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Downstream of Tyrosine Kinases-1 and Src Homology 2-Containing Inositol 5′-Phosphatase Are Required for Regulation of CD4+CD25+ T Cell Development

Masaki Kashiwada,*‡ Giorgio Cattoretti,† Lisa McKeag,*‡ Todd Rouse,§ Brian M. Showalter,* Umaima Al-Alem,* Masaru Niki,‖ Pier Paolo Pandolfi,‖ Elizabeth H. Field,‖§ and Paul B. Rothman2*‡

The adaptor protein, downstream of tyrosine kinases-1 (Dok-1), and the phosphatase SHIP are both tyrosine phosphorylated in response to T cell stimulation. However, a function for these molecules in T cell development has not been defined. To clarify the role of Dok-1 and SHIP in T cell development in vivo, we compared the T cell phenotype of wild-type, Dok-1 knockout (KO), SHIP KO, and Dok-1/SHIP double-knockout (DKO) mice. Dok-1/SHIP DKO mice were runted and had a shorter life span compared with either Dok-1 KO or SHIP KO mice. Thymocyte numbers from Dok-1/SHIP DKO mice were reduced by 90%. Surface expression of both CD25 and CD69 was elevated on freshly isolated splenic CD4+ T cells from SHIP KO and Dok-1/SHIP DKO, suggesting these cells were constitutively activated. However, these T cells did not proliferate or produce IL-2 after stimulation. Interestingly, the CD4+ T cells from SHIP KO and Dok-1/SHIP DKO mice produced higher levels of TGF-β, expressed Foxp3, and inhibited IL-2 production by CD3-stimulated CD4+CD25− T cells in vitro. These findings suggest Dok-1 and SHIP function in pathways that influence regulatory T cell development. The Journal of Immunology, 2006, 176: 3958–3965.

Lymphocyte development proceeds through a complicated series of cellular signaling events. The TCR, BCR, FcRs, and cytokine receptors are activated by their respective Ags or ligands to transduce appropriate signals into the cells that activate or repress expression of specific genes, resulting in cell activation, cell migration, and differentiation processes, or cell death. These signaling events involve a complicated array of signaling molecules, including adaptor molecules, kinase, and phosphatase enzymes. Recent studies using either knockout (KO)1 or transgenic mice have revealed the critical functions of many signaling molecules in the process of lymphocyte development.

The downstream of tyrosine kinases (Dok) protein family is a novel adaptor protein family characterized by an N-terminal pleckstrin homology domain, a central phosphotyrosine binding domain, and several YxxP motifs in the C-terminal region. Dok-1 (1, 2), Dok-2 (3) (also called Dok-R, IL-4 receptor-interacting protein (4, 5)), and Dok-3 (6) (also called DokL (7)) are mainly expressed in hemopoietic cells and are thought to be important for lymphocyte development. Dok-1 was initially identified as a downstream target for both v-Abl and BCR-Abl (1, 2). Dok-1 has several YxxP motifs that upon phosphorylation serve as binding sites for the Src homology 2 domain-containing protein, RasGAP. In B cells, Dok-1 has been shown to be a negative regulator of the Ras/MAPK pathway. Cross-linking of BCR and FcγRIIB induces the tyrosine phosphorylation of Dok-1 and its subsequent association with RasGAP (8, 9). In FcγRIIB signaling, Dok-1 is recruited to the receptor complex via SHIP; in this case, SHIP functionally serves as an adaptor protein (10). To date, Dok-1 has not been reported to be involved in T cell development. Two groups have established Dok-1 KO mice and have shown that these mice do not demonstrate any apparent phenotype in the T cell population (11, 12). However, in some cell line systems, Dok-1 is tyrosine phosphorylated and associates with RasGAP upon CD2 and CD28 stimulation, but not following CD3-TCR stimulation, indicating a possible role of Dok-1 in T cell signaling (13). Additionally, Dok-1 KO thymocytes have been shown to proliferate more extensively in response to Con A in the presence of IL-2 when compared with wild-type (WT) thymocytes (12). It has also been demonstrated that the overexpression of Dok-2 results in a dramatic reduction in both thymocytes and splenic T cell numbers, suggesting a negative role of Dok-2 in T cell development (14). The exact functional role for Dok family members in T cells remains to be elucidated.

