyzed by FACS®. The data were obtained from at least three 2–5-wk-old mice and are shown as averages. Cell numbers are shown as the number per mouse (two femurs and two tibias). Value is different from control $SHIP^{+/+}$ with a significance of [a]$P < 0.001$ and [b]$P < 0.05$ by Student's *t* test.

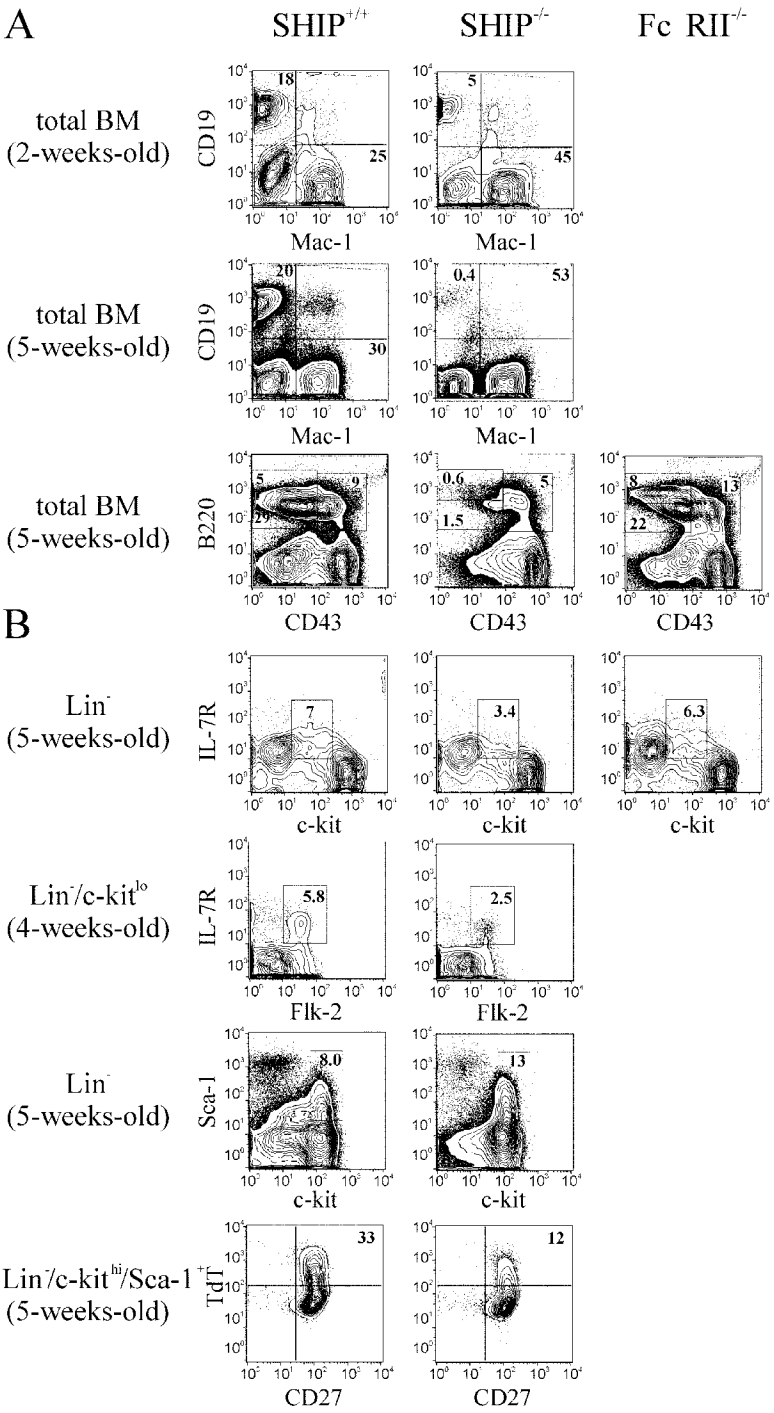


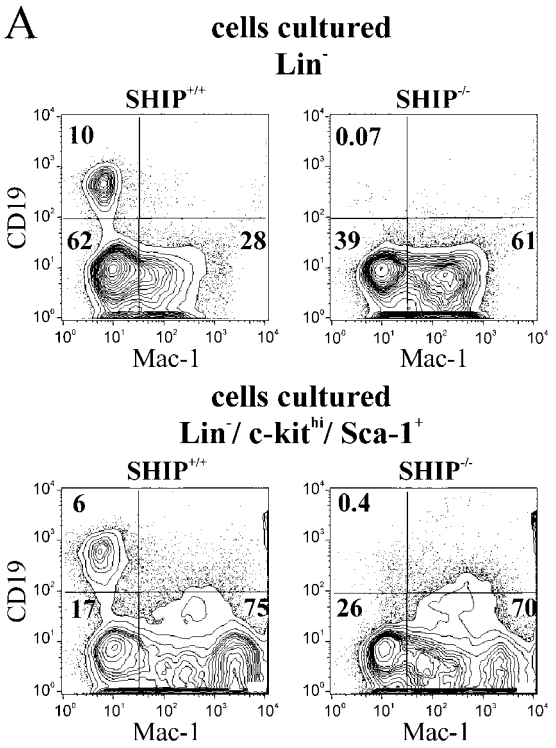
Figure 1. Flow cytometric analysis of bone marrow cells in SHIP$^{+/+}$ and SHIP$^{-/-}$ mice. Total bone marrow cells (A) or sorted Lin$^-$ cells (B) were isolated from 2–5-wk-old SHIP$^{+/+}$ and SHIP$^{-/-}$ mice. The staining protocol is described in Materials and Methods. Numbers represent the percentage of cells within each marked region. Results are representative of at least three independent experiments.

