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The role of SHIP in macrophages

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1. ABSTRACT

The SH2-containing inositol-5′-phosphatase, SHIP, represses the proliferation, survival, and activation of hematopoietic cells, in large part by translocating to membranes following extracellular stimulation and hydrolysing the phosphatidylinositol-3-kinase (PI3K)-generated second messenger PI-3,4,5-P_3 (PIP_3) to PI-3,4-P_2. SHIP-/- mice have, as a result, an increased number of monocyte/macrophages because their progenitors display enhanced survival and proliferation, as well as more rapid differentiation. Interestingly, SHIP-/- mice do not display lipopolysaccharide (LPS)- or CpG oligonucleotide-induced tolerance because this blunting of inflammatory mediator production is contingent upon LPS- and CpG-induced upregulation of SHIP in their macrophages and mast cells. This upregulation is mediated via the production of autocrine-acting TGFbeta which is induced via the MyD88-dependent pathway. The increased levels of SHIP then inhibit both MyD88-dependent and independent signaling. Intriguingly, SHIP-/- peritoneal and alveolar macrophages produce less nitric oxide (NO) than wild-type macrophages because they have constitutively high arginase I levels and this enzyme competes with inducible nitric oxide synthase (iNOS) for the substrate L-arginine. It is likely that, in the face of chronically elevated PIP_3 levels in their myeloid progenitors, SHIP-/- mice display a skewed development away from M1 (killer) macrophages towards M2 (healing) macrophages. This suggests that SHIP plays a critical role in programming macrophages.

2. INTRODUCTION

The phosphatidylinositol (PI)-3-kinase (PI3K) pathway plays a central role in regulating many biological processes through the generation of the potent second messenger PI-3,4,5-P_3 (PIP_3) (1). This membrane-associated phospholipid, which is present at low levels in resting cells, is rapidly synthesized from PI-4,5-P_2 by PI3K in response to a diverse array of extracellular stimuli and attracts pleckstrin homology (PH)-containing proteins such as the survival/proliferation enhancing serine/threonine kinase, Akt (also known as protein kinase B (PKB)), and the Rac and cdc42 guanine nucleotide exchange factor, Vav, to membranes to mediate its effects. To ensure that the activation of this pathway is appropriately suppressed/terminated, the ubiquitously expressed 54 kDa tumour suppressor PTEN (i.e., phosphatase and tensin homologue deleted on chromosome ten) hydrolyzes PIP_3 back to PI-4,5-P_2 while the 145 kDa hematopoietic-restricted SH2-containing inositol 5'-phosphatase, SHIP (also known as SHIP1), the 104 kDa stem cell-restricted SHIP (sSHIP) and the more widely expressed 150 kDa SHIP2 break it down to PI-3,4-P_2 (reviewed in (2)). The fact that many human cancers contain somatic activating mutations or gene amplification of the p110alpha subunit of PI3K (3) and that almost 50% of human cancers contain biallelic inactivating mutations of PTEN (4) highlights the importance of tightly regulating the PI3K pathway. In this review we will concentrate on the role that SHIP plays in macrophages.

