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SHIP Represses Th2 Skewing by Inhibiting IL-4 Production from Basophils

Etsushi Kuroda,*‡ Frann Antignano,*‡ Victor W. Ho,*¹ Michael R. Hughes,*¹ Jens Ruschmann,*‡ Vivian Lam,* Toshiaki Kawakami,‡ William G. Kerr,‡ Kelly M. McNagny,* Laura M. Sly,*‡¹ and Gerald Krystal*‡¹

We report that SHIP−/− mice, compared to SHIP+/+ mice, are Th2 skewed with elevated serum IgE and twice as many splenic CD4+ Th2 cells that, when stimulated with anti-CD3, produce more IL-4 and less IFN-γ. Exploring the reason for this Th2 skewing, we found that freshly isolated SHIP−/− splenic and bone marrow basophils are present in elevated numbers and secrete far more IL-4 in response to IL-3 or to FceRI stimulation than do WT basophils. These SHIP−/− basophils markedly skew wild-type macrophage colony stimulating factor–derived macrophages toward an M2 phenotype, stimulate OT-II CD4+ Th cells to differentiate into Th2 cells, and trigger SHIP−/− B cells to become IgE-producing cells. All these effects are completely abrogated with neutralizing anti-IL-4 Ab. Exploring the cell signaling pathways responsible for hyperproduction of IL-4 by SHIP−/− basophils, we found that IL-3–induced activation of the PI3K pathway is significantly enhanced and that PI3K inhibitors, especially a p110α inhibitor, dramatically suppress IL-4 production from these cells. In vivo studies, in which basophils were depleted from mast cell-deficient SHIP−/− and SHIP+/+ mice, confirmed the central role that basophils play in the Th2 skewing of naive SHIP-deficient mice. Taken together, these studies demonstrate that SHIP is a potent negative regulator of IL-4 production from basophils and thus may be a novel therapeutic target for Th1- and Th2-related diseases. *The Journal of Immunology, 2011, 186: 323–332.

The Th1/Th2 paradigm, although now considered somewhat simplistic with the discovery of Th17 and regulatory T cells, remains useful for subdividing T cell immune responses into those that are specialized for “cellular immunity” (i.e., Th1) to fight viruses and other intracellular pathogens and those responsible for “humoral immunity” (i.e., Th2) to defend against extracellular pathogens such as parasitic worms (1). Th1 responses are typically driven by IL-12 and IFN-γ and by the transcription factors STAT4 and T-bet (2, 3), whereas Th2 responses are driven by IL-4, IL-5, IL-13, IL-18, and IL-33 and the transcription factors STAT6, GATA3, c-maf, and Nfatc (4, 5). Notably, there is antagonism between Th1 and Th2 responses, with the cytokines they produce downregulating each other, and overactivation of either response can lead to inflammatory disorders. For example, asthma is considered to be the result of an overly active Th2 response, whereas many autoimmune diseases are thought to be due to an overly active Th1 response to autoantigens (6). Because type 2 dominance is critical for tolerance to both xenografts and the fetus during pregnancy (7) and because impaired Th1 or Th2 cell responses result in the inability to clear pathogens (8) or in inappropriate responses to innocuous Ags (9), there is a great deal of interest in understanding what regulates the differentiation of T cells into their different effector subsets (4, 5, 10).

SHIP (also known as SHIP1) is a hematopoietic-restricted enzyme that negatively regulates the PI3K pathway by hydrolyzing the PI3K-generated second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) to phosphatidylinositol (PI)-3,4-bisphosphate (11). We have shown previously that SHIP−/− mice display several immunological abnormalities, including an overabundance of alternatively activated M2 macrophages (Mɒs) in their lungs and peritoneal cavities as well as consolidated, inflamed, and fibrotic lungs that contain M2 Mɒs–associated Ym1 crystals (12). This latter finding has been substantiated by Oh et al. (13), who also showed that SHIP−/− mice spontaneously develop allergic airway inflammation with increased levels of Th2 cytokines, chemokines, and inflammatory cells (e.g., eosinophils and mast cells) in their lungs.

Because the generation of M2 Mɒs and Ym1 has been shown to be positively regulated, at least under some circumstances, by the Th2 cytokines IL-4 and IL-13 (14–16), we hypothesized that SHIP−/− mice might be spontaneously Th2 skewed. We show in this study that this is indeed the case and that the Th2 skewing of

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Abbreviations used in this paper: α, p110α; β, p110β; δ, p110δ; γ, p110γ; AG, AG490; Arg I, arginase I; BM, bone marrow; CBA, cytomteric bead array; DC, dendritic cell; HSA, human serum albumin; LY, LY294002; Mό, macrophage; M-CSF, macrophage colony stimulating factor; NP-40, Nonidet P-40; PD, PP98059; PI, phosphatidylinositol; PIP3, phosphatidylinositol-3,4,5-triphosphate; poly I:C, polyinosinic-polycytidylic acid; UO, UO126; W, wortmannin; WT, wild-type.

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SHIP+/− mice is due, at least in part, to hyperactive basophils. Our results indicate that the PI3K pathway, and SHIP in particular, negatively regulate IL-4 production by basophils and that SHIP may be a novel regulator of type 2 immune responses via its repression of basophil activation.

Materials and Methods

Mice

All mice were housed in specific pathogen-free facilities at the British Columbia Cancer Research Centre or The Biomedical Research Centre (Vancouver, British Columbia, Canada), and all animal experiments were carried out according to the guidelines for the care and use of animals approved by the University of British Columbia (Vancouver, British Columbia, Canada). OT-II TCR transgenic and SHIP+/− and SHIP−/− mice were used at 6–8 wk of age. B6.Cg-Kitw−/−H2b/NihJaeBsmJ (Wsh/Wsh) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in-house at The Biomedical Research Centre. The B6-congenic, inducible SHIP+/− strain SHIPFl/Fl.Mx1Cre (17) was crossed twice with Wsh/Wsh mice to obtain Wsh/Wsh.SHIP Fl/Fl.Mx1Cre (+ or −) mice.