Even more primitive lymphocyte progenitors have been identified within a small Lin$^-$ c-kit(high), Sca-1$^+$ CD27$^+$ fraction of bone marrow according to hormone sensitivity, TdT expression, synthesis of an immunoglobulin transgene, and activation of the RAG-1 locus (2, 6). These early lymphoid progenitors are not homogeneous, do not yet express transcripts for Pax-5 or IL-7Rα, and take longer to generate CD19$^+$ cells than prolymphocytes. In the present study, we enumerated Lin$^-$, c-kit(high), Sca-1$^+$, CD27$^+$, TdT$^+$ progenitors in SHIP$^{-/-}$ and wild-type bone marrow by flow cytometry (Fig. 1 B). Although percentages in SHIP$^{-/-}$ mice were significantly lower than in wild-type mice (12 versus 33%, respectively), absolute numbers of these fractions were not significantly different (Fig. 1 B and Table I). Hence, our findings show that SHIP regulates an additional and previously unappreciated transition stage—that of early lymphoid progenitors to prolymphocytes. Our observations that more primitive lymphoid precursors are affected in the SHIP$^{-/-}$ animal is in contrast to studies using models of RAG$^{-/-}$ complementation (15) or unirradi-

ated NOD/SCID (16) mice, where the contributions to lymphoid development by the SHIP-deficient myeloid cells were removed or diminished.

In B cells, SHIP function requires expression and B cell receptor coclustering of Fcγ receptor II (FcγRII) (21). However, we found no abnormality at any developmental stages in bone marrow of FcγRII$^{-/-}$ mice (Fig. 1, A and B). These observations indicate that SHIP functions independently of FcγRII to regulate B cell development in bone marrow. This function of SHIP is distinct from that used in mature B cells.

We then exploited serum-free, stromal cell-free cultures using Lin$^-$ cells or Lin$^-$, c-kit(high), Sca-1$^+$ cells as starting materials. In this culture system, B cell development is supported to the pro-B and pre-B cell stage because CD19$^+$ cells in the Lin$^-$ culture after 1 wk still express CD43 (5; unpublished data). Therefore, serum-free, stromal cell–free cultures provide a useful way to analyze early stages of lymphoid development. Using this model, we found in both cultures of Lin$^-$ cells and Lin$^-$, c-kit(high), Sca-1$^+$ cells that the number of CD19$^+$ cells were 20- (Lin$^-$) or 10- (Lin$^-$, c-kit[high], Sca-1$^+$) fold smaller than



B

cells cultured		cells recovered: CD19$^+$ cells (x 10^4)		cells recovered: Mac-1$^+$ cells (x 10^4)	
Lin$^-$	SHIP$^{+/+}$	5.04 ± 1.82	*	27.4 ± 12.0	**
	SHIP$^{-/-}$	0.254 ± 0.176		49.1 ± 30.9	
Lin$^-$ c-kit^{hi} Sca-1$^+$	SHIP$^{+/+}$	4.19 ± 1.51	*	54.4 ± 10.8	
	SHIP$^{-/-}$	0.432 ± 0.241		35.8 ± 10.4	

*P<0.005, **P<0.1

Figure 2. Cultures of Lin$^-$ or Lin$^-$, c-kit(high), Sca-1$^+$ cells of SHIP$^{+/+}$ and SHIP$^{-/-}$ mice. (A) Purified Lin$^-$ (top) or Lin$^-$, c-kit(high), Sca-1$^+$ (bottom) cells from SHIP$^{+/+}$ (left) or SHIP$^{-/-}$ (right) mice were incubated for 1 (Lin$^-$) or 2 wk (Lin$^-$, c-kit[high], Sca-1$^+$) in serum-free, stromal cell–free cultures. The CD19$^+$ and Mac-1$^+$ cells were analyzed by FACS® after culture. Numbers represent the percentage of cells within each marked region. (B) The absolute numbers of CD19$^+$ and Mac-1$^+$ cells in A were shown as the average of triplicate wells of six independent experiments for Lin$^-$ cells or three experiments for Lin$^-$, c-kit(high), Sca-1$^+$ cells. Student's *t* test (*) was used to assess statistical significance of the difference between CD19$^+$ and Mac-1$^+$ cells of the wild-type and SHIP$^{-/-}$ cultures.

