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SHIP Is Required for Dendritic Cell Maturation

Frann Antignano,* Mariko Ibaraki,* Connie Kim,* Jens Ruschmann,*† Angela Zhang,* Cheryl D. Helgason,‡ and Gerald Krystal*

Although several groups have investigated the role of SHIP in macrophage (Mφ) development and function, SHIP’s contribution to the generation, maturation, and innate immune activation of dendritic cells (DCs) is poorly understood. We show herein that SHIP negatively regulates the generation of DCs from bone marrow precursors in vitro and in vivo, as illustrated by the enhanced expansion of DCs from SHIP−/− GM-CSF cultures, as well as increased numbers of DCs in the spleens of SHIP-deficient mice. Interestingly, however, these SHIP−/− DCs display a relatively immature phenotype and secrete substantially lower levels of IL-12 after TLR ligand stimulation than wild type DCs. This, in turn, leads to a dramatically reduced stimulation of Ag-specific T cell proliferation and Th1 cell responses in vitro and in vivo. This immature phenotype of SHIP−/− DCs could be reversed with the PI3K inhibitors LY294002 and wortmannin, suggesting that SHIP promotes DC maturation by reducing the levels of the PI3K second messenger phosphatidylinositol-3,4,5-trisphosphate. These results are consistent with SHIP being a negative regulator of GM-CSF–derived DC generation but a positive regulator of GM-CSF–derived DC maturation and function. *The Journal of Immunology, 2010, 184: 000–000.

Dendritic cells (DCs) are bone marrow (BM)-derived leukocytes that reside in an immature state throughout the body and are especially concentrated in the skin and mucosal surfaces where pathogens are most often encountered (1). They are our most potent APCs, being highly specialized for the uptake, processing, and presentation of Ags to naive T cells. Upon encountering a pathogen, DCs typically secrete inflammatory cytokines and initiate a cellular or humoral immune response through Th cell polarization (2–4). Many components of these pathogens are bound by TLRs (5), and this leads to the activation of MyD88-dependent or -independent pathways, or both, depending on the TLR activated (6–9). This, in turn, triggers DC maturation, which results in decreased Ag-processing capacity and enhanced expression of MHC class I and MHC class II (mHCII) costimulatory and adhesion molecules, the chemokine receptor CCR7 and cytokines (in particular IL-12, a key inducer of Th1 cell responses) (5). These cells migrate to secondary lymphoid tissues where they bind and activate naive or memory T cells (10, 11). Several signaling pathways have been implicated in these events, one of which is the PI3K pathway. Class I PI3Ks are eukaryotic lipid kinases that are activated by a wide array of extracellular stimuli and generate the critical second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) (12, 13). PIP3, in turn, attracts signaling proteins that contain pleckstrin homology domains, such as the serine/threonine kinase Akt, which, when phosphorylated/activated, enhances cell survival and proliferation (12, 13). Although numerous studies have shown that the PI3K pathway affects TLR signaling, it remains uncertain whether it is a positive or negative regulator. However, the prevailing hypothesis is that PI3K is a negative regulator of TLR-induced pathways (14). The majority of these studies have focused on the role of PI3K in TLR-induced activation of macrophages (Mφs), with only a small subset investigating its role in DCs. One study within this small subset showed that splenic and BM-derived DCs from mice lacking the p85α regulatory subunit of class I PI3Ks produce more IL-12 upon stimulation with the TLR ligands LPS, peptidoglycan (PGN), or CpG DNA (15). In addition, treatment of wild type (WT) DCs with the PI3K inhibitor wortmannin results in increased LPS-induced IL-12 production. These results suggest that PI3K is a negative regulator of DC-derived IL-12 (15, 16).

The PI3K pathway is negatively regulated in hematopoietic cells, in part, by the lipid phosphatase SHIP (also known as SHIP1), which hydrolyzes the 5’ phosphate from PIP3 to generate PI-3,4-P2 (17–20). Although several groups have investigated the role of SHIP in Mφ development and function (21–25), SHIP’s contribution to the generation, maturation, and innate immune activation of DCs remains poorly understood. In this study, we examined the role of SHIP in the differentiation and innate immune activation of GM-CSF–derived DCs. We found that SHIP negatively regulates the generation of DCs from BM precursors in vitro and in vivo. Interestingly, however, these DCs display a relatively immature phenotype and secrete substantially lower levels of IL-12 after TLR ligand stimulation. This, in turn, leads to a dramatically reduced stimulation of Ag-specific T cell proliferation and reduced Th1 cell activation. These results suggest that SHIP is essential for TLR-induced maturation and function of GM-CSF–derived DCs.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; 5-FU, 5-fluorouracil; GM-DC, GM-CSF–derived bone marrow dendritic cell; LN, lymph node; LPS, lipopolysaccharide; mDC, myeloid dendritic cell; MFI, mean fluorescence intensity; MHCII, MHC class II; MTG, monothioglycercate; PGN, peptidoglycanc; PIP3, phosphatidylinositol-3,4,5 trisphosphate; PTEN, phosphatase and tensin homolog; W, wortmannin; WT, wild type.

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**Materials and Methods**

**Mice**

SHIP⁻/⁻ and SHIP⁺/⁺ mice, backcrossed onto a C57BL/6 background for ≥12 generations (provided by Dr. Frank Jirik, University of Calgary, Calgary, Alberta, Canada) and OTII transgenic mice, purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house, were used between 6 and 12 wk of age. Mice were maintained in the Animal Resource Centre of the British Columbia Cancer Research Centre under specific pathogen-free conditions and according to approved and ethical treatment of animal standards of the University of British Columbia.

