The Inositol 5′-Phosphatase SHIP-1 and the Src Kinase Lyn 
Negatively Regulate Macrophage Colony-stimulating 
Factor-induced Akt Activity*

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The M-CSF receptor (M-CSF-R) is a receptor tyrosine kinase, in vivo expression of which is limited to monocytes, macrophages, proliferating smooth muscle cells, and cells of the female reproductive tract (6, 7). Upon binding its ligand, M-CSF, the receptor dimerizes and undergoes auto- and trans-phosphorylation at specific tyrosine residues within the cytoplasmic domain of the receptor. These tyrosine residues, based upon the human M-CSF-R protein sequence, include tyrosines 561 (Tyr-561), Tyr-699, Tyr-708, Tyr-723, and Tyr-809, and bind specific signaling proteins once they become phosphorylated (8-17). Overall, M-CSF-R activation stimulates selective biochemical events.

We have previously shown that the M-CSF-R activates the serine/threonine kinase Akt in a PI 3-kinase-dependent manner to promote cellular survival (18). The activation of PI 3-kinase converts phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), leading to increased levels of the second messenger PI(3,4,5)P3. The SH2 domain-containing inositol 5′-phosphatase, SHIP-1, acts as a negative regulator of Akt phosphorylation by dephosphorylating PI(3,4,5)P3 to PI(3,4)P2 (19-21). The SHIP family of proteins contains two members: SHIP-1 and SHIP-2. SHIP-1 was first characterized as an ~145-kDa protein that became tyrosine-phosphorylated upon interleukin-3 stimulation of a murine hematopoietic cell line (19). Further studies showed that SHIP-1 has several alternatively spliced isoforms, including 135, 125, and 110 kDa (22, 23). Initially, SHIP-1 was shown to be involved in the negative regulation of B-cell and IgE receptor signaling (24, 25). A related gene product to SHIP-1 was also identified and was named SHIP-2 (26). SHIP-2 is characterized by the same 5′-phosphatase activity as SHIP-1. However, whereas both SHIP-1 and SHIP-2 hydrolyze PI(3,4,5)P3 in vitro, only SHIP-1 hydrolyzes phosphatidylinositol 1,3,4,5-tetraphosphate (27). Furthermore, unlike SHIP-1, which is limited to cells of the hematopoietic lineage, SHIP-2 is predominantly expressed in chronic myelogenous leukemia; WT, wild-type; DMEM, Dulbecco's modified Eagle's medium; BMM, bone marrow-derived macrophage; TBS, Tris-buffered saline.
heart and skeletal muscle and the placenta, and differs from SHIP-1 in both the N-terminal and C-terminal regions (26, 28). SHIP-1 has been extensively studied in B-cells, T-cells, and mast cells. However, little attention has been placed on the involvement of SHIP-1 in monocytes and the specific mechanisms used by this phosphatase to regulate M-CSF-induced cellular signaling.

Recently, the Src kinase Lyn has been shown to directly phosphorylate SHIP-1 both in vitro and in vivo (29). However, the biological significance of this phosphorylation remains unclear. Specifically, there is no clear consensus on whether the tyrosine phosphorylation affects either its enzymatic activity or its localization to the plasma membrane. However, membrane targeting of this phosphatase is critical, as the main target of SHIP-1, phosphatidylinositol 3,4,5-trisphosphate, is located in the plasma membrane. Because SHIP-1-deficient mice exhibit hyperplasia of myeloid cells and M-CSF is important in myeloid cell homeostasis, we hypothesized that SHIP-1 was involved in the regulation of M-CSF-induced cellular signaling in monocytes and sought to define the mechanism of this regulation.

