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SHIP Regulates the Reciprocal Development of T Regulatory and Th17 Cells

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Maintaining an appropriate balance between subsets of CD4⁺ Th and T regulatory cells (Tregs) is critical to maintain immune homeostasis and prevent autoimmunity. Through a common requirement for TGF-β, the development of peripherally induced Tregs is intimately linked to that of Th17 cells, with the resulting lineages depending on the presence of proinflammatory cytokines such as IL-6. Currently very little is known about the molecular signaling pathways that control the development of Tregs vs Th17 cells. Reduced activity of the PI3K pathway is required for TGF-β-mediated induction of Foxp3 expression and the suppressive activity of Tregs. To investigate how negative regulators of the PI3K pathway impact Treg development, we investigated whether SHIP, a lipid phosphatase that regulates PI3K activity, also plays a role in the development and function of Tregs. SHIP-deficient Tregs maintained suppressive capacity in vitro and in a T cell transfer model of colitis. Surprisingly, SHIP-deficient Th cells were significantly less able to cause colitis than were wild-type Th cells due to a profound deficiency in Th17 cell differentiation, both in vitro and in vivo. The inability of SHIP-deficient T cells to develop into Th17 cells was accompanied by decreased IL-6-stimulated phosphorylation of STAT3 and an increased capacity to differentiate into Tregs under the influence of TGF-β and retinoic acid. These data indicate that SHIP is essential for normal Th17 cell development and that this lipid phosphatase plays a key role in the reciprocal regulation of Tregs and Th17 cells.

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daptive immunity is controlled by subsets of CD4⁺ T cells that are polarized by cytokines into functionally diverse Th cell subsets. The classical paradigm that cellular immunity is mediated by Th1 cells and humoral immunity by Th2 cells has recently been revised upon recognition of a third lineage of Th cells that produce IL-17 (Th17 cells) and subsets of T regulatory cells (Tregs) that suppress immune responses. There is intense interest in defining how these different types of CD4⁺ T cells develop and function, as interventions to modulate their relative activities would have widespread clinical use to treat dysregulated immune responses.

PI3K is a critical regulator of tolerance. In conventional T effector (Teff) cells PI3K is activated upon TCR and CD28 costimulation, delivering signals that enhance cell cycle progression, survival, and proliferation (1). In contrast, Foxp3⁺ Tregs have reduced PI3K activity downstream of TCR or IL-2 receptor activation, a molecular requirement for their suppressive capacity (2) and expression of Foxp3 (3, 4). Negative regulation of the PI3K pathway in hematopoietic cells is primarily mediated by phosphatases, including phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and SHIP-1 and SHIP-2. These phosphatases hydrolyze the PI3K product phosphatidylinositol 3,4,5-triphosphate, a key second messenger in the PI3K pathway, and they negatively regulate the activity of downstream proteins such as Akt (5). Although both SHIP-1 and SHIP-2 are expressed in hematopoietic cells (6), it is thought that SHIP-1 (hereafter SHIP) is the major negative regulator downstream of cytokine (7), Fc (8), and B cell (9) receptors.

In T cells, TGF-β plays a major role in regulating the peripheral development of CD4⁺ T cell subsets, including peripherally induced Tregs (iTregs) (10) and Th17 cells (11), which are defined by their ability to produce IL-17A, IL-17F, TNF-α, IL-6, and IL-22, but not IFN-γ (11–13). Thus, depending on other cytokines in the environment, both iTreg and Th17 cells can be derived from a common precursor cell under the influence of TGF-β (14). Proinflammatory cytokines such as IL-6 promote Th17 differentiation via activation of STAT3 (15) and induction of the transcription factor retinoic acid-related orphan receptor (ROR)γT (16). In contrast, IL-2-mediated activation of STAT-5 prevents Th17 cell differentiation (17) and is critical for the development of iTregs (18). Recently it has been shown that TGF-β-induced activation of SMAD4 is required for iTreg but not Th17 cell differentiation, suggesting that the TGF-β-driven signaling events required for the development of these two lineages are distinct (19). This notion is further supported by the finding that enhancement of TGF-β-driven iTreg and inhibition of Th17 cell development by...
retinoic acid (RA) is associated with increased SMAD3 activity (20). We have shown that in macrophages and mast cells, SHIP expression is up-regulated by autocrine TGF-β (5), likely via SMAD-dependent signaling pathways (21), suggesting that SHIP may also be a key molecule in TGF-β-mediated responses.