**Reagents and cytokines**

Escherichia coli LPS serotype O127:B8, polyinosinic:polycytidylic acid, monothioglycollate (MTG), and 2-ME were purchased from Sigma–Aldrich (St. Louis, MO). HPLC-purified phosphorothioate-modified CpG-containing oligodeoxynucleotide (1826; 5'-TTCATAGCAGTTCCGACGT-3') was from Invitrogen (Burlington, Ontario, Canada). Pure recombinant, endotoxin-free H18 flagellin from enteraggregative E. coli strain 042 was provided by Dr. Ted Stein (University of British Columbia). PGN from Staphylococcus aureus was purchased from Fluka (Buchs, Switzerland). Recombinant mouse GM-CSF and tissue culture reagents were from StemCell Technologies (Vancouver, British Columbia, Canada). Unless otherwise stated, all other reagents were from Sigma–Aldrich.

**BM-derived DC cultures**

Ammonium chloride-lysed BM cells (6 × 10⁵ cells/l ml total volume/well) were cultured in 12-well plates in myeloid DC (mDC) base (IMDM + 10% FCS, 0.00125% (v/v) MTG, 2 mM glutamine, and 100 U/ml penicillin/streptomycin) containing 10 ng/ml recombinant mouse GM-CSF. On day 3 of culture, 1 ml fresh cytokine-containing mDC base was added; on days 5 and 7, half of the cell-free medium was replaced with fresh cytokine-containing mDC base. Nonadherent cells were harvested on days 6 or 8, and DCs were enriched by EasySep CD11c-PE positive selection (StemCell Technologies), according to the manufacturer’s instructions, unless otherwise indicated. DC purity, determined by flow cytometric enumeration of CD11c⁺ cells, was >95% after selection. To determine the number of DCs in mice, spleens were harvested, a single-cell suspension was prepared by passing the spleens through a 100-µm cell strainer, RBCs were lysed with ammonium chloride, and the cells were washed twice in HBSS (StemCell Technologies) + 5% FCS, counted on a hemocytometer, and analyzed for the expression of CD11c by flow cytometry.

**BM-derived DC cultures**

Survival and expansion studies

GM-CSF–derived BM DCs (GM-DCs) were harvested on day 8, washed three times in mDC base, and seeded at 1 × 10⁵ cells/200 µl total volume/well in a 96-well plate with or without the indicated cytokines. Cell survival was assessed by manually counting trypan blue-excluding cells using a hemocytometer. DC expansion was assessed by preparing GM-CSF cultures as described above or in microcultures of 100–200 µl in 96-well plates. Nonadherent and loosely adherent cells harvested from individual wells daily, counted on a hemocytometer, and analyzed by flow cytometry for the percentage of CD11c⁺ cells. In addition, mice were injected i.p. with 125 mg/kg 5-fluorouracil (5-FU); 6 d later, the resulting relatively synchronized population of primitive progenitors in the BM was isolated, labeled with 5 µM CFSE for 10 min at 37°C, and washed extensively. CFSE dilution was tracked in the CD11c⁺ cells over several days in culture by flow cytometry. At day 0, WT and SHIP⁻/⁻ cells were subjected to flow cytometry to ensure similar CFSE labeling and equal mean fluorescence intensity (MFI) at the commencement of the experiment.

**Cell stimulations and Western blot analysis**

For BM and GM-DC stimulations, cells were starved overnight in IMDM containing 0.1% BSA, 0.00125% (v/v) MTG, and 100 U/ml penicillin/streptomycin. Cells were resuspended at 1 × 10⁶ cells/ml, stimulated with the indicated concentration of GM-CSF for 10 min, washed with cold PBS, lysed in 1× SDS sample buffer, boiled for 2 min, and subjected to Western blot analysis, as described previously (26). Blots were probed for phosphorylated SHIP (StemCell Technologies), STAT5, and Akt (S473) (both from Cell Signaling Technology, Beverly, MA), as well as Grb2 (Santa Cruz Biotechnology, Santa Cruz, CA) as a loading control. In addition, blots of resting GM-DCs were probed for SHIP2, SHIP (Santa Cruz Biotechnology), phosphatase and tensin homolog (PTEN; StemCell Technologies), and GAPDH (Research Diagnostics, Flanders, NJ).

**SHIP IS REQUIRED FOR DENDRITIC CELL MATURATION**

**DC maturation**

To induce DC maturation, day-8 DCs were harvested, and CD11c⁺ cells were selected, where indicated, and stimulated at 5 × 10⁶ cells/ml with LPS, CpG, or dsRNA. After 24 h, the cells were subjected to flow cytometric analysis, and the supernatants were frozen at −20°C for subsequent determination of secreted cytokines by ELISA.

**Flow cytometry**

Cells were resuspended in HBSS + 2% FCS and 0.05% NaN₃ and incubated with anti-CD11b (2A2G2) ( StemCell Technologies) or 5% heat-inactivated goat serum on ice for 20 min to block FcRs before labeling. FITC-, PE-, or APC-conjugated Abs specific for mouse CD11c, CD1c, CD86, B220, CD80, CD86, CD40, or MHCII (I-Ab) (BD Biosciences, Mississauga, Ontario, Canada) were added at predetermined optimal concentrations for 20 min on ice. Unconjugated Abs to TLR4 (Santa Cruz Biotechnology) and CD14 (BD Pharmingen, Mississauga, Ontario, Canada) were used in conjunction with anti-rat PE secondary Ab. Data were collected using a FACS Calibur flow cytometer and CellQuest Pro software, and data were analyzed using FlowJo software.

**ELISA**

Tissue culture supernatants were assayed for the concentration of IL-4, -12p40, -6, and -10, TNF-α, and IFN-γ by ELISA, according to the manufacturer’s instructions (BD Biosciences).

**T cell proliferation assays**

SHIP⁻/⁻ or SHIP⁺/⁺ GM-DCs were resuspended in proliferation medium (IMDM + 10% heat-inactivated FCS, 0.00125% MTG, and 100 U/ml penicillin/streptomycin); seeded at 4 × 10⁵ cells/well in 96-well flat-bottom plates; stimulated with 100 ng/ml LPS, 0.3 µM CpG, 50 µg/ml dsRNA, 50 ng/ml flagellin, or 100 µg/ml PGN and OVA323-339 peptide (GeneScript, Piscataway, NJ); and incubated for 3 h at 37°C. CD4⁺ T cells, isolated from OTII spleens using an EasySep CD4⁺ selection kit, according to the manufacturer’s instructions (StemCell Technologies), were added to cultures at 4 × 10⁵ cells/well to a final volume of 200 µl. Cells were incubated at 37°C for 72 h, with [3H]-thymidine (1 µCi/well) added for the last 18 h. The contents of each well were harvested onto filterwats, and the radioactivity was determined using an LKB Betaplate Harvester and Liquid Scintillation Counter (LKB Wallac, Turku, Finland).