Here we report that SHIP-1 becomes tyrosine-phosphorylated upon M-CSF activation of THP-1 cells, and this phosphorylation occurred in a Src family kinase-dependent manner. Transfection of 3T3-Fms cells with WT SHIP-1 demonstrated the importance of SHIP-1 in regulating M-CSF-induced Akt activation. Additionally, following M-CSF stimulation of THP-1 cells, SHIP-1 associated with the Src kinase Lyn. This association occurred via the SH2 domain of SHIP-1, and was independent of the kinase activity of Lyn. WT SHIP-1 reduced NF-kB-dependent transcriptional activation of a reporter gene in M-CSF-activated THP-1 cells. Furthermore, in 3T3-Fms cells, Lyn enhanced the ability of SHIP-1 to regulate Akt activation by promoting the localization of SHIP-1 to the membrane. Finally, macrophages isolated from both SHIP-1- or Lyn-deficient mice exhibited enhanced Akt phosphorylation after M-CSF stimulation in comparison to macrophages isolated from wild-type mice. These data provide the first evidence of the involvement of both Lyn and SHIP-1 in M-CSF-R signaling, leading to a new paradigm regarding SHIP-1 activation, phosphorylation, and stability at the cell membrane.

MATERIALS AND METHODS

Cells—The THP-1 cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 1% penicillin-streptomycin (Invitrogen) at 37 °C, 5% CO2. NIH/3T3-Fms cells were a kind gift from Dr. Martine Roussel (St. Jude Children’s Research Hospital, Memphis, TN) and were maintained in DMEM (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 1% penicillin-streptomycin (Invitrogen). Recombinant human M-CSF was purchased from R&D Systems (Minneapolis, MN). The Src kinase inhibitors PP2 and its inactive analog PP3 were purchased from Calbiochem (La Jolla, CA). The monoclonal SHIP-1 (catalog no. sc-8425) and phosphotyrosine antibodies were purchased from Cell Signaling Technology (Beverly, MA), and the SHIP-1 antibody was purchased from Upstate Biotechnology.

Femoral and tibial bone marrow—Bone marrow derived from WT and SHIP-1-deficient mice (30) and 129/Sv wild-type and Lyn-deficient mice (Jackson Laboratories, Bar Harbor, ME). Bone marrow progenitor cells were flushed out with ice-cold RPMI and then plated in RPMI supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 10 μg/ml polymyxin B, and 20 ng/ml M-CSF. Cells were cultured in a 37 °C incubator for 5 days with addition of fresh M-CSF each day. Adherent mononuclear phagocytes were attached to the bottom of the plates and differentiated to macrophages. Those BMMs were serum-starved for 24 h at 37 °C before re-stimulation with 100 ng/ml M-CSF. Cells were lysed in Akt A buffer (50 mM Tris (pH = 7.5), 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 3 mM Na3VO4, 5 μg/ml aprotonin and leupeptin), and cell lysates were quantitated using the Bio-Rad DC assay (Hercules, CA) and used for Western analysis.

M-CSF Stimulation—THP-1 cells or NIH/3T3-Fms cells were serum-
Fig. 2. The phosphorylation of SHIP-1 following M-CSF activation is Src kinase-dependent. A, THP-1 cells were non-stimulated (−) or stimulated (+) with 100 ng/ml M-CSF, lysed, and immunoprecipitated for SHIP-1 or, as a control, normal IgG, separated via SDS-PAGE, and Western blotted for Lyn (top panel) and then reprobed for SHIP-1 (bottom panel) to show equal loading. B, SHIP-1 phosphorysine signals were quantitated and shown as fold increase of the M-CSF-treated cells over resting, either with or without PP2 preincubation. Data points are represented as mean ± S.E. of three independent experiments. Following 1 and 2 min of M-CSF treatment, the PP2-treated samples had less phosphotyrosine than did the Me2SO-treated samples (p < 0.005).

Fig. 3. The Src kinase Lyn interacts with SHIP1 after M-CSF activation and is independent of the kinase activity of Lyn. A, THP-1 cells were non-stimulated (−) or stimulated (+) with 100 ng/ml M-CSF, lysed, and immunoprecipitated for SHIP-1 or, as a control, normal IgG, separated via SDS-PAGE, and Western blotted for Lyn (top panel). Of note, the antibody used recognizes both the 53- and 56-kDa isoforms of Lyn. The same blot was re-probed for SHIP-1 (bottom panel) to show equal loading. B, THP-1 cells were treated with either PP2 (2 μg/ml) or PP3 (2 μg/ml) for 15 min prior to stimulation with 100 ng/ml M-CSF. Cells were then lysed, immunoprecipitated for SHIP-1, and Western blotted for Lyn (top panel). The same blots were re-probed for SHIP-1 (bottom panel) to show equal loading.

