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Role of SHIP1 in Invariant NKT Cell Development and Functions

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SHIP1 is a 5'-inositol phosphatase known to negatively regulate the signaling product of the PI3K pathway, phosphatidylinositol (3-5)-trisphosphate. SHIP1 is recruited to a large number of inhibitory receptors expressed on invariant NK (iNKT) cells. We hypothesized that SHIP1 deletion would have major effects on iNKT cell development by altering the thresholds for positive and negative selection. Germline SHIP1 deletion has been shown to affect T cells as well as other immune cell populations. However, the role of SHIP1 on T cell function has been controversial, and its participation on iNKT cell development and function has not been examined. We evaluated the consequences of SHIP1 deletion on iNKT cells using germline-deficient mice, chimeric mice, and conditionally deficient mice. We found that T cell and iNKT cell development are impaired in germline-deficient animals. However, this phenotype can be rescued by extrinsic expression of SHIP1. In contrast, SHIP1 is required cell autonomously for optimal iNKT cell cytokine secretion. This suggests that SHIP1 calibrates the threshold of iNKT cell reactivity. These data further our understanding of how iNKT cell activation is regulated and provide insights into the biology of this unique cell lineage. The Journal of Immunology, 2015, 195: 000–000.

Natural killer T cells are a heterogeneous subset of innate lymphocytes that express NK cell markers in addition to a TCR. There are multiple functionally distinct categories of NK cells, including invariant NKT (iNKT) cells, also known as type I NKT cells (1, 2). iNKT cells represent a small fraction of mature T cells within the thymus, spleen, and lymph nodes. However, iNKT cells also accumulate in non-lymphoid organs, including the blood, liver, and gut. In mice, iNKT cells make up a robust population within the liver, ranging between 25 and 40% of the lymphocytes (3). iNKT cell development occurs in the thymus from the same precursors as conventional T cells but diverges during positive selection (1, 2, 4). Although conventional T cells are selected and restricted by classical MHC peptide Ags presented by thymic cortical epithelial cells, iNKT cells are selected by CD4+CD8+ double positive (DP) cortical thymocytes that express CD1d (1, 2). CD1d is a nonclassical MHC class I-like molecule that preferentially binds glycolipid Ags (1, 2). iNKT cells are able to recognize presented glycolipid Ags because of their unique semi-invariant TCR, which consists of an invariant Vα14-Jα18 chain that preferentially dimerizes with a limited number of B-chains, mainly Vβ8.2, Vβ7, and Vβ2 (1, 2, 4–8).

In addition to their unique TCR repertoire, iNKT cells are characterized by their ability to rapidly secrete a wide array of cytokines upon stimulation, either through direct TCR activation or indirectly through cytokine signaling. This can include the production of large amounts of IFN-γ and IL-4 (1, 9), allowing iNKT cells to participate in either Th1- or Th2-polarized responses. Because of their rapid and diverse responses, iNKT cells are multifunctional and capable of augmenting the participation of other immune cells, including B cells, NK cells, macrophages, and other T cells (10–14).

The PI3K signaling pathway participates in a number of cellular processes, not limited to cellular activation, development, migration, proliferation, and survival (15, 16). PI3Ks phosphorylate PI(4,5)P2 to PI(3,4,5)P3. PI(3,4,5)P3 is a second messenger that attracts effector proteins containing a Pleckstrin homology domain and assists in their attachment to the inside of the plasma membrane, leading to downstream cellular responses (15, 17). Together with phosphatase and tensin homolog deleted on chromosome 10 (PTEN), SHIP1 is an important negative regulator of PI3K signaling. SHIP1 is expressed predominantly in hematopoietic cells, as well as mesenchymal stem cells and stromal cells (18, 19), and acts by dephosphorylating PI(3,4,5)P3 into PI(3,4)P2 (20). The Src homology 2 domain of SHIP1 allows it to associate with both ITAM- and ITIM-containing receptor tails, including SLAM family receptors and TCR-associated CD3 chains (21–23). Recently, our laboratory has shown that SHIP1 is recruited to the ITIM of KLRC1 receptors to negatively regulate intracellular signaling (24).

