SHIP Represses the Generation of IL-3-Induced M2 Macrophages by Inhibiting IL-4 Production from Basophils[1]

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There is a great deal of interest in determining what regulates the generation of classically activated (M1) vs alternatively activated (M2) macrophages (M𝜙s) because of the opposing effects that these two M𝜙 subsets have on tumor progression. We show herein that IL-3 and, to a lesser extent, GM-CSF skew murine M𝜙 progenitors toward an M2 phenotype, especially in the absence of SHIP. Specifically, the addition of these cytokines, with or without M-CSF, to adherence- or lineage-depleted (Lin⁻) SHIP⁻/⁻ bone marrow (BM) cells induces high levels of the M2 markers, arginase I, and Ym1 in the resulting mature M𝜙s. These in vitro-derived mature M𝜙s also display other M2 characteristics, including an inability to enhance anti-CD3-stimulated splenic T cell secretion of IFN-γ and low IL-12 and high IL-10 production in response to LPS. Not surprisingly, given that IL-3 and GM-CSF utilize STAT5 to trigger many downstream signaling pathways, this M2 phenotype is suppressed when STAT5⁻/⁻ BM cells are used. Unexpectedly, however, this M2 phenotype is also suppressed when STAT6⁻/⁻ BM cells are used, suggesting that IL-4- or IL-13-induced signaling might be involved. Consistent with this, we found that IL-3 and GM-CSF stimulate the production of IL-4, especially from SHIP⁻/⁻ Lin⁻ BM cells, and that neutralizing anti-IL-4 Abs block IL-3-induced M2 skewing. Moreover, we found that basophil progenitors within the Lin⁻ BM are responsible for this IL-3- and GM-CSF-induced IL-4 production, and that SHIP represses M2 skewing not by preventing skewing within M𝜙s themselves but by inhibiting IL-4 production from basophils. *The Journal of Immunology*, 2009, 183: 3652–3660.

The phenotypic heterogeneity of macrophages (M𝜙s)[4] is currently in the spotlight because of the dramatic effects that different subsets have on microbial infections, auto-immune disorders, and cancer (1–3). The initial inflammatory response to infectious agents and aberrant cells, for example, is typically conducted by classically activated (M1) M𝜙s, which attack invading microorganisms and tumor cells, and promote Th1-specific immune responses (3). In contrast, the resolution phase of inflammation is mediated by alternatively activated (M2) M𝜙s, which are hyporesponsive to inflammatory stimuli and are involved in debris scavenging, angiogenesis, tissue remodeling, wound healing, and the promotion of Th2-specific immunity (1–3). M𝜙 heterogeneity is induced by the microenvironment, with M1

M𝜙s typically developing in response to IFN-γ and microbial products such as LPS (3). M2 M𝜙s, on the other hand, are far more heterogeneous and have, to date, been subdivided into at least three subsets, that is, M2a M𝜙s, induced by type 2 cytokines, M2b M𝜙s, induced by immune complexes and LPS, and M2c M𝜙s, induced by deactivation factors such as IL-10, TGFβ, or glucocorticoids (3). Each M𝜙 subset has distinct functions within the innate immune system and contributes to the balance between "destruction" and "regeneration".

Similar to the Th1/Th2 balance, M1/M2 M𝜙 polarization is closely related to disease progression. In the case of cancer, infiltrating monocytes are often hijacked by tumors to become M2-like tumor-associated M𝜙s (TAMs) that enhance tumor growth and immune system evasion (1–3). In this regard, it has been suggested that the manipulation of M𝜙s toward an M1 phenotype could constitute a new form of therapy for cancer (1–3). In support of this, several recent reports have shown that manipulation of M1/M2 skewing has a profound effect on tumor growth. For example, the combination of CpG plus an anti-IL-10R Ab has been shown to switch infiltrating monocytes from an M2 to an M1 phenotype (4, 5). As well, a DNA vaccine against legumain, a member of the asparaginyl endopeptidase family overexpressed by M2-like TAMs, induces a strong CD8⁺ T cell response (6). A number of intracellular signaling proteins have also been implicated in M1/M2 skewing, with the inhibition of STAT3 or STAT6 skewing M𝜙s to an M1 phenotype (7–10). On the other hand, inhibition of NF-κB activity, by nuclear localization of the p50 NF-κB inhibitory homodimer, leads to M2 skewing and tumor progression (11). Some reports indicate that depletion of M𝜙s reduces angiogenesis and tumor progression in several experimental tumor models (12, 13). All of these findings are consistent with most TAMs having M2 M𝜙-like properties and that skewing these TAMs toward an M1 phenotype would have beneficial, antitumor activities. Because of this, there is currently a

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[4] Abbreviations used in this paper: M𝜙, macrophage; Arg I, arginase I; BM, bone marrow; Lin⁻, lineage depleted; TAM, tumor-associated macrophage; WT, wild type.

great deal of interest in understanding what determines M1 vs M2 Mφ skewing. In this regard, we recently reported that SHIP, which is a potent negative regulator of the PI3K pathway in hematopoietic cells (14, 15), represses the generation of M2 Mφs (16). This suggests that enhanced activation of the PI3K pathway facilitates M2 skewing in vivo and, consistent with this, we found that in vivo tumor growth is significantly faster in SHIP$^{-/-}$ mice and that tumor-infiltrated Mφs in these mice display a strong M2 phenotype (16). We (17) and others (18) have also recently found that IL-3 may, under some circumstances, be an important differentiation factor in the development of M2-like Mφs, and so we investigated whether IL-3 might have substantial M2 skewing ability in the absence of SHIP.

Specifically, in the present study, we have compared the ability of IL-3, GM-CSF, and IL-5 (which all utilize the common β-chain (βc) receptor subunit for intracellular signaling) to skew SHIP$^{+/+}$ and SHIP$^{-/-}$ Mφ progenitors to an M2 phenotype and found that IL-3, and to a lesser extent, GM-CSF, skew myeloid progenitors, especially from SHIP$^{-/-}$ bone marrow (BM), into M2 Mφs. Surprisingly, we found that these cytokines do not M2 skew myeloid progenitors directly but, instead, stimulate basophils within the BM cultures to secrete IL-4 and this, in turn, M2 skews the maturing Mφs. Moreover, we report herein that it is SHIP within the basophils, and not within the Mφs, that represses M2 skewing in response to IL-3 and GM-CSF, and it does so by inhibiting IL-3- and GM-CSF-induced IL-4 production. SHIP is thus an important negative regulator of basophil function.

Materials and Methods

Mice

SHIP$^{+/+}$ and SHIP$^{-/-}$ C57BL/6 × 129Sv mixed background mice were maintained in the Animal Research Centre at the British Columbia Cancer Research Centre under specific pathogen-free conditions. Bones from STAT5$^{-/-}$ C57BL/6 mice were provided by Dr. J. Ihle (St. Jude Children's Hospital, Memphis, TN). Bones from STAT6$^{-/-}$ C57BL/6 mice were from Dr. Richard A. Flavell (Yale University, New Haven, CT). All animal experiments were conducted according to the guidelines for the care and use of animals approved by the University of British Columbia.