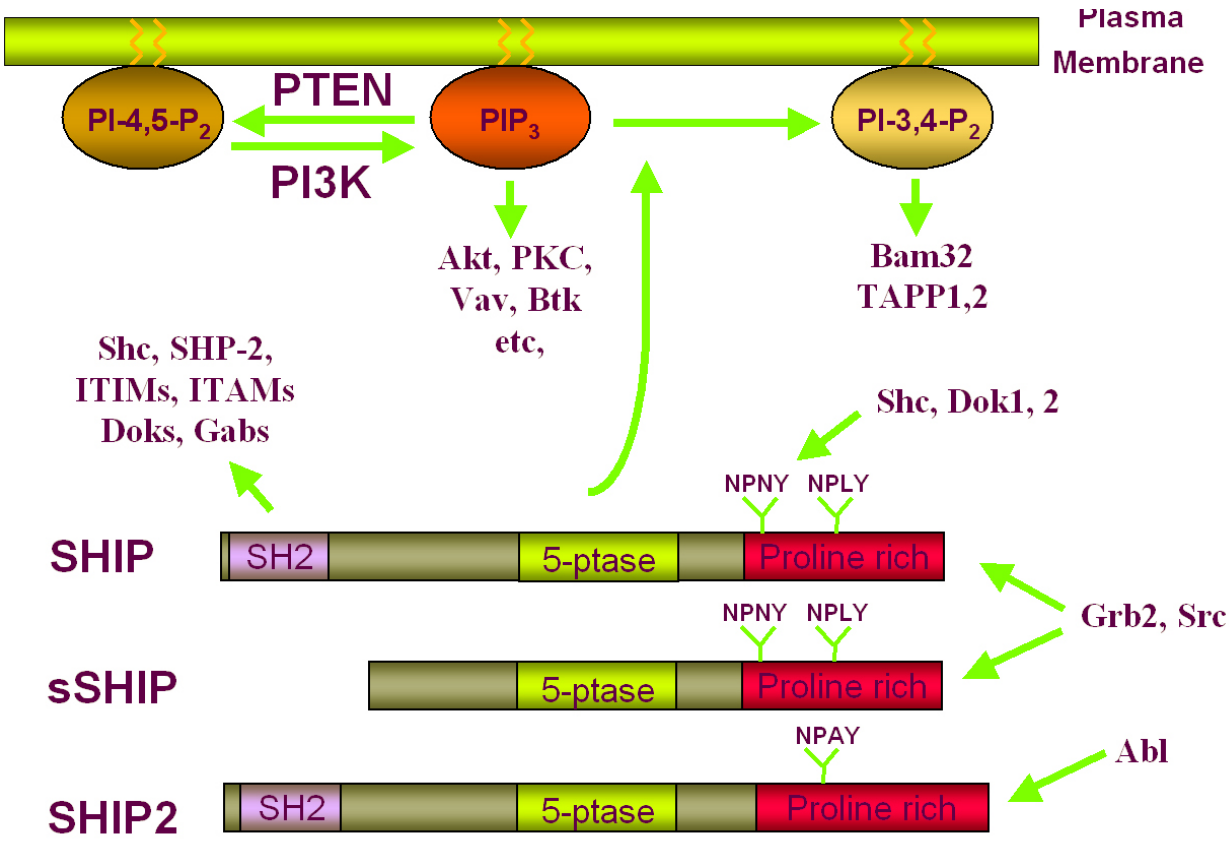

Figure 1. The structures of SHIP, sSHIP and SHIP2. Some of the known binding partners of SHIP, sSHIP and SHIP2 are shown as well as the PH-containing proteins that are attracted to the transiently generated PIP_3 and PI-3,4-P_2. One of SHIP's binding partners, Shc, has been shown in some receptor systems to facilitate the translocation of SHIP to membranes to hydrolyse PIP_3 to PI-3,4-P_2 (reviewed (132)).

3. THE STRUCTURE AND EXPRESSION OF SHIP

The gene for SHIP, which is located on chromosome 2q36/37 in the human and 1C5 in the mouse, codes for a 145 kDa protein that becomes both tyrosine phosphorylated (typically by members of the Src family ((5), and Hughes & Krystal, manuscript in preparation) and associated with the adaptor protein Shc in hematopoietic cells after exposure to a diverse array of extracellular stimuli. These stimuli include cytokines, growth factors, antibodies, chemokines, integrin ligands (2,6) and hypertonic and oxidative stress (Cao & Krystal, unpublished). It possesses, as shown in Figure 1, an amino-terminal Src homology 2 (SH2) domain that binds preferentially to the sequence pY(Y/S/T)L(M/L) (7) and has been shown to bind to the tyrosine phosphorylated forms of Shc, SHP-2, downstream of tyrosine kinases (Doks), Gabs, CD150, Cas, c-Cbl (8), certain immunoreceptor tyrosine based inhibitory motifs (ITIMs) and certain immunoreceptor tyrosine based activation motifs (ITAMs) (reviewed in (2)). SHIP also contains a centrally located phosphoinositol phosphatase domain that selectively hydrolyzes the 5'-phosphate from PIP_3 and inositol-1,3,4,5-tetrakisphosphate (IP_4), two NPXY sequences that, when phosphorylated, bind proteins with a phosphotyrosine binding (PTB) domain (eg, Shc, Dok2), and a critical proline rich C-terminus that binds a subset of SH3-containing proteins including Grb2, Src, Abl and PLCgamma1 (reviewed in (2,9,10)).

During murine development, SHIP is first detectable, by RT-PCR, in 7.5 day postcoitus mouse embryos (coincident with and dependent upon the onset of hematopoiesis) and its protein expression pattern in the embryo appears restricted to hematopoietic cells (11). In the adult mouse, SHIP protein expression is also restricted to hematopoietic cells (and to spermatids) (11) but there is some evidence for SHIP mRNA expression in endothelial cells (12) and this perhaps may become translated under certain physiological conditions. Within the hematopoietic system SHIP protein levels vary considerably (11,13). For example, SHIP is lost during erythropoiesis when erythroid cells become $Ter119^+$ (14) and there is some evidence that this loss may be required for terminal erythroid differentiation (15). In contrast, SHIP levels increase substantially with T cell maturation (11), show a bimodal expression pattern during B cell development and are dramatically increased when resting B cells are activated (16). SHIP is also present in mature granulocytes, monocyte/macrophages, mast cells, platelets and natural

killer (NK) cells (13,17-20). With regard to NK cells, there are two distinct subsets (ie CD56 bright and dim) in human peripheral blood and levels of SHIP are substantially lower in the CD56 bright cells, which may enable them to produce far more IFNgamma (20). Adding complexity to this expression pattern, there are two alternate splice forms of SHIP and the levels of these splice forms also change during hematopoiesis (21,22).

In addition to full length SHIP and its two splice forms, a 104 kDa stem cell SHIP (sSHIP) has been identified. This sSHIP was originally cloned from a human placental cDNA library and called SIP-110 (23). sSHIP was subsequently shown to be the only form of SHIP expressed in embryonic stem (ES) cells (24) and was co-expressed with full length SHIP in hematopoietic stem cells (HSCs) (24). In both cases, it disappears with differentiation. sSHIP mRNA is transcribed from a promoter within the intron between exons 5 and 6 of the full length SHIP gene and Rohrschneider *et al.* (25) recently demonstrated, using transgenic mice expressing this promoter attached to green fluorescent protein (GFP), that sSHIP promoter activity is restricted to a subset of stem/progenitor cells during mouse embryo development. These results suggest that expression of this promoter might serve as a useful marker for stem cell populations in different tissues. Since sSHIP protein is truncated at its N-terminus it lacks an SH2 domain and, as a result, is neither tyrosine phosphorylated nor associated with Shc following stimulation (24). However, it does bind constitutively to Grb2 and may be recruited via Grb2's SH2 domain to the plasma membrane to regulate PIP_3 levels in stem cells (24).