**Immune response assays**

For in vitro studies, SHIP⁻/⁻ and SHIP⁺/⁺ GM-DCs, at 5 × 10⁵ cells in proliferation medium/200 µl total volume/well in 96-well flat-bottom plates, were activated with 100 ng/ml LPS, 0.3 µM CpG, 50 µg/ml dsRNA, 50 ng/ml flagellin, or 100 µg/ml PGN and 1 µg/ml OVA323-339 peptide; a total of 5 × 10⁴ CD4⁺ OTII cells were added for 4 d, and coculture supernatants were collected for analysis of secreted cytokines by ELISA. For in vivo studies, SHIP⁺/⁺ and SHIP⁻/⁻ GM-DCs were incubated with 100 ng/ml LPS and 1 µg/ml OVA323-339 peptide for 18 h; cells were harvested, washed three times in proliferation medium, and injected i.v. into OTII transgenic mice (1 × 10⁶ DCs/250 µl/mouse). After 4 d, splenocytes were harvested, resuspended in proliferation medium, and cultured in 96-well flat-bottom plates at 5 × 10⁵ cells/well with 1 µg/ml OVA323-339 peptide. Alternatively, CD4⁺ T cells were enriched from these splenocytes and cultured (1:1 with LPS-activated SHIP⁺/⁺ or SHIP⁻/⁻ GM-DCs. Supernatants were collected from wells after 3 d of culture, and the concentration of secreted cytokines was determined by ELISA.

SHIP⁻/⁻ or SHIP⁺/⁺ mice were immunized s.c. with 100 µg OVA (50 µl) mixed 1:1 with CFA (Pierce, Rockford, IL) or i.p. (100 µl) with 100 µg OVA mixed (1:1 ratio) with alum (Pierce). After 10 d, cells from draining lymph nodes (LNs) and spleens were harvested and cultured in 96-well flat-bottom plates at 5 × 10⁵ cells/well with 100 µg/ml OVA. Alternatively, LN and splenic CD4⁺ T cells were isolated and cocultured (1:1 ratio, 2.5 × 10⁵ cells/well) with SHIP⁺/⁺ or SHIP⁻/⁻ GM-DCs preincubated with 100 µg/ml OVA for 18 h. After 3 d, supernatants were collected and analyzed for secreted cytokines by ELISA.

To measure Ag uptake, GM-DCs were incubated with 100 µg/ml FITC-labeled albumin at 4°C (background control) or 37°C for 2 h. The incorporation of FITC-albumin into CD11c⁺ cells was monitored by flow cytometry.

**RNA extraction and RT-PCR**

Total RNA was prepared using TRIzol reagent (Invitrogen), and genomic DNA contaminants were removed using a TURBO DNA-free kit (Ambion, Applied Biosystems, Foster City, CA), according to each manufacturer’s
instructions. cDNA synthesis was performed using Moloney murine leukemia virus RT (Invitrogen) with an oligonucleotide primer. The reactions were performed as per the manufacturer's instructions, except the reactions were linearly scaled to a 25-μl total volume. PCR amplifications were performed using the Phusion High-Fidelity DNA Polymerase kit (Finnzymes, Espoo, Finland). Internal control amplification reactions using GAPDH-specific primers were performed (5' TTAGGCCCCCTGGC-CAAGG and 5' CTTACTCCTTGAGGCCTAGT). Amplification of mouse TLRs was performed with RT primers from Invitrogen (version #67090-SV, Burlington, Ontario, Canada). PCR amplifications were performed at 98°C for 75 s, followed by 35 cycles of 98°C for 15 s, 58°C or 63°C for 20 s, and 72°C for 30 s, followed by a final step of 72°C for 10 min. All reactions were performed using a GeneAmp PCR System 9700 Thermo Cycler (PerkinElmer, Wellesley, MA).

Statistical analysis
Statistical significance was calculated using a two-tailed unpaired Student t test using Microsoft Excel (Microsoft, Redmond, WA). Differences were considered significant when p < 0.05.