starved on ice for 4.5 h in RPMI 1640 or DMEM without fetal bovine serum. Cells were then supplemented with 100 ng/ml M-CSF and incubated at 37 °C. Cells were then lysed in 1 ml of TN1 lysis buffer (125 mM NaCl, 50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 10 mM Na4P2O7, 10 mM NaF, 1% Triton X-100, 3 mM Na3VO4, 5 μg/ml aprotinin and leupetin) and centrifuged, and supernatants were used for analysis.

Immunoprecipitations and Immunoblotting—THP-1 cells or BMM lysates were immunoprecipitated overnight at 4 °C. 20 μl of a 50% suspension of Protein G-agarose (Amersham Biosciences) was added and incubated at 4 °C for 1 h. Beads were then washed twice in 1 ml of lysis buffer, resuspended in 45 μl of SDS-loading buffer (30 mM Tris (pH 6.8), 2% SDS, 0.01% w/v bromphenol blue, 10% glycerol, 1% mercaptoethanol), and boiled for 5 min. Samples were separated via 10% SDS-PAGE, transferred to nitrocellulose, and blocked in TBS (pH = 7.75) supplemented with 5% (w/v) nonfat dry milk (Bio-Rad) for 20 min at room temperature. Membranes were immunoblotted in TBS (pH = 7.75) supplemented with 0.1% Tween 20 overnight at 4 °C. Blots were thoroughly washed in TBS + 0.1% Tween 20 and probed with 0.6 μg of the appropriate horseshadish peroxidase-labeled secondary antibody at room temperature for 12 min. Blots were then thoroughly washed in TBS + 0.1% Tween 20 and developed by enhanced chemiluminescence (Amersham Biosciences).

GST Fusion Protein Pull-down Experiments—THP-1 cells were treated with or without 100 ng/ml M-CSF, lysed in 1 ml of TN1 lysis buffer, and incubated with 10 μg of the GST-SHIP-1 SH2 domain or GST alone and 50 μl of a slurry of glutathione-Sepharose. Samples were rocked overnight at 4 °C. Beads were washed two times with TN1 lysis buffer and treated in the same manner for Western blotting as described above.

Inhibitors—2 μg/ml amounts of PP2 or PP3 or an equivalent volume of Me2SO (vehicle control) were added to THP-1 cells in serum-free RPMI 1640 medium and incubated at room temperature for 15 min prior to stimulation with M-CSF.

Plasmids—NF-κB-luciferase reporter plasmid was purchased from Stratagene (La Jolla, CA), and pEGFP was purchased from Clontech. Wild-type SHIP-1 and the SHIP-1 SH2 domain cloned into pCDNA3 were a generous gift from Dr. K. Mark Coggeshall (Oklahoma Medical Research Facility, Oklahoma City, OK). WT Lyn cloned into pcDNA3 was a kind from Dr. Henry Metzger (National Institutes of Health, Bethesda, MD). The GST-AKT construct was a kind gift from Dr. R. B. Pearson (Trescowthick Research Laboratories, Melbourne, Australia).

Transfection of THP-1 Cells and Luciferase Assays—THP-1 cells were transfected by electroporation (310 V, 950 microfarads; Bio-Rad Gene Pulser II) with 20 μg of wild-type SHIP-1 or SHIP-1 SH2 domain, 5 μg of NF-κB-luc plasmid, and 2 μg of pEGFP to normalize for transfection efficiency. Transfectants were harvested 24 h later and activated by adding M-CSF for 6 h at 37 °C. The cells were lysed in 100 μl
ship-1-deficient mice suffer expansion and infiltration of macrophages and other myeloid cells into many organs, including the lungs (5, 30), we wanted to determine the molecular details of SHIP-1 function following M-CSF stimulation.