Reagents

All cytokines were purchased from StemCell Technologies. The following Abs were used for Western blot analysis: anti-Ym1 (StemCell Technologies), anti-STAT5 and anti-SHIP (P1C1; Santa Cruz Biotechnology), anti-arginase I (Arg I; BD Biosciences), and anti-GAPDH (Fitzgerald Industries International). The following Abs were used for neutralization studies: anti-IL-3, anti-IL-4, and anti-IL-13 (R&D Systems). For flow cytometry, Alexa Fluor 647-conjugated rat anti-mouse CD206 was from AbD Serotec; FITC-conjugated rat anti-mouse CD86 (clone GL1) and FITC-conjugated rat anti-mouse MHC-II (clone 2G9) were from BD Pharmingen; Alexa Fluor 647-conjugated anti-mouse gp49 receptor, PE-labeled anti-IL-4, FITC-labeled anti-FcεR1α, and allophycocyanin-labeled anti-c-kit were from eBioscience. The PI3K inhibitors LY294002 and wortmannin were purchased from EMD Biosciences.

In vitro derivation of macrophages

BM cells were obtained by flushing murine femurs and tibias and either adherence depleted (2 h at 37°C) to remove mature Mφs or lineage-depleted (Lin$^-$) using an EasySep mouse hematopoietic progenitor enrichment kit containing Abs against CD5, CD11b, CD19, CD45R, 7-4, Ly-6G (Gr-1), and Ter119 (StemCell Technologies). The resulting BM cells were cultured in a total volume of 2 ml in IMDM (StemCell Technologies) plus 10% FCS (HyClone), 150 μM monothioglycerol (Sigma-Aldrich), 50 U/ml penicillin, and 50 μg/ml streptomycin (StemCell Technologies) in 12-well plates (BD Biosciences). M-CSF, GM-CSF, IL-3, IL-4, IL-5, IFN-γ, IL-12, or a combination of these cytokines (at 10 ng/ml where not specified) were added on day 0. After 6 days, nonadherent cells were vigorously removed and adherent cells were used for analysis. These day 6 cells were >95% mature Mφs, as assessed by double-positive staining for F4/80 and Mac-1, using flow cytometric analysis. In some experiments, various concentra-

tions of LY294002, wortmannin, or neutralizing Abs against IL-3, IL-4, or IL-13 (typically at 2.5 μg/ml) were added on day 0.

In vitro stimulations

For ELISAs, BM-derived Mφs were cultured at 5×10^5 cells/ml/well in 24-well plates (BD Biosciences) and stimulated with LPS for 18 h at 37°C. Cell-free supernatants were used for IL-12 and IL-10 ELISAs using cyokine ELISA kits (BD Bioscience) according to the manufacturer's instructions.

For Western analyses, cells were lysed with Nonidet P-40 lysis buffer (50 mM HEPES, 100 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 2 mM Na$_3$VO$_4$, 2 mM EDTA, 2 mM NaMoO$_4$, 0.5% Nonidet P-40) containing protease inhibitors (2 μg/ml leupeptin, 10 μg/ml aprotinin, 500 μM PMSF) to assess protein concentrations (using a BCA protein assay kit (Pierce) standardized with BSA). The lysates were then supplemented with concentrated SDS sample buffer to give 1× SDS sample buffer. If protein concentrations were not required, cells were lysed directly with 1× SDS sample buffer. All samples were boiled for 5 min and subjected to Western analysis as described previously (19).

To determine the effects of various Mφ populations on T cell activation, in vitro-derived Mφs (5, 2.5, or 1×10^4 cells) were cultured with spleen cells (2×10^6 cells) and stimulated with soluble anti-CD3 Ab (1 μg/ml) in a total volume of 1 ml in 24-well plates for 24 h at 37°C. Cell-free culture supernatants were used for IFN-γ and IL-4 ELISAs (BD Biosciences).

Analysis of apoptotic cells

Apoptotic cells were detected as annexin V$^+$ cells. Briefly, 1×10^5 of Lin$^-$ BM cells were cultured with 10 μM of LY294002 or DMSO at 37°C for 24 or 72 h. Cells were harvested, washed, and labeled with PE-conjugated anti-annexin V Ab (BD Bioscience) or appropriate isotype control Ab. Labeled cells were analyzed using flow cytometry.

Flow cytometry

Intracellular staining of IL-4 was conducted with Cytofix/Cytoperm (BD Bioscience) according to the manufacturer's instructions. Briefly, freshly obtained adhesion-depleted (2 h at 37°C) BM or Lin$^-$ BM cells (1×10^5) in 0.5 ml of IMDM plus 10% FCS were cultured with 0.5 μl of Golgi Plug (BD Bioscience) at 37°C for 5 min, stimulated with 10 ng/ml IL-3 for 5 h, harvested, washed, and suspended in Cytofix/Cytoperm solution at 4°C for 20 min. Fixed and permeabilized cells were harvested, washed again, and resuspended in BD Perm/Wash buffer containing PE-labeled anti-IL-4 Ab, FITC-labeled anti-FcεRIα Ab, and allophycocyanin-labeled anti-c-Kit Ab or appropriate isotype control Ab. Labeled cells were analyzed using flow cytometry. For cytospin experiments, SHIP$^{-/-}$ adhesion-depleted BM cells were stained with FITC-labeled anti-FcεR1α and allophycocyanin-labeled anti-c-kit Abs, sorted using flow cytometry, centrifuged onto glass slides, and stained with Giemsa by Diff-Quik (Dade Behring).

Statistical analysis

Statistical analyses were performed using Student's t test. A confidence level of <0.05 was considered significant. All experiments were performed a minimum of three times.