Global loss of SHIP1 results in a pleiotropic phenotype because of its role in the development and function of a number of immune cells. Germline-deficient SHIP1 animals have increased myeloid cell number, attributed to heightened proliferation and survival, but
are conversely lymphopenic (25). B cell development and survival are also affected by SHIP1 regulation and BCR signaling is hypersensitive (26, 27). However, the role of SHIP1 in T cell development and functions is less clear. Some studies claim that deletion of SHIP1 affects T cell development, whereas others report no major developmental issues (25, 28, 29). Using germline-deficient mice, chimeric mice, and conditionally deficient mice, we revisited these studies with a special emphasis on iNKT cells, which are known to express inhibitory receptors capable of associating with SHIP1. We found that iNKT cell and T cell development are significantly impaired in mice with germline deletion of SHIP1. However, this phenotype could be rescued by extrinsic expression of SHIP1. In contrast, SHIP1 was required cell autonomously for optimal iNKT cell cytokine secretion, suggesting that SHIP1 calibrates the threshold of iNKT cell reactivity.

Materials and Methods

Mice

Inbred C57BL/6 mice and BALB/c mice were purchased from Taconic Farms (Germantown, NY). SHIP1 mice were described previously and bred to acquire SHIP1+/+, SHIP1−/−, and SHIP1+/− animals (30). C57BL/6.1J, 18 heterozygous mice were provided by Dr. M Taniguchi (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan) and bred to obtain Ly5.1−/− mice in our facility. B6-Thy1.1 mice were acquired from The Jackson Laboratory (Bar Harbor, ME). CD4CreSHIPfl/fl (referred to as CD4creSHIP) and SHIP1fl/fl (referred to as SHIP1) samples were described previously (31). All mice were maintained at Brown University in pathogen-free facilities.

Isolation of murine lymphocytes

Mice were sacrificed by isofluorane treatment, and a cardiac puncture was performed prior to 7 wk of age. Characterization of SHIP1-deficient animals was purchased from eBioscience (San Diego, CA). Ly90C/6-FITC, Ly49A/D-PE, and CD4-PerCP were purchased from BD Pharmingen. TCRVβ7-8 was purchased from BioLegend. CD1d tetramer loaded with α-GalCer, CD1d tetramer loaded with PBS-57 as well as unloaded controls were provided by the National Institutes of Health Tetramer Facility.

In vivo proliferation analysis

Recipient mice were sublethally irradiated with 7.5 Gy and placed on oral Sulfamethoxazole and Trimethoprime (Hi-tech Pharmacal) treatment. On day three postirradiation, donor mice were sacrificed. thymi were harvested, and thymocytes were isolated. iNKT cells were enriched by labeling samples with anti-CD8 magnetic beads and depleting CD8+ cells using an AutoMACS (Miltenyi Biotec). Cells were then stained for 10 min at 37°C in the dark with 10 μM C Cell Proliferation Dye eFluor450 (eBioscience) in PBS. Irradiated recipients were i.v. injected with 5–10 million cells. iNKT cell proliferation was analyzed in recipient spleen, liver, and blood samples on day 7 postinjection.

In vitro iNKT cell stimulation and cytokine analysis

A 96-well plate was coated with 0.5 μg/well anti-mouse CD3 and CD28 Abs (eBioscience). Thymic iNKT cells were sorted using a FACSAria (BD Biosciences) and plated in triplicate at a concentration of 10,000 or 30,000 cells/well, depending on the experiment. The supernatant was collected 24 h poststimulation, and cytokines were quantified using a Cytometric Bead Array Flex Sets (BD Biosciences) and analyzed with FCAP Array Software (BD Biosciences). For cytokine stimulation, 2 × 10^6 thymocytes were treated with PMA (200 ng/ml) and ionomycin (5 μg/ml) for 2 h at 37°C in RPMI 1640 cell culture medium with 10% FBS. Samples were stained with cell surface markers, fixed, and permeabilized, and stained with anti-cytokine Abs.