To determine whether SHIP has a role in the development and/or function of naturally occurring cells or iTregs, we have analyzed the phenotype and function of CD4+ T cells from mice lacking a functional SHIP gene (22). Ex vivo Tregs from SHIP-deficient mice retained normal suppressive capacity in vitro and in vivo. Deletion of SHIP, however, reduced the capacity of naive CD4+ T cells to differentiate into Th17 cells and, in parallel, promoted the development of iTregs. Our results suggest that SHIP controls the developmental balance between iTregs and Th17 cells.

Materials and Methods

Mice and cell isolation

Female mice aged 6–10 wk were used and maintained under specific pathogen-free conditions in accordance with ethics protocols approved by the University of British Columbia Animal Care Committee. C57BL/6 mice were stimulated with anti-CD3/CD28-coated microbeads (Invitrogen) or anti-CD2 (2 μg/ml; R&D Systems), anti-IL-2 (2 ng/ml; R&D Systems), IL-23 (10 ng/ml; R&D Systems), anti-IL-2 (2 ng/ml) for the indicated times at 37°C with gentle shaking.

Phospho-signaling assay

CD4+ T cells were incubated for 2 h in serum-free medium before stimulation. To measure phospho-AKT Ser473, cells were incubated with anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) mAbs on ice for 15 min, followed by crosslinking with prewarmed anti-hamster IgG F(ab’)_2 Ab (20 μg/ml; Cedarlane Laboratories) for the indicated times at 37°C with gentle shaking. Phosphorylation of STAT3 was initiated by addition of mIL-6 (100 ng/ml; R&D Systems) for the indicated times at 37°C with gentle shaking. Activation was arrested by addition of an equal volume of ice-cold 2× Foxp3-specific Fix/Perm buffer (eBioscience). Cells were permeabilized and stained with CD4-PE-Cy7 (RM4-5), CD25-allophycocyanin (PC61), and CD45RB-FITC (16A) from BD Biosciences.

RT-PCR analysis

Expression was measured in real time with a sequence detection system (GeneAmp 7300; Applied Biosystems). Primer sets were sequences as follows: RORγt, 5′-CCCGTGAGGAGGGTTTAC-3′ (forward) and 5′-TGCAAGGTAGGCCACATTACA-3′ (reverse); Foxp3, 5′-CCACACACACACCTTGCCAAAG-3′ (forward) and 5′-TCC CCAACAGCTGTTA CAGTT-3′ (reverse); and 5′-TTCCTCAACACCGCCACTG-3′ (reverse); 18S, 5′-CTGCCGACATCATGACCACTA-3′ (forward) and 5′-CTGGCCCATGAATGACACCACTA-3′ (reverse). The QuantiTect SYBR Green PCR kit (Qiagen) was used to quantify mRNA levels. Data presented are normalized to 18S using the comparative _C_ method (ΔΔ_ C_).

Cell culture

Medium was RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 1 mM MEM nonessential amino acids, 100 U/ml each of penicillin G and streptomycin. Cells were stimulated with anti-CD3/CD28-coated microbeads (Invitrogen) or plate-bound CD3 (10 μg/ml, 2C11) and soluble CD28 (1 μg/ml, 37.51). Tregs were differentiated from CD4+CD25+CD45RBhigh T cells in the presence of rhIL-2 (100 U/ml; Chiron) and rhTGF-β (1 ng/ml; R&D Systems). Retinoic acid (10 ng/ml; Sigma-Aldrich) was added where indicated. Th17 cells were differentiated from CD4+CD25−CD45RB− T cells by addition of TGF-β (0.5 μg/ml). IL-6 (20 ng/ml, unless otherwise indicated; R&D Systems), IL-23 (10 ng/ml; R&D Systems), anti-IL-2 (2 μg/ml; N96-1A12; BD Biosciences), anti-IL-4 (2 μg/ml; BVD4-11D1; BD Biosciences), and anti-IFN-γ (4 μg/ml; R&D Systems) and anti-IL-4 (2 μg/ml). Treg anergy was assessed by culture of Tregs (5 × 10^6/well) with or without 50 U/ml rhIL-2. To measure suppression CD4+CD25− naïve T cells (5 × 10^6/well) were stimulated with anti-CD3 (0.5 μg/ml), irradiated CD3-depleted splenocytes (1 × 10^6/well), and serially-diluted Tregs (5 × 10^6 to 0). Proliferation and suppression were measured by 3H-thymidine incorporation in the final 16 h of a 72-h assay.