SHIP is a novel inositol phosphatase expressed in hemopoietic cells and is tyrosine phosphorylated in response to the activation of Ag receptors and multiple cytokine-signaling pathways (15). SHIP hydrolyzes the 5′-phosphates from phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3) and inositol-1,3,4,5-tetraphosphates, negatively regulating the signaling mediated by the PI3K pathway (16, 17). In addition to its enzymatic function, SHIP functions as an adaptor molecule linking FcγRIIB and Dok-1, leading to the inactivation of the Ras/MAPK pathway (10). Interestingly, SHIP

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§ Abbreviations used in this paper: KO, knockout; DKO, double knockout; Dok, downstream of tyrosine kinases; PI-3,4,5-P3, phosphatidylinositol-3,4,5-triphosphate; Treg, regulatory T cell; WT, wild type.
has been shown to positively regulate the IL-4-induced proliferation of the 32D myeloid cell line expressing insulin receptor substrate-2 (18). The mechanism by which SHIP increases proliferation is still unclear. In T cells, SHIP has been shown to be tyrosine phosphorylated by CD28 cross-linking; it also plays a role in FcγRIIB-mediated inhibition of TCR signaling and CD4-mediated LFA-1-dependent adhesion (19–22). These observations suggest an important role of SHIP in T cell function. However, the SHIP KO mice apparently have normal CD4⁺ and CD8⁺ populations in the thymus (23, 24). These findings indicate that SHIP has multiple functions in the proliferation and the differentiation of hemopoietic cells.

Due to the similar expression profiles of both SHIP and Dok-1 and the implicated roles in regulating BCR and TCR signaling, we sought to investigate the possibility that SHIP and Dok-1 might collaborate in signaling events involved in B and T cell development. To demonstrate the function of SHIP and Dok-1 in T cell development, we compared WT, Dok-1 KO, SHIP KO, and Dok-1/SHIP double-knockout (DKO) mice. DKO mice show dramatically reduced number of T cells in both the thymus and peripheral tissues. Splenic T cells from SHIP KO and DKO mice express activation markers, are unresponsive to in vitro stimulation, show enhanced production of TGF-β and Foxp3 expression, and show suppressive activity in vitro. These phenotypes are the characteristics of CD4⁺CD25⁺ regulatory T cells (Tregs). SHIP KO and DKO mice also exhibit severe disorganization of splenic architecture. Furthermore, DKO mice demonstrate a dramatically expanded population of Mac-1⁺/Gr-1⁻ cell population in the bone marrow and spleen. Together, these findings suggest an important collaboration of SHIP and Dok-1 that is required for normal T and B cell development and for myeloid cell homeostasis.

Materials and Methods

Mice

All mice were maintained in a specific pathogen-free room. All mice experiments conformed to Columbia University’s Institutional Animal Care and Use Protocols. Generation of Dok-1 KO and SHIP KO mice has been described previously (12, 23). The genotyping was performed by PCR methods using tail DNA, and the sequences of primers and PCR conditions described previously (12, 23). The genotyping was performed by PCR and Use Protocols. Generation of Dok-1 KO and SHIP KO mice has been described previously (12, 23). All mice were maintained in a specific pathogen-free room. All mice experiment conformed to Columbia University’s Institutional Animal Care and Use Protocols. Generation of Dok-1 KO and SHIP KO mice has been described previously (12, 23). The genotyping was performed by PCR methods using tail DNA, and the sequences of primers and PCR conditions described previously (12, 23).

FACS analysis

Cell suspensions were prepared from thymus, spleen, bone marrow, and lymph nodes. Cells were stained using combinations of the following Abs from BD Pharmingen against: CD3 (FITC), CD4 (allophycocyanin, PE, FITC, or biotin), CD8 (PE or PerCP), B220 (allophycocyanin or PE), Mac-1 (FITC), Gr-1 (PE), CD69 (FITC), CD25 (PE, allophycocyanin), CD44 (FITC), CD45RB (biotin), TCR-β (allophycocyanin), CD62L (FITC), and, if necessary, avidin (PerCP). For intracellular cytokine and Foxp3 detection, cells were fixed and permeabilized after cell surface staining (FITC), or biotin), CD8 (PE or PerCP), and analyzed by FACS.