Reagents

All cytokines were purchased from StemCell Technologies (Vancouver, British Columbia, Canada). For Western analysis, Abs to Ym1 and phospho-SHIP were from StemCell Technologies (StemCell Technologies). PI3K and UO126) were from EMD Bioscience (La Jolla, CA). The subtype-specific inhibitor studies, sorted basophils were treated with inhibitors (10 μM LY294002 [LY], 30 nM wortmannin [W], 50 μM AG490, 20 μM PD98059, or 10 μM U0126) in the presence of IL-3 for 18 h at 37°C and IL-4 production determined. For PI3K subtype-specific inhibitor studies, sorted basophils were treated with inhibitors (50 nM PI3Ks inhibitor VIII or 10 μg TGX-221, SW18, SW30, and AS055240), unless otherwise indicated, and stimulated with IL-3 for 15 h. For signal transduction studies, sorted IL-3–starved basophils (4 × 105 cells/200 μl in 96-well plates) were stimulated with IL-3 for 0, 5, or 30 min, lysed with Nonidet P-40 (NP-40) lysis buffer (19), and used for the analysis of SHIP, STAT5, Akt, and Erk activation by Western blotting.

In vitro Th1/Th2 differentiation assays

Th1/Th2 differentiation was induced in vitro using OT-II mouse spleen cells as described previously (20) with slight modifications. Briefly, 1 × 106 OT-II mouse splenic CD4+ T cells were cultured with 5 × 105 T cell-derived wild-type (WT) spleen cells, as APCs, both prepared using an EasySep T cell sorting kit. Then, 2 × 105 highly enriched SHIP+/− or SHIP−/− in vitro-derived BM basophils, purified as FcεRI+ c-Kit+ cells using a FACSVantage SE, were added in the presence of 1 μg/ml OVA peptide residues 323–339 in 24-well flat-bottom plates. In some experiments, neutralizing anti-IL-4 Abs (5 μg/ml) were added during differentiation. Cells were harvested on day 4 and CD4+ cells sorted again and used for IL-4/INF-γ production or intracellular staining. For cytokine production, activated CD4+ T cells (5 × 105 cells in 1 ml) were restimulated with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 Abs for 18 h and culture supernatants used for IFN-γ and IL-4 ELISAs as described later. For intracellular cytokine staining, BD Cytofix/Cytoperm (BD Biosciences) was used according to the manufacturer’s instructions.Briefly, activated CD4+ T cells (1 × 105 cells in 1 ml) were restimulated with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 Abs plus 1 μl Golgi Plug (BD Biosciences) for 5 h. The cells were then harvested, washed, and suspended in Cytofix/Cytoperm solution at 4°C for 20 min. Fixed and permeabilized cells were stained with PE-labeled anti-IL-4, FITC-labeled anti-INF-γ, and allophycocyanin-labeled anti-CD4 or appropriate isotype control Abs. Labeled cells were used for flow cytometric analysis.

In vitro M1/M2 skewing assays

M0s were generated from SHIP+/− BM cells by incubation with 10 ng/ml M-CSF for 7 d. Resulting M0s (5 × 105 cells) were cultured with 1 × 105 highly purified SHIP+/− or SHIP−/− FcεRI+ c-Kit+ basophils with or without 10 ng/ml IL-3 in 24-well plates for a total volume of 1 ml for 4 d. In some experiments, 5 μg/ml neutralizing anti-IL-4 Ab was added on day 0. Four days later, culture supernatants were harvested and assessed for IgE levels by ELISA.
In vivo basophil depletion studies

To induce SHIP deletion in a mast cell-deficient mouse model, Wsh. SHIPFL/FL-Mx1Cre+ (or Mx1Cre−) mice were i.p. injected with 200 μg/mouse poly I:C (polynosinic–polycytidylic acid; Sigma-Aldrich) prepared in PBS at 48-h intervals (4 doses total). After the last poly I:C treatment, mice received 10 i.p. injections of either the anti-FcεRIa (MAR-1) Ab or isotype control Ab (50 μg/mouse i.v.) for 3 consecutive days. Three days after the final i.v. Ab treatment, the mice were sacrificed and spleens harvested for analysis.

Western analysis
Cell lysates, supplemented with concentrated SDS sample buffer to give 1 × SDS sample buffer, were boiled for 5 min and subjected to Western analysis as described previously (21). To determine protein concentrations, samples were first solubilized in NP-40 lysis buffer and protein levels assessed using a BCA protein assay kit (Pierce, Rockford, IL), standardized with BSA.

ELISAs and CBAs

The levels of cytokines in culture supernatants were measured with cytokine ELISA kits from BD Biosciences according to the manufacturer’s instructions. IgE levels were determined using purified anti-mouse IgE (clone R35-118) as detection Ab (BD Biosciences). Alternatively, a Th1/Th2 and an inflammatory CBA kit from BD Biosciences were used according to the manufacturer’s instructions.

Statistical analysis

Statistical analyses were performed between SHIP+/+ and SHIP−/− samples using the Student t test. A confidence level of p < 0.05 was considered significant.