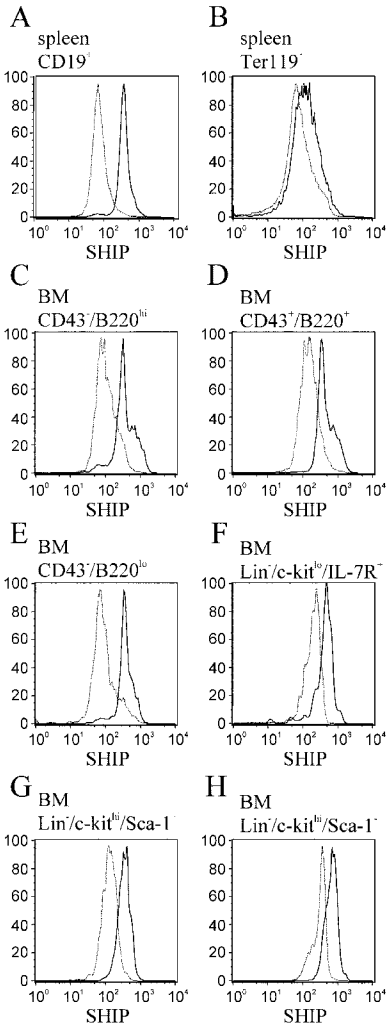
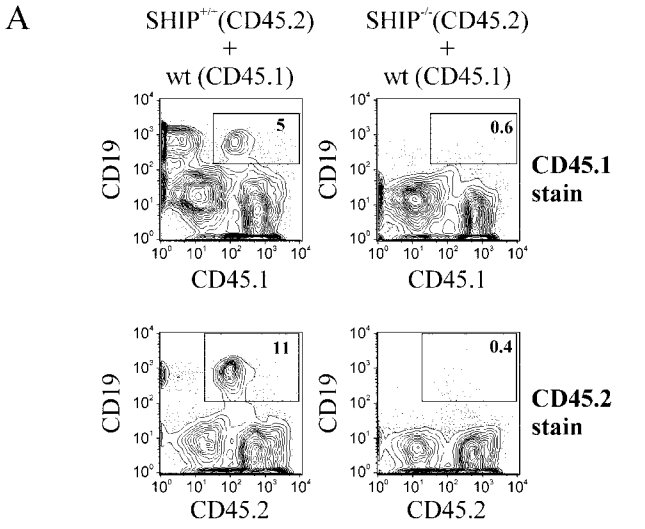


Figure 3. Expression of SHIP in subsets of bone marrow cells. Splenocytes (A and B), total bone marrow cells (C–E), and Lin$^-$ cells (F–H) were stained with the indicated antibodies and then permeabilized and stained with anti-SHIP monoclonal antibody. Solid lines and dashed lines indicate the cells from SHIP$^{+/+}$ and control staining of SHIP$^{-/-}$ mice, respectively.

those of wild-type (Fig. 2). The number of Mac-1$^+$ cells was twofold larger in Lin$^-$ culture. These results clearly demonstrated that SHIP deficiency alters early stages of B lymphoid development.

Expression of SHIP in Bone Marrow. Our findings indicating that the lack of the *SHIP* gene affects early B lymphoid and myeloid development predicted that SHIP is expressed in the precursor cell populations. To test this prediction, we stained marrow cells with markers defining lineage stages and performed intracellular staining of SHIP using a commercial monoclonal antibody. The stained cells were then analyzed by flow cytometry. The results shown in Fig. 3 indicate that SHIP is expressed in hematopoietic stem cell–enriched faction (Fig. 3 G), common myeloid progenitors (Fig. 3 H), prolymphocytes (Fig. 3 F), pro-B and large pre-B cells (Fig. 3 D), small pre-B cells (Fig. 3 E), and immature B cells in bone marrow (Fig. 3 C). Splenic B cells also expressed SHIP, whereas splenic erythrocytes showed only background staining (Fig. 3, A and B, respectively). These data indicate that SHIP is widely expressed in bone marrow subpopulations and therefore is capable of functioning at all stages of lymphoid and myeloid development.

Soluble Factor(s) Produced by the Cells Derived from SHIP$^{-/-}$ Mice Suppress B Cell Development In Vitro. There are two possibilities to explain the impairment in B lymphoid development in SHIP$^{-/-}$ mice. First, SHIP may intrinsically regulate the early stages of all lymphoid development in progenitor cells. Thus, expression of SHIP is required for the maturation of cells within the lymphoid compartment. Second, development of lymphoid precursors in SHIP$^{-/-}$ mice may be blocked by extrinsic factors, including a bystander effect caused by the presence of other cell types. These possibilities are not mutually exclusive. To test these possibilities, we established cocultures in which wild-type Lin$^-$ cells derived from C57Bl/6 SJL mice (CD45.1 background) were cultured together with the same number of Lin$^-$ cells from either SHIP$^{+/+}$ or SHIP$^{-/-}$ mice. The cells expressing CD45.1 and originating from C57Bl/6 SJL mice could be distinguished from the SHIP$^{+/+}$- or SHIP$^{-/-}$-derived cells expressing CD45.2 by flow cytometry. The results of the coculture are shown in Fig. 4. The data clearly show that the total number and percentage of CD45.1$^+$CD19$^+$ cells derived from Lin$^-$ cells of C57Bl/6 SJL mice were decreased when cocultured with SHIP$^{-/-}$ Lin$^-$ cells, whereas CD45.1$^+$CD19$^+$ cells cocultured with SHIP$^{+/+}$ Lin$^-$ cells developed normally. In contrast with CD19$^+$ cells, CD45.1$^+$Mac-1$^+$ cells were elevated threefold when cocultured with SHIP$^{-/-}$ Lin$^-$ cells. Thus, the ability of progenitors to develop into lymphoid-committed cells is suppressed when SHIP$^{-/-}$ marrow cells are present. Essentially the same results were obtained when Lin$^-$, c-kit(high), Sca-1$^+$ cells were used (Fig. 4 B). Hence, the presence of myeloid cells in the SHIP$^{-/-}$ culture appears to affect B lineage development. The results are consistent with the hypothesis that the myeloid hyperplasia in SHIP$^{-/-}$ animals could contribute to the loss of lymphoid precursors.