Histological analysis

All tissues were fixed in phosphate-buffered Formalin solution (PROTOCOL; Fisher Diagnostic), and embedded in paraffin. Sections were stained with H&E, anti-CD3 (DakoCytomation), anti-Pax5 (BD Pharmingen), anti-Mac-2 (Cedarlane Laboratories), and anti-Ki-67 (NovoCasta) Abs were used to identify T cells, B cells, macrophages, and proliferating cells, respectively. Species-specific secondary Abs were used for double staining.

Preparation of T cells

T cells were isolated from spleen and lymph nodes. T cell populations in DKO mice spleen were markedly reduced, and Mac-1⁺/Gr-1⁻ cells were predominant (Fig. 4, A and B, and Table 1). For this reason, cell suspensions from SHIP KO and DKO mice were first purified through Sephadex G-10 to remove large cells, and effluent cells were then incubated with anti-MHC class II microbeads to purify T cells, or anti-CD8 and anti-MHC class II microbeads to purify CD4⁺ T cells (Miltenyi Biotec). Cells were then washed and applied onto MACS column (Miltenyi Biotec), and unbound cells were collected. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were purified by use of CD4⁺CD25⁻ regulatory T cell Isolation Kit (Miltenyi Biotec). The purity of T cells was estimated to be >90% by FACS.

Proliferation assay

Purified T cells were labeled with 5 μM CFSE (Molecular Probes) in RPMI 1640 for 15 min at 37°C; labeling was stopped by adding an equal volume of FCS. After washing, labeled cells were plated in 96-well culture plates precoated with anti-CD3e Ab (10 μg/ml) in the presence or absence of anti-CD28 Ab (10 μg/ml) for 72 h at 37°C. Harvested cells were stained with either anti-CD4 (APC) or anti-CD8 (PerCP) and analyzed by FACS.

ELISA for TGF-β production

Purified CD4⁺ cells were stimulated with plate-bound anti-CD3e (10 μg/ml) and soluble anti-CD28 (10 μg/ml) Abs in a concentration of 1 × 10⁶ cells/ml using 96-well plates. After 72 h, culture supernatants were collected, acidified by HCl, and then neutralized by NaOH per the manufacturer’s protocol before using Quantikine TGF-β ELISA immunoassay kit (R&D Systems).

RNA preparation and RT-PCR

Total RNA from 1 × 10⁶ purified CD4⁺ T cells was extracted using TRIzol reagent (Invitrogen Life Technologies) and subjected to cDNA synthesis using oligo(dT) primer. The cDNAs were used for PCR as PCR templates using primers and PCR conditions described previously (26).

In vitro suppression assay

CD4⁺CD25⁻ T cells were stimulated in the presence or absence of CD4⁺CD25⁺ T cells at the ratio indicated together with T cell-depleted APC and anti-CD3e (0.5 μg/ml) in RPMI 1640 with 10% serum in 96-well culture plates, as described previously (27).

Results

Altered growth and survival in Dok-1/SHIP double-deficient mice

To examine the roles of Dok-1 and SHIP in vivo, we established Dok-1/SHIP DKO mice and compared them with WT, Dok-1 KO, and SHIP KO mice. As previously reported, Dok-1 KO mice develop normally (11, 12), and SHIP KO mice show a slight reduction in body weight (Fig. 1 B). Surprisingly, viability is profoundly altered in DKO mice; many DKO mice fail to thrive and show a 35% reduction in body weight at 6–8 wk of age (Fig. 1 B). Interestingly, viability is profoundly altered in DKO mice; many DKO mice fail to thrive and show a 30% reduction in body weight at 6–8 wk of age (Fig. 1 B). Although SHIP KO mice show a slightly diminished life span (23, 24) compared with WT and Dok-1 KO mice, only 63% of DKO mice survive to 13 wk (Fig. 1 C) and few survive to 6 mo of age (data not shown).