4. THE PHENOTYPE OF THE SHIP KNOCKOUT MOUSE

In 1998, SHIP-/- mice (26) (as well as B and T cell specific SHIP-/- mice (27)) were generated by deleting SHIP's first exon. Although SHIP-/- mice are viable they have a shortened lifespan, likely due to a massive infiltration of neutrophils and macrophages into their lungs (26). Interestingly, granulocytic and monocytic/macrophage progenitors from these SHIP-/- mice are substantially more responsive to suboptimal levels of cytokines, growth factors and chemokines than their SHIP+/+ counterparts (26,28) and, even in the absence of added growth factors, develop into small colonies (26). This likely explains, at least in part, the finding that these SHIP-/- mice overproduce granulocytes and macrophages, forcing erythropoiesis out of the bone marrow and into the spleen and elsewhere. As a result, these mice suffer from progressive splenomegaly, due to both extramedullary erythropoiesis and myelopoiesis (26). Because SHIP-/- mice were generated by deleting its first exon, sSHIP is still expressed in these mice (14). In fact, the expression of sSHIP is higher and more prolonged in SHIP-/- than +/+ ES cells when they are induced to differentiate (14) and it is likely that the SHIP-/- mouse phenotype would be more severe if both sSHIP and SHIP were deleted. Interestingly, although red blood cell numbers remain within the normal range (even though primitive erythroid progenitors are at twice normal levels (29)), B cells are reduced as the SHIP-/- mice age because the large number of monocyte/macrophages produce high levels of IL-6 which both inhibit B cell and enhance myeloid cell development (30). SHIP-/- mice also suffer from severe osteoporosis (due to an increased number of Paget-like, hyper-resorptive osteoclasts) (31). Interestingly, SHIP-/- mice also display a progressive perturbation of NK cell development, characterized by an abnormally high expression of inhibitory receptors, and this likely plays a major role in their reduced acute graft-versus-host disease and deficient allograft rejection (32). Related to this, SHIP-/- mice also possess a dramatically elevated number of $Gr\text{-}1^+Mac\text{-}1^+$ myeloid suppressor cells, which impair the priming of allogeneic T cells, and may therefore also play a role in suppressing graft-versus-host disease (33). Also related is the recent finding, described in more detail in Section 7.2, that macrophages in SHIP-/- mice tend to be skewed away from a killer M1 phenotype and toward a healer M2 phenotype as the mice reach adulthood (34).

Interestingly, SHIP-/- mice share many phenotypic characteristics with PTEN+/- mice (35). For example, it was shown in 2003 that a higher proportion of HSCs were in cycle in SHIP-/- mice and that competitive repopulating unit (CRU) recovery was 30-fold lower in mice that received transplants of SHIP-/- cells (36). This contrasts slightly with a more recent report suggesting that both HSC proliferation and numbers are increased in SHIP-/- mice but these HSCs exhibit a reduced capacity for long term repopulation because they inefficiently home to the bone marrow of irradiated recipients (37). Regardless, these findings are very similar to a recent report showing that conditional deletion of PTEN in hematopoietic cells promotes HSC proliferation and their subsequent depletion (38). These similarities in the phenotype of SHIP deficient and PTEN deficient mice suggest that it is the higher levels of PIP_3 in SHIP-/- mice that are primarily responsible for its phenotype. Related to this, mice heterozygous for both PTEN and SHIP develop a lymphoproliferative autoimmune syndrome more rapidly than do PTEN+/- mice (39,40). However, some caution should be exercised in ascribing all of the phenotype of SHIP-/- mice directly to the loss of SHIP since many of the characteristics of these mice appear, or worsen, with age and it is possible that they are the result of compensatory mechanisms to offset the damage induced by this deletion.

5. SHIP'S MECHANISM OF ACTION

Since SHIP's 5′-phosphatase activity does not appear to change following extracellular stimulation (41) or subsequent tyrosine phosphorylation (42), the current consensus is that SHIP mediates its inhibitory effects by translocating to sites of synthesis of PIP_3 (and perhaps IP_4). In support of this, GFP-tagged SHIP has been shown to translocate to the plasma membrane in response to stimulation (19,42,43) and both the SH2 domain and the proline rich C-terminus of SHIP are required for this translocation and for SHIP's biological effects (44-46). In terms of how SHIP translocates to the plasma membrane, it is likely that different mechanisms are employed, depending on the stimulus and cell type (47-53). For

example, it may utilize its own SH2 domain to take it to the $F_c$gammaRIIB, MAFA (mast cell function-associated antigen (Ag)) or the $F_c$epsilonRI in mast cells or use Shc's PTB domain to take it to the interleukin-3 receptor in mast cells or Shc's SH2 domain to take it to CD16 in NK cells (53). Interestingly, SHIP has been shown to translocate to the cytoskeleton as well (19,54). For example, thrombin stimulation of human platelets causes the tyrosine phosphorylation and translocation of SHIP to the actin cytoskeleton, perhaps via actin-binding filamins (19,54). Consistent with this finding, Lesourne *et al*. (55) recently reported that SHIP is constitutively associated with filamin-1 in the rat mast cell line, RBL-2H3, and that when the negative co-receptor, $F_c$gammaRIIB co-aggregates with the activated $F_c$epsilon receptor, they rapidly interact with the actin cytoskeleton and bind SHIP/filamin1 to inhibit IgE-induced signalling.