Results

IL-3 and GM-CSF skew SHIP$^{-/-}$ Lin$^-$ BM cells toward an M2 Mφ phenotype

We reported previously that peritoneal and alveolar Mφs from SHIP$^{-/-}$ mice, but not their SHIP$^{+/+}$ littermates, express Arg I and Ym1 levels, indicative of an M2 phenotype (16). However, in vitro M-CSF-derived BM Mφs from these same SHIP$^{-/-}$ mice did not express these M2 markers, suggesting that standard in vitro derivation conditions did not mimic in vivo differentiation (16). Since we (17) and others (18, 20–23) also found that IL-3 and GM-CSF were capable of supporting Mφ development from BM cells and that the resulting Mφs displayed characteristics that differed from M-CSF-derived Mφs, we asked if simply adding these cytokines during in vitro derivation of SHIP$^{-/-}$ Mφs might simulate in vivo differentiation conditions, that is, skew to an M2 phenotype. Specifically, Lin$^-$ progenitors from SHIP$^{+/+}$ and SHIP$^{-/-}$ BM were cultured for 6 days with M-CSF, IL-3, GM-CSF, or IL-5 or with M-CSF plus each of these three cytokines, all at 10 ng/ml, and the resulting adherent Mφs were analyzed for Arg
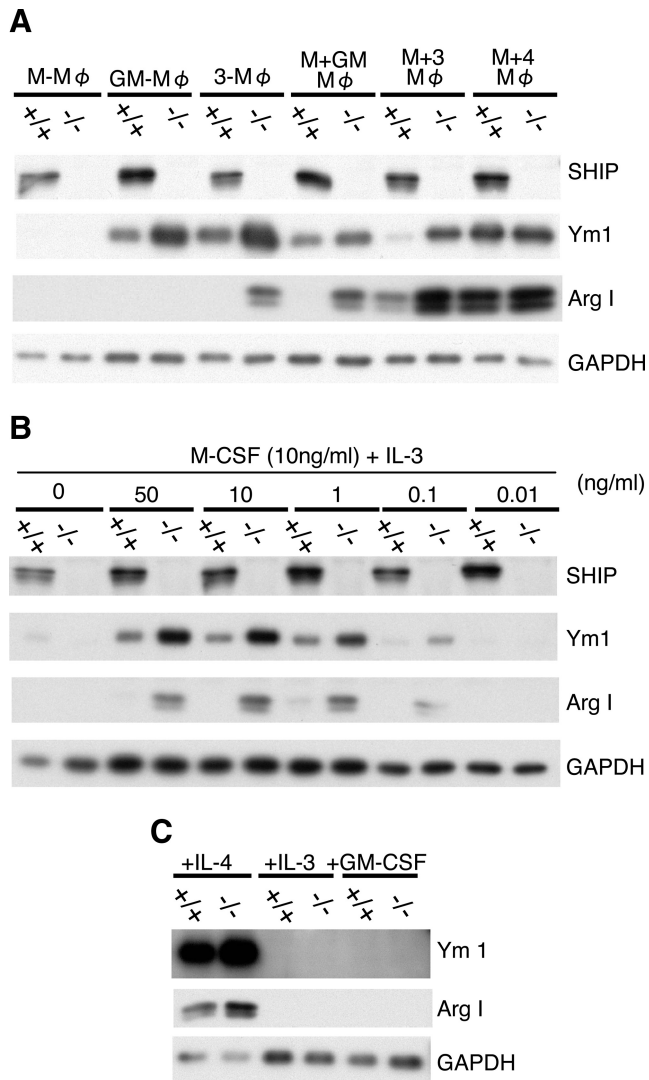
FIGURE 1. IL-3 and GM-CSF skew SHIP$^{-/-}$ Lin$^-$ BM cells to M2 Mϕs. *A*, Lin$^-$ progenitors from SHIP$^{+/+}$ and SHIP$^{-/-}$ BM cells were differentiated in the presence of M-CSF (M-Mϕ), GM-CSF (GM-Mϕ), IL-3 (3-Mϕ), M-CSF plus GM-CSF (M + GM-Mϕ), M-CSF plus IL-3 (M + 3-Mϕ) or M-CSF plus IL-4 (M + 4-Mϕ), all at 10 ng/ml, for 6 days. Cell lysates from mature adherent Mϕs were analyzed for SHIP, Ym1, Arg I, and GAPDH by Western blotting. *B*, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM progenitors were differentiated in the presence of 10 ng/ml M-CSF with the indicated concentrations of IL-3 for 6 days. Cell lysates from mature adherent Mϕs were analyzed for SHIP, Ym1, Arg I, and GAPDH by Western blotting. *C*, Day 6 M-CSF-derived SHIP$^{+/+}$ and SHIP$^{-/-}$ adherent Mϕs were treated with 10 ng/ml IL-4, GM-CSF, or IL-3 for 4 days. Cell lysates were analyzed for Ym1, Arg I, and GAPDH by Western blotting. Similar results were obtained for *A–C* in three independent experiments.

I and Ym1 expression. As shown in the first two lanes of Fig. 1*A*, M-CSF-derived SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs did not express Arg I or Ym1, consistent with our earlier findings (16). However, the presence of IL-3 and, to a lesser extent, GM-CSF led to the expression of both Arg I and Ym1 in the resulting mature SHIP$^{-/-}$ Mϕs, and some M2 skewing could also be seen, albeit at a much lower level, with wild-type progenitors. IL-5 had no effect (data not shown). Interestingly, adding M-CSF to IL-3- or GM-CSF-containing cultures increased Arg I but decreased Ym1 expression, suggesting that these M2 markers are differentially regulated. Also of note, when Lin$^-$ progenitors were cultured with M-CSF plus IL-4, as a positive control for M2 skewing (3), Arg I and Ym1

were prominently expressed at about the same level in SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs, and similar to that seen in M-CSF plus IL-3-derived SHIP$^{-/-}$ Mϕs. An IL-3 dose-response study revealed that as little as 0.1 ng/ml IL-3 was capable of stimulating detectable levels of Arg I and Ym1 in SHIP$^{-/-}$ Mϕs and that 10 ng/ml induced maximal expression (Fig. 1*B*). SHIP$^{+/+}$ progenitors, on the other hand, were far less responsive to IL-3.

We then asked if IL-3 or GM-CSF could M2 skew mature Mϕs, as has been reported for IL-4 (3, 16), or whether their effects were restricted to BM progenitors. To test this, M-CSF-derived mature Mϕs from SHIP$^{+/+}$ and SHIP$^{-/-}$ BM progenitors were treated with IL-4, IL-3, or GM-CSF for 4 days, and cell lysates were analyzed for M2 markers. As shown in Fig. 1*C*, IL-4, as expected, triggered the appearance of Ym1 and Arg I (16), but IL-3 and GM-CSF did not. Thus, it appeared that IL-3 and GM-CSF affected M2 skewing only during differentiation.

To determine whether it was SHIP's ability to hydrolyze phosphatidylinositol 3,4,5-trisphosphate and thus reduce PI3K activation that was responsible for restraining IL-3-induced M2 skewing in wild-type (WT) cells, Lin$^-$ SHIP$^{-/-}$ BM was differentiated with IL-3 in the presence of different concentrations of the PI3K inhibitor, LY294002 (LY). As shown in Fig. 2*A*, this prevented IL-3-induced M2 skewing of the resulting mature SHIP$^{-/-}$ Mϕs. Since the total number of mature Mϕs was significantly reduced following 6 days with 10 μM LY, we wanted to know if this was due simply to an overall reduced proliferation rate or altered differentiation, as might be expected, or apoptosis of a selected subset of progenitors with heightened M2 capacity by carrying out annexin V staining. As shown in Fig. 2*B*, we could not detect any apoptotic cells (annexin V$^+$ cells) during treatment with this concentration of LY.

Since Th1 and Th2 cytokines are known to modulate M1/M2 skewing (1–3), we then asked if the Th1 cytokines IFN-γ and IL-12 could prevent IL-3-induced M2 skewing. To test this, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM progenitors were differentiated in the presence of IL-3 with or without IFN-γ or IL-12 for 6 days, and the resulting mature Mϕs were analyzed for Arg I and Ym1 expression. As shown in Fig. 2*C*, IFN-γ dramatically suppressed IL-3-induced M2 generation of both SHIP$^{+/+}$ and SHIP$^{-/-}$ progenitors, while IL-12 had no effect.