Colitis T cell transfer, histology and scoring

Colitis was induced in 6- to 10-wk-old C57BL/6 mice (The Jackson Laboratory) by i.p. injecting CD4+CD25−CD45RBhigh T cells (4 × 10^6/mouse) alone or together with CD4+CD25high Tregs (2 × 10^7/mouse). Eight weeks after T cell transfer, distal and proximal colon specimens were fixed in 10% neutral buffered formalin, embedded in paraffin blocks, and 5-μm sections were stained with H&E and Alcian blue-periodic acid Schiff. Tissue sections were graded semiquantitatively in a blinded fashion from 0 to 5, representing no change to severe changes, as described (23). For Foxp3 immunohistochemistry, 5-μm-thick paraffin sections were dewaxed, rehydrated with alcohol, and Ags were retrieved by steaming in sodium citrate buffer (pH 6.0, 30 min). After blocking endogenous peroxidase (3% H2O2, 3 min), biotin-labeled Foxp3 (FJK-16s; eBioscience) was incubated overnight at 4°C. Biotin was detected with HRP-conjugated swine anti-biotin, rehydrated through alcohol, and mounted.

Intracellular staining and ELISA

Intracytoplasmic expression of cytokines was assessed after 6 h of stimulation with PMA (25 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml) during the final 4 h. Cells were fixed and permeabilized using Foxp3-specific kit reagents (eBioscience) and stained with anti-IFN-γ–PE-Cy5 (eBioscience), anti-IL-17–PE-Cy5 (eBioscience), and anti-CD25–PE (eBioscience). Sections were counterstained with hematoxylin, dehydrated through alcohol, and mounted.

Statistical analysis

Differences between colitis histology scores were analyzed using a one-way ANOVA. All other analyses were performed with two-tailed Student’s t test at a 95% confidence interval. Paired t tests were used for analysis of all cell cultures and unpaired t tests were used for analysis of data from colitis T cell transfer experiments. Values of _p_ < 0.05 were considered significant and are indicated on graphs as follows: ∗, _p_ < 0.05; ∗∗, _p_ < 0.01; ∗∗∗, _p_ < 0.001. Error bars represent SD.

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Mouse Tregs have reduced activation of Akt upon TCR stimulation

We first determined whether TCR-mediated activation of the PI3K pathway was less robust in Tregs than in Teff cells. CD4+ T cells were purified from WT mice, and phospho-Akt levels were measured in Foxp3+ Tregs and Teff cells (Fig. 1A). After 5 and 10 min of TCR-mediated stimulation, Teff cells displayed significantly increased levels of Akt phosphorylation at Ser473 compared with unactivated control cells. Consistent with results in human peripheral blood CD4+ T cells and Foxp3 transgenic mice (2, 24), anti-CD3 and anti-CD28-induced Akt phosphorylation was almost completely absent in Treg cells. In three independent experiments phospho-Akt Ser473 levels were on average 3.7- (p = 0.0038) or 6.3-fold (p = 0.0179) lower in Treg cells than in Teff cells after 5 or 10 min of TCR stimulation, respectively (Fig. 1B).

SHIP-deficient Tregs are anergic and suppressive in vitro

Reduced activity of the PI3K/Akt pathway is known to be necessary for Treg function (2–4), but the upstream molecular events that prevent normal activation of Akt in Tregs are unknown. We investigated whether reduced activity of Akt in Tregs was related to expression of SHIP, a lipid phosphatase known to limit PI3K activity (5). Pooled spleen and lymph node cells from 6- to 8-wk-old SHIP−/− and WT littermate controls were analyzed by FACS for expression of CD4, CD25, and Foxp3 (Fig. 2A). Six- to 10-wk-old SHIP−/− mice had a consistent reduction in the proportion of CD4+CD25+ T cells compared with WT littermates (SHIP−/− 24.98 ± 5.99% vs WT 9.24 ± 1.31%, n = 7, p = 0.0002) (25, 26). The increase in the proportion of Tregs also resulted in an increase in their absolute numbers (data not shown), consistent with previous reports (25, 26). We next determined whether the absence of SHIP altered the proliferative and/or suppressive capacity of Tregs. SHIP-deficient Teff cells proliferated to the same extent as WT Teff cells (Fig. 2B). In the absence of IL-2,
Tregs from both genotypes failed to proliferate, indicating that SHIP is not required for Treg anergy. Additionally, Tcell proliferation was suppressed by WT and SHIP−/− Tregs equally (Fig. 2C). These results are in agreement with previous findings that SHIP-deficient Tregs retain their suppressive capacity (25, 26).