While most SHIP-induced effects are likely mediated by its hydrolysis of PIP_3, SHIP may also hydrolyze IP_4 in some cell types (56) and thus could affect the levels of the higher inositol polyphosphates like IP_6, which has been shown to play an essential role in transporting mRNA out of the nucleus for translation (57) and IP_7, which has been shown to compete with PIP_3 for PH-domain containing proteins (58).

Interestingly, there is also evidence that PI-3,4-P_2 may act as a second messenger in some cells by attracting PH-containing proteins, such as Bam32 and TAPP1 and 2 (59-61). If so, one might expect a SHIP-/- hematopoietic cell (with its elevated PIP_3 and reduced PI-3,4-P_2 (62,63)) to be qualitatively different from a PTEN-/- hematopoietic cell (with its elevated PIP_3 and PI-3,4-P_2).

SHIP has also been shown to mediate some of its effects by acting as an adaptor, eg. in the activation of SHP-2 (64), PLCgamma 1 (10), and the Doks (65). In terms of the Doks, there is evidence that during $F_c$gammaRIIB-mediated inhibition of B cell activation, SHIP reduces Ras activity by recruiting $p62^{Dok}$ (i.e., Dok1) to RasGAP (65). As well, Andre Veillette's group has found that SHIP associates with Dok3 in B cells in response to BCR engagement and the Dok3/SHIP complex suppresses B cell activation via inhibition of the Jnk pathway (66). More recently, Paul Rothman's group has shown, using Dok1/SHIP double knock out mice, that the number of T cells from these mice is dramatically reduced and they possess a T regulatory cell phenotype (67). There is also evidence that SHIP (and SHIP2) may directly reduce Ras activation in some cells by competing with Grb2 for Shc (68,69).

6. THE BIOLOGICAL ROLES OF SHIP

As far as biological functions are concerned, SHIP appears to be the key negative regulator of IgE + Ag-generated PIP_3 levels in murine bone marrow derived mast cells (BMMCs) (70). By reducing the transient increase and duration of PIP_3 levels, SHIP restricts extracellular calcium entry and the activation of PDK1 (which plays a key role in the activation of PKC isoforms involved in the cytoskeletal changes important to mast cell degranulation) (71,72) (and reviewed in (73)). These results reveal a vital role for SHIP in limiting and setting the threshold for mast cell degranulation. As well, SHIP has been shown to play a role in reducing mast cell degranulation at supra-optimal IgE or Ag levels (74). SHIP also plays a central role in negatively regulating IgE- or IgE+Ag-induced inflammatory cytokine secretion from mast cells, at least in part by dampening down PIP_3-mediated activation of NFkappaB (reviewed in (75)). As well, IgE- or stem cell factor (SCF)-induced adherence of mast cells to fibronectin appears to be restrained by SHIP (76). These results suggest that SHIP negatively regulates degranulation, cytokine production and adhesion of mast cells. On the other hand, very recent studies looking into the non-specific desensitization that follows $F_c$epsilonRI induced activation of human basophils suggest that SHIP may not play a role in this specific phenomenon since the kinetics of SHIP phosphorylation do not correlate with the duration of desensitization (77). However, this conclusion is based in part on the assumption that tyrosine phosphorylated SHIP is synonomous with activated SHIP and this may not be the case. In fact, while the tyrosine phosphorylation of SHIP likely indicates that SHIP has at some point been recruited to hydrolyze PIP_3 (and this is consistent with evidence that Lyn is required for SHIP activation in neutrophils (78)), phosphorylation might serve to release SHIP from the plasma membrane in other receptor systems (52). In addition to its inhibitory role in mast cells and basophils, SHIP has been shown to be a negative regulator of B cell proliferation, chemotaxis and activation (reviewed in (2,28)). It has also been shown to negatively regulate alphaIIbbeta3 integrin-induced spreading/adhesion (6), thrombin- and collagen-induced activation and fibrinogen-induced spreading of platelets (6,18,79,80). SHIP has also been reported to inhibit the G-CSF-induced proliferation (81), Toll like receptor 2 (TLR2)-induced activation (82) and survival of neutrophils (78). As well, SHIP has been demonstrated to inhibit phagocytosis, superoxide generation and M-CSF-induced chemotaxis (via inhibition of Vav/Rac activation) by monocytes/macrophages (19,83,84) and early erythroid (BFU-E) colony formation (29).

Interestingly, although SHIP is not required for mast cell or macrophage development it restrains their differentiation since primitive progenitors or ES cells from SHIP-/- mice differentiate into mature mast cells and macrophages significantly faster than their wild type counterparts (85). This could suggest that elevated PIP_3 levels drive myeloid differentiation.

In apparent contradiction to studies showing that SHIP reduces hematopoietic cell survival, a very recent report suggests that expressing SHIP in the SHIP-negative Jurkat T cell line actually protects against H_2O_2-induced apoptosis (86). Complicating matters further is a recent study showing that the malignancy-enhancing serine/threonine kinase Akt may actually suppress tumor metastasis by degrading the calcium dependent transcription factor, NFAT (87,88). Thus, since SHIP inhibits Akt (by hydrolyzing PIP_3), it might on the one hand reduce tumorigenesis but increase invasion.