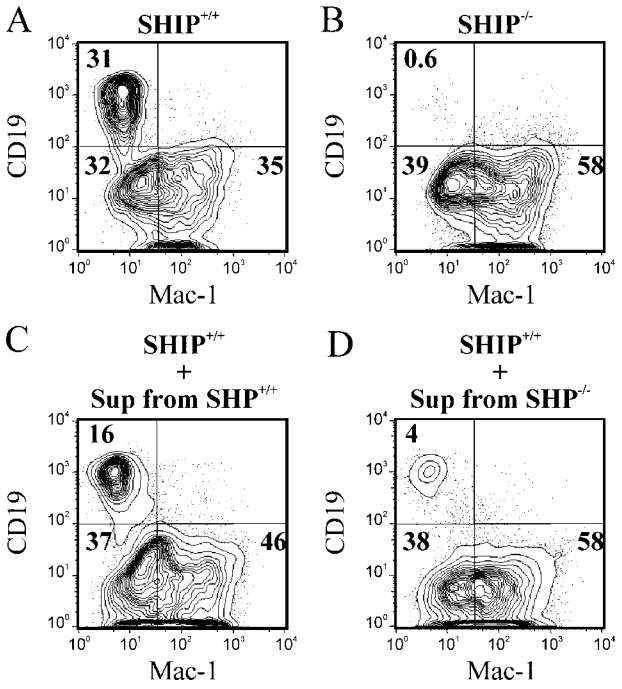
B

cells cultured		cells recovered: CD19$^+$ cells (x 10^3)	cells recovered: Mac-1$^+$ cells (x 10^3)
Lin$^-$ wt+SHIP$^{+/+}$	CD45.1$^+$	20.2 ± 3.1	10.5 ± 1.4
	CD45.2$^+$	51.6 ± 25.3	18.3 ± 0.5
Lin$^-$ wt+SHIP$^{-/-}$	CD45.1$^+$	9.6 ± 2.8	34.0 ± 4.7
	CD45.2$^+$	5.5 ± 0.8	49.8 ± 4.0
Lin$^-$/c-kit^{hi}/Sca-1$^+$ wt+SHIP$^{+/+}$	CD45.1$^+$	5.8 ± 0.2	12.9 ± 1.0
	CD45.2$^+$	7.5 ± 0.2	11.4 ± 1.7
Lin$^-$/c-kit^{hi}/Sca-1$^+$ wt+SHIP$^{-/-}$	CD45.1$^+$	0.9 ± 0.6	17.2 ± 4.6
	CD45.2$^+$	0.4 ± 0.05	13.8 ± 3.8

*P<0.05, **P<0.005

Figure 4. Cocultures of SHIP$^{+/+}$ or SHIP$^{-/-}$ Lin$^-$ cells with wild-type Lin$^-$ cells. Lin$^-$ cells from SHIP$^{+/+}$ or SHIP$^{-/-}$ mice were cocultured in vitro with Lin$^-$ cells from wild-type mice for 1 wk, and then CD19$^+$CD45.1$^+$ or CD19$^+$CD45.2$^+$ cells were analyzed by FACS®. The cells derived from wild-type mice were distinguished by staining with anti-CD45.1 antibody from those of SHIP$^{+/+}$ or SHIP$^{-/-}$ mice, expressing CD45.2. Numbers represent the percentage of cells within each marked region. (B) The absolute numbers of CD19$^+$ and Mac-1$^+$ cells in A were shown as the averages of triplicate wells and are representative of two independent experiments. Student's *t* test (*) was used to assess statistical significance of the difference between CD19$^+$CD45.1/2$^+$ and Mac-1$^+$CD45.1/2$^+$ cells derived from cultures containing SHIP$^{+/+}$ and/or SHIP$^{-/-}$ cells.

To examine whether the cells derived from the SHIP$^{-/-}$ culture suppress B cell development by cell–cell contact or by production of soluble factor(s), we cultured Lin$^-$ cells from wild-type mice with supernatants of the SHIP$^{-/-}$



E

cells cultured	supernatants	cells recovered	
		$CD19^+$ cells ($\times 10^4$)	$Mac\text{-}1^+$ cells ($\times 10^4$)
$SHIP^{+/+}$	none	3.15 ± 1.56	4.92 ± 0.20
$SHIP^{-/-}$	none	0.121 ± 0.101	34.2 ± 6.2
$SHIP^{+/+}$	$SHIP^{+/+}$	2.28 ± 0.95 *	13.1 ± 1.5 *
$SHIP^{+/+}$	$SHIP^{-/-}$	0.866 ± 0.756 *	53.2 ± 12.3 *