**SHIP-deficient Tregs suppress the adoptive transfer of colitis**

Since in vitro suppression assays do not necessarily predict in vivo function (27), we investigated whether the absence of SHIP alters the capacity of Tregs to prevent the adoptive transfer of inflammatory bowel disease (IBD). Whereas transfer of highly purified CD4+ CD25+ CD45RBhigh Teff cells into rag1−/− mice induces colitis, cotransfer of Tregs prevents disease (28). Mice that received Teff cells from WT mice developed severe colitis (Fig. 3, A and B). In contrast, mice that received WT Teff cells in the presence of a 2:1 ratio (Teff cells-Tregs) of Tregs, from either WT or SHIP−/− mice, showed no apparent clinical signs of disease, had normal colon morphology, and significantly reduced proximal colitis scores (WT Teff cells and WT Tregs, 0.58 ± 0.34; WT Teff cells and SHIP−/− Tregs, 0.67 ± 0.56, n = 6, p < 0.001) compared with transfer of WT Teff cells alone (3.13 ± 0.72, n = 8).

The ability of mesenteric lymph node (MLN) cells to produce IFN-γ and TNF-α was also measured as an indication of the severity of inflammation (Fig. 3C). Cotransfer of WT Tregs suppressed the development of MLN cells capable of producing IFN-γ compared to T cell transfer of Teff cells alone (% CD4 cells IFN-γ+; WT Teff cells, 29.53 ± 3.61% vs WT Teff cells and WT Tregs, 12.05 ± 4.52%, n = 4; p = 0.0009), as did the cotransfer of SHIP−/− Treg cells (14.87 ± 4.78%, n = 3; p = 0.0055). In contrast, neither WT (% CD4 cells TNF-α+; 32.98 ± 6.18%, n = 4; p = 0.168 (NS)) nor SHIP−/− Tregs (41.66 ± 15.09%, n = 3; p = 0.868 (NS)) significantly altered TNF-α production from WT Teff cells (43.43 ± 11.82%, n = 4).

**SHIP regulates the colitogenic potential of T eff cells**

Surprisingly, during the course of these experiments we consistently observed that the transfer of SHIP−/− Teff cells appeared to induce less severe changes in colon morphology including more moderate mucosal inflammatory cell infiltration and epithelial hyperplasia and reduced mucin depletion from goblet cells (Fig. 3A). This difference was statistically significant when colitis scores in the proximal colon were compared (SHIP−/− Teff cells, 1.88 ± 0.40 vs WT Teff cells, 3.13 ± 0.71, n = 8; p < 0.001, Fig. 3B). Furthermore, analysis of inflammatory cytokines present in serum showed that mice that received SHIP−/− Teff cells had significantly lower levels of systemic IFN-γ (SHIP−/− Teff cells, 32.30 ± 6.42 pg/ml (n = 5) vs WT Teff cells, 65.98 ± 11.91 pg/ml (n = 4); p = 0.0009) and TNF-α (SHIP−/−, 132.6 ± 76.91 pg/ml (n = 5) vs WT, 530.3 ± 243.4 pg/ml (n = 4); p = 0.010) (Fig. 4A). This reduction in systemic IFN-γ and TNF-α is indicative of reduced Th1 cell development and inflammation, and it correlates with the reduced severity of colitis induced by transfer of SHIP-deficient Teff cells.

**Negative regulation of iTreg development by SHIP is T cell intrinsic**

More detailed analysis of recipients of SHIP−/− Teff cells revealed that MLN from these mice contained significantly fewer numbers of Foxp3+ cells than did MLN from recipients of WT Teff cells (SHIP−/−, 10.69 ± 2.20% vs WT, 1.82 ± 0.32%, n = 6–8; p < 0.0001) (Fig. 4B). Notably, the percentage of MLN cells expressing Foxp3 in recipients of SHIP−/− Teff cells was not significantly different from that in mice that received WT Teff and Treg cotransfer (10.12 ± 1.32%, n = 9; p = 0.53). Although the WT Teff plus WT Treg population contained ~25% Foxp3+ cells at the time of transfer, after 8 wk in vivo this was reduced to ~10%. This reduction in Foxp3+ cells reflects the normal homeostatic level of Tregs and is consistently seen in the T cell transfer model of colitis (29).

Immunohistochemical staining of colon tissue samples (Fig. 4C) revealed that Foxp3+ cells were also located within the colonic tissue of mice that received SHIP−/− Teff cells: Foxp3+ cells were found scattered throughout inflammatory infiltrates and also immediately below the epithelial basement membrane of intact epithelium. In contrast, very few Foxp3+ cells were present in mucosal inflammatory infiltrates of mice that received WT Teff cells and no inflammation or Foxp3+ cells were found in PBS control mice.