Results
SHIP−/− mice are spontaneously Th2 skewed

Based on our previous study showing that SHIP−/− peritoneal and alveolar Møs possess an M2 phenotype (12) (i.e., express high Arg I and Ym1 levels) and on the finding of Oh et al. (13) that the lungs of SHIP−/− mice are spontaneously inflamed, we first asked if SHIP−/− mice might be constitutively Th2 skewed. To address this, we assessed several Th2 immune markers. Specifically, we measured the IgE levels in serum from SHIP+/+ and SHIP−/− mice and found serum IgE levels were ~4-fold higher in SHIP−/− mice than those in WT mice (Fig. 1A). We also assessed IL-4 and IFN-γ production from SHIP+/+ and SHIP−/− splenic T cells. As shown in Fig. 1B, CD3-activated spleen cells from SHIP−/− mice produced higher levels of IL-4 and lower levels of IFN-γ than those of spleen cells from WT littermates. Also, because T1/ST2 has recently been shown to be a cell surface marker for Th2 CD4+ T cells (22, 23), we compared the frequency of T1/ST2+ CD4+ T cells in the spleens of SHIP+/+ and SHIP−/− mice and found they were two times higher in SHIP−/− spleens (data not shown). Given that there is ~1.5 times more nucleated spleen cells in SHIP−/− mice than that in WT mice, SHIP−/− mice contain three times more splenic T1/ST2+ CD4+ T cells. Taken together, these data suggest that SHIP−/− mice are more Th2 skewed than their WT littermates.

SHIP−/− splenic and BM basophils secrete higher levels of IL-4
To explore the underlying mechanism(s) responsible for the Th2 skewing of SHIP−/− mice, we first compared the cytokines secreted by SHIP+/+ and SHIP−/− BM and spleen cells, with or without IL-3, using inflammatory and Th1/Th2 CBAs, and found that SHIP−/− cells produced substantially more IL-10, TNF-α, MCP-1, IL-6, and IL-4 than that produced by their WT counterparts (Supplemental Fig. 1). Cytokines that were not detectable in either SHIP+/+ or SHIP−/− BM or spleen cells included IL-5, IL-2, IFN-γ, and IL-12p70. Because of the well-established role of IL-4 in Th2 skewing (24, 25), we then focused on IL-4 by first confirming our CBA results using IL-4 ELISAs. As shown in Fig. 2A, we found that SHIP−/− spleen and BM cells produced detectable levels of IL-4 even in the absence of any stimulation, unlike WT cells, and produced substantially higher levels of IL-4 in response to IL-3 than those produced by cells from WT mice.

Because basophils have been reported to produce IL-4 in response to IL-3 (20, 26) and to reside in the spleen and BM, we enriched for SHIP+/+ and SHIP−/− splenic and BM basophils by sorting FcεRIα+ cells and assessed their IL-4 production. As shown in Fig. 2B, basophil-enriched splenic and BM SHIP−/− cells produced substantially higher levels of IL-4 in response to IL-3 than those produced by WT cells. To determine if there were more basophils (i.e., FcεRIα+ c-Kit+ cells) in SHIP−/− spleen and BM, we carried out FACS analysis and found higher percentages (~5-fold) in both the spleen and BM of SHIP−/− mice (Fig. 2C). We also compared absolute basophil numbers in spleen, BM, and in the peritoneal cavities of SHIP−/− and SHIP+/+ mice and found that SHIP−/− mice displayed a marked basophilia (Fig. 2D). Of note, we found that FcεRIα+ c-Kit− cells isolated from SHIP+/+ and SHIP−/− BM were of a similar size and morphology, with multilobular nuclei and Giemsa staining granules (Supplemental Fig. 2A), consistent with recent reports for marine basophils (27, 28).

To obtain sufficient numbers of highly purified basophils for more detailed studies, we incubated SHIP+/+ and SHIP−/− BM for 10 d in the presence of IL-3 (29, 30). As shown in Fig. 3A (left panel), we found that in vitro-derived SHIP−/− BM cells, like freshly isolated BM and spleen cells, produced substantially more IL-4 in response to IL-3 than that of WT cells. Notably, both the proportion and total yield of SHIP−/−/basophils were less than half that from WT BM after 10 d of culturing whole BM with IL-3 (Fig. 3A, right panel, Supplemental Fig. 2B).

To verify that the basophils in these day-10 cultures were responsible for the IL-4 production, we compared IL-4 secretion from equal numbers of SHIP+/+ and SHIP−/− FcεRIα+ c-Kit+ basophils, FcεRIα+ c-Kit+ mast cells, and FcεRIα+ c-Kit− non-basophil, non-mast cells treated with or without IL-3 (Fig. 3B, left panel).
Flucytomycin of SHIP+/+ and SHIP−/− stimulated with IL-3 for 18 h. IL-4 levels were then determined by ELISA. Because, in addition to IL-3, FcεRI activation enhances IL-3-induced IL-4 production from basophils (10), we next asked what effect the absence of SHIP might have on FcεRI-induced IL-4 production. As shown in Fig. 4A, in vitro-derived SHIP−/− basophils, and to a lesser extent SHIP+/+ cells, produced IL-4 in response to IgE (SPE-7) alone or anti-FceRI Ab (MAR-1). Of note, the combination of IL-3 with IgE or with anti-FceRI Ab alone, and this was especially true for SHIP+/+ mice and SHIP−/− basophils (Fig. 4C), mast cells (FceRIα−c-Kit−), and other cells (FceRIα−c-Kit−) were sorted using flow cytometry. Right panel, The three cell subsets were then stimulated with IL-3 for 18 h and IL-4 levels in cell supernatants determined by ELISA. C, The FceRIα−c-Kit− cells from B were stimulated with the indicated concentrations of IL-3 for 18 h and IL-4 levels in cell supernatants determined by ELISA. D, Freshly isolated SHIP+/+ and SHIP−/− BM basophils obtained by NKG2D− and then DX5+ selection (left panel) or in vitro-derived BM basophils obtained by DX5+ selection (right panel) were stimulated with IL-3 for 18 h and IL-4 levels in cell supernatants determined by ELISA. *p < 0.05 (significantly different between SHIP+/+ and SHIP−/− cells).

Because, in addition to IL-3, FceRI activation has been shown to stimulate IL-4 production from basophils (10), we next asked whether SHIP−/− basophils on a per cell basis, produced 10-fold more IL-4 in response to IL-3 than that of WT basophils and produced more IL-4 in the absence of IL-3 than that produced by WT cells in the presence of IL-3. The other two cell fractions did not contribute significantly to the total IL-4 produced. IL-3 dose-response studies revealed that all doses of IL-3 tested induced higher levels of IL-4 in SHIP−/− than those in SHIP+/+ basophils (Fig. 3C).