*P<0.05

Figure 5. Effects of culture supernatants of $SHIP^{-/-}$ Lin^- cells on the cultures of wild-type Lin^- cells. 2×10^4 Lin^- cells from $SHIP^{+/+}$ (A, C, and D) or $SHIP^{-/-}$ (B) mice were cultured for 1 wk in the absence (A and B) or presence (C and D) of culture supernatants (Sup) from 1-wk cultures of $SHIP^{+/+}$ (C) or $SHIP^{-/-}$ (D) Lin^- cells. Supernatants were used at a concentration of 30% vol/vol. The $CD19^+$ and $Mac\text{-}1^+$ cells were analyzed by FACS®. Numbers represent the percentage of cells within each marked region. (B) The absolute numbers of $CD19^+$ and $Mac\text{-}1^+$ cells in A were shown as the averages of triplicate wells and are representative of two independent experiments. Student's *t* test (*) was used to assess statistical significance of the difference between cultures containing $SHIP^{+/+}$ or $SHIP^{-/-}$ supernatant.

culture, and the number of $CD19^+$ and $Mac\text{-}1^+$ cells was determined. We found that the culture supernatant of $SHIP^{-/-}$ Lin^- cells reduced the total number and percentage of $CD19^+$ cells to 30% of the control, whereas that of $SHIP^{+/+}$ Lin^- cells had only a small effect (Fig. 5). In parallel, total $Mac\text{-}1^+$ cells were >10-fold increased when the culture supernatant of $SHIP^{-/-}$, Lin^- cells were added. These results demonstrated that soluble factor(s) produced by $SHIP^{-/-}$ bone marrow cells are capable of suppressing B lymphoid and enhancing myeloid development in $SHIP^{-/-}$ culture.

IL-6 Is Overproduced by Myeloid Cells in $SHIP^{-/-}$ Culture and Is a Candidate Cytokine for the Suppression of B Cell Development and Expansion of Myeloid Cells in $SHIP^{-/-}$ Mice. To identify the soluble factor(s) which suppresses the early stages of B cell development and increases the number of myeloid cells, we performed RNase protection assay and RT-PCR analyses. $Mac\text{-}1^+$ cells in 1-wk culture of $SHIP^{-/-}$ Lin^- cells were sorted and subjected to RNase protection assay and RT-PCR. The results of RNase protection assay and the quantified data are shown in Fig. 6, A and B. The data revealed that only IL-6 mRNA was significantly up-regulated in $SHIP^{-/-}$ $Mac\text{-}1^+$ cells among nine different cytokines examined. The level of IL-6 mRNA expression was fivefold higher than that of wild-type $Mac\text{-}1^+$ cells. To confirm the results of RNase protection assay, we performed semiquantitative RT-PCR to detect inflammatory cytokines TNFα, LTβ, IL-1β, and IL-6. Consistent with the results of RNase protection assay, the level of TNFα, LTβ, and IL-1β were essentially identical between $SHIP^{-/-}$ and wild-type $Mac\text{-}1^+$ cells, whereas IL-6 mRNA was 3.5-fold increased relative to wild-type (Fig. 6, C and D). These results indicate that IL-6 is a possible suppressor of B lymphoid development in $SHIP^{-/-}$ mice.

To test whether IL-6 is capable of suppressing B lymphoid and enhancing myeloid development, we added neutralizing antibody to IL-6 to cultures of wild-type Lin^- cells in the presence of $SHIP^{-/-}$ culture supernatants. We found that the reduced number of $CD19^+$ cells in the wild-type Lin^- culture in the presence of the $SHIP^{-/-}$ culture supernatant was restored to control levels by the addition of neutralizing anti–IL-6 antibodies (Fig. 7 A). There was no effect of anti–IL-6 antibody on B cell development in wild-type culture in the presence of $SHIP^{+/+}$ culture supernatant or on cultures lacking culture supernatants. The enhancing effect of the $SHIP^{-/-}$ culture supernatant on the total number of $Mac\text{-}1^+$ cells in the culture was also reversed by the addition of anti–IL-6 antibody (Fig. 7 A). There was no significant effect on myeloid development when the anti–IL-6 antibody was added to the culture of wild-type Lin^- cells in the presence or absence of culture supernatants of wild-type Lin^- cells.

To confirm that IL-6 has the potential to regulate the balance of lymphopoiesis and myelopoiesis in vitro, we examined the effect of recombinant IL-6 on B lymphoid development and myeloid development in wild-type Lin^- cell cultures. Recombinant IL-6 reduced the number of $CD19^+$ cells in 1-wk cultures in a dose-dependent manner (Fig. 7 B). In contrast, the number of $Mac\text{-}1^+$ cells was fivefold increased at 10 ng/ml of recombinant IL-6 (Fig. 7 B). Together, these findings show that IL-6 is a potential candidate cytokine that can account for the observed repression of lymphoid and expansion of myeloid development in $SHIP^{-/-}$ animals.