Table 1. T cell, B cell, and myeloid cell population in Dok-1/SHIP DKO mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total Cell No.</th>
<th>CD3⁺</th>
<th>CD4⁺</th>
<th>CD8⁺</th>
<th>CD4⁺:CD8⁺ Ratio</th>
<th>B220⁺</th>
<th>Mac-1⁺ /Gr-1⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>13.5 (±1.3)</td>
<td>5.0  (±0.2)</td>
<td>3.2  (±0.1)</td>
<td>1.4  (±0.1)</td>
<td>3.0</td>
<td>0.4  (±0.2)</td>
<td></td>
</tr>
<tr>
<td>Dok-1 KO</td>
<td>13.7 (±1.6)</td>
<td>3.8  (±0.3)</td>
<td>2.5  (±0.2)</td>
<td>1.1  (±0.1)</td>
<td>2.3</td>
<td>6.5  (±0.3)</td>
<td></td>
</tr>
<tr>
<td>SHIP KO</td>
<td>15.5 (±1.6)</td>
<td>3.7  (±0.4)</td>
<td>2.8  (±0.2)</td>
<td>0.6  (±0.1)</td>
<td>4.7</td>
<td>6.2  (±0.2)</td>
<td></td>
</tr>
<tr>
<td>DKO</td>
<td>16.5 (±2.2)</td>
<td>1.5  (±0.2)</td>
<td>1.2  (±0.1)</td>
<td>0.2  (±0.1)</td>
<td>6.0</td>
<td>2.3  (±0.3)</td>
<td></td>
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were compared up to 13 wk. The percentages of survival of WT (n = 23, circles), SHIP KO (n = 23, circles), and Dok-1/SHIP DKO (n = 21, diamonds) were compared up to 13 wk.

**Abnormal T cell development in DKO mice**

As Dok-1 and SHIP are expressed in hemopoietic cells (6, 15), we examined T cell development in DKO mice. Thymi from Dok-1 KO and SHIP KO mice are of normal size compared with those of WT mice (Fig. 2A), and histologic analysis did not reveal any abnormalities (data not shown). However, DKO mice show a significant reduction in thymus size and a 90% reduction in thymocyte numbers (Fig. 2, A and B). Interestingly, total thymocyte number from Dok-1 KO mice and SHIP KO mice is also slightly reduced (4 and 15%, respectively). Moreover, in DKO mice, the cortex area appeared greatly reduced in size, although in Dok-1 KO mice and SHIP KO mice the cortex:medullary ratio appeared preserved and the histologic structure undistinguishable (data not shown). These observations suggest that thymic T cell development is affected by the combined loss of both Dok-1 and SHIP expression.

To examine thymic T cell development in detail, FACS analysis was performed on T cell subsets. As shown in Fig. 2C, normal percentages of CD4$^+$ cells, CD8$^+$ cells, and CD4$^+$CD8$^+$ cells were observed in Dok-1 KO and SHIP KO mice. However, in DKO mice, the percentage of CD4$^+$CD8$^+$ cells is greatly reduced, while the percentage of CD4$^+$ and CD8$^+$ single-positive cells was increased. The percentage of CD4$^+$CD8$^+$ cells was also increased; however, FACS analysis demonstrated that these cells were Gr-1$^+$ cells, but not Thy-1.2$^+$ cells (data not shown). These results suggest that expression of both Dok-1 and SHIP proteins is necessary for the normal early T cell development in the thymus, and that these defects cannot be compensated for by other Dok and SHIP family members.

**Severe splenomegaly and neutrophilia in DKO mice**

Dok-1 and SHIP are both implicated in signal transduction pathways required for proper development and function of lymphocytes and myeloid cells (23, 24, 28–30). We examined secondary lymphoid organs taken from mice deficient in Dok-1, SHIP, or doubly deficient mice. Spleens from the SHIP KO animals are enlarged 2- to 3-fold by weight over WT and Dok-1-deficient spleens, as previously shown (23, 24) (Fig. 3A). In the DKO spleens, an even greater degree of splenomegaly was observed (Fig. 3A): spleens from these mice were 4- to 7-fold larger than controls, and the splenomegaly is apparent as early as 2 wk of age. Despite the increased size of SHIP KO and DKO spleens, no large difference in total cellularity was observed (Table I).

