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Differential roles for the inositol phosphatase SHIP in the regulation of macrophages and lymphocytes

Wai-Hang Leung, **Tatyana Tarasenko**, and **Silvia Bolland**
Laboratory Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn drive, Twinbrook 2, Room 217, Rockville, MD 20852, USA

Abstract

The SH2 domain-containing inositol 5′-phosphatase (SHIP) negatively regulates antigen, cytokine, and Fc receptor signaling pathways in immune cells. Our knowledge of the function of SHIP largely derives from in vitro studies that utilized SHIP-deficient cell lines and immune cells isolated from SHIP null mice. To avoid the pleiotropic effects observed in mice with germline deletion of SHIP, we have used the Cre-lox system to generate SHIP conditional knockout mice with deletion in specific immune cell populations. In this review we summarize our observations from mice with deletion of SHIP in lymphocyte and macrophage lineages and contrast them with earlier data gathered by the analysis of SHIP null mice.

Keywords

SHIP; Lymphocytes; Macrophages; T-bet; Conditional knockout

Introduction

The SH2 domain-containing inositol 5′-phosphatase (SHIP) is a hemopoietic-specific inhibitory molecule that has been extensively studied for its regulatory role in B cells, T cells, macrophages, and mast cells [1–5]. SHIP was first described as a phosphatase linked to the fms/CSF pathway in macrophages, identified by its binding to the adaptor molecules Shc and Grb2 [6,7]. Subsequently, SHIP was found to be an important component of the inhibitory signaling pathway triggered by the IgG receptor FcγRIIB in mast cells and B cells [1]. Recruitment of SHIP by the phosphorylated ITIM in the cytoplasmic domain of FcγRIIB was shown to inhibit recruitment of PH-domain-containing proteins and prevent extracellular calcium influx, overall reducing transcription activation, and cytokine release [8]. Experiments performed in cell lines have implicated SHIP in signaling pathways triggered by the antigen receptor in B cells [3,9,10], and by the TCR and CD28 in T cells [11,12]. Additionally, SHIP plays an important regulatory role in establishing endotoxin tolerance in macrophages [13]. Overall, SHIP can regulate a variety of signaling pathways related to cytokine, antigen, and IgG engagement in both lymphocytes and myeloid cells [4,14]. Our current experiments using mice with conditional deletion of SHIP intend to dissect pathways and cell populations that are directly affected by the removal of SHIP rather than by the general immune system dysregulation occurring as a consequence of the pathological SHIP-deficient environment.

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SHIP protein structure and function

SHIP's polypeptide has a molecular weight of 145 kDa and comprises several functional domains [2]. It includes a Src homology 2 (SH2) domain at the N-terminus, a proline-rich region, and two NPXY amino acid sequences at the C-terminus. All of these domains have been shown to act as important docking sites for the interaction with several signaling molecules, adaptor proteins, and the cytoplasmic portion of a number of immune receptors [15,16]. The SH2 domain of SHIP preferentially interacts with proteins containing the amino acid sequence pY(Y/D)X(L/I/V), which defines an inhibitory tyrosine immuno-regulatory motif or ITIM [17]. The proline-rich region of SHIP favors the interaction with proteins that possess an SH3 domain, such as Grb2 and PLCγ [18]. The NPXY motif in SHIP can become phosphorylated at the tyrosine residue to facilitate binding to the phosphotyrosine base domain (PTB) in Shc [19]. The central domain in SHIP confers 5′-phosphatase enzymatic activity, which selectively hydrolyzes the 5′ phosphate from PI-3,4,5-P3 to produce PI-3,4-P2 [19]. Since PI-3,4,5-P3 is a key secondary messenger generated by PI3-kinase upon the activation of numerous signaling pathways, the removal of PI-3,4,5-P3 by SHIP is therefore considered the major negative inhibitory function of the protein.

Upon stimulation, SHIP is recruited from the cytoplasm to the membrane through its interaction with the cytoplasmic tail of immune receptors such as the phosphorylated ITIM of the inhibitory receptor FcγRIIB in B cells [17]. In other cases of receptor activation, such as the B cell receptor (BCR) or cytokine receptors that do not contain ITIM sequences, it is unclear how SHIP is recruited to the signaling complex. Once SHIP is brought to the site of activation, its 5′-phosphatase hydrolyzes PI-3,4,5-P3 and prevents membrane localization of PH-domain-containing signaling molecules such as Tec kinases, Akt, and PLCγ [20–22]. The inhibition of PLCγ activation leads to a reduction of calcium influx, which is the crucial activation signal in many immune cells such as mast cells and B cells [9,14]. No significant change in SHIP's 5′-phosphatase activity has been detected after receptor stimulation, thus suggesting that SHIP exerts its downstream regulatory effects via interacting with different proteins and translocating to the sites of synthesis of PI-3,4,5-P3 [14]. In this regard, the tyrosine-phosphorylation of the NPXY motifs of SHIP is generally considered a sign of activity of the protein [23]. On the other hand, it has been shown that Shc binds the phosphorylated NPXY motif in SHIP and in this way competes for its binding with the receptor [16].