Generation of mixed bone marrow chimera

Jo18−/− recipient mice (CD45.2+) were lethally irradiated with 10.5 Gy and placed on oral sulfamethoxazole and trimethoprim (Hi-tech Pharmacal) treatment for 2 wk. One day postirradiation, donor bone marrow cells were harvested under sterile conditions from SHIP1−/− and SHIP1+/− animals (CD45.1+) and control C57BL/6 animals (CD45.2+). Anti-CD5 and anti-DX5 magnetic beads were used to deplete mature T and NK cells using an AutoMACS (Miltenyi Biotec). Mixed bone marrow chimeric mice were generated by pooling cells from SHIP1+/− or SHIP1−/− animals with cells from C57BL/6 animals in a 1:1 ratio. A total of 1 × 10^7 cells were injected i.v. into recipients and allowed to reconstitute for 8–10 wk.

Statistical analysis

All statistical analyses were accomplished with Prism Version 5.0 (GraphPad Software) or Excel (Microsoft) using unpaired two-tailed Student t tests. A paired two-tailed Student t test was used for samples from mixed bone marrow chimera. **p < 0.0001, ***p = 0.0001–0.001, ****p = 0.001–0.01, *p = 0.01–0.05, and p > 0.05 is not significant.

Results

Germline deletion of SHIP1 hinders iNKT cell development

SHIP1 (encoded by INPP5D) expression is an important regulator of P13K activity. Loss of SHIP1 in mice leads to a number of hematopoietic defects, such as myeloid cell infiltration in the lungs and a severe inflammation of the small intestine that resembles human Crohn’s disease (25, 31). Given its proposed role as a negative regulator of TCR signaling (32, 33) and because iNKT cell development is critically dependent on unique signals emanating from its semi-invariant TCR (34), we hypothesized that SHIP1 may play a role in iNKT cell development and functions. To minimize any pleiotropic effects, SHIP1−/− animals were sacrificed prior to 7 wk of age. Characterization of SHIP1-deficient mice revealed an increased frequency of iNKT cells in the thymus yet decreased frequencies in the spleen and liver, compared with SHIP1+/− and SHIP1+/+ animals (Fig. 1A, Supplemental Fig. 1A). In contrast, the total number of iNKT cells was significantly decreased in the thymus and spleen (Fig. 1B). To exclude this being because of a relocation to other organs, we determined that the frequency of iNKT cells in the bone marrow, blood, intestinal lymph nodes, and mesenteric lymph nodes was also decreased (Supplemental Fig. 2A, 4).
We next wanted to determine whether SHIP1 deletion was causing iNKT cells to arrest at a specific stage of maturation. However, there was no difference in iNKT cell maturation between stages 1 through 3, distinguished by NK1.1 and CD44 expression (Fig. 1C, Supplemental Fig. 1B). Notably, the frequency of stage 0 iNKT cells in SHIP1-deficient animals was modestly increased, whereas the total number was comparable to littermate controls (Supplemental Fig. 1C). These results indicated that residual iNKT cells are capable of undergoing normal development. We also examined whether the absence of SHIP1 influenced the ratio of NKT1, NKT2, and NKT17 cell lineages. iNKT cells have recently been phenotypically classified according to their function and transcription factor expression and are distinguished using anti-PLZF, anti-RORγt, and anti–T-bet mAbs (35). When comparing thymic iNKT cells from SHIP1-deficient mice and littermate controls, we found a modest skew toward the NKT1 lineage (Fig. 2). A BALB/c positive control was used to define the gating strategy for these experiments because they have robust populations of the three NKT lineages.

Interestingly, when we examined the thymic T cell compartment, it revealed that T cell development was being affected globally. SHIP1−/− mice had increased frequencies of double-negative (DN) and CD4 and CD8 single-positive (SP) T cell populations and a decreased frequency of CD4+CD8− DP T cells, compared with SHIP1−/+ and SHIP1+/+ controls (Fig. 3). However, although the mice had been crossed more than 15 times onto the B6 background, there was a spectrum in the severity of the impairment observed in the T cell compartment, most likely because of the pleiotropic effects of global SHIP1 loss (Fig. 3B). Similarly to T cells, iNKT cell frequency was also quite variable in the thymus of SHIP1-deficient animals (Fig. 1A). Interestingly, SHIP1−/− mice with less severely affected T cell development were also those with less serious defects in their iNKT cell population (data not shown). Overall, the data demonstrated that germline deletion of SHIP1 hindered both T cell and iNKT cell development.