Results
SHIP−/− BM progenitors proliferate and differentiate into DCs faster and survive longer than WT DCs
Because we reported previously that SHIP−/− myeloid progenitors display enhanced proliferation of Mδ and granulocyte lineages in vitro and in vivo, because of hyperresponsiveness to cytokines and growth factors (27, 28), we asked whether SHIP−/− DC progenitors were also hyperresponsive to growth-promoting factors. To test this, freshly isolated SHIP+/+ and SHIP−/− BM cells were cultured with various concentrations of GM-CSF. After 8 d in culture, the nonadherent and loosely adherent cells from both cultures showed the same characteristic DC morphology (Supplemental Fig. 1A), expressed similar levels of cell surface CD11c and CD11b (characteristic of mDCs), and lacked expression of the granulocyte marker Gr-1 or CD8α and B220, which are present on other DC subsets (Supplemental Fig. 1B). However, SHIP−/− BM cells generated a higher frequency of CD11c+ cells, in keeping with a report by Neill et al. (29). However, in contrast with their report, we found that total cell numbers from SHIP−/− DC cultures were also consistently higher than those from WT cultures and, when combined with the increased percentage of DCs, generated ~50% more DCs at all concentrations of GM-CSF tested (Fig. 1A, left panel). We then compared the frequency of CD11c+ cells generated by SHIP+/+ and SHIP−/− BM cells with different concentrations of GM-CSF on days 3–8; the frequency was consistently greater in SHIP−/− cultures, particularly during the earlier days of culture (days 3 and 4) and with low concentrations of GM-CSF (Fig. 1A, middle panel). Expressed this way, it can be seen that when SHIP−/− BM cells are cultured with 2 ng/ml GM-CSF, almost 100% of the cells are CD11c+ by day 6, whereas only 50% of the WT BM cells are CD11c+ by day 6. Time-course studies using 10 ng/ml GM-CSF, and taking into account total cell numbers in the cultures, revealed that more DCs were generated from SHIP−/− BM cells on each day of culture (Fig. 1A, right panel). Importantly, by day 5, the number of DCs generated from SHIP−/− BM cells had plateaued, whereas DCs from WT BM cells had only reached 50% of their maximal production. These results suggest that SHIP−/− DC precursors survive better or proliferate more quickly and differentiate faster in response to GM-CSF than do WT DC progenitors. However, interpreting these results was complicated because of a previous study showing that SHIP−/− mice have 4-fold greater numbers of BM DC progenitors than WT mice (29). Thus, to eliminate this variable, we examined the proliferation of individual cells by tracking the division of CD11c+ DCs using CFSE dilution. To do this, we injected mice with 125 mg/kg 5-FU i.p. to increase primitive hematopoietic progenitors; 6 d later, we isolated these primitive BM cells, labeled them with 5 μM CFSE, and cultured and monitored the appearance of CD11c+ cells for several days. Our results suggest that the generation of CD11c+ cells from SHIP−/− BM precursors was more rapid than from WT BM precursors, with the CFSE dilution (indicative of cell divisions) in day-3 SHIP−/− cultures being similar to that observed in day-5 WT cultures (Fig. 1B). Thus, the SHIP−/− BM cells showed an increased rate of cell division, as well as accelerated differentiation into CD11c+ cells. To determine whether SHIP specifically regulated GM-CSF–induced signaling in DC progenitors, we stimulated SHIP+/+ and SHIP−/− BM cells with increasing concentrations of GM-CSF for 10 min; we found, by Western blot analysis, that SHIP was phosphorylated/activated in response to GM-CSF stimulation of WT cells, as expected. STAT5 phosphorylation was not enhanced in SHIP−/− BM cells, whereas Akt was more phosphorylated/activated in SHIP−/− BM cells, particularly at low concentrations of GM-CSF (Fig. 1C). This elevated activation of Akt in SHIP−/− DC progenitors likely contributes to their enhanced cell division.

Because the greater numbers of CD11c+ cells from SHIP−/− whole BM cultures could also be the result of enhanced survival rather than, or in addition to, enhanced proliferation, we tested the survival of differentiated day-8 SHIP+/+ and SHIP−/− DCs in the presence and absence of GM-CSF. We found that SHIP−/− DCs displayed enhanced survival (Fig. 1D). Specifically, SHIP−/− DCs survived better than WT DCs in the presence and absence of GM-CSF. Strikingly, the survival of SHIP−/− DCs in the absence of GM-CSF surpassed that of WT DCs in the presence of GM-CSF. Because the PI3K pathway is known to be important for cell survival, we investigated whether day-8 GM-DCs also had enhanced phosphorylation of Akt in response to GM-CSF signaling. We found that, much like in BM progenitors, SHIP−/− GM-DCs had comparable STAT5 phosphorylation but greatly enhanced Akt phosphorylation, particularly at low concentrations of GM-CSF (Fig. 1E). This enhanced Akt pathway activation likely accounts for the improved survival of SHIP−/− DCs.

SHIP−/− mice possess higher numbers of splenic DCs
To determine whether the increased numbers of DCs that we obtained from SHIP−/− BM cells in vitro also occurred in vivo, we assessed splenic DCs by flow cytometry. SHIP−/− spleens contain a greater proportion of Gr-1−, CD11b−, and CD11b+Gr-1− cells and lower levels of B220+, CD4+, and CD8− cells than do WT spleens (27, 30). More importantly, the percentage of CD11c+ splenocytes from SHIP−/− mice was greater than in age- and sex-matched WT littermates (9.4% ± 2.5% versus 4.9% ± 0.8% in WT; n = 5). Because the total number of splenocytes was also greater in SHIP−/− mice (198 ± 57 × 106 versus 170 ± 27 × 106 in WT; n = 5), this translated into an overall 2-fold greater total number of DCs in the spleens of SHIP−/− mice (17.9 ± 3.8 × 106 versus 8.4 ± 2.0 × 106 in WT; n = 5). Thus, SHIP seems to be a negative regulator, in vivo and in vitro, of DC proliferation, differentiation, and survival.

To gain some insight into the specific subtypes of DCs that were overproduced in SHIP−/− mice, we compared the levels of various cell surface markers on SHIP+/+ and SHIP−/− splenic CD11c+ cells (Fig. 2). Interestingly, although SHIP+/+ splenic DCs seemed to contain a single homogeneous population of CD11b+ cells (CD11bhigh), SHIP−/− splenic DCs contained a population of CD11bhigh and CD11blast DCs. Also of interest, the SHIP−/− DCs consistently contained slightly more CD8α+ and slightly fewer B220+ plasmacytoid DCs, although these differences were not statistically significant. Most interesting, however, was the finding that the SHIP−/− splenic DCs displayed substantially lower levels of MHCII on their surface, suggesting a less mature phenotype.
TLR-induced cytokine secretion is altered in SHIP-/− DCs

Because DCs secrete inflammatory cytokines upon TLR activation by pathogens, and these cytokines play important roles in regulating subsequent immune responses, we compared the secretion of IL-12 and -6 and TNF-α, as well as the anti-inflammatory cytokine IL-10 from SHIP+/+ and SHIP-/− GM-DCs after stimulation with a variety of TLR ligands. Over a wide range of LPS, CpG DNA, and dsRNA concentrations, SHIP-/− GM-DCs secreted a lower level of IL-12p40 (Fig. 3, top panels). In contrast, IL-6 and TNF-α levels were significantly enhanced in SHIP-/− DCs in response to LPS and dsRNA, but not CpG, stimulation (Fig. 3, middle panels). In addition, we found that SHIP-/− DCs tended to produce lower levels of IL-10 in response to CpG, dsRNA, and LPS (Fig. 3, bottom panels). Similar results to those observed with LPS and dsRNA were obtained with flagellin and PGN (Supplemental Fig. 2). Thus, in general, the absence of SHIP resulted in decreased TLR-induced production of IL-12 and -10 but increased production of IL-6 and TNF-α.