IL-3- and GM-CSF-derived SHIP$^{-/-}$ Mϕs display M2 properties

To confirm that IL-3- and GM-CSF-derived SHIP$^{-/-}$ Mϕs possess an M2-like phenotype, we tested them for their ability to enhance T cell production of the Th1 cytokine IFN-γ. Specifically, since M2 Mϕs have been reported to be incapable of enhancing cytokine production from Th1 cells (1–3), we co-cultured M-CSF-, GM-CSF-, and IL-3-derived SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs, at three different cell concentrations, with SHIP$^{+/+}$ spleen cells and then treated with anti-CD3 Ab to stimulate splenic T cell activation. Preliminary dose-response studies established that Mϕ derivation with 10 ng/ml of each cytokine yielded plateau responses. As shown in Fig. 3*A*, M-CSF-derived SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs stimulated splenic T cells to secrete IFN-γ to the same degree, that is, substantially more than that elicited by anti-CD3-stimulated splenic T cells alone. Of note, none of the Mϕ populations secreted IFN-γ in the absence of spleen cells. Importantly, neither GM-CSF- nor IL-3-derived SHIP$^{-/-}$ Mϕs could enhance SHIP$^{+/+}$ splenic T cell IFN-γ production, consistent with their being M2 skewed. Of interest, the order of enhancing ability for the WT Mϕs, when 2.5 $\times$ 10^4 Mϕs were used, from strongest to weakest, was GM-CSF-, M-CSF-, and IL-3-derived Mϕs.
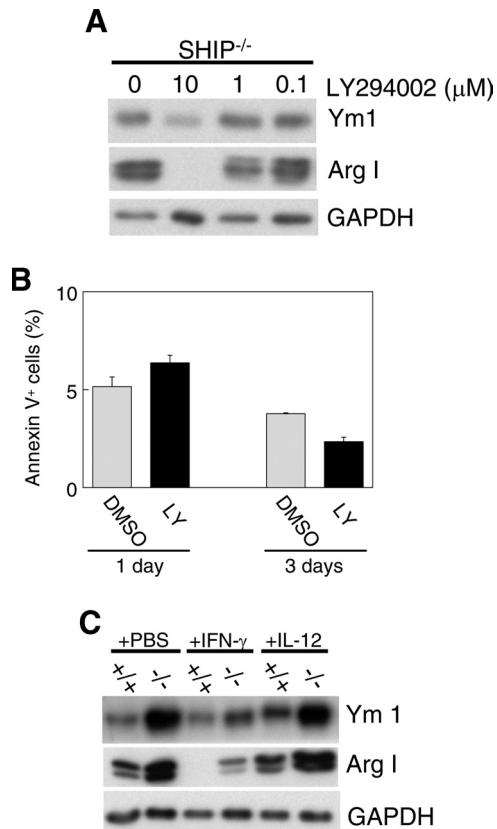
FIGURE 2. The IL-3-induced skewing of SHIP$^{-/-}$ Lin$^-$- BM cells to M2 Mϕs is inhibited by LY294002 and by IFN-γ but not by IL-12. *A*, SHIP$^{-/-}$ Lin$^-$ BM progenitors were differentiated in the presence of 10 ng/ml IL-3 and DMSO (LY vehicle control) or 10, 1, or 0.1 μM LY294002 (LY) for 6 days. Cell lysates from adherent Mϕs were analyzed for Ym1, Arg I, and GAPDH by Western blotting. *B*, SHIP$^{-/-}$ Lin$^-$ BM progenitors were treated with 10 μM LY294002 or DMSO for 24 or 72 h, labeled with PE-conjugated anti-annexin V, and subjected to flow cytometric analysis. Data shown are the means $\pm$ SEM of duplicate determinations. *C*, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM progenitors were differentiated in the presence of IL-3 with or withour IFN-γ or IL-12 (all at 10 ng/ml) for 6 days. Cell lysates from adherent Mϕs were analyzed for Ym1, Arg I, and GAPDH by Western blotting. Similar results were obtained for *A–C* in three independent experiments.

As well, we examined LPS-induced IL-12 and IL-10 secretion since LPS-stimulated M2 Mϕs have been reported to secrete low levels of the proinflammatory cytokine IL-12 and high levels of the antiinflammatory cytokine IL-10 (1–3). Specifically, we compared LPS-stimulated production of IL-12 and IL-10 from M-CSF-, GM-CSF-, and IL-3-derived Mϕs. As shown in Fig. 3*B*, GM-CSF-derived Mϕs secreted very high levels of IL-12, both from SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs, with significantly more from the SHIP$^{+/+}$ cells. The GM-CSF-derived SHIP$^{-/-}$ Mϕs, on the other hand, produced substantially more IL-10 than did their WT counterparts, and thus the IL-12-to-IL-10 ratio was dramatically higher in SHIP$^{+/+}$ cells, consistent with the GM-CSF-derived SHIP$^{-/-}$ Mϕs being M2 skewed. The IL-3-derived Mϕs secreted more modest levels of IL-12 in response to LPS, both from SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs, with the SHIP$^{-/-}$ Mϕs secreting very low levels of IL-12 and slightly higher levels of IL-10 than SHIP$^{+/+}$ controls, yielding a low IL-12-to-IL-10 ratio, consistent with our Western blot results showing that IL-3-derived SHIP$^{-/-}$ Mϕs have the highest levels of Arg I and Ym1 (Fig. 1*A*). Interestingly, however, we found that M-CSF-derived Mϕs secreted by far the highest levels of IL-10 in response to LPS and only moderate levels of

IL-12. As well, there was very little difference between SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs in the levels of these cytokines secreted, and this resulted in M-CSF-derived Mϕs having the lowest IL-12-to-IL-10 ratio. Given that the IL-3- and GM-CSF-derived SHIP$^{-/-}$ Mϕs appear more M2 skewed than do M-CSF-derived SHIP$^{-/-}$ Mϕs, based on Arg I and Ym1 expression and the inability of these cells to stimulate IFN-γ production from activated T cells, this calls into question the concept that a low IL-12-to-IL-10 ratio coincides with an M2 phenotype.

To further characterize IL-3-derived Mϕs, we compared the cell surface expression of CD206 (the mannose receptor), gp49 (aka LILRB4), MHC-II. and CD86 on M-CSF-, IL-3- and M-CSF plus IL-4-derived Mϕs. As shown in supplemental Fig. 1,[5] CD206 was present at higher levels on IL-3-derived than on M-CSF-derived SHIP$^{-/-}$ Mϕs, consistent with the former being more M2 skewed (24, 25). As expected, M-CSF plus IL-4-derived Mϕs also expressed higher levels of CD206 than did M-CSF-derived Mϕs. Interestingly, IL-3-derived SHIP$^{-/-}$ Mϕs also displayed higher surface levels of MHC-II than did M-CSF-derived Mϕs but less than on IL-4-derived Mϕs. On the other hand, the cell surface levels of CD86, which were very low, and gp49 were similar on M-CSF- and IL-3-derived SHIP$^{-/-}$ Mϕs and slightly less than on IL-4-derived Mϕs.

STAT5 and STAT6 are both required for IL-3-induced M2 skewing

Since both IL-3 and GM-CSF are potent activators of STAT5 (26), and several reports have indicated that STAT5 is involved in Mϕ activation and differentiation (27, 28), we next asked whether STAT5 was required for IL-3-induced M2 skewing. Specifically, adherence-depleted BM progenitors from STAT5$^{+/+}$ and STAT$^{-/-}$ mice were cultured for 6 days with IL-3, and then adherent Mϕs were analyzed for Arg I and Ym1 expression. As can be seen in Fig. 4*A*, the levels of Ym1 and Arg I in IL-3-derived Mϕs were substantially lower in the STAT5$^{-/-}$ Mϕs. As expected, IL-4-induced M2 skewing did not require STAT5, that is, the levels of IL-4-induced Ym1 and Arg I were similar in STAT5$^{+/+}$ and STAT$^{-/-}$ Mϕs (Fig. 4*A*). As a negative control, we also investigated the role of STAT6, which is typically activated via IL-4 but not by IL-3. As a positive control, we first determined whether IL-4-induced M2 skewing was reduced with adherence-depleted or Lin$^-$ BM cells from STAT6$^{-/-}$ mice and, as expected, it was totally abolished, that is, there was no induction of Arg I or Ym1 in STAT6$^{-/-}$ Mϕs (Fig. 4*B*). Surprisingly, however, we found that STAT6$^{-/-}$ progenitors that were differentiated with IL-3 also gave markedly reduced levels of Arg I and Ym1 (Fig. 4*B*). These results indicated that both STAT5 and STAT6 were required for IL-3-induced M2 skewing of BM progenitors.