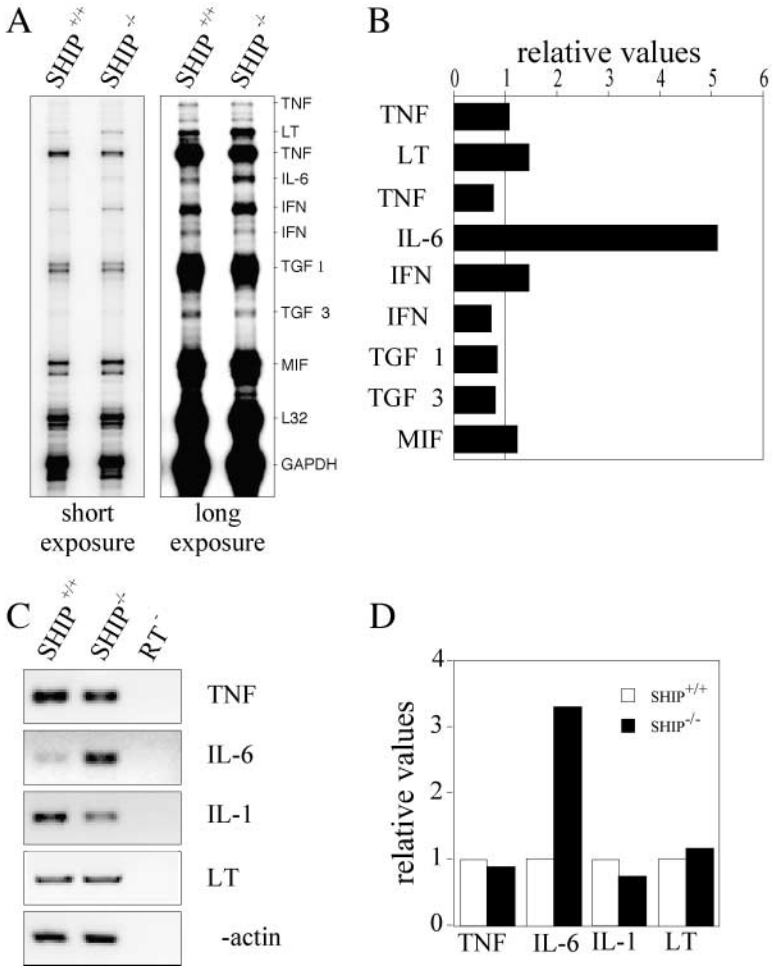


Figure 6. IL-6 mRNA levels are increased in SHIP$^{-/-}$ Mac-1^{+} cells. (A). Cytokine mRNAs were analyzed by RNase protection assay using total RNAs from Mac-1^{+} cells isolated from 1-wk culture of SHIP$^{+/+}$ or SHIP$^{-/-}$ Lin^{-} cells. Results from 1-d exposure (left) and 1-wk exposure (right) are shown. The positions of protected fragments corresponding to the mRNAs of cytokines were indicated on the right. (B). The amount of mRNA of each cytokine to L32/GAPDH shown in A was quantified. The amounts in the SHIP$^{-/-}$ cells are shown as values relative to those of SHIP$^{+/+}$ cells. (C). The mRNAs for TNFα, IL-6, IL-1β, and LTβ were analyzed by RT-PCR using the total RNAs described in A. The PCR products without reverse transcription are shown as negative controls (RT^{-}). (D). The amount of mRNA of each cytokine to β-actin shown in C was quantified and shown as relative values to those of SHIP$^{+/+}$ cells.

In Fig. 1 A, we showed that the number of CD19^{+} cells declines with increasing age of SHIP$^{-/-}$ mice. The age effect may be due to a lack of IL-6 sensitivity of fetal lymphoid precursors. To test this possibility, we examined the ability of recombinant IL-6 to suppress lymphoid development of Lin^{-} cells derived from fetal liver. We found that production of B lymphoid precursors in cultures of Lin^{-} fetal liver cells of both wild-type and SHIP$^{-/-}$ were equally sensitive to exogenous IL-6 (unpublished data). Thus, the age effect is not due to a lack of IL-6 sensitivity in fetal liver-derived precursors. One possible explanation is that the SHIP$^{-/-}$ mice need to accumulate sufficient Mac-1^{+} cells and IL-6 levels before the affect on lymphoid development is apparent.

Discussion

SHIP$^{-/-}$ animals exhibit a reduction in mature peripheral B cells and increases in myeloid cell number (9, 10). Previous experiments identified a defect in the transition from pro-B to pre-B cell stage (15, 16). No defect in the development of primitive lymphoid progenitors was apparent from these experiments. However, those studies used models in which contributions to lymphoid development by the myeloid fraction of SHIP$^{-/-}$ bone marrow were minimized by complementation of RAG$^{-/-}$ blastocysts or the use of unirradiated NOD/SCID mice. We demonstrate here the important contributions of SHIP and of the myeloid expansion in SHIP$^{-/-}$ mice toward the early stages of lymphoid development in bone marrow. Our results further identified an important role of IL-6 in affecting lymphoid and myeloid cell development. In bone marrow of SHIP$^{-/-}$ animals, we found dramatic alterations in populations pro-lymphocytes (Lin^{-}, c-kit[low], IL-7R^{+}, Flk-2^{+}) and pro-B cells (CD43^{+}B220^{+}). Lastly, we found that deficiencies in early lymphoid development are associated at least in part with overproduction of IL-6 secreted by Mac-1^{+} myeloid cells. The suppression of lymphoid progenitors in the SHIP-deficient mouse is unlikely to involve lineage choice decisions, since equal total numbers of Lin^{-} c-kit(high) Sca-1^{+} CD27^{+} TdT^{+} cells were present in wild-type and SHIP$^{-/-}$ mice. Recent studies revealed that while these early lymphoid progenitors are very primitive, they have little potential for non-lymphoid lineage differentiation (2, 6). Thus, our findings strongly support our current hypothesis that SHIP expression affects expansion and/or survival of lymphoid-committed progenitors.