SHIP-/− DCs are less mature than WT DCs before and after TLR activation

The development of an appropriate DC response to an inflammatory agent depends on its maturation, typically induced by pathogens or damage signals and immune-stimulating cytokines. To examine the ability of SHIP-/− DCs to mature following stimulation with TLR ligands, we treated SHIP+/+ and SHIP-/− BM cells with or without 100 ng/ml LPS, 0.3 μM CpG, or 50 μg/ml dsRNA for 24 h at 37˚C. The in vitro-derived SHIP-/− DCs, like in vivo-derived SHIP-/− splenic DCs (Fig. 2), were far less mature, even before TLR ligand-induced maturation. For example, unstimulated SHIP-/− CD11c+ cells expressed very little MHCII, whereas unstimulated WT CD11c+ cells expressed high levels (Fig. 4, top left panel). As well, in response to all three TLR

FIGURE 1. SHIP+/− BM cells generate more DCs than WT BM cells in response to GM-CSF. A, SHIP+/+ and SHIP+/− BM cells were cultured in 96-well plates with the indicated concentrations of GM-CSF for 8 d (left panel). SHIP+/+ and SHIP+/− BM cell differentiation was determined daily during 8 d of culture with 2, 10, or 50 ng/ml GM-CSF by measuring the percentage of CD11c+ cells (middle panel). SHIP+/+ and SHIP+/− BM cells were cultured with 10 ng/ml GM-CSF; and CD11c+ DCs were enumerated by hemocytometer and flow cytometry daily for 8 d (right panel). B, BM cells isolated from 5-FU–treated mice were labeled with CFSE and cultured in 10 ng/ml GM-CSF. On days 3, 4, and 5, CFSE dilution was determined in the CD11c+ population by flow cytometry. MFI for the CD11c+ cells is indicated. C, SHIP+/+ and SHIP+/− BM cells were cultured for 10 min with increasing concentrations of GM-CSF and subjected to Western blot analysis. D, SHIP+/+ and SHIP+/− GM-CSF–derived DCs were purified from day-8 cultures, washed, and cultured in mDC base ± 10 ng/ml GM-CSF; survival was assessed by trypan blue exclusion.

FIGURE 2. Analysis of splenic DC populations. Spleens were isolated from age- and sex-matched littermates. Flow cytometric analysis of CD11c+ splenic DCs was carried out for the expression of the indicated surface molecules. Isotype control is indicated by the gray area; the percentage of positive cells are indicated. Data shown are representative of three independent experiments.

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ligands, SHIP<sup>−/−</sup> DCs were incapable of achieving MHCII levels comparable to even unstimulated WT DCs (Fig. 4, top left panel). As shown in the remainder of the panels in Fig. 4, cell surface levels of CD40, CD80, and CD86 were also reduced, before and after TLR agonist stimulation, on GM-CSF–derived SHIP<sup>−/−</sup> DCs compared with WT DCs.

**FIGURE 3.** SHIP<sup>−/−</sup> GM-CSF–derived DCs produce less IL-12 and -10 but more IL-6 and TNF-α after TLR activation. GM-DCs were purified from day-6 cultures and treated with the indicated concentration of LPS, CpG, or dsRNA for 24 h at 37°C. Supernatants were collected and assayed for IL-6,-10, and -12 and TNF-α by ELISA. Results are mean ± SEM of three independent experiments performed in duplicate. ■, WT DC supernatants; □, SHIP<sup>+/+</sup> DC supernatants. *p < 0.05; **p < 0.01; †p < 0.001 (WT DCs versus SHIP<sup>−/−</sup> DCs).

**FIGURE 4.** SHIP<sup>−/−</sup> DCs are less mature before and after TLR stimulation. Day-8 DC cultures were stimulated with LPS, CpG, or dsRNA for 24 h. The maturation of CD11c<sup>+</sup> cells in control (thin line) and TLR ligand-treated (thick line) cultures was assessed by the expression of MHCII, CD40, CD80, and CD86 by flow cytometry. The isotype control is represented by the gray area. The increase in the percentage of positive cells from control to stimulated is indicated by the region markers. Data shown are representative of three independent experiments. *p < 0.05; **p < 0.01; †p < 0.001 (SHIP<sup>+/+</sup> DCs versus SHIP<sup>−/−</sup> DCs); for TLR-stimulated samples. In addition, SHIP<sup>−/−</sup> DCs expressed significantly reduced levels of MHCII, CD40, CD80, and CD86 in unstimulated samples.

Because one of the primary functions of activated DCs is to induce Ag-specific T cell proliferation and activation, we next assessed the relative abilities of WT and SHIP<sup>−/−</sup> DCs to trigger T cell proliferation. Splenic CD4<sup>+</sup> T cells isolated from OTII transgenic mice were cocultured for 72 h with SHIP<sup>+/+</sup> or SHIP<sup>−/−</sup> GM-DCs that were loaded with OVA<sub>233-339</sub> peptide and activated with a TLR ligand. We found that induction of Ag-specific T cell proliferation was markedly reduced in response to SHIP<sup>−/−</sup> GM-DCs stimulated with LPS, CpG, dsRNA, flagellin, or PGN compared with WT GM-DCs (Fig. 5), most likely because of the inability of the SHIP<sup>−/−</sup> DCs to upregulate MHCII and costimulatory receptors following TLR stimulation.