Since STAT6 is thought to be activated only by IL-4 and IL-13 (19), the above results suggested that IL-4 or IL-13 might be participating in IL-3-induced M2 skewing. To test this, Lin$^-$ cells from SHIP$^{+/+}$ and SHIP$^{-/-}$ BM were cultured with IL-3 in the presence and absence of neutralizing anti-IL-4 or anti-IL-13 Abs for 6 days, and then Ym1 and Arg expression was analyzed in the resulting mature Mϕs. As shown in Fig. 4*C*, Arg I expression was completely abolished and Ym1 markedly reduced if SHIP$^{-/-}$ Mϕs were derived in the presence of IL-3 plus anti-IL-4 Ab. However, no reduction in M2 markers was seen with anti-IL-13. These results suggested that IL-4, but not IL-13, was a major player in IL-3-induced M2 skewing.

Since Xiao et al. recently reported that BM progenitors from Lyn/Hck$^{-/-}$ or SHIP$^{-/-}$ mice were capable of producing low levels

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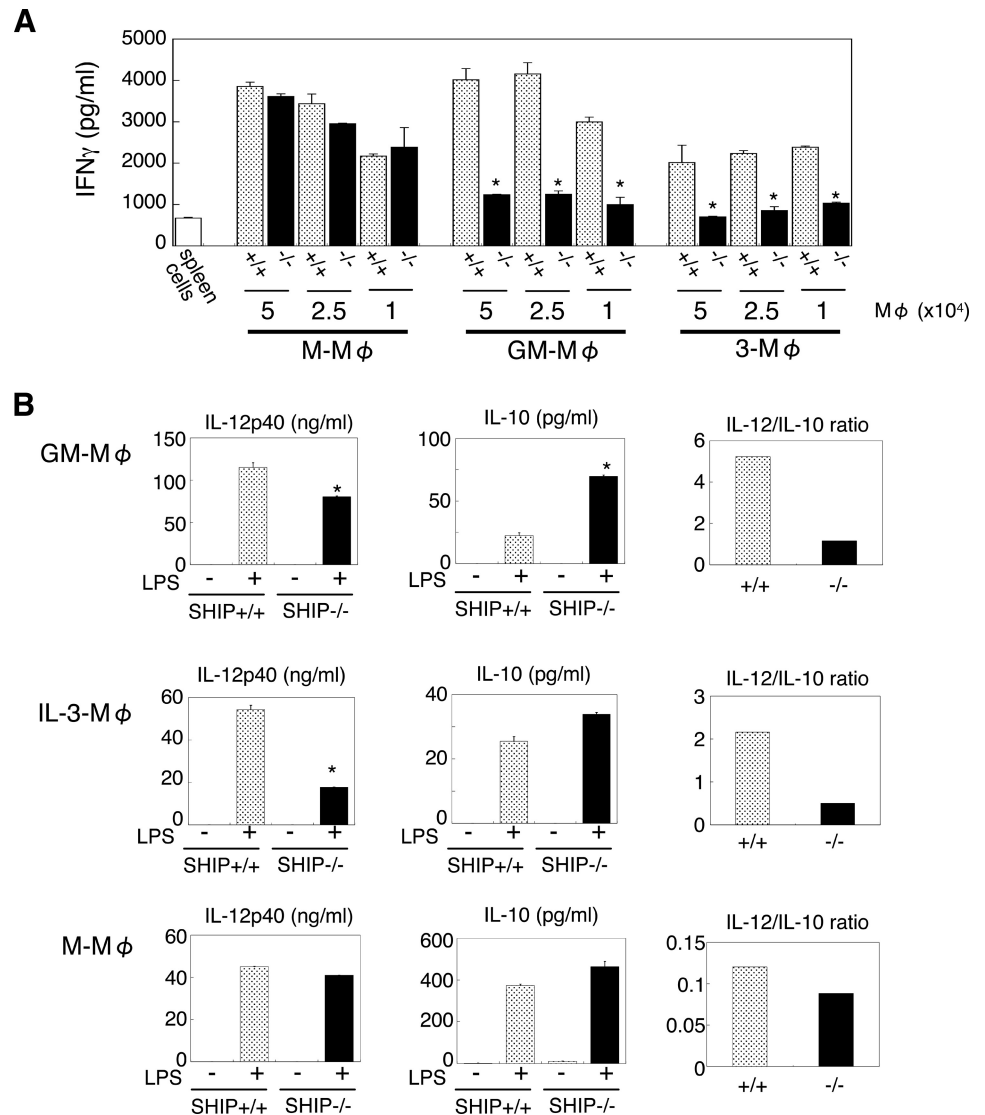[5] The online version of this article contains supplemental material.

FIGURE 3. IL-3- and GM-CSF-derived SHIP$^{-/-}$ Mϕs display M2 properties. *A*, M-CSF-, GM-CSF-, or IL-3-derived SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs (5, 2.5, or 1 × 10^4 cells) were cocultured with SHIP$^{+/+}$ spleen cells in the presence of anti-CD3 Abs for 24 h. Culture supernatants were assessed for IFN-γ levels via ELISA. Data shown are the means ± SEM of duplicate determinations. *B*, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM progenitors were differentiated in the presence of GM-CSF (GM-Mϕ), IL-3 (3-Mϕ) or M-CSF (M-Mϕ) for 6 days and the resulting adherent Mϕs were treated with or without 1 μg/ml LPS for 18 h. Culture supernatants were subjected to IL-12 and IL-10 ELISAs. Similar results were obtained for *A* and *B* in three independent experiments. ∗, $p < 0.05$.

of IL-3 and GM-CSF constitutively, that is, in the absence of exogenous cytokines, and that they activated STAT5 and promoted the expansion of myeloid cells in an autocrine and paracrine manner (29), we conducted similar studies and found that SHIP$^{-/-}$, but not SHIP$^{+/+}$, Lin$^-$ BM cells spontaneously matured into viable Mϕs without exogenously added growth factors, that is, with no IL-3, GM-CSF, or M-CSF added. The number of mature SHIP$^{-/-}$ Mϕs obtained in the absence of exogenous factors was ~10% of that obtained with saturating levels of IL-3, and Western analysis of these SHIP$^{-/-}$ mature Mϕs revealed they were M2 skewed (Fig. 4*D*). Interestingly, this M2 phenotype was suppressed with anti-IL-4 Ab, similar to that seen with IL-3-derived progenitors (Fig. 4*C*) and suggested, given Xiao et al.'s results, that IL-3 was likely involved in the spontaneous generation of M2 Mϕs from SHIP$^{-/-}$ BM progenitors. To confirm this, we added neutralizing anti-IL-3 Ab to SHIP$^{-/-}$ BM cultures growing without exogenous cytokines and found that it partially suppressed M2 generation, although not as effectively as anti-IL-4 Ab (Fig. 4*D*).