Because using anti-FceRI Abs to purify basophils might induce their activation, we also enriched basophils from freshly isolated BM based on their NKG2D− CD49b+ phenotype and in vitro-derived basophils based on their CD49b+ or simply c-Kit+ phenotype. Once again, using these different enrichment strategies, we found that SHIP−/− cells produced far more IL-4 in response to IL-3 than that produced by their WT counterparts (Fig. 3D, Supplemental Fig. 2C).

FceRI activation enhances IL-3-induced IL-4 production from SHIP−/− basophils

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Because basophils have been reported to induce type 2 immune responses under certain circumstances, via IL-4 production (25), we asked if SHIP−/− basophils were more capable of activating type 2 responses than were WT basophils. To address this, we first cocultured SHIP+/+ or SHIP−/− basophils with WT M-CSF–derived BM Mφs with or without IL-3 for 48 h. The adherent cells (Mφs) were then subjected to Western analysis and, as shown in Fig. 5A, the addition of IL-3–activated SHIP−/− basophils for 48 h was far more potent than WT basophils at skewing the WT Mφs to an M2 phenotype (i.e., expressing high levels of the M2 markers Arg I and Ym1). Treatment with neutralizing anti–IL-4 Ab blocked this M2 skewing. These results indicate that SHIP−/− basophils, which produce higher levels of IL-4, are strong M2 skewers.

To investigate specifically the relative effects of SHIP+/+ and SHIP−/− basophils on Th1 versus Th2 cell development, CD4+ tested two non-cytokinergic IgEs (i.e., clone 27-74 and C38-2), and found they did not enhance IL-4 production from either SHIP+/+ or SHIP−/− basophils (data not shown). Because Ag-dependent stimulation, which is induced by the aggregation of IgE/FcεRI complexes with multivalent Ags, is also thought to play an important role in basophil and mast cell activation, we also stimulated SHIP+/+ and SHIP−/− basophils with IgE plus Ag. As shown in Fig. 4B, basophils produced IL-4 in response to Ag-specific cross-linking, and enhanced IL-4 production was detected from SHIP−/− basophils. We carried out similar experiments with freshly isolated BM basophils but found that IgE and anti-FcεRIα were ineffective at augmenting IL-3–induced IL-4 production from either SHIP+/+ or SHIP−/− basophils (Fig. 4C). We also found IgE plus Ag was a weak stimulator of IL-4 production from these cells (Fig. 4D). Related to this we found that, unlike in vitro-generated basophils (and BM-derived mast cells; data not shown), freshly isolated basophils already had IgE on their surfaces (Fig. 4E, 4F), and it is possible that the presence of this IgE prevented the binding of the highly cytokinergic SPE-7 IgE from attaching. This would explain why neither SPE-7 IgE nor SPE-7 IgE plus Ag (DNP–HSA) could stimulate IL-4 production, because the Ag was specific for the SPE-7 IgE.

**FIGURE 4.** FcεRI stimulation augments IL-3–induced IL-4 production from in vitro-derived but not freshly isolated basophils. A and B, SHIP+/+ and SHIP−/− in vitro-derived basophils were stimulated (A) with 1 μg/ml IgE (SPE-7) or anti-FcεRIα Ab with or without IL-3 or (B) with 1 μg/ml IgE (SPE-7) plus DNP–HSA for 18 h and IL-4 levels in cell supernatants determined by ELISA. C and D, Freshly isolated NKG2D−DX5+ basophils from SHIP+/+ and SHIP−/− BM were stimulated (C) with IL-3 alone or in combination with 1 μg/ml IgE (SPE-7) or anti-FcεRIα Ab or (D) with IgE (SPE-7) with or without DNP–HSA for 18 h and IL-4 levels in cell supernatants determined by ELISA. Similar results were obtained in two independent experiments for A–D. E and F, In vitro-derived (E) and freshly isolated (F) BM basophils were stained with FITC–anti-IgE Ab and allophycocyanin–anti–c-Kit (E) or with FITC–anti-IgE Ab and allophycocyanin–anti-CD49b (F) and subjected to flow cytometric analysis. *p < 0.05 (significantly different between SHIP+/+ and SHIP−/− cells).

**FIGURE 5.** SHIP−/− basophils promote type 2 immune responses. A, SHIP+/+ or SHIP−/− in vitro-derived basophils were cocultured with SHIP+/+ M-CSF–derived BM Mφs with or without IL-3 for 48 h, and then nonadherent basophils were washed out and adherent Mφs subjected to Western analysis. B and C, CD4+ T OT-II spleen cells were cocultured for 4 d with APCs in the presence of OVA-peptide and in vitro-derived basophils. Expanded CD4+ T cells were sorted again and either restimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab for 18 h and IFN-γ and IL-4 levels in cell supernatants assessed (B) or stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab in the presence of Golgi Plus for 5 h for the analysis of IL-4/IFN-γ–producing cells (C). D, SHIP+/+ or SHIP−/− B220+ B cells were stimulated with anti-CD40 Ab for 4 d with or without IL-3–stimulated, in vitro-derived SHIP+/+ or SHIP−/− basophils and IgE levels in culture supernatants determined by ELISA. *p < 0.05 (significantly different between SHIP+/+ and SHIP−/− basophils).
T cells from OVA-TCR transgenic OT-II mice were cocultured with T cell-depleted APCs and OVA-peptide with or without SHIP+/+ or SHIP−/− basophils. As shown in Fig. 5B, in the absence of added basophils, both Th1 and Th2 skewing occurred as suggested by the secretion of both IFN-γ and IL-4 from activated CD4+ T cells. The addition of SHIP+/+ basophils augmented the generation of Th2 cells, indicated by the enhanced IL-4 and reduced IFN-γ production (Fig. 5B). However, SHIP−/− basophils were far more effective than SHIP+/+ basophils at stimulating IL-4 production, and this Th2 skewing was totally abolished with anti-IL-4 Abs (Fig. 5B). We also assessed the population of Th1 and Th2 cells in these experiments by intracellular flow cytometry. As shown in Fig. 5C, more IL-4–producing CD4+ T cells were generated by coculture with SHIP−/− basophils (4.0%) than with SHIP+/+ basophils (3.1%) or with no basophils (2.2%).