It has been reported previously that both SHIP and Dok-1 are involved in IL-3 and GM-CSF signaling (12, 23, 24). Consistent
with a negative role for SHIP in these pathways, increased numbers of myeloid cells were found in spleen of SHIP KO mice (Table I). Immunohistochemistry revealed in DKO and rarely in SHIP KO the presence of Mac-2⁺ large, nonproliferating multinucleated epithelioid histiocytes, scattered or forming microgranulomas, in addition to an exuberant population of mature and maturing Mac-2⁺ macrophages, histiocytes, and monocytes (Fig. 3, B and C). These observations are consistent with a previous report, which showed that deletion of SHIP in myeloid cells caused marginal zone destruction (31).

The immunohistochemical findings were expanded by FACS analyses. The percentage of Mac-1⁺Gr-1⁺ cells was dramatically increased in SHIP KO (~17% of the splenocyte population) and DKO mice (>50%) compared with WT (~3%) and Dok-1 KO mice (~5%) (Fig. 4A). These results suggest that SHIP plays a role in regulating Mac-1⁺Gr-1⁺ cell development. The above evidence shows that deletion of Dok-1 in mice lacking SHIP expression causes a profound increase in Mac-1⁺Gr-1⁺ cells, leading to severe splenomegaly. Thus, the SHIP KO and DKO mice have disrupted splenic architecture and an overabundance of Mac-1⁺Gr-1⁺ cells in peripheral lymphoid tissues (23, 24).

**B and T cell numbers are simultaneously reduced in spleen**

FACS analyses of spleen cells were performed to determine the effect of Dok/SHIP deficiency on splenic B cell and T cell population. As expected, the percentage of B cells and T cells in the spleen is dramatically reduced in DKO mice (Fig. 4B and Table I). Associated with the reduced numbers of B cells in DKO mice, the B cell population is skewed toward mature cells, as indicated by surface IgM/IgD expression (data not shown). This is consistent with accelerated B cell development previously reported in SHIP KO mice (28, 30). These results suggest that both Dok-1 and SHIP are necessary for normal B cell maturation.

As described above (Fig. 3, B and C), SHIP KO and DKO mice showed disrupted splenic architecture. We therefore examined B cell and T cell distribution in these spleens by double immunostaining with anti-Pax5 (B cell marker) and anti-CD3 (T cell marker) Abs. Staining revealed that WT and Dok-1 spleens had normal B cell/T cell zones organized in lymphoid follicles, with B cells closely associated around the T cell zone. However, in DKO mice, there was a decrease in the numbers of follicles and no apparent segregation of B cell and T cell zones (Fig. 3D). We found an intermediate phenotype in SHIP KO spleens; B cell and T cell zones were disorganized, but overall follicle numbers are comparable to WT spleen. The marginal sinus lined by Mac-2⁺ macrophages was not observed in the spleen of SHIP KO and DKO mice (Fig. 3B). Taken together, these data suggest that SHIP expression is required for the normal development of lymphocyte and myeloid cells and for the formation of splenic architecture.

The absence of both SHIP and Dok-1 caused even more severe disruption of splenic architecture.

We next examined the CD4⁺ and CD8⁺ proportions in the spleen. Surprisingly, both SHIP KO and DKO mice exhibited an increased percentage of CD4⁺ cells concomitant with reduced percentages of CD8⁺ T cells (Fig. 4C). This contrasted with the Dok-1 KO spleens that showed normal CD4⁺:CD8⁺ ratios. These results suggest that SHIP is required to maintain a normal ratio of CD4⁺ and CD8⁺ T cells, while Dok-1 does not affect CD4⁺:CD8⁺ ratios in the spleen.

**Peripheral SHIP KO and DKO T cells express activation markers**

We more closely examined the surface phenotypes of splenic T cells from these mice. Typically, CD69 and CD25 are highly expressed on activated T cells. Splenic T cells from SHIP KO and DKO mice show dramatically increased percentages of CD69- and CD25-positive cells compared with either WT or Dok-1 KO mice (Fig. 5A). This phenotype suggests that SHIP KO and DKO T cells are constitutively activated. This observation also led us to examine whether SHIP KO and DKO T cells are naive or differentiated. We also examined the CD45RB and CD62L expression levels on
these T cells as markers for naive cells. Most freshly isolated WT and Dok-1 KO T cells were found to be CD45RB and CD62L positive. However, most of SHIP KO and DKO T cells were CD45RB and CD62L negative (Fig. 5B), suggesting that SHIP KO and DKO T cells are not naive cells, but are phenotypically effector/memory T cells (32). Taken together, these data suggest that the lack of SHIP expression in T cells affects the activation state of T cells in the spleen.