Perhaps one of the most exciting findings that has taken place in the last few years is the finding in Jean-Jacques Lebrun's lab that TGFbeta and activin, which are potent inhibitors of hematopoietic cell proliferation and survival, elicit their effects on hematopoietic cells, in part, by dramatically upregulating SHIP mRNA and protein levels (56). Interestingly, this TGFbeta/activin-induced upregulation in hematopoietic cells appears to be limited to SHIP since neither SHIP2 nor PTEN levels change significantly (89).

As far as SHIP's role in human disorders is concerned, there is evidence that it is downregulated within human basophils of a subset of patients with severe allergies (90). As well, there is a subset of patients with familial Paget-like osteoporosis that shows a loss of heterozygosity at chromosome 2q36 (91), the chromosomal location of human SHIP (92) and this could suggest that the osteoporosis in these patients is due to a reduction in SHIP levels. Recently, SHIP has also been shown to be upregulated in the oral mucosa of patients with chronic periodontitis. This upregulation may serve to induce tolerance to the Gram negative bacteria present within the plaques of these patients (93) (see Section 7.1). Also of interest, inactivating mutations of SHIP have recently been reported in the blast cells of patients with acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL) (94,95), suggesting that SHIP normally acts as a tumor suppressor in hematopoietic progenitors. Related to this, both SHIP and SHIP2 have been shown to be constitutively tyrosine phosphorylated and associated with Shc in chronic myelogenous leukemic (CML) progenitors (9) and SHIP levels are reduced in both mature primary cells from leukemic patients (96,97) and in Ba/F3 cells following induced expression of BCR-ABL (96). In fact, there is an inverse relationship between the expression of BCR-ABL and SHIP (98), suggesting that reduced SHIP activity might be a prerequisite for the proliferative advantage of some CML and AML clones. Interestingly, a similar inverse relationship has just been reported between a constitutively active oncogenic c-kit receptor (ie, KIT^{K641E}) and SHIP in Ba/F3 cells (99). This mutant receptor has no effect on SHIP2 or PTEN levels and inhibition of its intrinsic tyrosine kinase activity with Gleevec reversibly raises SHIP levels (99).

7. THE ROLE OF SHIP IN MACROPHAGES

Despite arising from a common myeloid progenitor, both macrophages and the peripheral blood monocytes from which they are derived are very heterogeneous, both in phenotype and function. As far as the macrophages are concerned, their phenotype is very dependent on both the tissue in which they differentiate and the inflammatory state of that tissue (reviewed in (100-102)). Together with mast cells, resident macrophages are the first cells within the innate immune system to respond, via their TLRs and other pathogen recognition receptors (PRRs), to invading microorganisms and to tissue damage. They typically respond by releasing pro-inflammatory molecules such as TNFalpha, interleukin-12, nitric oxide (NO), reactive oxygen species and arachidonic acid metabolites. These inflammatory agents directly and indirectly destroy both infectious agents and surrounding host tissues. As well, like dendritic cells, macrophages can phagocytose and present processed microbial peptides, via major histocompatibility class II (MHCII), to T cell receptors on primed T cells and thus act as a bridge between the innate and adaptive immune systems. However, in the late stages of an inflammatory response, macrophages with a helper or M2 phenotype predominate. These typically express scavenger receptors to phagocytose debris and produce anti-inflammatory and growth promoting molecules (102). Intriguingly, in tumor tissues, infiltrating macrophages tend to be skewed, via the tumor microenvironment, into M2 helper-like macrophages and these cells typically promote tumor growth and angiogenesis (103).

As mentioned earlier, SHIP is normally present in myeloid progenitors as well as in mature monocytes and macrophages (17) and when it is deleted, myeloid progenitors survive and proliferate better in suboptimal growth conditions (i.e., in less than optimal levels of macrophage colony stimulating factor (M-CSF, aka CSF-1) (26). As also mentioned earlier, in the absence of SHIP, myeloid progenitors differentiate more rapidly into Mac1+ macrophages (85) and one of the hallmarks of the SHIP knockout mouse phenotype is a marked elevation of monocytes and macrophages (17). As well, in the absence of SHIP, the phagocytosis, H_2O_2 generation and M-CSF-induced chemotaxis of monocyte/macrophages is more robust (19,83,84). Related to this, studies in Clay Marsh's lab have shown that Lyn binds and likely tyrosine phosphorylates SHIP in human monocytes following M-CSF stimulation and that SHIP negatively regulates Akt and NFkappaB activity in both these cells and mouse macrophages (5).

Worthy of note is that while SHIP is likely the major negative regulator of PIP_3 levels in activated macrophages there is recent evidence that SHIP2 also plays a role. For example, results from Clay Marsh's lab indicate that SHIP2 is recruited to the cell membrane upon M-CSF stimulation and negatively regulates M-CSF-induced signalling (104). Also, studies in Susheela Tridandapani's lab demonstrate that SHIP2 down regulates $F_c$gammaR-mediated phagocytosis in macrophages, independently of SHIP (105).