We also compared the ability of SHIP+/+ and SHIP−/− basophils to induce IgE production from CD40-stimulated WT splenic B cells and found that SHIP−/− basophils induced far more IgE production from WT B220+ B cells than that induced by SHIP+/+ basophils (Fig. 5D). Once again, this was totally abolished with anti-IL-4 Abs (Fig. 5D).

The PI3K pathway positively regulates IL-4 production from basophils.

To address why SHIP−/− basophils produced more IL-4 in response to IL-3 or to IgE (with or without Ag), we first asked if it was due to higher IL-3R or FcεRI levels at their cell surfaces. As shown in Supplemental Fig. 3A and 3B, the cell surface levels of IL-3Rα and the common β receptor (βc) and FcεRIα were similar on the surfaces of SHIP+/+ and SHIP−/− basophils. We next looked intracellularly and found that SHIP became tyrosine phosphorylated after 5 min of stimulation with IL-3, IgE, IgE plus Ag or anti-FcεRIα, with the greatest phosphorylation occurring with IL-3 (Fig. 6A). We then focused on IL-3 stimulation and analyzed the activation of the STATs5, Erk, and PI3K pathways. As shown in Fig. 6B, a 5-min exposure to IL-3 stimulated maximal phosphorylation of SHIP, STAT5, Akt, and Erk. STAT5 and Erk were phosphorylated to the same degree in SHIP+/+ and SHIP−/− basophils. However, as expected, there was a far stronger phosphorylation/activation of Akt in the SHIP−/− basophils, consistent with SHIP being a negative regulator of the PI3K pathway, via hydrolysis of PIP3 to PI-3,4-P2 (Fig. 6B). To determine if the enhanced activation of the PI3K pathway might be responsible for the enhanced production of IL-4 in SHIP−/− basophils, we stimulated SHIP−/− basophils with IL-3 with or without the PI3K inhibitors, LY or W. As shown in Fig. 6C, both LY and W dramatically suppressed IL-4 production from SHIP−/− basophils, whereas a Jak2 inhibitor, AG490, and the Erk pathway inhibitor, PD98059 and UO126, at concentrations that completely inhibited phosphorylation of their target proteins (data not shown), only partially reduced IL-4 production from SHIP−/− basophils.

The three catalytic subunits of class IA PI3Ks are p110α, β, and δ and that of class IB PI3Ks is p110γ. We found that all of these proteins were expressed in both SHIP+/+ and SHIP−/− basophils (Fig. 6D). To test which isoform(s) was involved in IL-3–induced IL-4 production, SHIP−/− in vitro-derived basophils were stimulated with IL-3 with or without isoform-specific inhibitors and their effects on IL-4 production assessed. Initial dose-response studies with these inhibitors established their relative potencies (Supplemental Fig. 4A, 4B). As shown in Fig. 6E, treatment with the p110α inhibitor at 50 nM dramatically suppressed IL-3–induced IL-4 production from SHIP−/− basophils, whereas 10 μM concentrations of the p110β and δ inhibitors only partially inactivated basophils. Therefore, we stimulated SHIP+/+ and SHIP−/− basophils with IL-3 with or without 10 μM p110α (α), 10 μM p110β (β), 10 μM p110γ–dual (γ), 10 μM p110δ (δ), and 10 μM p110γ–dual (β, δ) inhibitors for 15 h and IL-4 levels in cell supernatants determined by ELISA. *p < 0.05 (significantly different between inhibitor treated and nontreated cells). F, SHIP+/+ and SHIP−/− in vitro–derived basophils were stimulated with 10 ng/ml IL-3 with or without 10 μM LY, 300 nM W, 50 μM AG490 (AG), 20 μM PD98059 (PD), or 10 μM UO126 (UO) for 18 h and IL-4 levels in cell supernatants determined by ELISA. *p < 0.05 (significantly different between inhibitor-treated and nontreated cells). G, Cell lysates of SHIP+/+ and SHIP−/− in vitro–derived basophils were subjected to Western analysis using anti-p110α, β, δ, and γ Abs. E, SHIP+/+ and SHIP−/− in vitro–derived basophils were stimulated with 10 ng/ml IL-3 with or without 10 μM MLY, 50 nM p110α (α), 10 μM p110β (β), 1 μM p110γ–dual (β, δ), 10 μM p110δ (δ), and 10 μM p110γ–dual (β, δ) inhibitors for 15 h and IL-4 levels in cell supernatants determined by ELISA. *p < 0.05 (significantly different between inhibitor treated and nontreated cells). F, SHIP+/+ and SHIP−/− in vitro–derived basophils were stimulated with 10 ng/ml IL-3 with or without 10 μM MLY, 50 nM p110α (α), 10 μM p110β (β), 1 μM p110γ–dual (β, δ), 10 μM p110δ (δ), and 10 μM p110γ–dual (β, δ) inhibitors for 15 h and IL-4 levels in cell supernatants determined by ELISA. *p < 0.05 (significantly different between inhibitor treated and nontreated cells).
hhibited and 10 μM of the γ inhibitor had no effect, because IL-3R is not a G-coupled receptor. Even taking into consideration the 10- to 20-fold lower IC_{50} of the α inhibitor (Supplemental Fig. 4C), this inhibitor was at least 10-fold more potent at inhibiting IL-3–induced IL-4 production from SHIP^{−/−} basophils. However, an important caveat in the signaling studies shown in Fig. 6A–E is that they were carried out with in vitro-derived basophils because it was difficult to obtain sufficient freshly isolated basophils for Western analysis. Because the signaling characteristics of basophils derived from 10–12 d in vitro with IL-3 might not reflect those of in vivo-derived basophils, we also examined the effects of LY and the PI3K isoform specific inhibitors on freshly isolated, IL-3–stimulated basophils. As shown in Fig. 6F, both LY and the p110α inhibitor markedly inhibited freshly isolated BM basophils.