Extrinsic expression of SHIP1 rescues T cell development

As mentioned above, germline deletion of SHIP1 results in massive myeloid infiltration of the lung, making it difficult to study the role of SHIP1 in lymphocytes without the influence of an inflammatory environment. In addition, the intrinsic role of SHIP1 in iNKT cell development cannot be examined in SHIP1-deficient animals. To circumvent these two issues, we generated a series of mixed bone marrow chimeras. SHIP1−/− bone marrow, which is on a congenic
background, and wild-type C57BL/6 competitor bone marrow were transferred at a 1:1 ratio into lethally irradiated Jα18−/− recipients (Fig. 4A). Jα18−/− animals lack the Jα18 gene segment necessary for generation of the iNKT cell invariant α-chain. Therefore, all iNKT cells present in mixed bone marrow chimeric mice were derived from the two donor populations. Analysis of the chimeric mice indicated that conventional T cell development was globally intact. In contrast to the germline-deficient mice, the thymic T cell compartment of the chimera did not reveal significant differences in the DP, DN, or CD4 and CD8 SP populations after the lymphoid gate in SHIP1+/+ (Fig. 3B), SHIP1−/−, and SHIP1−/−− mice. Black circles and bars, SHIP1+/+ (n = 14); gray circles and bars, SHIP1+/− (n = 18); and white circles and bars, SHIP1−/− (n = 16). Data are pooled from at least three independent experiments, and each dot is representative of one mouse. Error bars indicate SEM. ****p < 0.0001, ***p = 0.0001–0.001.

To confirm these results, we also analyzed mice that were conditionally deficient for SHIP1. In these mice (CD4CreSHIP1fl/fl, referred to as CD4Cre), a transgene for Cre recombinase driven by the cd4 enhancer/promoter/silencer (36, 37) allows specific gene deletion at the double negative 4 (DN4)/DP stages of T cell development. To verify that SHIP1 was efficiently deleted from T cells, splenic T cells and non-T cell populations from CD4Cre and CD4CreSHIP1fl/fl donor populations had comparable frequencies in all the organs tested, suggesting that extrinsic expression of SHIP1 was sufficient for normal development of the iNKT cell compartment as well (Fig. 4C).

FIGURE 3. SHIP1−/− mice have impaired conventional T cell development. (A and B) Frequencies of thymic lymphocyte populations that are DN (CD4−CD8−), DP (CD4+CD8+), CD4 SP, and CD8 SP from SHIP1+/+, SHIP1−/−, and SHIP1−/−− mice. (C) Representative staining of thymic T cell development, illustrating DN (CD4−CD8−), DP (CD4+CD8+), CD4 SP, and CD8 SP populations after the lymphoid gate in SHIP1+/+, SHIP1−/−, and SHIP1−/−− mice. Black circles and bars, SHIP1+/+ (n = 14); gray circles and bars, SHIP1+/− (n = 18); and white circles and bars, SHIP1−/− (n = 16). Data are pooled from at least three independent experiments, and each dot is representative of one mouse. Error bars indicate SEM. ****p < 0.0001, ***p = 0.0001–0.001.

was also intact in the thymus, spleen, and liver of CD4Cre and Control animals (Fig. 5A). In addition, analysis of the different developmental thymic iNKT cell stages did not show any abnormalities (Fig. 5B). Taken together, these data further illustrated that extrinsic expression of SHIP1 was sufficient to rescue iNKT and T cell development.

Decreased number of iNKT cells in mice with germline deletion of SHIP1 is due to impaired proliferation

SHIP1 has been shown to have opposing roles on myeloid and lymphoid cell proliferation (25, 38). Therefore, the reduced number of iNKT cells in the thymus and the spleen could potentially be the result of impaired proliferation in SHIP1-deficient animals. To evaluate the role of SHIP1 on iNKT cell proliferation, thymic iNKT cells from SHIP1−/− and wild-type C57BL/6 animals were labeled with Cell Proliferation Dye eFluor450 and injected intravenously into sublethally irradiated recipients. This lymphopenia-induced in vivo proliferation assay has shown that iNKT cells undergo extensive proliferation (39). We found that iNKT cells from SHIP1−/−− animals had a decreased proliferative capacity on Day 7 compared with control wild-type cells in the liver, spleen, and blood (Fig. 6A). We also investigated steady state iNKT cell proliferation on Day 0 using intracellular Ki67 expression. In agreement with the impaired proliferation, we observed that SHIP1−/−/− thymic iNKT cells had significantly decreased Ki67 expression compared with C57BL/6 thymic iNKT cells (Supplemental Fig. 3A). Interestingly, SHIP1−/−− animals with less affected T cell development and iNKT cell populations had comparable Ki67 expression to C57BL/6 controls (Supplemental Fig. 3B). We also observed thymic iNKT cells from CD4Cre and Control animals to have comparable Ki67 levels (Supplemental Fig. 3C). Notably, CD69 expression, a marker of early activation, was not affected in SHIP1-deficient iNKT cells (data not shown). Taken
together, these data indicated that the lower number of iNKT cells in SHIP1/−/− mice was due to a decreased proliferative potential, which could be rescued with extrinsic expression of SHIP1.