**GM-CSF–derived SHIP<sup>−/−</sup> DCs do not induce Ag-specific T cell proliferation**

Because DCs play a pivotal role in determining the Th1/Th2 cell characteristics of an immune response, we next asked whether DCs lacking SHIP might also be abnormal in this regard, and we tested this in vitro and in vivo. Specifically, OTII transgenic CD4<sup>+</sup> T cells were activated in vitro by TLR ligand-treated DCs loaded with OVA peptide. Supernatants of the cocultures were collected and assayed for IFN-γ and IL-4 levels by ELISA. As shown in Fig. 6A, the production of IFN-γ was significantly reduced when T cells were cocultured with SHIP<sup>−/−</sup> DCs compared with WT DCs, regardless of the TLR agonist used, except for PGN, for which no difference between WT and SHIP<sup>−/−</sup> DCs was detected. IL-4 production was not detectable in any of the cultures.

**SHIP<sup>−/−</sup> DCs skew to a Th2 cell response**

To study T cell polarization in vivo, we injected OVA peptide-loaded and LPS-activated SHIP<sup>+/+</sup> or SHIP<sup>−/−</sup> GM-CSF–derived DCs i.v. into OTII transgenic mice. The Th cell responses were measured 4 d later by culturing total splenocytes isolated from DC-immunized mice with OVA peptide or by isolating CD4<sup>+</sup>T cells and coculturing them with LPS-activated OVA peptide-loaded WT or SHIP<sup>−/−</sup> DCs. Similar to our in vitro results, the production of IFN-γ was significantly reduced in whole
splenocytes and CD4+ T cells from OTII mice immunized with SHIP−/− DCs (Fig. 6B). The production of IL-4 was only detectable in whole-splenocyte cultures, with SHIP−/−-immunized OTII splenocytes producing more IL-4 (22.7 ± 2.8 pg/ml versus 14.6 ± 1.6 pg/ml for WT).

In another set of experiments, we examined the immune responses of WT and SHIP-deficient mice by immunizing them with whole OVA protein mixed with CFA (which is Th1 cell biasing) or alum (Th2 cell biasing). We found that SHIP−/− splenocytes and LN cells produced less IFN-γ when immunized with OVA in CFA (Fig. 6C, left panel), whereas the production of IL-4 was increased in splenocytes from SHIP−/− mice immunized with OVA in alum (Fig. 6C, right panel). To ensure that these results accurately reflected DC-induced T cell proclivity, and not simply differences in the number of T cells or Ag-presenting B cells and other IFN-γ-producing cells in LNs and spleens that could impact secreted cytokine levels, we isolated CD4+ T cells from OVA-immunized WT and SHIP-deficient mice. Isolated splenic and LN CD4+ T cells were cocultured with SHIP+/+ or SHIP−/− GM-DCs pulsed overnight with OVA. Consistent with our in vivo immunization results, purified LN and splenic CD4+ T cells from SHIP−/− mice produced significantly less IFN-γ (Fig. 6C, left panel) in response to OVA-presenting SHIP−/− GM-DCs. In addition, we found that purified LN and splenic CD4+ T cells from SHIP−/− mice also produce less IFN-γ compared with SHIP+/+ CD4+ T cells, when restimulated with OVA-presenting SHIP+/+ GM-DCs, although the levels were slightly higher than that triggered by SHIP−/− GM-DCs (Supplemental Fig. 3). IL-4 production was less than the limit of detection from SHIP+/+ and SHIP−/− cells. Notably, we found that SHIP−/− DCs were fully competent in the capture of Ag, because SHIP−/− DCs had a similar, if not enhanced, ability to take up FITC-labeled albumin (Fig. 6D).

SHIP−/− DCs are less mature than WT DCs because of a failure to repress the PI3K pathway

To gain some insight into why SHIP−/− DCs were less mature than WT DCs, we first asked whether other negative regulators of the PI3K pathway were upregulated in DCs when SHIP is absent. Specifically, we compared the levels of SHIP2 and PTEN, two PI3K pathway lipid phosphatases, in WT and SHIP−/− DCs, and found that SHIP2 and PTEN levels were not elevated in SHIP−/− mice. To ensure that these results accurately reflected DC-induced T cell proclivity, and not simply differences in the number of T cells or Ag-presenting B cells and other IFN-γ-producing cells in LNs and spleens that could impact secreted cytokine levels, we isolated CD4+ T cells from OVA-immunized WT and SHIP-deficient mice. Isolated splenic and LN CD4+ T cells were cocultured with SHIP+/+ or SHIP−/− GM-DCs pulsed overnight with OVA. Consistent with our in vivo immunization results, purified LN and splenic CD4+ T cells from SHIP−/− mice produced significantly less IFN-γ (Fig. 6C, left panel) in response to OVA-presenting SHIP−/− GM-DCs. In addition, we found that purified LN and splenic CD4+ T cells from SHIP−/− mice also produce less IFN-γ compared with SHIP+/+ CD4+ T cells, when restimulated with OVA-presenting SHIP+/+ GM-DCs, although the levels were slightly higher than that triggered by SHIP−/− GM-DCs (Supplemental Fig. 3). IL-4 production was less than the limit of detection from SHIP+/+ and SHIP−/− cells. Notably, we found that SHIP−/− DCs were fully competent in the capture of Ag, because SHIP−/− DCs had a similar, if not enhanced, ability to take up FITC-labeled albumin (Fig. 6D).

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To gain some insight into why SHIP−/− DCs were less mature than WT DCs, we first asked whether other negative regulators of the PI3K pathway were upregulated in DCs when SHIP is absent. Specifically, we compared the levels of SHIP2 and PTEN, two well-established negative regulators of the PI3K pathway, in WT and SHIP−/− GM-DCs by Western blot analysis. As shown in Fig. 7A, PTEN and SHIP2 levels were not elevated in SHIP−/− GM-DCs, indicating that loss of SHIP does not result in a compensatory increase in these two lipid phosphatases.