Because the PI3K pathway is important, in general, for cell survival, we wanted to rule out that the inhibitory effects on IL-4 production that we observed with the p110α inhibitor was not simply due to reduced viability of the basophils. To do this, we stimulated in vitro-derived basophils with IL-3 for 15 h with or without the PI3Kα inhibitor, as in Fig. 6F, and saw no significant differences in either total trypan blue-negative or annexin V-positive cell numbers among nontreated, LY-treated, or PI3Kα-treated cells (Supplemental Fig. 4D, 4E). These results suggest that enhanced activation of the PI3K pathway is responsible for the overproduction of IL-4 from SHIP^{−/−} basophils and that the PI3Kα isoform of PI3K likely plays a critical role in activating this pathway. Also, because Xiao et al. (32) recently reported that mice deficient in both Lyn and Hck, which are thought to phosphorylate/activate SHIP, show a similar phenotype to SHIP^{−/−} mice, including M2 MΦ expansion in vivo, we assessed the IL-4 production from BM cells from Lyn^{−/−}, Hck^{−/−}, and Lyn/Hck^{−/−} BM cells. As shown in Fig. 6G, Lyn/Hck^{−/−} BM cells produced higher levels of IL-4 in response to IL-3 than those produced by Lyn^{−/−} or Hck^{−/−} BM cells. This suggests that IL-3–induced IL-4 production from basophils is regulated by Hck/Lyn-dependent SHIP activation.

In vivo depletion of basophils reduces the Th2 skewing of SHIP^{−/−} mice

In an attempt to test the in vivo contribution of SHIP^{−/−} basophils to the Th2-skewed phenotype of naïve SHIP^{−/−} mice, we treated mice with anti-FcεRI (MAR-1) Ab to deplete basophils [as in standard protocols (10)]. Unfortunately, the SHIP^{−/−} mice died of anaphylactic shock soon after the first MAR-1 treatment, likely because of hyperdegranulation of mast cells in these mice (Supplemental Fig. 5A–C) (33). We therefore generated a mast cell-deficient SHIP^{−/−} mouse model by crossing the Wsh/Wsh strain with a previously described inducible SHIP^{−/−} strain (SHIP^{FL/FL}, Mx1Cre) (17) to generate triple-transgenic mice homozygous for the Wsh andloxP-flanked SHIP alleles and with or without an inducible Cre transgene (Mx1Cre). After induction of Cre expression with a course of poly I:C treatments, and after a 4-wk period, Wsh/Wsh.SHIP^{FL/FL}.Mx1Cre^{−} (but not Mx1Cre^{−}) mice

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**FIGURE 7.** Basophil depletion attenuates Th2 skewing in SHIP^{−/−} mice. Wsh/Wsh (mast cell-deficient) mice homozygous for the floxed-SHIP allele (with or without Mx1Cre) were treated with poly I:C to induce SHIP-deletion and the mice were further treated with anti-FcεRI Ab (MAR-1) (or with an isotype control Ab) to deplete basophils in vivo. A, Splenocytes harvested from SHIP-deficient Wsh mice (Wsh-SHIP^{−/−}) or SHIP^{+/+} controls (Wsh-SHIP^{+/+}) were stimulated with (+) or without (−) IL-3 and the concentration of IL-4 secreted into the supernatant over 18 h determined. Basophil-depleted splenocytes (w/o basophils) and nondepleted mice (w/basophils) are compared. B, Separate splenocyte cultures (as in A) were stimulated with (+) or without (−) anti-CD3 to determine the Th proclivity of SHIP-deficient Wsh/Wsh splenocytes with (w/) and without (w/o) basophils. Open symbols represent SHIP-deficient Wsh splenocytes; closed symbols represent SHIP^{+/+} Wsh controls. The mean IL-4 concentration for each condition is indicated with a horizontal line, and the error bars are the SEM (n = 4 to 6 mice per group). *p < 0.05 (significantly different). WshSHIP^{−/−} and WshSHIP^{+/+} are abbreviations for Wsh/Wsh.SHIP^{FL/FL}.Mx1Cre^{−} and Wsh/Wsh.SHIP^{FL/FL}.Mx1Cre^{+} after poly I:C treatments. C, In the left panel, IL-4 levels were assessed by ELISA of culture supernatants from DX5^{+} or DX5^{−} plus DX5^{+} cells obtained after 6 d of in vitro culture of EasySep fractionated, adherence-depleted SHIP^{−/−} BM with 10 ng/ml IL-3 plus 10 ng/ml M-CSF. p < 0.06 between DX5^{+} and DX5^{−} plus DX5^{+} cells. The right panel shows a Western blot analysis of the adherent macrophages from this same study, using Abs to Arg I, Ym1, and SHC.
developed splenomegaly and showed no expression of SHIP in peritoneal MØs by Western analysis (data not shown) and a Th2-skewed phenotype similar to conventional SHIP<sup>−/−</sup> mice. We next treated these SHIP-deficient Wsh/Wsh mice (designated Wsh-SHIP<sup>−/−</sup>) with MAR-1 to deplete basophils in vivo. As shown in Fig. 7A, ex vivo stimulation of Wsh-SHIP<sup>−/−</sup> splenocytes with IL-3 resulted in enhanced IL-4 production compared with that of Wsh-SHIP<sup>+/+</sup> controls, consistent with a SHIP-null phenotype. More importantly, in vivo depletion of basophils attenuated the enhanced IL-4 production in IL-3–stimulated Wsh-SHIP<sup>−/−</sup> splenocyte cultures (Fig. 7A). These results suggest that basophils (and not mast cells) are responsible for the enhanced IL-4 production of SHIP<sup>−/−</sup> splenocytes in response to IL-3. To determine directly the Th-proliferacy of Wsh-SHIP<sup>−/−</sup> and Wsh-SHIP<sup>+/+</sup> mice, we next stimulated splenocytes with anti-CD3. As shown in Fig. 7B, the enhanced IL-4 production of anti-CD3–treated Wsh-SHIP<sup>−/−</sup> splenocytes was neutralized by basophil depletion. Complicating the interpretation of these results, however, are very recent studies by Hammad et al. (34), Ohnmacht et al. (35), and Phythian-Adams et al. (36) suggesting that a previously uncharacterized subpopulation of dendritic cells (DCs) (i.e., CD11c<sup>+</sup> FcεRI<sup>+</sup> CD11b<sup>+</sup> DX5<sup>+</sup> cells) may also be depleted by MAR-1 treatment and play an important role in Th2 induction, at least under some circumstances. To assess the role of these inflammatory DCs in our system, we compared the ability of DX5<sup>−</sup> (containing putative FcεRI<sup>+</sup> DCs and MØs) versus DX5<sup>+</sup> (MØs) plus DX5<sup>+</sup> (basophils) BM cells to secrete IL-4 and M2-skw our Mφ progenitors and found that only the DX5<sup>−</sup> plus DX5<sup>+</sup> cells were capable of doing this (Fig. 7C). This is consistent with our data showing that DX5<sup>+</sup> freshly isolated or in vitro-derived basophils secrete IL-4 in response to IL-3 (Fig. 3D) and/or FcεRI stimulation (Fig. 4C, 4D). Also of note, both Hammad et al. (34) and Phythian-Adams et al. (36) found that their FcεRI<sup>−/−</sup> BM cells were only induced after allergen exposure or infection [with house dust mite extract (34) or the helminth Schistosoma mansoni (36), respectively] and were not detectable in naive mice. Because our studies were carried out with naive mice, our results do not preclude a prominent role for FcεRI<sup>−/−</sup> DCs in inflammation-induced Th2 polarization.