We speculated that the increase in iTregs in mice receiving SHIP−/− Teff cells may be due to insufficient purification and...
anti-CD3 (1 H9262 with either 10 cells from WT or SHIP stimulation (31). We also tested the effect of TGF-

tion since iTreg development is influenced by the strength of TCR

experiments were performed with different modes of TCR activa-

tion autocrine TGF-

The absence of M2 macrophages in recipients of SHIP mice did not differ in the percentages of Foxp3 cells (30). However, analysis of the cells pretransfer revealed that CD4+CD25−CD45RBhigh T cells from WT and SHIP−/− mice did not differ in the percentages of contaminating Foxp3+ cells (Fig. 4D) or CD4+ cells (<1%). The absence of M2 macrophages in recipients of SHIP−/− T cells was confirmed by flow cytometric analysis for CD11b+ macrophages and investigation of arginase I activity in spleen populations (data not shown). We also considered the possibility that the small fraction (≤1%) of SHIP−/− Foxp3+ Tregs that were transferred had a significant growth advantage compared with WT Tregs, but since we did not see a difference in the in vitro proliferative capacity of SHIP−/− Tregs (Fig. 2B), preferential proliferation of these cells is unlikely to be the major factor leading to their increase in vivo. Thus, somewhat counterintuitively, in the absence of this negative regulator of the PI3K pathway, Teff cells appear to have an intrinsic capacity to differentiate more frequently into iTregs.

**SHIP inhibits in vitro Treg differentiation in the presence of strong TCR stimulation**

To directly test the possibility that there is a T cell-intrinsic role for SHIP in iTreg development, we tested the capacity of CD4+CD25−CD45RBhigh T cells from WT and SHIP−/− mice to develop into Foxp3+ cells in vitro in the presence of TGF-β. These experiments were performed with different modes of TCR activation since iTreg development is influenced by the strength of TCR stimulation (31). We also tested the effect of TGF-β in the presence of RA, the active metabolite of vitamin A, since it enhances TGF-β-induced Foxp3 expression (31, 32) and enables Treg differentiation even in the presence of high levels of costimulation (31). Upon weak TCR stimulation with immobilized anti-CD3 and soluble CD28, similar percentages of Foxp3+ Treg cells were elicited by TGF-β from WT (75.53 ± 8.02%, n = 4) and SHIP−/− (75.22 ± 12.98%, n = 4) T cells (Fig. 5A). The addition of RA to this condition did not reveal a difference in iTreg differentiation (% Foxp3+ cells: WT, 76.98 ± 16.01% vs SHIP−/−, 68.81 ± 21.18, n = 3; p = NS). Similarly, upon mild TCR stimulation with a low (1:5) ratio of anti-CD3/CD28-coated beads, TGF-β induced similar Foxp3 expression in WT and SHIP−/− T cells (% Foxp3+ cells: WT, 45.14 ± 9.68% vs SHIP−/−, 33.27 ± 15.62, n = 3; p = 0.575 (NS)) and the addition of RA enhanced this conversion in both genotypes (% Foxp3+ cells: WT, 61.39 ± 7.50% vs SHIP−/−, 58.99 ± 29.94, n = 3; p = 0.191 (NS)).

In contrast, under conditions of strong TCR stimulation, with one anti-CD3/CD28-coated bead for each T cell, iTreg differentiation was significantly increased in SHIP-deficient cells in the presence of TGF-β alone (% Foxp3+ cells: WT, 0.21 ± 0.06% vs SHIP−/−, 4.48 ± 1.54%, n = 4; p = 0.018) or TGF-β and RA (% Foxp3+ cells: WT, 48.50 ± 13.95% vs SHIP−/−, 75.03 ± 9.37%, n = 4; p = 0.037). Culture of SHIP−/− Teff cells with CD3/CD28-coated beads and RA in the absence of TGF-β did not result in any detectable Foxp3 expression (data not shown), indicating that elevated autocrine TGF-β production in the absence of SHIP is unlikely to be responsible for the increased iTreg differentiation.

We also tested whether SHIP−/− iTregs induced by TGF-β, in the absence or presence of RA, were functional. These experiments were performed with cells differentiated with weak TCR activation so that the proportion of induced Foxp3+ cells was similar between WT and SHIP−/− T cells. We found that the SHIP−/− iTreg cells were functional and did not significantly differ from