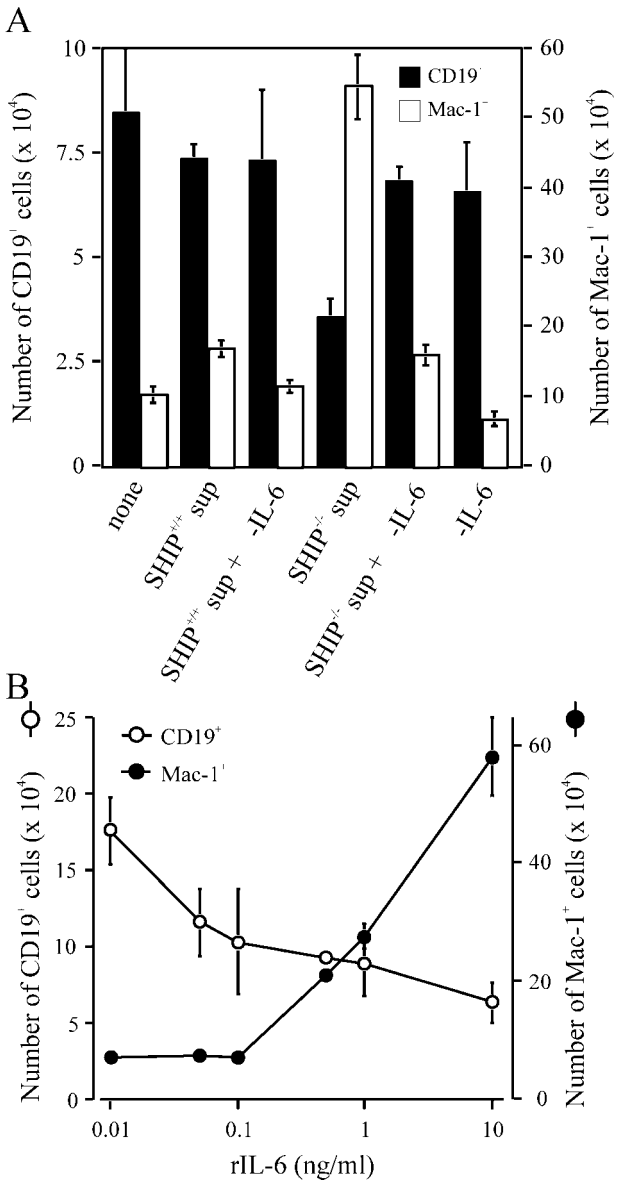


Figure 7. IL-6 suppresses B lymphoid and enhances myeloid development. (A) Lin^- cells of wild-type C57Bl/6 mice were cultured with the combination of anti–IL-6 antibody and culture supernatants (30% in volume) of 1-wk cultures of $SHIP^{+/+}$ or $SHIP^{-/-}$ Lin^- cells as indicated. Numbers of $CD19^+$ cells (white bars) and $Mac\text{-}1^+$ cells (black bars) are shown as averages of triplicate wells and represent two independent experiments. (B) Lin^- cells of wild-type mice were cultured for 1 wk with different concentrations of recombinant IL-6 as indicated. Numbers of $CD19^+$ (○) and $Mac\text{-}1^+$ (●) cells were shown as averages of duplicate wells. Bars represent SDs of duplicate samples.

Our findings are consistent with the 70% reduction in IL-7–responsive precursor cells in bone marrow of SHIP-deficient mice (10) but appeared at first to contradict earlier reports showing that the number of $B220^+CD43^+$ pro-B cells was normal in $SHIP^{-/-}$, $RAG^{-/-}$ (15) or $SHIP^{-/-}$, NOD–SCID chimeras (16). In the $SHIP^{-/-}RAG^{-/-}$ chimeric mice, all lymphocytes were derived from $SHIP^{-/-}$ embryonic stem cells, but myeloid cells were generated from both $SHIP^{-/-}$ embryonic stem cells and $RAG^{-/-}$ blastocysts. Similarly, the NOD–SCID study used animals that had not been irradiated, thereby retaining host myeloid cells. The few myeloid cells derived from bone marrow of $SHIP^{-/-}$ mice in the NOD–SCID chimeras may have been unable to produce sufficient IL-6 to suppress early B cell development, or myeloid cells from $SHIP^{-/-}$ progenitors may have failed to differentiate. Indeed, the $Mac\text{-}1^+$,$Gr\text{-}1^+$ population from $SHIP^{-/-}$ donors did not expand in the unirradiated NOD/SCID model (16). It is not clear why we found a decrease in pro-B cells (Fig. 1 A and Table I) in $SHIP^{-/-}$ bone marrow, whereas an early study using the same $SHIP^{-/-}$ animals found normal levels of pro-B cells (16). One possibility is that the $SHIP^{-/-}$ animals used earlier were F2 or F3 generation of backcrossing with C57Bl/6. At F2/3, the chimeras still have a considerable contribution from the strain 129 mouse genome. We received the animals after an additional 1 yr of backcrossing to C57Bl/6, although the precise number of backcrossings could not be determined. In any case, the results reported here used a strain that is well beyond F2/F3. The myeloid phenotype becomes more severe with backcrossing to C57Bl/6 (Krystal, G., personal communication), and the affect on lymphoid development appears to be due to myeloexpansion in $SHIP^{-/-}$. Therefore, the difference between these findings and the earlier reports is likely due to a greater genetic contribution from C57Bl/6 in the $SHIP^{-/-}$ strain tested here.