Experimental models to study the function of SHIP

In order to study the inhibitory function of SHIP genetically, Ono et al. [24] generated a SHIP-deficient DT40 chicken B cell line by homologous recombination of the target gene construct. This in vitro model has provided much of the mechanistic analysis of signaling pathways regulated by SHIP in B cells [8,24–27]. The DT40 system was used to show how SHIP is essential for inhibitory pathways triggered by FcγRIIB but not by KIR receptors, even though both receptor types contain ITIM sequences in their cytoplasmic domains [24]. Experiments with SHIP-deficient DT40 cells proved that SHIP can directly regulate pathways triggered by the BCR because these cells were found to have diminished calcium oscillatory response upon BCR stimulation [9]. Complementation studies in DT40 cells indicated that SHIP's phosphatase enzymatic activity was essential for its inhibitory function, and that a major role of this enzyme was to prevent recruitment of the kinase Btk [8].

SHIP's role in T cell biology was first studied in vitro using various T cell lines. Edmunds et al. [11] showed that SHIP is tyrosine phosphorylated upon colligation of TCR and CD28 in vitro. SHIP was found to regulate PI3K effectors and to interact with and to negatively regulate Tec kinases in T cells [28]. It has also been suggested the SHIP participates in a negative signaling complex by association with LAT [29].

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The first in vivo mouse model to study the function of SHIP was established in 1998 by Humphries' group [30]. The first exon from the SHIP gene was genetically replaced with the neomycin resistance gene so that the resulting homozygous mice lacked SHIP in all immune cells. SHIP-null knockout mice were viable and fertile but experienced shortened lifespan. SHIP deficiency in mice led to higher number of granulocytes and macrophages, progressive splenomegaly, and massive myeloid infiltration of the lungs, ultimately resulting in lethality. Immune cells isolated from these SHIP-null mice were used to prove SHIP's important role in regulating lymphoid and myeloid cell development and activation. Thus, it was shown that macrophages [31], mast cells [32], B cells [33], dendritic cells [34], and NK cells [35] isolated from SHIP-null mice exhibit functional deficiencies. The limitation of these studies is that pleiotropic effects of the ubiquitous deletion of SHIP hinder the elucidation of in vivo regulatory roles of SHIP in specific cell types. In order to precisely address the intrinsic function of SHIP in different immune cells both in vitro and in vivo, we have generated conditional knockout mice with deletion of SHIP in various specific immune cell types using the Cre-Lox system [36]. In the following sections we will summarize what we have learned from mice with conditional deletion of SHIP in T cells, B cells, and macrophages, and contrast these results with what was earlier found in the characterization of SHIP null mice.

SHIP is not essential for proper T cell development and TCR signaling

Mice with T cell-restricted deletion of SHIP were generated by crossing mice containing a loxP-flanked SHIP gene [37] with mice transgenic for the Cre recombinase driven by the CD4 promoter (CD4cre) [38]. The resulting mice were healthy and had a normal lifespan comparable to SHIP-proficient littermates [39]. The T cell conditional deletion of SHIP effectively deleted SHIP at the thymocyte double positive stage and beyond. No reduction in SHIP expression was observed in immune cells other than T cells. No deficiencies in T cell development in the thymus or periphery was observed in these SHIP conditional CD4cre mice, neither when in polyclonal backgrounds nor in transgenic mice with single TCR specificities. This result contrasts with the slight reduction in the peripheral T cell numbers in germline SHIP-deficient mice reported earlier [30]. Furthermore, mice with germline deletion of SHIP have increased number of $CD4^+CD25^+$ regulatory and $CD62L^{low}$ effector T cells [40]. In contrast, we found no differences in regulatory or effector T cell numbers in the T cell conditional deletion of SHIP. This fact indicates that SHIP does not play an essential intrinsic role in thymic T cell selection or in T cell development and spontaneous activation. This result also suggests that it is in fact the environment of SHIP-null mice that promotes the expansion of regulatory and effector T cells [34].