To determine whether the absence of SHIP was preventing the maturation of naïve DCs through enhanced activation of the PI3K pathway, we treated SHIP−/− BM progenitors grown in GM-CSF for 8 d with and without low levels of LY294002 or wortmannin and assessed MHCIi levels by flow cytometry. As shown in Fig. 7B, the addition of LY294002 or wortmannin increased MHCIi levels of the
SHIP−/− GM-DCs in a dose-dependent manner, consistent with the possibility that the absence of SHIP in DC progenitors results in an elevated activity of the PI3K pathway and this, in turn, prevents normal DC maturation. Treatment of SHIP+/+ BM progenitors with LY294002 during differentiation also modestly increased MHCII levels from 40% to a maximum of 53% (data not shown). This increased maturation of SHIP+/+ and SHIP−/− GM-DCs was seen in the absence of significantly increased cell death or decreased cell numbers (data not shown).

Lastly, to gain some insight into why SHIP−/− DCs were less mature than WT DCs after TLR agonist stimulation, we compared the expression of TLRs in SHIP+/+ and SHIP−/− DCs. As shown in Fig. 7C, the LPS receptors TLR4 and CD14 were expressed at comparable levels in SHIP+/+ and SHIP−/− GM-DCs, as assessed by flow cytometry. As well, the levels of TLR3, TLR4, and TLR9 mRNA, as assessed by RT-PCR (Fig. 7D), were comparable in WT and SHIP−/− GM-DCs. In addition, we found that SHIP+/+ and WT GM-DCs expressed similar levels of TLRs 1, 2, 6, 7, and 8 mRNA. Although only barely detectable levels of TLR5 were found in WT and SHIP−/− DCs, these cells were still capable of responding to flagellin stimulation, albeit weakly (Supplemental Fig. 2). Therefore, we propose that an enhanced PI3K pathway activity is responsible for the immature DC phenotype of SHIP−/− GM-CSF–derived DCs and when these DCs are stimulated with TLR ligands, they cannot overcome this immature phenotype, despite expressing comparable levels of TLRs.

Discussion
DCs are sentinels of the immune system. After encountering pathogens, these DCs mature into highly efficient APCs that stimulate T cell proliferation and the subsequent initiation of adaptive immune responses (1). Our data suggest that SHIP negatively regulates DC progenitor proliferation, differentiation, and survival. These results differ from a previous report that found no difference in the number of GM-CSF–derived DCs generated from SHIP+/+ and SHIP−/− BM cells (29), possibly because of differences in mouse background (we used mice backcrossed to C57BL/6 for ≥12 generations, whereas the previous study used F2 or F3 on a mixed 129/Sv and C57BL/6J background) or differences in the culture methods used (they added IL-4 and did not change the medium). Because SHIP-deficient mice have increased numbers of myeloid progenitors (27), we determined SHIP’s effect on DC generation via CFSE dilution and demonstrated a more rapid generation of SHIP−/− DCs from BM cells via enhanced dilution of CFSE in CD11c+ cells. This enhanced generation of GM-DCs was also evident in vivo, where a higher frequency and total number of CD11c+ cells were present in SHIP−/− spleens.

The hyperactivation of the PI3K pathway in SHIP−/− BM progenitors (Fig. 1C) and mature DCs (Fig. 1E) in response to low levels of GM-CSF is likely responsible for the enhanced proliferation, differentiation, and survival of these cells, because the activation of the downstream serine/threonine kinase, Akt, was shown to promote cell growth, survival, and cytokine production in many cell types (31). A similar phenomenon was documented in Møs derived from SHIP−/− mice (32). Lyn is another protein that has been implicated in the negative regulation of Møs (33). Lyn-deficient mice, like SHIP-deficient mice, have perturbed myelopoiesis (34), and Lyn−/− DCs, like SHIP−/− DCs, are hypersensitive to GM-CSF, display an immature phenotype, and produce less IL-12 upon TLR stimulation (35). Given that SHIP is a substrate for Lyn (33, 36), these similarities are not surprising.

The principal function of DCs is to activate T cells, and their ability to do this is closely linked to their maturation status (1). We showed herein that although SHIP negatively regulates DC progenitor proliferation, differentiation, and survival, it is essential for DC maturation and the induction of Ag-specific T cell proliferation. Specifically, in the absence of SHIP, naive CD11c+ GM-DCs express very low levels of MHCII and, in response to various TLR ligands, they fail to mature appropriately and secrete less IL-12 and -10 and more IL-6 and TNF-α than WT DCs. Although these results are in agreement with a previous study showing reduced induction of allogeneic responses by SHIP−/− splenic GM-DCs (29), they contrast with another study showing that SHIP−/− splenic DCs are as capable as WT splenic DCs at priming allogeneic and Ag-specific T cell responses (37). Related to this, we found that naive and LPS-treated SHIP−/− splenic CD11c+ DCs are not as capable as naive and LPS-treated SHIP+/+ DCs to stimulate T cell proliferation, although they were still capable of stimulating Ag-specific T cell responses.