7.1. SHIP is essential for TLR-induced tolerance

Lipopolysaccharide (LPS, also known as endotoxin) is a major glycolipid in the outer membrane of Gram-negative bacteria (106) that potently triggers inflammation, by stimulating innate immune cells, via TLR4, to produce pro-inflammatory molecules, including pro-inflammatory cytokines and NO (reviewed in (106)). The production of high levels of NO by macrophages, in particular, is a major defense mechanism against bacterial and viral infections and also is responsible in large part for the anti-tumor effects of macrophages (107). LPS triggers this inflammatory response by binding to a soluble LPS binding protein (LBP) and this LPS/LBP complex binds CD14 on the cell surface. CD14 then presents the LPS/LBP

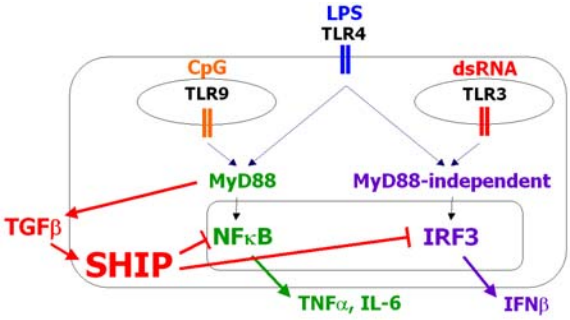

Figure 2. The role of SHIP in TLR-induced events. While LPS activates both the MyD88-dependent TIRAP/IRAK/TRAF6/NFkappaB and MyD88-independent TICAM/IRF3/IFNbeta/JAK/Stat1 pathways, CpG only activates the former and dsRNA only activates the latter. TGFbeta expression is upregulated by the MyD88-dependent pathway and this anti-inflammatory cytokine then activates the transcription of SHIP, increasing its protein levels about 10 fold in BMmacs over a 24 h period. A second dose of LPS or CpG does not activate the production of NO and pro-inflammatory cytokines because of the increased level of SHIP. As well, the elevated level of SHIP represses dsRNA-induced IFNbeta mRNA levels.

complex to the MD-2/TLR4 complex and this triggers the dimerization of TLR4. Upon dimerization, the cytoplasmic tail of TLR4 recruits several intracellular adaptor proteins triggering a MyD88 dependent IRAK4/TRAF6/TAK1/ NFkappaB pathway and a MyD88-independent TRAM/TRIF/TBK1/IRF3/IFNbeta pathway (Figure 2). The production of IFNbeta via the MyD88-independent pathway then acts in an autocrine manner to activate STAT1 and this synergizes with the NFkappaB pathway to promote pro-inflammatory cytokine and inducible nitric oxide synthase (iNOS) (and thus NO) synthesis (reviewed in (89)).

Although LPS-induced pro-inflammatory molecules are indispensable for destroying bacteria, overproduction can lead to endotoxic shock ie, sepsis syndrome, which is responsible for almost 50% of the deaths that occur in intensive care units in North America (108). However, if animals are first exposed to a low, non-lethal dose of LPS, this makes them transiently (2-3 week) refractory to a second, high LPS exposure, such that far less pro-inflammatory cytokines and NO are produced following the second exposure and the animals survive. This phenomenon, referred to as endotoxin tolerance (108), is thought to protect the host from cellular damage and may represent an adaptation to a persistent bacterial infection. Although all the pathways underlying this phenomenon have not as yet been fully elucidated, it is known that it is not simply a global shut-down of LPS-induced events since normal or even elevated levels of anti-inflammatory molecules, such as interleukin-10 and TGFbeta are typically produced by LPS-stimulated tolerized cells (108). Relevant to this, SHIP-/- bone marrow derived macrophages (BMmacs) and BMMCs do not display endotoxin tolerance (89). Moreover, an initial LPS treatment of wild-type BMmacs or BMMCs increases the level of SHIP, but not SHIP2 nor PTEN, about 10 fold and this increase is critical for the refractoriness to subsequent LPS stimulation since SHIP antisense oligonucleotides, given at the same time as the initial dose of LPS to block the increase in SHIP protein levels, totally prevent endotoxin tolerance (89). Interestingly, this increase in SHIP protein is mediated by the LPS-induced production of autocrine-acting TGFbeta (89), which is reminiscent of previous work showing that TGFbeta and activin elicit their inhibitory effects on hematopoietic cells, in part, by dramatically upregulating SHIP, but not SHIP2 or PTEN (56).

Although SHIP plays a crucial role in promoting endotoxin tolerance, it obviously does not do so in isolation but acts in concert with many other negative regulators. These include IRAK-M, SOCS1, SOCS3, Twist 1 and 2, MyD88s, the p50 subunit of NFkappaB and osteopontin (reviewed in (89)). However, these other molecules can not invoke tolerance in the absence of SHIP, suggesting, perhaps, that the elevated PIP_3 levels which occur in the absence of SHIP raise the threshold required for hypo-responsiveness invoked by these other negative regulators.

To determine if SHIP is induced via the MyD88-dependent or -independent pathway and to see if it plays a role in the tolerance observed for other TLRs (109), the responses of SHIP+/+ and -/- BMmacs to dsRNA (ie, poly IC) which binds to TLR3 and triggers the MyD88-independent pathway and to CpG oligonucleotides (ie, unmethylated DNA), which bind to TLR9 and trigger a MyD88-dependent pathway were recently compared (110). It was found that CpG, like LPS, markedly increases SHIP levels but dsRNA does not and this correlates with the ability of CpG, but not dsRNA, to stimulate the production of TGFbeta. Thus the production of TGFbeta and its subsequent triggering of SHIP expression likely occur via the MyD88-dependent pathway and this has been confirmed using MyD88-/- BMmacs (L. Sly and G. Krystal, submitted). Importantly, pre-treatment of SHIP+/+ (but not -/-) BMmacs with CpG leads to tolerance when these cells are stimulated a second time with either CpG (or LPS, i.e., cross-tolerance). As well, CpG (or LPS) pre-treatment reduces subsequent dsRNA-induced IFNbeta expression, consistent with the MyD88-dependent upregulation of SHIP negatively impacting MyD88-independent induced events (L. Sly and G. Krystal, submitted) (see Figure 2).