In the monocytic-like cell line, THP-1, which endogenously expresses both SHIP-1 and the M-CSF-R, Akt becomes rapidly phosphorylated after 1 min on threonine 308 (Thr(П)-308) following M-CSF activation (Fig. 1A, top panel), an event that is associated with Akt activity following growth factor stimulation (31–34). Additionally, SHIP-1 becomes rapidly tyrosine-phosphorylated after 1 min following M-CSF activation of THP-1 cells (Fig. 1B, top panel), indicating that THP-1 cells are a good model for studying M-CSF-R signaling. Each blot was subsequently re-probed to ensure equal loading (Fig. 1, A and B, bottom panels).

Recent studies investigating the functional consequence of tyrosine phosphorylation on SHIP-1 enzymatic activity revealed two interesting findings. First, Phee et al. (29) demonstrated that membrane localization, not phosphorylation, of SHIP-1 was the critical event for induction of its enzymatic activity. Second, it has been shown that FcγRIIb1 phosphorylation results in SHIP-1 phosphorylation and subsequent enzymatic activity. This concept was supported in a recent study published by Hibbs et al. (35, 36), which demonstrated that SHIP-1 hyperphosphorylation correlated with FcγRIIb1 hyperphosphorylation in B-cells, leading to the inhibition of signaling events. Taken together, these events indicate that signaling through the M-CSF-R stimulates both positive signaling events, in terms of Akt activation, and negative signaling events, in terms of SHIP-1 phosphorylation. Because Akt utilizes P(3,4,5)P3 to localize to the plasma membrane, and SHIP-1 reduces the availability of P(3,4,5)P3 by converting P(3,4,5)P3 to P(3,4)P2, SHIP-1 may play a role in limiting Akt activation after M-CSF activation.

To further investigate the importance of SHIP-1 in M-CSF-R-induced cellular signaling, we decided to use 3T3-Fms cells. These cells, which have been utilized in previous studies to study M-CSF-R signaling (18, 37), are stably transfected with the human M-CSF-R but do not contain SHIP-1, therefore making it a useful model for this study. These cells were transiently transfected with or without wild-type SHIP-1 plus GST-Akt, and Thr(П)-308 GST-Akt levels were measured. Fig. 1C shows that transfection of 3T3-Fms cells with wild-type SHIP-1 (top panel, lanes 1 and 2) reduced Thr(П)-308 GST-Akt levels following M-CSF activation in comparison to cells that were not transfected with SHIP-1 (top panel, lanes 3 and 4). This blot was then re-probed for total Akt to show equal loading (Fig. 1C, middle panel). These data are quantitated and represented in graph form in Fig. 1D. The graph compares the fold increase in Thr(П)-308 GST-Akt levels, following M-CSF stimulation, of the SHIP-1-transfected and non-transfected lanes relative to non-stimulated. In the absence of SHIP-1, the level of Thr(П)-308 GST-Akt after M-CSF stimulation is ~7.7-fold greater than non-stimulated. In the presence of SHIP-1, the -fold difference is only ~5.3-fold for M-CSF-stimulated versus non-stimulated. To ensure that 3T3-Fms cells do not endogenously express SHIP-1, we also performed immunoprecipitations of SHIP-1-transfected and non-transfected cells and saw evidence of SHIP-1 expression only in the SHIP-1-transfected cells (Fig. 1C, bottom panel). Furthermore, SHIP-1 became tyrosine-phosphorylated following M-CSF activation of 3T3-Fms cells (data not shown). These data indicate the importance of SHIP-1 in the negative regulation of M-CSF-induced Akt activation.

The Src Kinase Inhibitor PP2 Decreases the Phosphorylation of SHIP-1 following M-CSF Activation of THP-1 Cells—Because SHIP-1 is phosphorylated following M-CSF activation of THP-1 and 3T3-Fms cells, we next wanted to determine the possible mediator of this phosphorylation. Previous studies of signaling through the M-CSF-R have indicated the involvement of the Src kinases, specifically via tyrosine 561 (9). Similarly, Phee et al. (29) have recently shown that the Src kinase Lyn phosphorylates SHIP-1 both in vitro and in vivo. Further-
more, Li et al. (38) have shown that Lyn-deficient B-cells displayed hyperactivation of Akt, indicating a negative regulatory role for this Src kinase and a possible candidate responsible for the phosphorylation of SHIP-1. Therefore, we next investigated whether SHIP-1 was phosphorylated in a Src kinase-dependent manner.