Our model of IL-3–induced hyperproduction of IL-4 from SHIP<sup>−/−</sup> basophils is shown in Fig. 8 and is discussed later.

**Discussion**

In this study, we demonstrate a role for SHIP as a repressor of Th2 skewing. Our results suggest that it does this, at least in part, by inhibiting IL-3– and IgE-induced IL-4 production from basophils. These findings extend recent work from our laboratory showing that IL-3 promotes the survival/proliferation and differentiation of SHIP<sup>−/−</sup> basophil progenitors within the BM and also stimulates these basophils, via STAT5, to produce high levels of IL-4. This IL-4, in turn, skews Mφ progenitors to an M2 phenotype (19). Together with our current study, this suggests that IL-3 plays a more prominent role than previously recognized in both M2 and Th2 skewing and may do so, in large part, by promoting the survival and activation of basophils. This is supported by recent studies showing that IL-3, produced by T cells, is critical for the dramatic increase in blood basophils that occurs in mice after nematode infections (37, 38). In addition, BALB/c mice deficient in both βc and the IL-3–specific β receptor (β<sub>βc</sub>-IL-3) have been reported to display reduced type 2 immune responses, including reduced allergic airway inflammation (39), and it has been proposed that allergen challenge of allergic patients may lead to blood basophils entering the sites of inflammation and interacting with activated tissue mast cells (40). This would allow IL-3, secreted by the activated mast cells, to trigger IL-4 production from the incoming basophils and thus stimulate Th2 polarization (40). Related to this, it has been recently shown that the specific loss of SHIP in mast cells, which increases their cytokine production (41), increases allergic inflammation in vivo (42). Thus, in the SHIP knockout mouse, SHIP<sup>−/−</sup> mast cells, by producing elevated levels of IL-3, may collaborate with SHIP<sup>−/−</sup> basophils, which produce elevated levels of IL-4 in response, to skew to a Th2 phenotype.

In terms of how IL-3–induced IL-4 production is regulated in basophils, we propose, as shown in our model in Fig. 8, that IL-3 binds to an IL-3R/FcεR complex on basophils, as Hida et al. (30) recently demonstrated that the FcεR-chain is a constitutive component of the IL-3Rβ chain on the surface of basophils and is required for IL-3–induced IL-4 production. This binding activates Jak2 to tyrosine phosphorylate both the IL-3Rβ–chain, which attracts STAT5 and PI3K (43), and the FcεR-chain on its ITAM, to attract Syk (30). These attracted proteins all contribute to the induction of IL-4 gene expression. At the same time, IL-3 also activates Lyn and Hck to tyrosine phosphorylate SHIP, which then translocates to the plasma membrane to hydrolyze PI3K-generated PIP<sub>3</sub> to PI-3,4-P<sub>2</sub> and thus dampen down IL-4 production. Consistent with this model, Rivera’s group (10) recently published an elegant study showing that Lyn-deficient mice are Th2 skewed and this skewing is dependent on basophils, IgE, and IL-4. Because we find that Lyn/Hck double knockout BM cells produce substantially higher levels of IL-4 in response to IL-3 than those produced by either Lyn or Hck single knockout BM cells (Fig. 6G), we predict that Lyn/Hck double knockout mice would be far more Th2 skewed than Lyn<sup>−/−</sup> mice. Related to this, Xiao et al. (32) have recently shown that Lyn/Hck double knockout mice possess a similar...
phenotype to SHIP-deficient mice, with both suffering from a myeloproliferative disorder, M2 Mb expansion, and severe lung inflammation. More importantly, these same authors found that Lyn and Hck are upstream plasma membrane recruiters/activators of SHIP and that the Lyn/Hck phenotype can be corrected by targeting SHIP to the membrane (32). Lyn and Hck, in turn, have been shown to be activated, via Jak2, by IL-3, IL-5, or GM-CSF and can directly bind to the tyrosine phosphorylated βc receptor (44, 45). As well, we found enhanced phosphorylation of Akt in IL-3–induced SHIP+/− basophils and inhibition of IL-3–induced IL-4 production with both classic PI3K pathway inhibitors and isoform-specific PI3K inhibitors. The activation of p110α (PI3Kα), in particular, may be important for the enhanced production of IL-4 from SHIP−/− basophils. We also find that IL-3–induced IL-4 production is augmented by costimulation with highly cytokinergic IgE or with anti-FcεRI Abs in both SHIP+/+ and SHIP−/− basophils and that IgE plus Ag, like IL-3, induces higher levels of IL-4 from SHIP−/− than from WT basophils. These data suggest that SHIP restrains IL-4 production in response to innate (IL-3–induced) and acquired (IgE plus Ag) stimuli.