Ex vivo analysis of T cells from mice with T cell-restricted deletion of SHIP established that SHIP does not noticeably regulate the TCR strength of signal. We observed no differences between SHIP-deficient and wild-type T cells in levels of phosphorylation of ERK, Akt, Zap-70, and PLCγ1, or in calcium influx upon TCR activation. SHIP-deficient T cells were also comparable to wild-type cells in ex vivo proliferation induced by a wide variety of antigen presenting cells with a wide variety of TCR transgenic systems and peptide concentrations [39]. Moreover, in vivo antigen-driven expansion of OVA-specific SHIP-deficient T cells was equal to SHIP-proficient controls. Thus, even though earlier experiments using cell lines showed that SHIP becomes phosphorylated upon TCR stimulation, we see no evidence of a definitive role for SHIP regulation of TCR signaling in vivo.

SHIP's function impedes a bias toward Th1 skewing in helper and cytotoxic T cells

We observed that mice with T cell-restricted deletion of SHIP failed to respond efficiently to immunizations with Alum as adjuvant, which would normally bias the immune response

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toward the Th2 type. Meanwhile, responses to immunization with Ribi adjuvant, which provide a Th1 bias, were shown to be similar or even enhanced in these mice [39]. We subsequently analyzed the cytokine production of SHIP-deficient T cells under different polarizing conditions. Major differences were observed under Th2 skewing conditions, in which SHIP-deficient T cells produced significantly lower levels of IL-4, IL-5, and IL-13. In vivo challenge of CD4cre-SHIP conditional mice with *S. mansoni* eggs showed diminished capacity to respond to this type 2 response-inducing helminth. In vitro stimulation of purified T cells from challenged mice with *S. mansoni* egg antigen (SEA) demonstrated lower proliferative response and lower IL13/IL4 production. Since we established that TCR-mediated signaling is normal in the absence of SHIP, we speculate that the reduced Th2 response in SHIP-deficient T cells may result from the enhanced sensitivity to activation through a type 1 cytokine, such as IFN-γ. In agreement with this hypothesis, SHIP-deficient T cells have increased basal expression of T-bet and augmented phosphorylation of STAT1, both events likely linked to type 1 responses and both sensitive to IFN-γ activation [41,42]. Altogether, these results suggest an essential role of SHIP in diminishing Th2 responses in helper T cells due to its role in regulating cytokine sensitivity.

Not only T helper cells, but also $CD8^+$ T cells isolated from mice with T cell-restricted deletion of SHIP demonstrated elevated levels of T-bet. We decided to test cytotoxic function of SHIP-deficient T cells in light of the fact that T-bet had earlier been shown to regulate effector $CD8^+$ function [43]. Our results confirmed an additional role for SHIP in dampening Th1 bias in cytotoxic cells: $CD8^+$ T cells purified from CD4cre–SHIP conditional mice were more efficient than wild-type cells in cytotoxic assays [39].

The role of SHIP in B cell development

A large body of in vitro studies provides evidence for an inhibitory role of SHIP in the regulation of B cell receptor (BCR) signaling through its interaction with the phosphorylated ITIM of the IgG receptor FcγRIIB [1]. SHIP's function has also been linked directly to the regulation of the BCR pathway itself [9,44]. Liu et al. [10] used the system of RAG complementation with SHIP-deficient bone marrow to prove a role for SHIP in B cell development and the humoral response. Furthermore, Helgason et al. [44] showed that germline SHIP-deficient mice experience a decrease in B cell populations beyond the Pre-B cell stage in the bone marrow and an increase in the number of IgM^+IgD^+ mature B cells in spleen. Our approach has been to generate mice with B cell-restricted deletion of SHIP by breeding floxed SHIP mice to mice expressing the Cre recombinase driven by the CD19 promoter [45]. We believe these mice, in which SHIP is deleted at the pro-B stage, provide an optimal system for the in vivo functional testing of B cell responses. We observe that CD19cre SHIP-conditional mice develop no obvious pathologies and no alterations in B-cell development in the bone marrow (Leung and Bolland, unpublished results). Once again, our experiments suggest that the lymphopenia detected in SHIP-null mice is likely due to the pathological environment of the total SHIP deletion. This view could be explained by the finding by Coggeshall et al. that higher levels of IL6 in the bone marrow of SHIP-null mice enhance myelopoiesis while reducing levels of lymphopoiesis [46].

More concordant data between total SHIP deletion and B cell-restricted deletion was found at the level of recirculating B cells, which were lower in both types of mice, and in the spontaneous formation of germinal centers and antibody producing cells in both cases. [10,44]. The reduction in recirculating B cells could be explained by a change in CD22 function in these mice: First, it has been suggested that CD22 plays an essential role in homing of mature IgM^+IgD^+ cells from spleen into bone marrow [47]. And second, two reports have reported on the interaction between SHIP and the ITIM motif of CD22 [17,48].