Fig. 2A shows that preincubation of THP-1 cells with PP2, an inhibitor of the Src kinases, prior to M-CSF activation reduced the phosphorylation of SHIP-1 (top panel, lanes 6–10) compared with the vehicle control (Me$_2$SO-treated samples (top panel, lanes 1–5). Quantitation of these bands is shown in Fig. 2B and indicates that preincubation of THP-1 cells with PP2 resulted in a ~2-fold reduction in SHIP-1 phosphorylation after 1 min of M-CSF treatment compared with the Me$_2$SO-treated lane. Although these data do not signify that Lyn is responsible for the phosphorylation of SHIP-1, they do indicate that the Src kinases are an important component following M-CSF activation.

SHIP-1 Associates with Lyn after M-CSF Activation via the SH2 Domain of SHIP-1—Next, because of the published data regarding the importance of Lyn in both phosphorylating SHIP-1 and negatively regulating Akt activation, we sought to determine whether SHIP-1 associated with Lyn following M-CSF activation. By utilizing THP-1 cells, we show that SHIP-1 associated with Lyn following M-CSF activation (Fig. 3A, top panel, lane 2). The same blot was re-probed for SHIP-1 to show equal loading (Fig. 3A, bottom panel). Furthermore, this association was not inhibited by preincubation of THP-1 cells with PP2 (Fig. 3B, top panel, lane 3) or the PP2-inactive analog PP3 (lane 4). The same blot was also re-probed with SHIP-1 to show equal loading (Fig. 3B, bottom panel). These data indicate that the association of SHIP-1 and Lyn is M-CSF-dependent but independent of both the kinase activity of Lyn and the phosphorylation of SHIP-1.

Given that the association of SHIP-1 and Lyn was activation-dependent, we next sought to determine whether the SH2 domain of SHIP-1 was involved in binding Lyn. Therefore, we utilized a GST fusion protein containing the SH2 domain of SHIP-1. We added this GST-SH2 domain to resting or M-CSF-stimulated THP-1 cell lysates and found that the SH2 domain of SHIP-1 interacted with Lyn following M-CSF activation of THP-1 cells (Fig. 4A, lane 2). Consistent with our previous study with endogenous SHIP-1, this interaction was not inhibited by preincubation of THP-1 cells with PP2 or PP3 (Fig. 4B, lanes 3 and 4, respectively), indicating the involvement of the SHIP-1 SH2 domain in M-CSF-R signaling.

The SHIP-1 SH2 Domain Is Necessary for NF-κB Transcriptional Activity—To assess the functional relevance of the SH2 domain of SHIP-1, we took advantage of a SHIP-1 SH2 domain construct. We transiently transfected THP-1 cells with a NF-κB-luciferase construct along with either an excess of functional SHIP-1 or the SHIP-1-SH2 domain, which competes with native SHIP-1. We chose to assess the activity of SHIP-1 via the promotion of NF-κB activity for two reasons. First, the transfection efficiency of THP-1 cells is extremely low, and the luciferase assay is extremely sensitive and has been utilized in other published studies investigating the involvement of phosphatases in regulating NF-κB activation (39, 40). Second, it has been shown that Akt is capable of promoting NF-κB activation, thus assessing SHIP-1 in this pathway may give us a better understanding of downstream targets (41–43).

Fig. 5 shows that transfection of THP-1 cells with wild-type SHIP-1 reduced NF-κB transcriptional activity after M-CSF activation. In contrast, transfection of the SH2 domain of SHIP-1 resulted in a ~3.8-fold increase in NF-κB transcriptional activity after M-CSF activation compared with WT SHIP-1, indicating that SHIP-1 is also important in the negative regulation of M-CSF-induced NF-κB activation, and that the SHIP-1 SH2 domain is an important component of this pathway.