Worthy of note is that there is considerable controversy concerning the role of the PI3K pathway in LPS-induced pro-inflammatory mediator production and in endotoxin tolerance, with some investigators claiming this pathway is a positive and others, a negative regulator of these events (111-113). Possible reasons for these opposing conclusions include differences in the ability of LY294002 and wortmannin, two often used PI3K inhibitors in these studies to inhibit kinases other than PI3K (we have found, for example, that LY294002 is far more potent than wortmannin at inhibiting mTOR (M. Rauh & G. Krystal, unpublished)). As well, it has recently been shown that the TLR-induced production of interleukin-10, which is

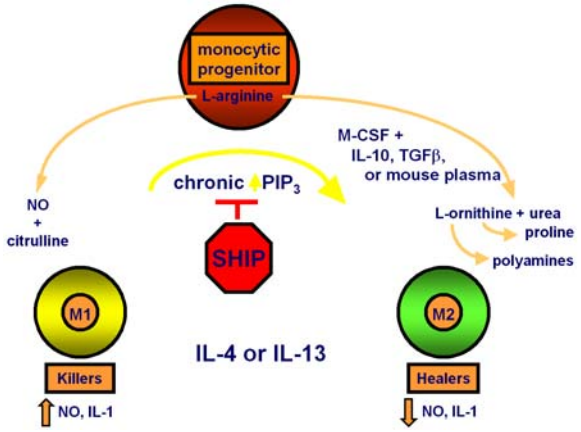

Figure 3. A model suggesting that chronically elevated PIP_3 levels skew monocytic progenitors towards M2 "healer" macrophages. Bone marrow from SHIP-/- mice when cultured in the presence of M-CSF and mouse plasma, interleukin-10 ot TFGbeta, yield M2-like macrophages displaying constitutively high levels of arginase 1 whereas SHIP+/+ bone marrow under the same conditions yield M1-like macrophages. Interleukin-4 or interleukin-13, on the other hand (but not mouse plasma, interleukin-10 or TGFbeta) convert both SHIP+/+ and -/- mature M1-like macrophages into M2-like macrophages.

positively regulated by PI3K, quickly suppresses interleukin-12 production (114,115). Related to this, SHIP-/- mature peritoneal macrophages, as well as SHIP-/- bone marrow progenitors, secrete far more interleukin-10 and TGFbeta than their SHIP+/+ counterparts (34) and this may explain why SHIP sometimes appears to be a positive regulator of pro-inflammatory cytokines (34). Regardless of the outcome of this controversy, LPS and CpG-treated BMmacs upregulate SHIP levels and this upregulation leads to the dampening down of inflammatory cytokine release upon a subsequent exposure to either LPS or CpG (L. Sly & G. Krystal, submitted).

7.2. SHIP skews myeloid differentiation towards M1 killer macrophages

Simplistically speaking, macrophages can be sub-divided into “killer”, classically activated, M1 macrophages and “healer”, alternately activated, M2 macrophages (116). The M1 macrophages are characterized by being able to rapidly generate high levels of iNOS, at least in the mouse (34,117), in order to convert the substrate L-arginine into NO to kill bacteria, viruses and tumour cells (118,119). The M2 macrophages, on the other hand, which are very heterogeneous in nature, are characterized by constitutively high levels of arginase 1 (which sequesters L-arginine away from iNOS and generates ornithine) and are thought to play an important role in “cleaning up” after an infectious agent is destroyed by phagocytosing cellular debris and stimulating host cell proliferation (via ornithine-derived polyamines) and collagen synthesis (via ornithine-derived proline) (see Figure 3). Both M1 and M2 macrophages are derived from a common monocytic progenitor and there is considerable interest in understanding what regulates development into one type versus the other and how easily they can be interconverted. This is especially of interest given that successfully growing tumours (in which macrophages are prominent (i.e., tumour associated macrophages (TAMs)) appear to have manipulated the infiltrating macrophages so that they possess an M2 phenotype and actually help the tumour cells grow (120). In keeping with this notion, there is a growing body of evidence that stimulation of TLRs, which skews macrophages to an M1 phenotype, may help to eradicate tumours (121).

Although, as mentioned above, there is some controversy about whether the PI3K pathway is a positive or negative regulator of LPS-induced pro-inflammatory mediator production in macrophages (122,123), SHIP-/- BMmacs generated at low (but not high) cell densities produce higher levels of NO and inflammatory cytokines (consistent with the PI3K pathway being a positive regulator of either the NFkappaB or STAT1 pathways). However, when in vivo derived macrophages (peritoneal and alveolar macrophages) from SHIP-/- mice were examined, we were surprised to find that they produce 5-10-fold less NO than their wild type counterparts. Subsequent studies revealed that this was because the macrophages within SHIP-/- mice, unlike those in wild type littermates, develop an M2 phenotype as they reach 5 weeks of age (34). In spite of this, 5 week old SHIP-/- mice are substantially more susceptible than SHIP+/+ mice to LPS-induced death (89), likely because SHIP-/- mice are only modestly M2-skewed at 5 weeks of age, they possess ten fold more macrophages than SHIP+/+ mice and, most importantly, they do not display endotoxin tolerance (89).