Poor response of SHIP and DKO T cells to stimulation

SHIP KO and DKO T cells display cell surface markers characteristic of activated T cells in the absence of stimulation, suggesting that these T cells may be basally activated (Fig. 5). We therefore examined the effect of SHIP and Dok-1 deficiency on the proliferative responses of these cells. The proliferation of SHIP and DKO T cells in response to TCR stimulation was diminished compared with WT or Dok-1 KO T cells (Fig. 6A). This result is different from the SHIP KO lymph node T cell proliferation, which was previously described (28). One possible explanation for this difference is that the T cells used in the previous experiments were produced in RAG KO environment, wherein SHIP expression was maintained in nonlymphoid cells (28). This anergic phenotype of SHIP KO and DKO T cells can be abrogated by addition of high dose of IL-2 (data not shown). Interestingly, T cells from WT, Dok-1 KO, and SHIP KO mice divided only once after 72 h of culture without T cell stimulation, whereas DKO T cells divided twice under these conditions, indicating that DKO T cells may exist in an activated state and may spontaneously proliferate without TCR stimulation.

To more fully characterize SHIP KO and DKO T cells functionally, we next examined cytokine production and T cell activation. Freshly isolated WT T cells produce IL-2, but not IFN-γ or IL-4 after stimulation with PMA/ionomycin, and Dok-1 KO T cells exhibited a similar cytokine production profile (Fig. 6B and data not shown). This result indicates that Dok-1 is not required for cytokine production induced by PMA/ionomycin in unprimed T cells. Surprisingly, both SHIP KO and DKO T cells did not produce IL-2 and IFN-γ in response to PMA/ionomycin stimulation, even though they appeared constitutively activated (Fig. 6B). We next addressed T cell activation after TCR stimulation (Fig. 6C). As expected, the levels of CD69, CD25, and CD44 expression on WT and Dok-1 KO T cells were up-regulated, suggesting that these T cells respond normally to TCR stimulation. In contrast, the expression levels of CD25, CD69, and CD44 on both SHIP KO and DKO T cells were unchanged after TCR stimulation, despite comparable levels of surface TCR expression on these cells. This suggests that the poor response of SHIP KO and DKO T cells is not due to the low expression of TCR. Again, these results indicate that SHIP KO and DKO T cells are defective in TCR responses.

**FIGURE 6.** T cells of SHIP KO and Dok-1/SHIP DKO mice are unresponsive to T cell stimulation. A, CD4⁺ T cells labeled with CFSE were cultured in 96-well plate precoated with anti-CD3 Ab (10 μg/ml) in the presence or absence of soluble anti-CD28 Ab (10 μg/ml). After 72 h, the viable CD4⁺ T cells were gated and the cell divisions were estimated by CFSE fluorescence intensity. The fluorescence of undivided cell (day 0) is indicated as opened histogram. B, Freshly isolated T cells were incubated with PMA and ionomycin in the presence of GolgiStop (BD Pharmingen) for 4 h at 37°C. Harvested cells were stained with anti-CD4 and anti-CD8 Abs and permeabilized, and stained intracellular cytokine with Abs to indicated cytokine. Unstimulated cells and stimulated cells are indicated in thin histogram and thick histogram, respectively. C, CD4⁺ T cells were cultured in 96-well plate precoated with anti-CD3 Ab (10 μg/ml) in the presence of soluble anti-CD28 Ab (10 μg/ml). After 72 h, cells were stained with anti-CD4, CD8, CD69, CD25, CD44, and TCR-β Abs. The viable CD4⁺ T cells were gated, and these surface markers were examined. Thin and thick histograms indicate unstimulated and stimulated cells, respectively.
level of Foxp3 mRNA expression than WT CD4+ T cells. Moreover, the majority of CD4+ T cells from SHIP KO and DKO spleen express Foxp3 protein, whereas those of WT and Dok-1 KO spleen CD4+ T cells do not (Fig. 7C). These results indicate DKO CD4+CD25+ T cells are characteristically Tregs. Interestingly, some SHIP KO and DKO CD4+CD25+ T cells also express increased levels of Foxp3, although further analyses of these populations are needed (Fig. 7C).