The Src Kinase Lyn Enhances the Ability of SHIP-1 to Reduce Thr(P)-308 Levels by Stabilizing SHIP-1 at the Membrane—The experiments above primarily focused on the effects of SHIP-1 in regulating either Thr(P)-308 Akt levels or NF-κB activation following M-CSF stimulation. We next wanted to demonstrate the importance of Lyn in this process.

We again utilized the 3T3-Fms cells because they do not endogenously express SHIP-1 or Lyn. Transfection of these cells with or without Lyn along with wild-type SHIP-1 and GST-Akt allowed us to determine the role of Lyn in the regulation of M-CSF-induced Akt activation. Fig. 6A shows that, in the presence of Lyn, Thr(P)-308 GST-Akt levels are ~1.6-fold higher than in cells with Lyn. Cells were also immunoblotted for Lyn (Fig. 6B) to demonstrate that Lyn was incorporated into these cells after transfection. Interestingly, the tyrosine phosphorylation of SHIP-1 in cells that were co-transfected with Lyn (Fig. 6C, top panel, lanes 1 and 2) was much greater than the phosphorylation of SHIP-1 in cells not transfected with Lyn (Fig. 6C, top panel, lanes 3 and 4). The low level phosphorylation of SHIP-1 without Lyn mirrors that described in Fig. 1 (data not shown), with minimal but apparent phosphorylation, indicating that SHIP-1 becomes phosphorylated following M-CSF stimulation of 3T3-Fms cells to a much greater extent, even in the resting state, in the presence of Lyn.

To further indicate the importance of Lyn, we next wanted to determine what effect Lyn has on the membrane localization of SHIP-1. We utilized 3T3-Fms cells that were transfected with
SHIP-1 and Lyn or SHIP-1 alone, and isolated the membrane fraction. We then immunoprecipitated SHIP-1 from the membrane fraction and determined the phosphotyrosine level of SHIP-1 within the membrane in the presence or absence of Lyn. In the presence of Lyn, the tyrosine phosphorylation of SHIP-1 following M-CSF activation was greatly enhanced when compared with the sample without Lyn (Fig. 6E, top panel, compare lanes 2 and 4). Furthermore, the amount of

**Fig. 6.** Lyn enhances SHIP-1 phosphorylation and reduces Thr(P)-Akt levels following M-CSF stimulation of 3T3-Fms cells. A, 3T3-Fms cells were transfected with or without a Lyn construct plus WT SHIP-1 and GST-Akt. Cells were harvested 24 h after transfection, serum-starved for 4 h, and then stimulated with 100 ng/ml M-CSF for 5 min. Cells were then lysed, immunoprecipitated for Akt, separated via SDS-PAGE, and Western blotted for Thr(P)-308 GST-Akt (top panel). The same blots were re-probed for total Akt (bottom panel) to show equal loading. B, cells were also immunoprecipitated for Lyn, separated via SDS-PAGE, and Western blotted for Lyn to ensure proper transfection. C, lysates from A were also immunoprecipitated for SHIP-1 and Western blotted for Tyr(P) (top panel) and subsequently re-probed for SHIP-1 (bottom panel) to show equal loading. D, Thr(P)-308 GST-Akt bands in A were quantitated and are graphed as -fold induction of M-CSF treated lanes over resting in the presence or absence of Lyn. Data points are represented as mean ± S.E. for three independent experiments. Following M-CSF treatment, Thr(P)-308 Akt levels were reduced in the samples transfected with SHIP-1 and Lyn versus samples transfected with SHIP-1 alone (p < 0.005). E, 3T3-Fms cells were transfected with SHIP-1 plus Lyn or with SHIP-1 alone, or were not transfected (C). Cells were then lysed without detergent to separate the membrane fraction, immunoprecipitated for SHIP-1, separated via SDS-PAGE, and Western blotted for either Tyr(P) (top panel) or total SHIP-1 (bottom panel). F, 3T3-Fms transfectants (as described in E) were also lysed in the presence of detergent to demonstrate equal transfection. Lysates were immunoprecipitated for SHIP-1, separated via SDS-PAGE, and Western blotted for SHIP-1.
SHIP-1 present within the membrane was greater in the cells transfected with Lyn (Fig. 6E, bottom panel). Fig. 6F shows that 3T3-Fms cells were effectively transfected with SHIP-1 even in the absence of Lyn. These data indicate that Lyn may function to phosphorylate and stabilize SHIP-1 at the membrane following M-CSF activation, demonstrating a novel functional role for this Src kinase in regulating M-CSF-induced Akt activation.