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In the periphery, mice with B cell-restricted deletion of SHIP have B cells with enhanced activation traits, increased level of spontaneous isotype switching, and a shifted repertoire toward low affinity receptors [10,44]. Overall this phenotype would be consistent with the view that SHIP-deficient B cells display enhanced responses to antigen stimulation through the BCR.

SHIP regulates B cell positive selection

We observed that B cell-restricted deletion of SHIP led to the generation of spontaneous germinal centers and augmented isotype switching. But unexpectedly, these mice responded very poorly to T-independent and T-independent immunizations, or even to viral challenges (Leung and Bolland, unpublished). In stark contrast, SHIP-null mice had earlier demonstrated enhanced humoral responses [44]. Again, this implies that the SHIP-null environment is a potent stimulator of lymphocyte activation, a fact that underscores the relevance of studies using SHIP conditional mice. In the case of conditional B cell deletion of SHIP, the reduced T-independent responses may be explained by the fact that these mice have reduced number of marginal zone B cells, a population that has been proposed to be majorly responsible for immediate B cell responses [49–52]. But the reduced T-dependent antibody responses in CD19cre SHIP-conditional mice points toward a fundamental alteration in B cell positive selection at the germinal center stage. Our analysis of antibodies produced upon NP-CGG$^+$ Alum stimulation revealed that while SHIP-deficient germinal center B cells exhibit normal rate of somatic hypermutation, they are not selected for high affinity sequences as occurs in wild-type B cells (Leung and Bolland, unpublished). Thus, SHIP plays an essential role in regulating the positive selection of high affinity antigen-specific B cell clones in GC upon T-dependent immunization. These experiments illustrate the fact that enhanced signaling through the BCR may lead to a shift in antibody repertoire that could be deleterious for an efficient immune response.

SHIP is essential for the suppressor activity of macrophages on inflammatory and IL17-driven responses

The discordance between the phenotype of SHIP-null mice and that of mice with lymphocyte-restricted deletion of SHIP indicates that the activated phenotype of lymphocytes in SHIP-null mice occurs as a secondary effect of the dysregulation of SHIP-deficient myeloid cells. We have confirmed this hypothesis by the analysis of mice with SHIP deletion restricted to the macrophage–granulocyte lineage. These mice were generated by breeding SHIP floxed mice to mice with Cre recombinase expression driven by the LysMcre promoter [37]. LysMcre SHIP conditional mice develop a myeloproliferative pathology that reduces their lifespan, although the severity of disease is not as dire as in SHIP null mice (Tarasenko and Bolland, unpublished). One possible explanation as to why the LysMcre deletion of SHIP does not completely recapitulate the pathology observed in SHIP-null mice is that the deletion of SHIP in LysMcre occurs in already differentiated macrophages but not in earlier precursors that could be potentially be more pathogenic in the absence of SHIP. Another possible explanation is that cells other than macrophages and granulocytes contribute to the overall phenotype in SHIP-null mice.

Deletion of SHIP in macrophages not only leads to myeloid hyperproliferation, but also to lymphocyte activation as a secondary effect. LysMcre SHIP conditional mice develop splenomegaly with a large number of activated lymphocytes. We determined that T cells in these mice are biased toward the Th17 phenotype and expand the regulatory population (T.Tarasenko and B. Bolland, unpublished data). This result explains the phenotype observed in T cells from SHIP-null mice as resulting from overt macrophage hyperactivity as opposed to a clear role for SHIP in T cell development. Specifically, our data is consistent with the view that SHIP-deficient macrophages are hypersensitive to growth factor stimulation (e.g. CSF)

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and release inflammatory cytokines (IL6 and others) that promote T cell IL17 bias. Additionally, we observed that LysMcre SHIP conditional mice lack the type of suppressor macrophages that has been reported to keep immunogenic dendritic cells in check [53]. Further analysis of these mice with conditional deletion of SHIP in macrophages will give insight into how spontaneous activation of myeloid cells can influence the entire immune response and lead to a chronic inflammatory pathology.

Concluding remarks

To avoid the pleiotropic effects of the SHIP germline deletion, we have generated conditional knockout mice with deletion of SHIP restricted to T cell, B cells, or macrophages. We believe these mice are the optimal system to address SHIP's intrinsic roles in a variety of immune cells. Our data show that SHIP is essential for the regulation of Th1/Th2 responses by T cells, in determining antibody affinity and isotype switching in B cells, and for the formation of suppressor macrophages that can limit IL17 and other inflammatory responses. By comparing phenotypes of these mice with that of the total SHIP deletion in SHIP-null mice, we have been able to assess the impact that myeloid dysregulation can inflict on the lymphocyte activation status.

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