Next, we performed in vitro suppression assays to examine whether relatively increased CD4+CD25+ T cells from SHIP KO and DKO mice have suppressive activity, which is the most significant function of Tregs (33, 37). As shown in Fig. 7D, CD4+CD25+ T cells from both SHIP KO and DKO spleen inhibited IL-2 production by CD3-activated CD4+CD25+ T cells (responder cells) in a dose-dependent manner. These results clearly suggest that SHIP KO and DKO CD4+CD25+ T cells have suppressive activity. Taken together, our data demonstrate that the absence of SHIP or SHIP/Dok-1 expression in the T cell lineage affects the development of Tregs.

**Discussion**

The role of SHIP in B cell signaling, development, and myeloid cell function has been well documented (23, 24, 28, 29), and the molecular mechanism by which SHIP regulates B cell signaling has been previously described by several groups (10, 38–41). In this study, we have demonstrated that SHIP plays a critical role in the regulation of T cell development. Although the adaptor protein Dok-1 by itself does not appear to be important for T cell function, our results clearly show that expression of Dok-1 modulates SHIP’s role in thymic development.

Previous studies have shown that SHIP KO mice have significantly reduced numbers of B cells in the bone marrow (23, 24, 28, 29), while analysis of several cell surface markers, including surface IgM and surface IgD, revealed that their splenic B cells appear both activated and accelerated in development (28, 30). With regard to T cell development, thymocyte numbers are dramatically reduced in DKO mice. Splenic T cells are also significantly reduced in number, but appear activated. These results suggest that SHIP’s regulation of T cells may be analogous to its role in B cells, in which biphasic control over SHIP function accounts for reduced numbers of B cells or T cells during development, but activation in the periphery. Although the mechanism responsible in the case of T cells remains to be elucidated, this control of SHIP function appears to be dependent on Dok-1. The Src homology 2 domain of SHIP can bind to the CD3 complex of a TCR via both an ITIM and an ITAM (42). Costimulation via CD28 leads to tyrosine phosphorylation of SHIP, suggesting that SHIP does play an important role in T cell development (20).

Early T cell development appeared to be unaffected in SHIP KO mice (23, 24); however, splenic T cells are activated, as shown by CD25 and CD69 expression levels. Moreover, CD62L and CD45RB expression on SHIP KO and DKO T cells was dramatically down-regulated. This surface phenotype in the SHIP KO and DKO mice is characteristic of an effector or memory T cell subset (32). However, neither SHIP KO nor DKO T cells proliferate in response to TCR stimulation, or produce cytokines by primary stimulation using PMA/ionomycin, suggesting that these activated T cells may not be typical effector/memory T cells. The other unique CD4+CD25+ T cell population is the CD4+CD25+ Tregs, which are an important T cell population that maintains immunological tolerance (33, 37). In this study, we demonstrated that SHIP KO and DKO CD4+CD25+ T cells are phenotypically similar to naturally occurring Tregs’ phenotypes, including expression of cell surface markers, such as CD25, CD45RB, glucocorticoid-induced TNFR (data not shown), CD45RB, and anergy to TCR stimulation, which can be abrogated by high dose of IL-2 (data not shown), and increased TGF-β production and Foxp3 expression. We also demonstrated that CD4+CD25+ T cells from SHIP KO and DKO mice suppressed IL-2 production by CD3-activated CD4+CD25+ T cells in vitro. The results presented in this work suggest that CD4+CD25+ T cells in SHIP KO and DKO mice are phenotypically similar and functionally Tregs. Although the actual numbers of CD4+CD25+ T cells are decreased in both SHIP KO and DKO spleen because of the dramatic decrease of total T cell numbers, the ratio of CD4+CD25+ : CD4+CD25− cells is dramatically increased. These findings suggest that SHIP alone, or in cooperation with Dok-1, negatively regulates the development of Tregs, which are important for maintaining peripheral immune tolerance.
The molecular mechanisms by which Dok-1 and SHIP regulate CD4⁺CD25⁺ Treg development are still unclear. One possibility is that the relative increase of Tregs is caused by the profoundly expanded Mac-1⁺Gr-1⁺ cells in DKO spleen, which may affect T cell development in periphery. It has been suggested that hyporesponsiveness to TCR stimulation could be acquired through prolonged exposure to proinflammatory cytokines, such as TNF-α, which is largely produced by activated macrophages (43). Therefore, Tregs in these mice might be abnormally generated through exposure to inflammatory cytokines produced by the expanded Mac-1⁺ cell population. Another possibility is that some unknown stress induced by the absence of Dok-1 and SHIP affects Treg development. It has been reported that treatment with glucocorticoids increases Foxp3 expression in CD4⁺ T cells and initiates differentiation toward Tregs (44). We observed normal thymocyte development in the DKO newborn mice (data not shown), suggesting that early T cell development is not altered by the loss of Dok-1 and SHIP expression. The appearance of Tregs expressing Foxp3 is delayed compared with non-Treg CD4⁺ T cells during ontogeny (45). The significant expansion of Foxp3-expressing Treg population was observed between days 3 and 4. Accordingly, the thymocyte development in day 4 newborn DKO mice seems not to be affected at this stage. Therefore, it is possible that some stress factors induced with age cause abnormal T cell development. A third possibility is that Dok-1 and/or SHIP may directly regulate the downstream signaling pathways regulating the balance of Tregs and the other CD4⁺ T cells. Interestingly, Foxp3 expression levels in both CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were increased in DKO and SHIP KO mice. It has been reported that the Foxp3-transduced naive CD4⁺ T cells can be converted to have a Treg phenotype (26). Therefore, up-regulation of Foxp3 without SHIP expression in the peripheral CD4⁺ T cells may potentially induce Tregs.