**Macrophages Derived from Either SHIP-1- or Lyn-deficient Mice Show Enhanced Akt Phosphorylation**—Throughout our studies, we have done multiple transfections of either THP-1 cells or 3T3-Fms cells to determine the influence of SHIP-1 and Lyn on M-CSF-induced Akt activation. In addition, we have initiated characterized the association of SHIP-1 and Lyn in THP-1 cells following M-CSF activation. Therefore, we next wanted to examine the physiological roles of SHIP-1 and Lyn in regulating Akt activity.

Bone marrow-derived macrophages from WT and SHIP-1-deficient mice were serum-starved and activated with M-CSF for the indicated times. The cells were then lysed, and the lysates were immunoprecipitated for Akt, separated via SDS-PAGE, and immunoblotted for Thr(P)-308 Akt (Fig. 7). Macrophages derived from SHIP-1 wild-type mice showed a strong phosphorylation of threonine 308 Akt after 5 min followed by a decrease in phosphorylation after 30 min (Fig. 7A, top panel, lanes 1–5). However, macrophages isolated from SHIP-1-deficient littermates exhibited a residual phosphorylation of Akt, even in the absence of re-stimulation with M-CSF, and a markedly enhanced phosphorylation of Akt after M-CSF treatment up to 60 min (Fig. 7A, top panel, lanes 6–10). The same blots were re-probed for total Akt to show equal loading (Fig. 7A, bottom panel). These data are represented graphically in Fig. 7C. Band intensities of Thr(P)-308 Akt are plotted as percentage of decrease over time relative to the maximal Thr(P)-308 Akt time point (5 min for both wild-type SHIP-1 and SHIP-1-deficient macrophages). Data points are represented as mean and deviation from the mean of two independent experiments.

To investigate the physiological importance of Lyn, we also isolated bone marrow progenitor cells from Lyn-deficient and wild-type mice in the same manner as described for the SHIP-1 studies. Macrophages isolated from wild-type mice show a strong Thr(P)-308 Akt level after 5 min, followed by a decrease after 30 min (Fig. 8A, top panel, lanes 1–6). Macrophages isolated from Lyn-deficient mice also showed enhanced Thr(P)-308 Akt levels after M-CSF treatment compared with their wild-type littermates. The same blots were re-probed for total Akt to show equal loading (Fig. 8A, bottom panel). Again, these data are represented graphically in Fig. 8C and are plotted in the same manner as the data for the SHIP-1-deficient mice. Lyn-deficient macrophages exhibit ~22% (~1.6-fold) greater Thr(P)-308 Akt level compared with their wild-type littermates. Fig. 8B confirms that Lyn-deficient cells do not express native SHIP-1. These data indicate the physiological importance of SHIP-1 in regulating M-CSF-induced Akt activity.
DISCUSSION

The goal of this paper was to elucidate the role of SHIP-1 in M-CSF-induced myeloid cell activation and to determine the mechanism by which SHIP-1 regulates M-CSF-induced Akt activity. Using the monocytic cell line THP-1, we found that SHIP-1 bound the Src kinase Lyn following M-CSF stimulation via the SHIP-1 SH2 domain. Interestingly, the interaction between SHIP-1 and Lyn was much stronger after M-CSF stimulation than after non-selective tyrosine phosphorylation with pervanadate or FcγR cross-linking (data not shown), suggesting a level of specificity to this molecular interaction via M-CSF-R activation. Furthermore, the interaction between SHIP-1 and Lyn was independent of the Src kinase activity of Lyn, as the Src kinase inhibitor PP2 did not disrupt binding. In contrast, PP2 reduced the tyrosine phosphorylation of SHIP-1, not only suggesting that this Src kinase inhibitor was active but also that the kinase activity of Lyn and the phosphorylation of SHIP-1 were not necessary for this interaction. Further studies will need to be done to determine the possible tyrosine residue in Lyn that is involved in binding the SHIP-1 SH2 domain.