**iNKT cell cytokine production is decreased in the absence of SHIP1**

We next sought to determine whether deletion of SHIP1 affected iNKT cell functions. iNKT cells are unusually poised to produce cytokines and can secrete a large amount ex vivo without priming. Thymic iNKT cells were sorted from SHIP1/−/− and SHIP1+/− animals and stimulated with a combination of anti-CD3 and anti-CD28 mAbs for 24 h and culture supernatants were then collected to measure cytokine production. Ligation of CD3 or CD28 on T cells has been shown to induce SHIP1 tyrosine phosphorylation (40). Using cytometric bead array flex sets, we measured the amount of cytokines produced by activated thymic iNKT cells. We found that SHIP1 is not required intrinsically for iNKT cell development but is essential for optimal iNKT cell cytokine production (45). We next wanted to determine whether SHIP1-deficient iNKT cells were capable of producing cytokines irrespective of TCR stimulation. To accomplish this, we stimulated thymocytes from SHIP1/−/− animals and littermate controls for 2.5 h with PMA and Ionomycin, which act downstream of SHIP1. Interestingly, there were also significant decreases in the frequency of iNKT cells producing IFN-γ and TNF-α (Supplemental Fig. 3D, 3E). Altogether, these data demonstrate that SHIP1-deficient iNKT cells are hyporesponsive.

**Discussion**

The PI3K signaling pathway participates in a number of cellular processes, including activation, development, proliferation, and survival (15, 16). PI3K phosphorylates the D3 position of PI(4,5)P2 molecules to yield PI(3,4,5)P3 (42). PTEN and SHIP1 can prevent the initiation of these signals by converting PI(3,4,5)P3 to PI(4,5)P2 and PI(3,4)P2, respectively (43, 44). It has been shown that the absence of PTEN in T cells impairs the development of iNKT cells (45). Loss of regulation by SHIP1 has been shown to have differential effects on myeloid and lymphoid cells, including both NK and T cells (28, 30, 41, 43, 46, 47). However, the role of SHIP1 on iNKT cells is not well characterized. We found that SHIP1 is not required intrinsically for iNKT cell development but is essential for optimal iNKT cell cytokine production. We also report that SHIP1 expression in trans is
C57BL/6 (CD45.2+) mice. iNKT cells are defined from SHIP1 stimulation of the TCR (32). It has also been shown that T cells membrane, which is involved in cell proliferation following T cell signaling by affecting localization of Tec Kinase at the lymphoid gate. Data are representative of three independent experiments. (A) Cytokine production was assessed in vitro using sorted thymic iNKT cells from SHIP1+/− and SHIP1−/− animals. Represents pooled data from four independent experiments, normalized to heterozygous control samples. (B) Cytokine production was assessed in vitro using sorted thymic iNKT cells from SHIP1−/− and competitor C57BL/6 populations from mixed chimera. Represents pooled data from two independent experiments, normalized to C57BL/6 control samples. Error bars indicate SEM. ***p = 0.0001–0.001, **p = 0.001–0.01, *p = 0.01–0.05.

We found that SHIP1-deficient iNKT cells only skew slightly to a specific iNKT cell subset. However, this is unlikely because the absence of SHIP1 did not appear to have a greater effect on iNKT cell development than conventional T cells. Notably, cell surface expression of Ly49 receptors on iNKT cells was not influenced by absence of SHIP1 (data not shown). Altogether, our data indicate that extrinsic expression of SHIP1 indirectly influences iNKT cell development. The reduction in the iNKT compartment is analogous to the impaired B and NK cell cellularity that is also observed in SHIP1−/− animals (46, 52). However, there are discordant findings regarding an autonomous role for SHIP1 in peripheral NK cell homeostasis using NCR1CreSHIP flox/flox mice (41) and mixed bone marrow chimera (46).