**FIGURE 5. TGF-β- and retinoic acid-mediated Treg differentiation is increased in the absence of SHIP under strong TCR stimulatory conditions.** A, CD4+CD25−CD45RBhigh T cells from WT or SHIP−/− mice were cultured for 4 days under neutral (100 U/ml IL-2), TGF-β (IL-2 and 1 ng/ml TGF-β), or RA and TGF-β (IL-2, TGF-β, and 10 ng/ml RA) conditions with either 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28- or anti-CD3/CD28-coated beads at a ratio of one bead per cell or one bead to five cells. Data are representative of three independent experiments. B, To test for suppressive capacity, WT CD4+ T cells were stimulated with anti-CD3 (1 μg/ml) and T-depleted irradiated splenocytes (5 × 10^5) in the presence or absence of iTregs differentiated with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 with TGF-β or TGF-β and RA. After 72 h, [3H]thymidine was added to the cultures for the final 16 h before harvesting. Data are expressed as percentage suppression and represent the average ± SD of two independent experiments.
WT iTregs in terms of their capacity to proliferate (data not shown) or to suppress the proliferation of WT Teff cells (Fig. 5B).

SHIP is required for optimal Th17 cell differentiation in vitro

It is now known that TGF-β is involved in the development of both iTregs and Th17 cells, with the latter developing in the presence of TGF-β and proinflammatory cytokines such as IL-6 (33). We therefore hypothesized that SHIP may be involved in the reciprocal development of these two cell types. To test this, Teff cells from WT or SHIP−/− mice were cultured under Th17, Th1, or neutral differentiation conditions. In agreement with Tarasenko et al. (34), the absence of SHIP resulted in enhanced Th1 cell differentiation, as evidenced by an increase in the percentage of cells capable of producing IFN-γ (Fig. 6A). In contrast, the absence of SHIP significantly impaired Th17 cell differentiation, resulting in fewer cells expressing IL-17 by intracellular staining analyses (% IL-17+ cells: SHIP−/−, 15.16 ± 0.23% vs WT, 30.70 ± 0.97%, n = 3; p = 0.0017).

FIGURE 6. SHIP-deficient T cells are deficient in Th17 differentiation in vitro. A, CD4+CD25+CD45RBhigh T cells from WT or SHIP−/− mice were stimulated for 6 days with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 under neutral, Th1, or Th17 polarizing conditions. B, CD4+CD25+CD45RBhigh T cells were stimulated for 6 days with the indicated ratio of anti-CD3/CD28-coated beads to cells under Th17 polarizing conditions. C, The amount of IL-17 in triplicate culture supernatants from B was measured by ELISA. D, Naive cells were stimulated with plate-bound anti-CD3 (10 μg/ml) and soluble anti-CD28 (1 μg/ml) under Th17 differentiation conditions with increasing amount of IL-6 for 4 days. In all cases cells were restimulated with PMA and ionomycin and the percentage of cells producing IL-17, IFN-γ, or expressing Foxp3 was assessed by flow cytometry. E, CD4+ T cells from SHIP−/− or WT controls were stimulated with mIL-6 (100 ng/ml) for the indicated times. Cells were stained for phospho-STAT3 and CD4 and analyzed by flow cytometry. F, CD4+CD25+ T cells were purified and analyzed for expression of RORγt, Foxp3, and T-bet mRNA by quantitative RT-PCR. Data in A–E are representative of a minimum of three individual experiments. For E and F, data are the average ± SD from three independent experiments.
Since a differential requirement for SHIP depending on the strength of TCR activation was found in iTreg differentiation, Th17 cell differentiation was compared under strong (one bead to one cell) and mild (one bead to five cells) TCR activation (Fig. 6B). Th17 cell development was most efficient under strong stimulatory conditions, and in the absence of SHIP there was a 2-fold reduction in IL-17 expression in cells that were IL-6-expressing and an 8-fold reduction in IL-17 secretion (SHIP−/−, 1.48 ± 1.65 ng/ml vs WT, 11.8 ± 2.8 ng/ml, n = 3; p = 0.0074) (Fig. 6C). With mild TCR activation, SHIP−/− Teff cells failed to differentiate into Th17 cells, and levels of secreted IL-17 were below the limits of detection (Fig. 6, B and C).

To determine whether reduced Th17 cell development was due to altered responsiveness to IL-6 in SHIP−/− Teff cells, IL-6 was titrated into differentiation assays. These experiments revealed that Th17 cell differentiation was reduced in SHIP−/− Teff cells regardless of the concentration of IL-6 present (Fig. 6D). Under these conditions, in the absence of IL-2, IL-6 inhibited expression of Foxp3 equally in WT and SHIP−/− Teff cells, indicating that SHIP is not required for IL-6-mediated suppression of Foxp3 expression.