FIGURE 7. SHIP−/− DCs are less mature than WT DCs because of a failure to repress the PI3K pathway. A, SHIP+/+ and SHIP−/− DCs were purified from day-8 cultures; probed with specific Abs to SHIP2, SHIP1, and PTEN; and subjected to Western blot analysis. GAPDH served as a loading control. Data are representative of at least three independent experiments in which comparable levels of SHIP2 and PTEN were seen in SHIP+/+ and SHIP−/− GM-DCs. B, SHIP−/− BM cells were derived for 8 d with 10 ng/ml GM-CSF ± DMSO (vehicle control); 1, 2.5, or 5 μM LY294002 (LY); or 50 nM wortmannin (W) (all added each day). The resulting CD11c+ cells were assessed for MHCII expression by flow cytometry. Data shown are representative of two independent experiments performed in triplicate. C, The expression of TLR4 and CD14 was determined for the CD11c+ population of day-8 GM-CSF–derived cultures (black line). Isotype control is represented by the gray area. The MFI and the percentage of positive cells are indicated. D, Total RNA was isolated from day-8 WT and SHIP−/− DCs, and the levels of TLR transcripts were determined using RT-PCR. GAPDH internal control is shown for equal cDNA input. Data shown are representative of at least three independent experiments.
splenic CD11c+ DCs at inducing an Ag-specific T cell response (Supplemental Fig. 4A, 4B). These results are consistent with our findings using in vitro-generated GM-DCs. The discrepancy between our results and those of Ghanasah et al. (37) could be due to the source of DCs used; we used in vitro-generated GM-DCs, as well as whole CD11c+ splenic DCs, whereas they used a highly purified subset of splenic DCs. Thus, it is possible that our splenic CD11c+ cells contain an immunoregulatory DC subset that represses T cell proliferation and cytokine production, although we could not detect a significant difference in the expression of CD80+ or B220+ subsets between SHIP+/+ and SHIP−/− splenic CD11c+ DCs, nor was either of these markers expressed on the rather uniform CD11c+ GM-DC population used in our studies. It is also possible that the population used in their study (CD11c+ CD86+Lin−) only contains the most mature SHIP−/− DCs and lacks those DCs that are more immature and, thus, less capable of inducing T cell proliferation. As well, studies showed that naive GM-DCs can promote T cell hyporesponsiveness (38, 39). It is possible that SHIP−/− GM-DCs are intrinsically suppressive, as a result of their immature phenotype, and cannot acquire T cell-activating capabilities, even when stimulated by TLR ligands. The immature phenotype of our SHIP−/− DCs, which our LY294002 and wortmannin studies suggest is due to an overly active PI3K pathway, was also observed in vivo with splenic SHIP−/− DCs and is likely responsible for the profound impairment of Ag-specific T cell proliferation induced by SHIP−/− DCs, as well as the reduced priming of a Th1 cell response. These results also suggest that the PI3K pathway is a negative regulator of IL-12 production but is a positive regulator of IL-6 and TNF-α in GM-CSF-derived DCs.

The reduced IL-12 and increased IL-6 produced by SHIP−/− DCs likely contribute to the impaired Th1 cell polarization observed when OVA peptide-loaded and TLR-stimulated SHIP−/− DCs are cultured in vitro or in vivo with OTII transgenic T cells. This finding suggests that SHIP-deficient mice would show defects in Th1 cell-type responses. This was indeed the case, because splenocytes and LN cells from SHIP-deficient mice immunized with OVA in CFA showed greatly reduced IFN-γ secretion (a Th1 cell marker) upon restimulation with OVA. This result was also confirmed when CD4+ T cells were purified from spleens and LNs and cocultured with WT or SHIP−/− DCs, thus avoiding the problem of different contributions of B, T, and other cell types in SHIP+/+ and SHIP−/− spleens to T cell responses. Under conditions designed to skew toward a Th2 cell response (OVA in alum), SHIP-deficient mice had enhanced responses as measured by IL-4 production from splenocytes. These results match previous studies from our group showing that peritoneal Mψs from SHIP−/− mice are M2 skewed (40), perhaps, in part, as a consequence of enhanced IL-4 production by SHIP−/− CD4+ T cells. In addition, SHIP−/− mice were also shown to have enhanced tumor growth (40), indicative of a defect in Th1 cell activation and programming. Furthermore, SHIP-deficient mice suffer from a chronic asthmatic disorder, accompanied by infiltration of Mψs, lymphocytes, neutrophils, and eosinophils into their lungs and enhanced production of the Th2 cell cytokines IL-4 and -13 (41). This suggests that SHIP negatively regulates Th2 cell signaling pathways. Although we showed that the lung pathology in SHIP-deficient mice is likely due primarily to an increase in mast cell numbers and their enhanced responsiveness (42), DCs may also play a role. A study using Pten-deficient DCs revealed that they also produced lower amounts of IL-12 than WT DCs following LPS stimulation (43). However, unlike that study (43), we found that SHIP−/− DCs, in general, also produce lower levels of IL-10 in response to TLR activation. Because LPS-stimulated PTEN−/− DCs should yield higher PIP3 and PI-3,4-P2 levels than WT DCs, whereas LPS-stimulated SHIP−/− DCs should generate higher PIP3, but lower PI-3,4-P2, levels than WT DCs (44), it is possible that the lower PI-3,4-P2 level in SHIP−/− DCs is responsible for the lower IL-10 production (i.e., PI-3,4-P2 might promote IL-10 production in DCs). As well, enhanced IL-10 production was shown to contribute to reducing IL-12 levels (45), suggesting that other mechanisms may be responsible for the lower levels of IL-12 produced by SHIP−/− DCs.

Although it was previously shown that the environment in which DCs develop plays an important role in determining the phenotype that they acquire, as illustrated by swap/transfer studies with WT and SHIP−/− DCs (29), we demonstrated that an intrinsic elevated PI3K pathway also contributes to the immature phenotype seen in vitro-derived SHIP−/− GM-DCs. However, the expansion of other immune cell types in vivo, such as myeloid-derived suppressor cells, likely also plays a role to suppress Th1 cell responses (37, 46). Thus, as they become available, it will be very interesting to assess the DC phenotype and Th1/Th2 cell proclivity of DC-lineage specific SHIP knockout mice and to test the function of SHIP−/− DCs in vitro and in vivo in the absence of the influence of a pan-hematopoietic SHIP−/− milieu.

In summary, we provide evidence that SHIP plays a negative and positive role in GM-CSF-derived DC generation and maturation, respectively. Because DCs modulate the nature and intensity of the adaptive immune response, they provide an attractive target for cancer therapy, as well as control of autoimmune diseases. Recent advances in the development of small molecule activators of SHIP (47) provide evidence that the activation of SHIP could be used as an alternative to PI3K inhibition, with the attractive quality that SHIP is hematopoietic restricted, whereas PI3K is more ubiquitous. Such pharmacological modulators of SHIP activity could be used to modify DC activity in vitro and in vivo and may be useful in DC-based cell therapies.

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Disclosures
G.K. is a founding member and Chief Scientific Advisor of Aquinox Pharmaceuticals, which is dedicated to identifying small molecule activators and inhibitors of SHIP.

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