SHIP1 has been shown to participate in negatively regulating T cell signaling by affecting localization of Tec Kinase at the membrane, which is involved in cell proliferation following stimulation of the TCR (32). It has also been shown that T cells from SHIP1−/− animals have a poor proliferative response to TCR stimulation ex vivo (28). We found that SHIP1−/− thymic iNKT cells (or a subset of iNKT cells) have a decreased proliferative capacity in vivo and decreased Ki67 expression. However, animals with the least affected iNKT cell compartment had comparable Ki67 expression to C57BL/6 controls. In addition, CD4Cre and Control animals had comparable Ki67 expression. Therefore, SHIP1 is required in trans for normal iNKT cell proliferation, similar to conventional T cells (29). This explains the decreased number of iNKT cells found in SHIP1−/− animals. Although less likely, the decreased number of total DP cells could additionally result in poor positive selection of NKT cells.

Although iNKT cell development was unaffected in chimeric mice, we found that intrinsic absence of SHIP1 had an impact on iNKT cell cytokine secretion. The poor cytokine response of SHIP1-deficient iNKT cells was somewhat unexpected because SHIP1 is a negative regulator of intracellular signaling but in line with work showing that it promotes, rather than inhibits, IFN-γ production by NK cells (41, 53). However, there are several nonmutually exclusive possibilities that could explain this phenotype. First, it is conceivable that inhibitory receptor signaling via SHIP1 calibrates the threshold of iNKT cell reactivity. In fact, NK cells were also shown to be hyporesponsive in the absence of the phosphatases SHIP1 (46) and SHP-1 (54). Similarly to NK cells (30, 41, 55), the absence of SHIP1 may render iNKT cells hyporesponsive. Alternatively, the lower cytokine release from stimulated SHIP1-deficient iNKT cells could be due to a different differentiation program. For instance, it has been shown that a large proportion of SHIP1-deficient conventional T cells become phenotypically similar to regulatory T cells by expressing Foxp3 (28, 47). We first confirmed the previously observed increase of the regulatory T cell population in SHIP1-deficient animals (Supplemental Fig. 4A, 4C). However, even though other conditions can cause regulatory iNKT cells (56, 57), we found that SHIP1-deficient iNKT cells do not acquire Foxp3 expression (Supplemental Fig. 4B, 4C). Another possibility is that the impaired iNKT cell cytokine production is the result of the expansion of a specific iNKT cell subset. However, this is unlikely because we found that SHIP1-deficient iNKT cells only skew slightly toward the NKT1 lineage. Taken together, our data indicate that
deletion of the negative regulator SHIP1 leads to a decrease of iNKT cell cytokine secretion potential because of an alteration in the threshold for iNKT cell reactivity.