The development of iTregs vs Th17 cells is known to require cytokine-induced activation of STAT5 and STAT3, respectively (35). Although IL-2-induced activation of STAT5 did not differ significantly between WT and SHIP−/− CD4+ T cells (data not shown), SHIP−/− CD4+ T cells had a significant defect in IL-6-stimulated phosphorylation of STAT3 (Fig. 6E). Additionally, ex vivo CD4+CD25− T cells had reduced basal expression of RORyt and enhanced expression of T-bet and Foxp3 mRNA (Fig. 6F). Combined with evidence that SHIP−/− T cells have enhanced IFN-γ-induced phosphorylation of STAT1 (34), these data suggest that SHIP may have an underappreciated role in cytokine-mediated regulation of STAT activation and control over the expression of Th cell lineage-defining transcription factors.

**FIGURE 7.** SHIP deficiency impairs Th17 cell differentiation in vivo. MLN and spleen cells isolated 8 wk after transfer of the indicated T cell populations were stimulated with PMA and ionomycin. A, Intraglomerular IFN-γ and IL-17 production by MLN cells in gated CD4+ cells. Data are representative of three mice per group. B, Amounts of IL-17 in culture supernatants from stimulated MLN and spleen cells isolated 8 wk after transfer of the indicated T cell populations. The development of iTregs vs Th17 cells is known to require cytokine-induced activation of STAT5 and STAT3, respectively (35). Although IL-2-induced activation of STAT5 did not differ significantly between WT and SHIP−/− CD4+ T cells (data not shown), SHIP−/− CD4+ T cells had a significant defect in IL-6-stimulated phosphorylation of STAT3 (Fig. 6E). Additionally, ex vivo CD4+CD25− T cells had reduced basal expression of RORyt and enhanced expression of T-bet and Foxp3 mRNA (Fig. 6F). Combined with evidence that SHIP−/− T cells have enhanced IFN-γ-induced phosphorylation of STAT1 (34), these data suggest that SHIP may have an underappreciated role in cytokine-mediated regulation of STAT activation and control over the expression of Th cell lineage-defining transcription factors.

**Discussion**

We have shown here that SHIP has a key role in regulating CD4+ T cell differentiation via its capacity to promote Th17 and limit iTreg development. In vitro and in vivo analyses revealed that in the absence of SHIP, T cells have an enhanced capacity to develop into iTregs and a parallel decrease in Th17 cell development. These data suggest that modulation of the PI3K pathway is central to T cell-mediated immune regulation and suggest that pharmacological manipulation of SHIP activity may represent a new strategy for manipulating the balance between Tregs and Th17 cells.

We previously reported that human peripheral blood Foxp3+ Tregs show reduced activation of the PI3K pathway downstream of the TCR (2). We show herein that murine Tregs share the same alteration in PI3K pathway activation, as demonstrated by phosphorylation of Akt at Ser473. Mounting evidence indicates that limitation of Akt activity in Tregs is essential for the development of the normal Treg transcriptional signature (4), induction, but not maintenance, of Foxp3 expression (3, 4) and suppressive capacity (2). On the other hand, at least some level of PI3K activity appears to be critical for Treg suppression, since Tregs with a kinase dead mutant of the main PI3K isoform activated downstream of TCR stimulation, p110δ, are unable to prevent the spontaneous development of colitis (36), and IL-2-mediated preservation of Treg function is dependent on PI3K (37).

We, like others (25, 26), observed a dramatic increase in the ratio of Teff cells to Tregs as well as an increase in the absolute number of Treg cells in the spleen and lymph nodes of SHIP−/− mice. Collazo et al. (26) recently reported that SHIP−/− mice also have an increase in the proportion, but not the absolute number, of thymic Foxp3+ Tregs, suggesting that increased thymic output likely does not account for the expansion of peripheral Foxp3+ Treg cells. Indeed, since T cell-specific deletion of SHIP does not result in an increase in the number of splenic Foxp3+ cells, there is clearly a T cell extrinsic role for SHIP in the regulation of Treg development (34). Nevertheless, our data support the conclusion that there is also a T cell-intrinsic role for SHIP in the peripheral development of iTregs since in vivo adoptive transfer experiments revealed that a significantly higher proportion of SHIP−/− Teff cells became Foxp3+ in comparison to WT Teff cells.

Further investigation into how SHIP may be involved in Treg development revealed a critical role for this lipid phosphatase in...
the de novo differentiation of iTregs. Thus, under conditions of strong TCR activation, SHIP-deficient Teff cells differentiated more readily into Foxp3+ T cells upon stimulation with TGF-β in the absence or presence of RA in vitro. These Treg differentiation conditions are predicted to recapitulate the strong inflammatory conditions in the colon, where dendritic cells capable of processing retinol to RA are known to reside (38), and thus correlate with our in vivo data. Additionally, it has been suggested that RA promotes Treg differentiation by attenuating costimulation (31), consistent with the recent report that premature termination of TCR activation leads to the generation of Tregs (3). Interestingly, up-regulation of Foxp3 induced by premature termination of TCR signaling was contingent upon PI3K pathway inactivation (3), suggesting that RA may act by inhibiting PI3K pathway activity.