It is interesting to speculate that membrane-anchored Lyn may represent a mechanism to target and anchor SHIP-1 to the membrane after M-CSF stimulation to allow SHIP-1 to perform its catalytic activities. Of note, Lyn-deficient macrophages exhibited a time-dependent reduction in SHIP-1 phosphorylation compared with the wild-type littermates (data not shown). Because SHIP-1 reduces membrane targeting of a number of important PH domain-containing proteins, including the serine/threonine kinase Akt, targeting of SHIP-1 at the membrane is important. The decrease in SHIP-1 phosphorylation in the absence of Lyn may result from reduced stability of SHIP-1 at the membrane following M-CSF stimulation. Furthermore, we also speculate that M-CSF activation may induce the phosphorylation of a specific tyrosine residue in Lyn, therefore facilitating the interaction with SHIP-1. Further studies are now being done to address these issues.

These data provide further evidence of the role of Lyn as a negative regulator of M-CSF-induced cellular signaling. Initial work signifying the importance of Lyn in both positive and negative regulation of signaling events substantiated our hypothesis regarding the role of this Src kinase in M-CSF-R signaling (35, 44–49). Along these same lines, Gresham et al. (50) have shown that the Src kinase Fgr has a negative regulatory role in murine macrophages with respect to phagocytosis. The evidence provided here is the first indication of a negative regulatory function of the Src kinase Lyn in M-CSF-R signaling.

The importance of SHIP-1 in cellular regulation and survival is apparent in the SHIP-1-deficient animals. These animals suffer expansion and infiltration of myeloid cells into many organs, including the lungs, and may die of precocious respiratory insufficiency (30). The cellular constituency of this infiltrate seems to predominantly consist of macrophages. Strikingly, our results reveal that the absence of SHIP-1 results in prolonged activation of Akt after M-CSF stimulation and may play a key role in the observed massive accumulation of macrophages in SHIP-1-deficient mice. Of interest, in our experiments with the SHIP-1-deficient mice, the bone marrow-derived cells were incubated with M-CSF for 4 days, serum-starved, and then lysed either with or without restimulation with M-CSF. In contrast to the wild-type animals, macrophages from SHIP-1-deficient animals displayed threonine 308 phosphorylation of Akt even after 24 h of serum starvation, likely a residual effect of M-CSF stimulation from the preceding days. Therefore, SHIP-1 likely plays a role in the normal
regulation of monocyte and macrophage survival in response to M-CSF stimulation. Furthermore, because macrophages from Lyn-deficient mice also had enhanced threonine-308 phosphorylation of Akt, it can be concluded that, in addition to SHIP-1, Lyn is also involved in the regulation of M-CSF-induced Akt activation. The extent to which Akt phosphorylation is enhanced in the Lyn-deficient macrophages is similar to the extent that it is enhanced in the SHIP-1-deficient macrophages, defining the importance of Lyn in this process. Furthermore, because Thr(P)-Akt levels are still moderately reduced in SHIP-1-deficient mice, other phosphatases, such as PTEN, may aid in regulating the availability of phosphatidylinositol 3,4,5-trisphosphate necessary for Akt activation.

It is also important to note in malignancies like chronic myelogenous leukemia (CML) that the suppression of SHIP-1 appears to play a powerful role in cellular transformation. This was determined in elegant studies showing that a variety of cells, including those transformed with the bcr-abl oncogene and cells from patients with CML, demonstrated SHIP-1 suppression (51). These cells also have constitutive activation of Akt, suggesting a common mechanism for supporting cellular survival through a lack of regulation of PI(3,4,5)P3. Furthermore, in bcr-abl-transformed cell lines, the re-expression of SHIP-1 led to cell death. And because the malignant cells in CML are myeloid in origin, these data support a critical role for SHIP-1 in suppression of myeloid cell survival.

In summary, this paper demonstrates, for the first time, that both SHIP-1 and Lyn negatively regulate M-CSF-induced Akt activation. Further studies are now under way to investigate the role of the SHIP-1 interaction with Lyn.

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