Basophil progenitors within the Lin$^-$ BM population produce IL-4 in response to IL-3 or GM-CSF, and SHIP represses this IL-4 production

We next determined if IL-3 could actually be stimulating the production of IL-4 from SHIP$^{-/-}$ Lin$^-$ BM cells by incubating

SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM cells for 24 h with GM-CSF or IL-3 and assessing IL-4 levels in the cell supernatants. As shown in Fig. 5*A*, we found that IL-3 and, to a lesser extent, GM-CSF stimulated the production of IL-4 from SHIP$^{+/+}$ progenitors but far more from SHIP$^{-/-}$ progenitors. M-CSF was unable to trigger any IL-4 production. To determine whether all SHIP$^{-/-}$ Lin$^-$ BM cells were producing IL-4 or only a subpopulation, we conducted intracellular flow cytometry following IL-3 stimulation for 4 h in the presence of brefeldin A. As shown in Fig. 5*B*, not all SHIP$^{-/-}$ Lin$^-$ BM cells were synthesizing IL-4, just those that were c-kit$^-$ and FcϵRIα^+, that is, cells recently identified as basophils and their progenitors (30). We conducted similar intracellular IL-4 staining studies with SHIP$^{-/-}$ adherence-depleted BM cells to determine whether FcϵRIα^+ c-kit$^-$ cells are the major IL-4-producing cells in whole BM as well. As shown in supplemental Fig. 2*A*, this was indeed the case. Giemsa staining of these SHIP$^{-/-}$ FcϵRI$^+$c-kit$^-$ cells revealed a morphology consistent with previous reports for immature and mature basophils (supplemental Fig. 2*B*) (31).

These results suggested that IL-3 and GM-CSF stimulated SHIP$^{-/-}$ basophil progenitors within the Lin$^-$ BM population to secrete high levels of IL-4 and this IL-4, in turn, skewed SHIP$^{-/-}$ progenitors toward an M2 phenotype. To determine whether IL-3 and GM-CSF might be triggering a higher IL-4 production from
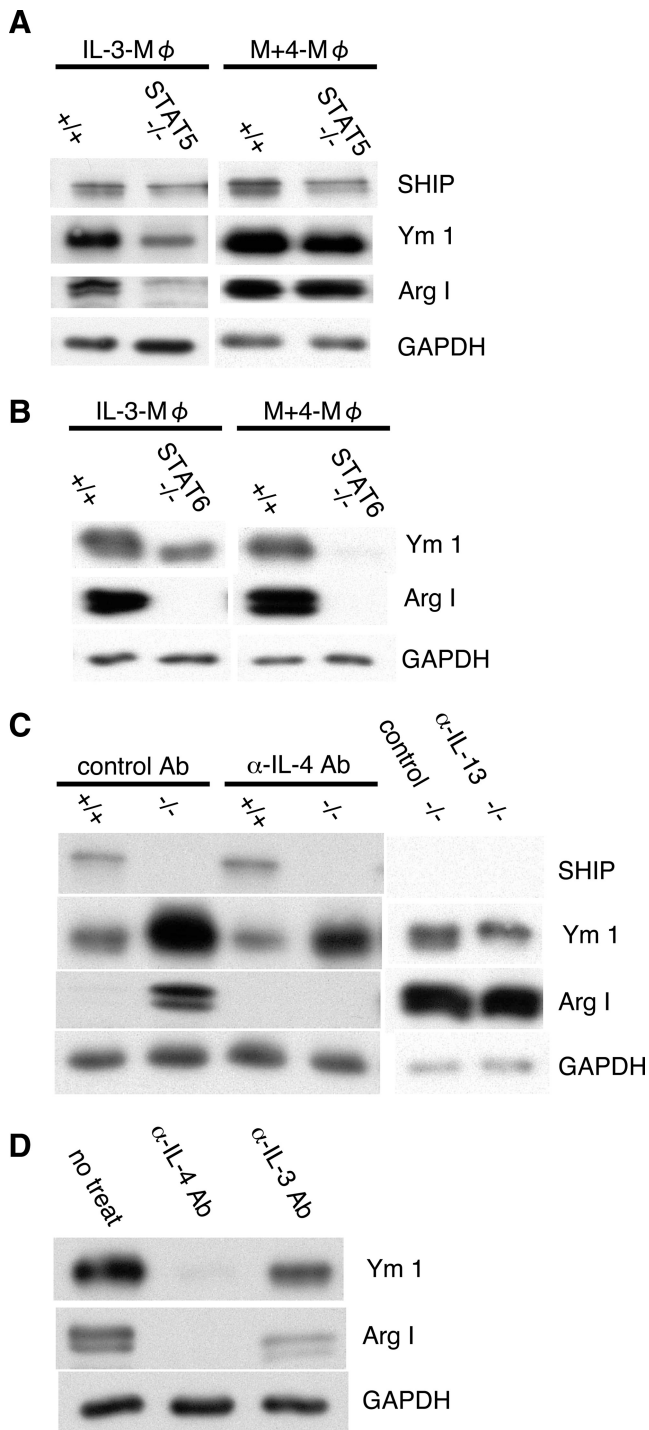
FIGURE 4. Both STAT5 and STAT6 are required for IL-3-induced M2 skewing. Adherence-depleted BM cells from WT (+/+), STAT5a/b$^{-/-}$ (A), or STAT6$^{-/-}$ (B) mice were cultured with IL-3 (IL-3-Mϕ) or M-CSF plus IL-4 (M + 4-Mϕ) for 6 days. Mature Mϕs were analyzed for SHIP, Ym1, Arg I, and GAPDH expression by Western blotting. C, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM cells were cultured with IL-3 with or without neutralizing anti-IL-4, neutralizing anti-IL-13, or irrelevant control Ab for 6 days. The resulting adherent Mϕs were subjected to Western analysis using anti-Ym1, anti-Arg I, and anti-GAPDH Abs. For D, SHIP$^{-/-}$ Lin$^-$ BM progenitors were cultured without exogenous cytokines with or without neutralizing anti-IL-4 or anti-IL-3 Ab for 6 days. Mature Mϕs were then used for the analysis of Ym1, Arg I, and GAPDH expression by Western blotting. Similar results were obtained for A–D in three independent experiments.

SHIP$^{-/-}$ than from SHIP$^{+/+}$ Lin$^-$ BM cells due to SHIP's dampening effects on the PI3K pathway, we incubated SHIP$^{-/-}$ Lin$^-$ BM cells in the presence and absence of LY294002 or wortmannin and found that they both inhibited IL-4 production (Fig. 5C). These results are consistent with SHIP repressing IL-3-induced IL-4 production from Lin$^-$ basophil progenitors via inhibition of the PI3K pathway.