The roles of other phosphatases, such as tyrosine phosphatases SHP-1 and SHP-2, may also contribute to iNKT cell activation. In addition, isoforms of SHIP1 such as s-SHIP and SHIP-2 may participate in immune cell development and function. SHIP-2 is also expressed on hematopoietic cells and contains a 5-phosphatase activity (58), whereas embryonic stem cells and progenitor cells express s-SHIP (59). ENU-induced SHIP1 mutants (SHIP1<sup>1226</sup>) also affect s-SHIP and result in an enhanced impairment, compared with SHIP1<sup>−/−</sup> animals, including reduced T cell populations (60). The contribution of SHIP-1, SHP-2, SHIP-2, and other phosphatases on iNKT cell development and functions still warrant further investigation.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Loss of thymic and peripheral iNKT cells in SHIP1-/- mice. (A) Representative staining of iNKT cell populations in the thymus, spleen, and liver from SHIP1+/+, SHIP1+/−, and SHIP1−/− mice. (B) Representative staining of thymic iNKT cell maturation: CD44loNK1.1− (Stage I), CD44hiNK1.1− (Stage II), and CD44hiNK1.1+ (Stage III). iNKT cells are defined as HSAloCD1tet+ in the thymus and TCRβ+CD1tet+ in the spleen and liver within the lymphoid gate. (C) Frequency and absolute number of thymic Stage 0 iNKT cells from SHIP1+/+, SHIP1+/−, and SHIP1−/− mice. Data are pooled from 2 independent experiments (n=5). Black circles: SHIP1+/+, gray circles: SHIP1+/−, white circles: SHIP1−/−, dotted line: unloaded CD1 tetramer control. Error bars indicate SEM.
Supplementary Figure 2. Loss of thymic and peripheral iNKT cells in SHIP1+/− mice, but normal T cell populations in CD4CreSHIP1fl/fl mice. (A) Representative staining of iNKT cell populations in the bone marrow, blood, inguinal lymph nodes (iLN), and mesenteric lymph nodes (mLN) from SHIP1+/+, SHIP1+/−, and SHIP1−/− mice. (B) Frequency of iNKT cells in each indicated organ from SHIP1+/+, SHIP1+/−, and SHIP1−/− mice. Data are pooled from 3 independent experiments (n=6-8). (C) SHIP1 protein expression from splenocytes was determined using Western blot. T cells (TCRβ+CD4+) and Non T cells (TCRβ−CD4−) were sorted from CD4Cre mice and littermate Controls. Cell lysate was blotted with α-SHIP1 and β-actin antibodies. (D) Frequencies of thymic lymphocyte populations that are DN (CD4−CD8−), DP (CD4+CD8+), CD4 SP, and CD8 SP from CD4Cre mice (n=10) and littermate controls (n=10). Data are pooled from 3 independent experiments and each dot is representative of 1 mouse. iNKT cells are defined as TCRβ+CD11e+ within the lymphoid gate. Black circles: SHIP1+/+, gray circles: SHIP1+/−, and white circles: SHIP1−/− mice. Black bars: Control mice and grey bars: CD4Cre. Error bars indicate SEM.
Supplementary Figure 3A-C. SHIP1 deficiency influences the proliferative capacity and cytokine production of thymic iNKT cells from SHIP1−/− mice. Intracellular Ki67 expression was used to determine steady state proliferative capacity of thymic iNKT cells. (A) Frequencies of Ki67+ thymic iNKT cells from C57BL/6 (n=8) and SHIP1−/− mice (n=14). Data are representative of 4 independent experiments. (B) Representative staining of Ki67+ thymic iNKT cells from C57BL/6 control mice and SHIP1−/− mice with impaired iNKT cell frequency and normal iNKT cell frequency. (C) Frequencies of Ki67+ thymic iNKT cells from CD4Cre mice (n=5) and littermate controls (n=5) and representative staining. Data are representative of 2 independent experiments. iNKT cells are defined as HSAloCD1tet+ in the thymus within the lymphoid gate. Error bars indicate SEM. (D) Cytokine production of thymic iNKT cells from SHIP1+/+ (n=6) and SHIP1−/− (n=6) mice following 2.5 hours of PMA and Ionomycin stimulation, compared to unstimulated controls (n=4). Data are pooled from two independent experiments. (E) Representative TCRβ MFI of thymic iNKT cells following PMA and Ionomycin stimulation from SHIP1+/+ and SHIP1−/− mice and unstimulated control. Numbers in upper right corner indicate MFI for each histogram. iNKT cells are defined as TCRβ+CD1tet+ within the lymphoid gate. Black bars: SHIP1+/+, gray bars: SHIP1−/−, and white bars: unstimulated control. Error bars indicate SEM.
**Supplementary Figure 4. Increased Foxp3 expression of conventional T cells, but not iNKT cells, in SHIP1−/− animals.** (A) Regulatory T cells in the thymus, spleen, and liver of SHIP1+/+ and SHIP1−/− mice. (B) Frequency of Foxp3+ iNKT cells in the thymus, spleen, and liver of SHIP1+/+ and SHIP1−/− mice. (C) Representative staining of Foxp3 and CD25 expression of splenic CD4+ T cell and iNKT cell populations in SHIP1+/+ (n=7) and SHIP1−/− mice (n=7). Data are representative of 3 independent experiments. Regulatory T cells are defined as Foxp3+CD4+TCRβ+ and iNKT cells are defined as TCRβ+CD1Tet+ within the lymphoid gate. Error bars indicate SEM.