It is not clear why the phenotype worsens with age. We found that fetal liver cells of both wild-type and $SHIP^{-/-}$-derived precursors were equally sensitive to added IL-6. One possibility is that IL-6 levels in utero or in young animals are not present at sufficient levels to suppress B lymphoid development. Since the origin of the elevated IL-6 is through myeloid cells in the $SHIP^{-/-}$ animal, it may be that the myeloid cells have not accumulated in fetal or neonatal mice.

IL-6 is a cytokine produced during inflammation (for review see reference 22). Knockout studies indicate that the IL-6 receptor is required for terminal differentiation of myeloid cells but not for formation of myeloid precursors (23). Administration of IL-6 to irradiated transplant patients (24) or animals (25) accelerates recovery of myeloid cells. Thus, IL-6 supports myeloid development and function in vivo. Furthermore, deregulation of IL-6 production is implicated in the pathology of several disease processes. IL-6 supports the growth of myelomas (26), and IL-6 transgenic mice develop a fatal plasmacytosis (27). Consistent with an effect of IL-6 on mature lymphocytes, animals expressing high IL-6 levels show increased peripheral mature lymphocytes (28, 29) and the development of lymph node-like structures at sites of IL-6 production (30). It is possible that the mature B cells of $SHIP^{-/-}$ animals might recover from the reduced output of lymphoid progenitors by overproduction of IL-6 in peripheral organs. Indeed, total B cell number in $SHIP^{-/-}$ spleens seems to be normal (10, 16; unpublished data). Increased IL-6 levels are found in several autoimmune dis-

eases (for review see reference 22), including rheumatoid arthritis (31). Accordingly, neutralizing IL-6 reagents have proven effective therapies for autoimmune diseases (32, 33). Lastly, transgenic animals harboring a Y to F point mutant IL-6 receptor spontaneously develop rheumatoid arthritis (34). Thus, unregulated IL-6 signaling or production promotes autoimmune disease.

There is little information on the role or the effects of IL-6 in the formation or survival of early lymphoid and myeloid progenitors. Our data identified a novel function of IL-6 to inhibit early lymphoid development and accelerate myeloid development. Other cytokines associated with inflammation such as IL-1 (35), IFN (36), TNF (37), and TGF-β (38) produce a phenotype similar to our observations of SHIP$^{-/-}$ animals: suppression of lymphoid but not myeloid precursor growth or survival. Furthermore, chronic inflammation caused by various autoimmune diseases promotes a lymphopenic state (39–41). The mechanism of lymphopenia that accompanies autoimmunity is unclear, but the observation is consistent with the findings reported here. Thus, inflammation may block development of lymphoid precursors and result in lymphopenia. Similar defects in lymphoid development (42) or lymphopenia (43, 44) are associated in patients with IL-6–dependent multiple myeloma or with graft versus host disease (45). Despite the lack of direct information on lymphopoiesis, the clinical data on IL-6 and the animal models of IL-6 transgenics and knockouts suggest that IL-6 plays an important but unappreciated role in lymphopoiesis.

Although we found elevated IL-6 production in SHIP$^{-/-}$ Mac-1^{+} cells, it is still unclear whether SHIP directly regulates IL-6 production in myeloid cells. IL-6 production is induced by IL-1 and by LPS in macrophages, but the possible involvement of SHIP in those signaling pathways has not been studied. Others have shown a marked increase of serum IL-6 levels in SHIP$^{-/-}$ mice (46). Observations that IL-6 is up-regulated in mast cells derived from SHIP$^{-/-}$ mice supports our finding in Mac-1^{+} cells of the same animal (47). NF-κB is a major transcription factor that regulates IL-6 production. It was reported that NF-κB activation is blocked by the overexpression of SHIP in macrophages stimulated with anti–Fc receptor antibodies (48). The role of SHIP in NF-κB signaling pathways remains to be elucidated.

SHIP is thought to function by opposing the activation effects of phosphatidylinositol 3-kinase (PI-3K) through the hydrolysis of its lipid product, phosphatidylinositol 3,4,5-trisphosphate (PIP3). Mice deficient in the p85α regulatory subunit of PI-3K exhibit a mild *Xid*-like phenotype and a significant increase in pro-B cell (B220^{+}CD43^{+}) population in the bone marrow (49, 50). Together with our findings, these data show that an early stage of lymphoid and myeloid development in marrow is tightly regulated by PIP3 production through PI-3K and PIP3 hydrolysis through SHIP.

We thank Gerry Krystal (British Columbia Cancer Agency, Vancouver, B.C., Canada) for providing SHIP$^{-/-}$ mice.

M. Coggeshall is supported by National Institutes of Health grants AI49264, CA64268, and RR015577. Dr. Coggeshall is a Scholar of the Leukemia and Lymphoma Society.

Submitted: 17 July 2003
Accepted: 2 December 2003

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