Since SHIP is generally thought to be a negative regulator of PI3K, and reduced activity of the PI3K pathway is strongly correlated with Foxp3 expression and normal Treg function (2–4), it was surprising that more iTregs developed in the absence of SHIP. Increasing evidence, however, indicates that SHIP may not simply “switch off” the PI3K pathway, but rather redirect signaling by specifically recruiting proteins containing pleckstrin homology domains to bind to the product of SHIP catalytic activity, PI(3,4)P₂, rather than to phosphatidyl inositol 3,4-di-phosphate (39). Additionally, although SHIP is involved in TCR signaling (39), evidence that TCR-mediated activation of Akt is unchanged in the absence of SHIP (34) suggests that this protein is dispensable for TCR-mediated activation of PI3K. Therefore, it is possible that modulation of reciprocal Treg and Th17 development by SHIP is mediated via a cytokine-dependent pathway, possibly related to distinct requirements for TGF-β-induced SMAD4 (19).

T cell-specific deletion of SHIP has been demonstrated to enhance Th1 differentiation due to increased sensitivity to cytokine-mediated T-bet induction (34). Indeed, the enhanced potential to differentiate into Th1 cells coupled with elevated PI3K signaling led us to predict that transfer of SHIP-deficient Teff cells would lead to exacerbated colitis. In striking contrast, however, SHIP−/− Teff cells induced less severe colitis and developed into an increased proportion of Foxp3+ cells in the colon, MLNs, and spleen. Although we cannot exclude the possibility that the increased proportion of Foxp3+ Tregs in the absence of SHIP is related to an increase in their proliferative capacity, evidence that ex vivo Tregs remained anergic and that TGF-β-induced Tregs proliferated at the same rate as their WT controls suggests that this is not the case. Moreover, since SHIP−/− Teff cells do not have enhanced proliferation in vitro or in vivo (34), it seems unlikely that there would be Treg-specific changes in this respect.

Although the proportion of Foxp3+ iTregs that differentiated in vivo from SHIP−/− Teff cells did not differ significantly from mice who received WT Teff cells and WT Tregs, recipients of SHIP−/− Teff cells had an intermediate colitis score. Since the in vitro suppressive capacity of SHIP−/− iTregs was similar to that of WT iTregs, this intermediate colitic phenotype is likely related to the kinetics of iTreg development in vivo. A major difference between the SHIP−/− Teff and WT Teff plus WT Treg groups is that the latter has fully developed natural Tregs at the time of transfer whereas the de novo development of iTregs in the former condition would take time, likely allowing for activation of pathogenic cells and the observed increase in colitis.

A more detailed analysis of cytokine production revealed that SHIP−/− naive T cells had a significant defect in their capacity to differentiate into Th17 cells in vivo, correlating with the known pathogenic role of Th17 cells in IBD (40). Parallel in vitro assays to test cytokine-induced Th17 cell differentiation demonstrated that SHIP-deficient Teff cells produced dramatically less IL-17 than their WT counterparts. This deficiency in IL-17 production was independent of the concentration of IL-6 present, and it was not due to increased inhibitory effects of IFN-γ on Th17 cell development, since the amounts of IFN-γ under Th17 polarizing conditions were equal in WT and SHIP−/− T cells. Nevertheless, since T-bet is known to negatively regulate Th17 differentiation (11, 41, 42) and SHIP−/− CD4+ T cells have high basal levels of T-bet and low RORγt mRNA, this imbalance in expression of lineage-defining transcription factors likely contributes to the molecular basis for the altered T cell differentiation in the absence of SHIP.

An outstanding question is which signaling pathways require SHIP for normal Th17 cell development. Evidence that SHIP−/− Teff cells do not have altered TCR-mediated activation of Erk, Akt, Zap-70, or phospholipase C-γ1 (34) suggests that the relevant pathways may be cytokine driven. Indeed, we found that IL-6-stimulated phosphorylation of STAT3 was significantly impaired in SHIP−/− CD4+ T cells. Moreover, TGF-β is known to induce SHIP expression and lipid phosphatase activity (21) and changes in this signaling pathway could contribute to the development of both naturally occurring Tregs and iTregs (43). Further investigation will be required to define how SHIP controls cytokine-mediated regulation of the STAT and PI3K pathways and thereby acts as a critical determinant in TH17 vs iTreg development.

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