Having established that SHIP plays a major role in restricting IL-3- and GM-CSF-induced IL-4 production from Lin$^-$ BM cells, we then asked if SHIP also played a role within the Mϕ progenitor by stimulating SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM cells with different concentrations of IL-4 and assessing the level of M2 skewing of the resulting mature Mϕs. As shown in Fig. 5D, the level of M2 skewing was similar in the SHIP$^{+/+}$ and SHIP$^{-/-}$ mature Mϕs at all IL-4 concentrations tested, indicating that SHIP represses IL-3- and GM-CSF-induced M2 skewing by inhibiting the production of IL-4 from Lin$^-$ BM cells and not by interfering with IL-4-induced skewing in the target cells.

Discussion

Taken together, our data suggest a model in which IL-3 promotes the survival/proliferation and differentiation of basophil progenitors within Lin$^-$ BM (Fig. 6). Additionally, IL-3 (and, to a lesser degree, GM-CSF) stimulates these basophils, via STAT5, and, as recently shown by Hida et al., FcRγ and Syk (32) to produce IL-4. This IL-4 production is negatively regulated by SHIP. Our results with LY294002 and wortmannin are consistent with SHIP repressing IL-3- and GM-CSF-induced IL-4 production from Lin$^-$ basophil progenitors by limiting PI3K pathway activation. The secreted IL-4 then skews the Mϕ progenitors to an M2 phenotype. Moreover, since IL-4 alone does not give rise to any mature Mϕs from Lin$^-$ BM, whereas IL-3 does, we propose that IL-3 also acts directly on Mϕ progenitors to promote their survival/proliferation and differentiation (Fig. 6). In partial support of this model, two recent studies have shown that IL-3, produced by T cells during nematode infection, is critical for the dramatic increase in blood basophils that occurs in mice following these infections (33, 34). As well, Schneider et al. have shown that both total BM and Lin$^-$ BM progenitors contain basophils and their precursors, which could be identified as c-kit$^-$ cells that produce IL-4 in response to IL-3 (35, 36).

Although basophils account for <1% of blood leukocytes in normal humans and mice, their numbers increase dramatically during parasitic infections, as mentioned above, and during allergic inflammation, and they are potent producers of IL-4 (30, 37). They are currently in the limelight because of their recently appreciated roles in promoting Th2 differentiation (37, 38) and in IgG-induced systemic anaphylaxis (39). Upon allergen challenge of allergic patients, blood basophils can enter sites of inflammation and interact 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 subsequent Th2 polarization. In this regard it has just been shown that human basophils activated by mast cell-derived IL-3 also secrete the Th2 mediator, retinoic acid (41). Importantly, while positive regulators of basophil mediator release have been described, as well as one negative intracellular regulator of basophil proliferation, that is, IRF-2 (42), our study is the first to report a negative regulator of basophil function, that is, SHIP. Of note in this regard, we have found that SHIP also negatively regulates IgE$^-$ and IgE$^+$ Ag-induced IL-4 production from basophils (data not shown).

We reported previously that in vivo-derived peritoneal and alveolar Mϕs from SHIP$^{-/-}$ mice have an M2 phenotype (16). However, the mechanisms responsible for this M2 skewing have

FIGURE 5. IL-3 and GM-CSF stimulate the production of more IL-4 from SHIP$^{-/-}$ than SHIP$^{+/+}$ Lin$^-$ BM cells. *A*, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM cells were cultured with M-CSF, IL-3, or GM-CSF for 24 h and supernatants were subjected to IL-4 ELISAs. Data shown are the means ± SEM of duplicate determinations. *, $p < 0.05$ compared with unstimulated cells. *B*, SHIP$^{-/-}$ Lin$^-$ BM cells were stimulated with IL-3 for 5 h in the presence of brefeldin A (Golgi Plug), fixed, and permeabilized and then stained with anti-IL-4, anti-c-kit, and anti-FcεRIα Abs. IL-4-producing cells were detected by flow cytometry. *C*, SHIP$^{-/-}$ Lin$^-$ BM cells were stimulated with IL-3 for 24 h with or without the indicated concentrations of LY294002 (LY) and wortmannin (WM) and IL-4 levels were determined by ELISA. Data shown are the means ± SEM of duplicate determinations. *D*, SHIP$^{+/+}$ and SHIP$^{-/-}$ Lin$^-$ BM cells were cultured with 10 ng/ml M-CSF plus the indicated concentrations of IL-4 for 6 days. Cell lysates from mature adherent Mϕs were analyzed for SHIP, Ym1, Arg I, and GAPDH by Western blotting. Similar results were obtained for *A–D* in three independent experiments.
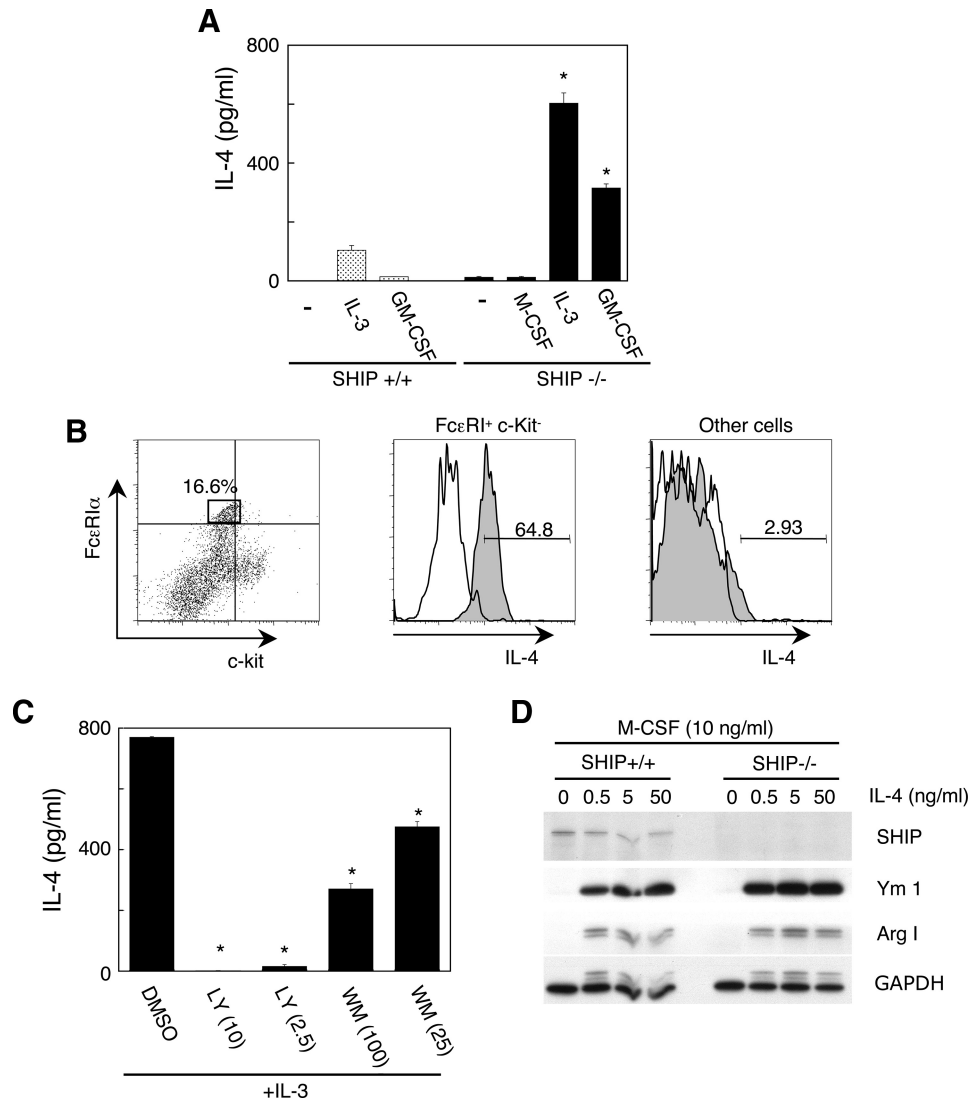
not been elucidated. Related to this, we report herein that SHIP$^{-/-}$, but not SHIP$^{+/+}$, progenitors spontaneously differentiate in the absence of exogenous growth factors into Mϕs. Furthermore, the resulting mature Mϕs display an M2 phenotype that can be suppressed with either anti-IL-4 or anti-IL-3. It is thus possible that BM progenitors in SHIP$^{-/-}$ mice spontaneously differentiate into M2 Mϕs in vivo via IL-4-secreting basophils. Consistent with this possibility, Xiao et al. elegantly demonstrated that BM progenitors from SHIP- or Lyn/Hck-deficient mice spontaneously form Mϕ colonies in the absence of growth factors. They also showed that progenitors from both knockout mice produce IL-3 and GM-CSF, and these cytokines participate in the spontaneous formation of Mϕ colonies (29). The fact that SHIP$^{-/-}$ Mϕs are not M2 skewed if M-CSF is added during in vitro derivation suggests that this M-CSF can override the influence of the low levels of endogenous IL-3.