Interestingly, this M2 phenotype is not observed if macrophages are derived in vitro from SHIP-/- bone marrow using standard culture conditions (i.e., M-CSF + fetal calf serum). However, this in vivo skewing towards M2 macrophages could be duplicated in vitro by simply adding mouse plasma or human serum to the standard culture conditions. However, no skewing is observed if addition is delayed until the third day of culture, suggesting that they may be acting only on early macrophage progenitors (34). Importantly, this in vitro skewing does not occur with wild type bone marrow cells, suggesting that in vivo conditions are being mimicked. To gain some insight into the factor(s) present in mouse or human serum (but not in FCS) responsible for this in vivo M2 skewing of SHIP-/- progenitors, various T_H2-derived cytokines that have been shown to shift mature BMmacs from an M1 to an M2 phenotype, i.e., IL-4, IL-13, TGFbeta and IL-10 (124,125) were tested. From these studies it was concluded that interleukin 4 and 13 are not the factors in serum since they could significantly “convert’’ mature (i.e. Mac1+, F4/80+) BMmacs from an M1 to an M2 phenotype, whereas mouse plasma or human serum could not and they could skew both SHIP+/+ progenitors into an M2 phenotype to the same degree as SHIP-/- progenitors. However, both TGFbeta and IL-10 could only skew SHIP-/- progenitors, making them potential candidates for the M2-skewing factor in serum. Further studies using neutralizing antibodies to TGFbeta and IL-10 suggested that TGFbeta is

a critical factor in mouse plasma responsible for skewing towards an M2 phenotype.

Based on these results, a possible model for M1 versus M2 macrophage development is that elevated PIP_3 levels drive progenitors down an M2 pathway even in the presence of cytokines that are poor activators of the PI3K pathway (eg, TGFbeta and IL-10 (126-128). This is consistent with inhibition of PI3K in SHIP-/- BMmacs reducing M2 skewing and constitutive activation of the PI3K pathway (with P110alpha-CAAX) in SHIP+/+ BMmacs increasing M2 skewing (34). We also suggest that cytokines that are potent inducers of the PI3K pathway (eg, IL-4 and IL-13) (128-130) can skew progenitors down the M2 pathway in the absence of intrinsically elevated PIP_3 levels (i.e., in wild type macrophage progenitors (see Figure 3). The fact that SHIP-/- mice only develop an M2 phenotype after 3-4 weeks of age suggests that this skewing is unlikely due solely to a cell autonomous deletion of SHIP in macrophages or macrophage progenitors. This is consistent with in vitro derived BMmacs not showing this skewing unless mouse plasma or human serum is added. Interestingly, the tolerant NK phenotype of SHIP-/- mice observed by Bill Kerr's group also occurs with age (32). This could suggest that a "healer", more tolerant phenotype occurs as SHIP-/- mice age as an attempt to counter the inflammatory phenotype of these mice.

8. SUMMARY AND PERSPECTIVE

As discussed in this review, SHIP appears, in general, to negatively regulate the survival and proliferation of hematopoietic progenitors and there is mounting evidence that it acts as a suppressor of leukaemias and lymphomas. This is suggested not only by the presence of phosphatase-reduced forms of SHIP in AML and ALL but also by its ability to act as an adaptor for the tumor suppressors, Dok1 and Dok2 (3,131). Moreover, the increased numbers of myeloid suppressor cells (33,67), M2 healer macrophages (34), and T regulatory cells (67), in SHIP-/- mice suggest that SHIP plays a prominent role in skewing both innate and acquired immune cells into cancer eliminating cell types. On the other hand, SHIP has also been shown to dampen down the activation of many mature myeloid cells and thus restrain the inflammatory response. Obviously, much remains to be learned about SHIP but the results to date suggest that therapeutic targeting of SHIP could be very useful for treating solid tumors, leukemias and lymphomas, infections and inflammatory disorders.

9. ACKNOWLEDGMENTS

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Abbreviations: Ag: antigen, ALL: acute lymphoblastic leukemia, AML: acute myelogenous leukemia, BMmacs: bone marrow derived macrophages, BMMCs: bone marrow mast cells, CML: chronic myelogenous leukemia, CRU: competitive repopulating units, Doks: downstream of tyrosine kinases, ES: embryonic stem, GFP: green fluorescent protein, HSCs: hematopoietic stem cells, iNOS: inducible nitric oxide synthase, IP_4: inositol-1,3,4,5-tetrakisphosphate, ITAMs: immunoreceptor tyrosine based activation motifs, ITIMs: immunoreceptor tyrosine based inhibitory motifs, LBP: LPS binding protein, LPS: lipopolysaccharide, MAFA: mast cell function-associated antigen, M-CSF: macrophage colony stimulating factor, MHCII: major histocompatibility class II, NO: nitric oxide, PI3K: phosphatidylinositol-3-kinase, PIP_3: phosphatidylinositol 3,4,5-trisphosphate, PRRs: pathogen recognition receptors; PTB: phosphotyrosine based, PTEN: phosphatase and tensin homologue deleted on chromosome ten, SCF: stem cell factor, SHIP: Src homology 2 containing inositol 5'-phosphatase, TLRs: Toll like receptors.

Key Words: SHIP, Macrophages, Endotoxin tolerance, LPS, TLR3, TLR4, TLR9, M1/M2 Macrophages, Review

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