In contrast to our results, it has been reported previously that SHIP KO T cells reconstituted in RAG KO mice produce normal levels of IL-2 (28). This discrepancy may be attributed to differences in KO system or the environment in which these mice were maintained. After escape from negative selection in the thymus, some T cells are rendered anergic and others dominantly regulate the self-reactive T cells (33, 37). However, results from the present study would suggest that these unusual SHIP KO and DKO T cells are in fact Tregs.

The role of SHIP is to hydrolyze PI-3,4,5-P3 to phosphatidylinositol-3,4-biphosphate and thus terminate PI3K-dependent pathways (16, 17). As a result, PI3K-dependent signaling in SHIP-deficient cells is enhanced in response to several receptor stimuli, including Ag receptor and cytokine receptor activation (23, 24, 28, 29). The other inositol phosphatase, PTEN, which catalyzes the hydrolyzation of phosphatidylinositol-4,5-biphosphate to PI-4,5-P2 at 3'-phosphate, also contributes to the regulation of PI3K-dependent pathways (46). In a conditional PTEN KO mouse, T cell-specific loss of PTEN produces some opposite phenotypes to our SHIP KO or Dok-1/SHIP DKO mice (47). The spleens of these mice are enlarged due to dramatically increased CD4⁺ T cell numbers, whereas SHIP KO and Dok-1/SHIP DKO showed splenomegaly due to the Mac-1⁺/Gr-1⁺ cell expansion with a reduction in the T cell population. Strikingly, the T cell-specific PTEN KO mouse has an enlarged thymus due to impaired negative selection (47), but the Dok-1/SHIP DKO thymus showed atrophy associated with reduction in number of thymocytes. Despite these differences, there is at least one common phenotype between T cell-specific PTEN KO and SHIP KO, or Dok-1/SHIP DKO T cells; the ratio of CD4⁺ to CD8⁺ cells was elevated in the spleen and lymph node in these mouse models (47). This suggests that the regulation of PI-3,4,5-P3 is critical for T cell signaling leading to normal T cell CD4⁺CD8⁺ ratio. We have not yet determined which is more important for normal T cell development, the phosphatase function or adaptor function of the SHIP molecule. Further studies together with biochemical analyses may shed light on the molecular mechanism by which SHIP and Dok-1 molecules regulate T cell function.

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**References**

The inositol polyphosphate 5-phosphatase SHIP is a crucial negative regulator of B cell development, activation, and death by the src homology 2 domain-containing inositol 5-phosphatase (SHIP), dephosphorylation of the linker of activated T-cells (LAT) and inhibition of calcium mobilization. Biochem. Soc. Trans. 29: 840–846.