Given our finding that basophils and their progenitors are critical mediators of IL-3-induced skewing, we had to reappraise our initial conclusion that mature Mϕs are unaffected by IL-3 and GM-CSF (Fig. 1*C*). Specifically, since it is standard practice to remove nonadherent cells from adherent Mϕs before carrying out mature Mϕ studies, we added basophils back to mature M-CSF-derived Mϕs and found that now IL-3 and GM-CSF could indeed M2 skew these cells (data not shown). To resolve whether IL-4 skews both

Mϕ progenitors and mature Mϕs to an M2 phenotype or just mature Mϕs, we removed IL-4 on different days during the in vitro differentiation assay and found that only low levels of M2 skewing were seen if IL-4 (or IL-3) was removed before day 4. While this is consistent with IL-4 acting primarily on mature Mϕs, we have also found that the IL-4-induced M2 skewing of mature Mϕs is reversible if IL-4 is removed for 2 days (data not shown). Thus, the issue remains open. Of note, however, we have found that SHIP$^{-/-}$ monocytes express Arg I (our manuscript in preparation), suggesting that M2-like monocytes can occur in vivo before they enter tissues and mature into resident Mϕs.

IL-3 is a pleiotropic hematopoietic cytokine (43) that is typically used for the in vitro generation of mast cells from BM progenitors. However, little attention has been paid to its role in Mϕ development. Recently, Hibbs et al. reported that mice lacking M-CSF, GM-CSF, and G-CSF still produce Mϕs (44), and they hypothesized that IL-3 might contribute to Mϕ generation in vivo. We show herein that IL-3-derived Mϕs are phenotypically distinct from M-CSF- or GM-CSF-derived Mϕs. This is consistent with studies by Saha et al. showing that IL-3-derived Mϕs have unique functions and preferentially activate Th2 responses after *Leishmania major* infection in BALB/c mice (18). Interestingly, a model has recently been put forward that GM-CSF, which is increased at sites of inflammation, skews to an M1 phenotype while M-CSF,
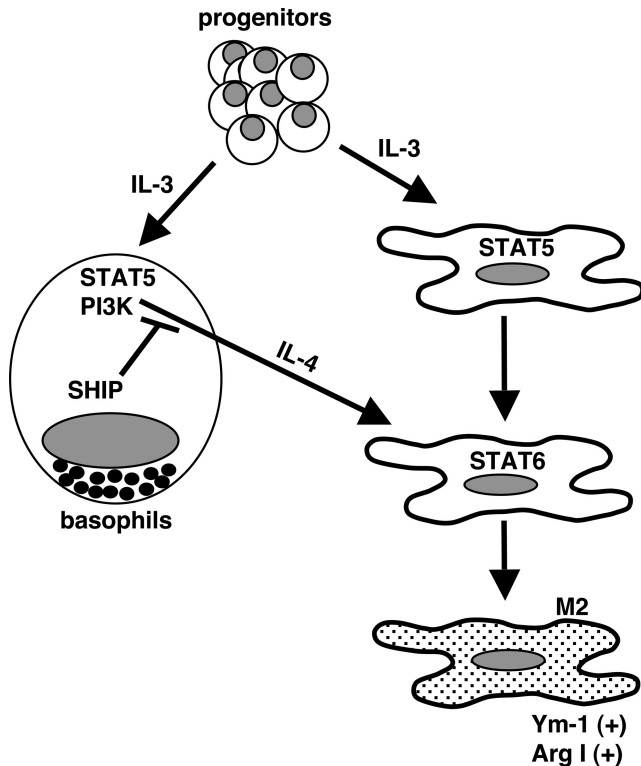
FIGURE 6. Model of IL-3-induced M2 skewing and the role that SHIP plays in this process. IL-3 stimulates the proliferation and differentiation of both basophil precursors and monocyte/macrophage progenitors. IL-3 also stimulates the production of IL-4 from basophils and basophil progenitors in a STAT5-dependent manner. SHIP within the basophils represses this IL-4 production. The secreted IL-4, in turn, skews, via STAT6, the maturing and mature Mϕs to an M2 phenotype.

which is produced constitutively, tends to polarize to a more M2-like state (45). Our data suggest that IL-3 should be added to this model as a cytokine that is increased at sites of inflammation and skews to an even more M2-like state than M-CSF. As far as GM-CSF is concerned, we reported previously that the lungs of SHIP$^{-/-}$ mice are consolidated, inflamed, and fibrotic, and this has subsequently been confirmed by others (16, 29). Moreover, we found Mϕ-associated Ym1 crystals in the bronchoalveolar lavage fluid of SHIP$^{-/-}$ mice (16). Related to this, it has recently been shown that *Cryptococcus neoformans* infection induces allergic bronchopulmonary mycosis and skews alveolar Mϕs to Ym1-containing cells (46). However, this M2 phenotype was not induced in the lungs of infected GM-CSF$^{-/-}$ mice. This suggests that GM-CSF controls the lung microenvironment by regulating alveolar Mϕ skewing (46). In our present study, we found that in vitro derivation with GM-CSF led to the expression of Ym1 in the resulting WT mature Mϕs (Fig. 1*A*), and we also detected higher levels of secreted Ym1 in culture supernatants from SHIP$^{+/+}$ and SHIP$^{-/-}$ Mϕs derived with GM-CSF or IL-3 than with M-CSF (data not shown). Given previous reports with GM-CSF-deficient mice (47, 48), it is possible that the lung consolidation and Ym1 crystal formation in our SHIP$^{-/-}$ mice are mediated by the action of GM-CSF on basophils within the lung environment.

The mechanisms underlying M1 vs M2 skewing are of considerable importance because of the potential use of this information in designing future therapies for cancers, infections, and autoimmune disorders. The studies presented herein suggest that the manipulation of basophil numbers and/or function, perhaps via SHIP, could be part of these future therapies.

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