Although basophils are the least common leukocyte, representing less than 1% of blood leukocytes, BM cells, and spleen cells in normal human and mice, their numbers increase dramatically during parasitic infections and allergic inflammation (46, 47). Similar to mast cells, they are derived from BM progenitors in response to IL-3 and express the FcεRI and various inflammatory mediators such as histamine and serotonin. Because of this, basophils were considered for many years as minor circulating mast cells. However, several recent reports indicate a clear distinction between mast cells and basophils as the latter rapidly produce far higher levels of IL-4 after cytokine stimulation or IgE cross-linking (48). Related to this, a recent report suggests that IL-4 transcription is regulated by different enhancer regions in Th2 cells, mast cells, and basophils (49). Basophils are currently in the limelight because of their recently appreciated role in promoting Th2 differentiation via their production of IL-4 (47, 50, 51) and their ability to present allergen and helminth Ags within MHC class II to CD4+ T cells in draining lymph nodes (25, 52–54). Related to this, a recent report suggests that SHIP restrains IL-4 production in response to innate (IL-3–induced) and acquired (IgE plus Ag) stimuli.

Disclosures

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

References


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**Supplementary Fig 1.** IL-3 stimulated SHIP-/- BM and spleen cells produce higher levels of various cytokines. SHIP+/+ and -/- BM and spleen cells were stimulated or not with IL-3 for 18 hrs and the cell supernatants subjected to inflammatory and Th1/Th2 CBAs. *p< 0.05, significantly different between SHIP+/+ and -/- cells. Similar results were obtained in 2 independent experiments.

**Supplementary Fig 2.** (A). Cytospin morphology of FcεRIα+ c-kit- basophils from BM. (B) The yield of FcεRIα+ c-kit- cells from 10 day in vitro-derived BM cells. SHIP+/+ and -/- BM cells (5 x 10^6 cells) were cultured with IL-3 for 10 days, and FcεRIα+ c-kit- cell numbers determined by flow cytometry. SHIP-/- BM cells generate reduced numbers of basophils because many progenitors differentiate into macrophages or macrophage precursors. (C) IL-4 production by enriched, in vitro-derived basophils. SHIP+/+ and -/- in vitro-derived BM cells were starved and then c-kit positive cells depleted using anti-c-kit Abs and EasySep. C-kit depleted cells were stimulated with IL-3 for 18 hrs and IL-4 in cell supernatants determined by ELISA. *p< 0.05, significantly different between SHIP+/+ and -/- cells. Similar results were obtained in 2 independent experiments.

**Supplementary Fig 3.** Flow cytometric analysis of the cell surface expression of (A) FcεRIα and (B) IL-3Rα and βc (shaded histograms) on freshly isolated and in vitro-derived BM basophils. Basophils were gated as FcεRI (FITC)+ CD49b(APC)+ and then IL-3Rs were detected with PE-labeled IL-3R Ab. Unshaded histograms are isotype-matched controls. These results demonstrate that the FcεRIα levels as well as IL-3Rα and βc levels are similar on the cell surface of in vitro-derived or freshly isolated SHIP+/+ and -/- BM basophils.

**Supplementary Fig 4.** PI3K inhibitors suppress IL-3-induced IL-4 production from SHIP-/- basophils. (A) SHIP+/+ and -/- in vitro-derived basophils were treated ± 10 ng/ml IL-3 ± 10 μM LY or the p110α and β inhibitors at the indicated concentrations for 15 hrs and IL-4 levels in cell supernatants determined by ELISA. (B) SHIP+/+ and -/- in vitro-derived basophils were treated ± 10 ng/ml IL-3 ± 10 μM LY or the p110α, β, δγ, δ or γ inhibitors at the indicated concentrations for 15 hrs and IL-4 levels in cell supernatants determined by ELISA (D & E) Enriched basophils were stimulated with IL-3 ± PI3K inhibitors for 15 hrs and trypan blue negative cells were
counted (D) and the cells were then stained with PE-annexin V Abs. Annexin V positive cells were determined by flow cytometric analysis (E) The number of apoptotic cells was not significantly increased by treatment with the PI3Kα inhibitor.

Supplementary Fig 5. Anti-FcεRI (MAR-1) administration causes SHIP-/- mast cell degranulation and anaphylaxis in SHIP-/- mice. (A) SHIP+/+ (stippled boxes) and -/- (black boxes) BMMCs were starved overnight, treated ± IgE (SPE-7) or the indicated concentration of MAR-1, and degranulation determined as previously described (33). (B) SHIP+/+ and -/- BMMCs were starved overnight in the presence of 2 μg/ml non-cytokinergic IgE (clone 27-74), stimulated with the indicated concentration of MAR-1 and degranulation determined. (C) SHIP+/+ (■) and SHIP-/- (△) mice (6-8 weeks old) were given 5 μg/mouse MAR-1 i.p. and temperatures measured using a mouse rectal thermometer at the indicated times.
Cytokines produced by BM cells

Cytokines produced by Spleen cells

Supplementary Fig 1

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Supplementary Fig 2
Supplementary Fig 4  

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