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SHIP1 Negatively Regulates Proliferation of Osteoclast Precursors via Akt-Dependent Alterations in D-Type Cyclins and p27

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Osteoclasts arise from macrophage progenitors in bone marrow (BMMs) as a consequence of signaling events elicited by M-CSF and receptor activator of NF-κB ligand, acting on their unique receptors, via c-Fms and receptor activator of NF-κB. Both receptors activate the PI3K and MAPK pathways, which promote cell proliferation and survival. SHIP1 is essential for normal bone homeostasis, as mice lacking the protein exhibit osteoporosis resulting from increased numbers of hyper-resorptive osteoclasts. In this study, we show that BMMs from SHIP1 null mice respond to M-CSF, but not receptor activator of NF-κB ligand, by increasing Akt activation. In consequence, there are up-regulation of D-type cyclins, down-regulation of the cyclin-dependent kinase inhibitor p27, and, therefore, increased phosphorylation of the retinoblastoma protein and cell proliferation. Surprisingly, cell survival of wild-type and knockout BMMs is unaltered. Finally, osteoclastogenesis and periaricular bone erosions are markedly increased in SHIP1−/− mice with inflammatory arthritis, a condition characterized by increased M-CSF expression. The SHIP1/Akt pathway therefore suppresses bone loss in pathological states associated with an excess of the cytokine. The Journal of Immunology, 2006, 177: 8777–8784.

In response to M-CSF, the hemopoietic specific SHIP1 becomes tyrosine phosphorylated and associates with c-Fms via the adaptor protein Shc, leading to generation of negative regulatory signals (9, 10). SHIP1 contains an N-terminal Src homology 2, a central inositol-5-phosphatase, and a C-terminal proline-rich domain, within which are two phosphotyrosine binding sites (NPXY). SHIP1 specifically removes γ phosphate from phosphatidylinositol 3,4,5-trisphosphate (11, 12) and thus down-regulates PI3K-mediated signaling.

SHIP1−/− mice contain increased numbers of granulocyte-macrophage progenitors (13, 14). Their marrow, when cultured in the presence of GM-CSF, M-CSF, IL-3, or steel factor, yields increased numbers of myeloid colonies (13), which contain osteoclast precursors. In fact, we find SHIP1 regulates osteoclast number and function (15). Furthermore, SHIP1−/− BMMs undergo robust osteoclastogenesis in response to M-CSF and RANKL in vitro, and osteoclasts generated from these cells exhibit prolonged survival. Consequently, SHIP1−/− mice are osteoporotic due to increased numbers of hyperresorptive osteoclasts (15). In contrast, the mechanism underlying the enhanced osteoclastogenesis and hyperplasia of myeloid cells in SHIP1 null mice is unknown.

In this study, we identify the means by which SHIP1 deficiency accentuates M-CSF-induced macrophage proliferation and osteoclastogenesis. We demonstrate that enhanced BMM proliferation, but not decreased apoptosis or increased differentiation, contributes to stimulated osteoclast formation by SHIP1−/− macrophages. This hyperproliferation arises from marked Akt activation, with consequent elevated expression of D-type cyclins, down-regulation of p27, and hyperphosphorylation of retinoblastoma (Rb). Importantly, we find SHIP1 plays a central role in moderating bone loss in a state of excess M-CSF, namely inflammatory arthritis. Enhancing SHIP1 expression and/or activity therefore represents a means of arresting M-CSF-induced pathological bone loss.
Materials and Methods

Reagents and Abs

LY294002 and wortmannin were obtained from Cell Signaling Technology. The Abs against c-Fms, cyclin D2, cyclin D3, SHIP1, c-Fos, goat anti-mouse, or rabbit IgG conjugated with HRP were purchased from Santa Cruz Biotechnology. Abs for Akt, phospho-Akt, ERK, phospho-ERK, phospho-Rb (807/811), cyclin D1, and p27 were purchased from Cell Signaling Technology. Anti-/H9252-actin Ab was obtained from Sigma-Aldrich. Human rM-CSF was provided by D. Fremont (Washington University, St. Louis, MO). Murine rGST-RANKL was described previously (16).

Cell culture

Whole bone marrow cells were isolated from long bones of 4- to 8-wk-old wild-type (WT) and SHIP1−/− mice, as described (17). These cells were grown in α-MEM with 10% FBS and 1:10 CMG14-12 culture supernatant (18) containing 1.2 μl/ml M-CSF for 3 days to generate BMMs. To generate osteoclasts, RANKL and M-CSF were added to α-MEM medium containing 10% FBS. Osteoclasts were stained for tartrate-resistant acid phosphatase (TRAP) as per the manufacturer’s instructions (Sigma-Aldrich).

Plasmids

The retroviral vector pMX-puro (19) and the hemagglutinin (HA)-tagged SHIP1 cDNA expression construct HA-SHIP1-BSKS+ (20) were described previously. To generate pMX-SHIP1, HA-tagged SHIP1 was removed from HA-SHIP1-BSKS+ by sequential digestion with XhoI and NotI, and ligated to pMX-puro sequentially digested with BamHI and NotI sites. The sticky ends generated by initial digestion were filled in using the large fragment of DNA polymerase I (Invitrogen Life Technologies) before the second digestion was performed. All constructs were verified by sequencing in both directions.

Infection of BMMs

The control vector and one containing a WT SHIP1 construct were transfected into Plat-E cells (21) to produce virus, as previously described (22). BMMs were infected with viral supernatants in the presence of 4 μg/ml polybrene for 24 h. Infected cells were replated and selected in medium containing 2 μg/ml puromycin for at least 3 days.

Western blot analysis

BMMs were grown in α-MEM with 10% FBS and 1/10 CMG14-12 supernatant for 3 days. Cells were then starved of serum and M-CSF for 6 h

FIGURE 1. SHIP1−/− BMMs proliferate at an accelerated rate in response to M-CSF. A and B, Equal numbers of WT (+/+) and SHIP1-deficient (−/−) BMMs (1 × 10⁵ per 100-mm plate) were grown for the indicated time periods in the presence of 20 ng/ml M-CSF. Cells were trypsinized, stained with trypan blue, and counted for viable (A) and dead (B) cells. C, Equal numbers of WT and SHIP1−/− BMMs (1 × 10⁴/well) were grown in medium supplemented with the indicated concentrations of M-CSF for 2 days. Cells were labeled with BrdU for the last 4 h and assayed for BrdU incorporation. D, Similar to C, except 10 ng/ml M-CSF with or without 25 ng/ml RANKL was used (*, p < 0.05; **, p < 0.005 vs +/−; #, p < 0.05, only vs M-CSF).

FIGURE 2. SHIP1 deficiency results in enhanced M-CSF-dependent activation of Akt, but not ERK, JNK, or p38. BMMs were grown in the absence of serum and M-CSF for 6 h and then stimulated with 20 ng/ml M-CSF for the indicated time periods (A and B) or with the indicated concentrations of M-CSF for 5 min (C) or 100 ng/ml RANKL for indicated time periods (D). Cell lysates were subjected to immunoblotting with Abs, as indicated.
before being stimulated with rM-CSF. To analyze protein expression during cell proliferation, BMMs were grown in α-MEM with 10% FBS lacking M-CSF for 16–24 h and then stimulated with 20 ng/ml M-CSF for various times. Western blot analysis was conducted using 20–30 μg of total proteins, as previously described (23).

**Proliferation assay and cell death ELISA**

BMMs plated in 96-well plates at a density of $1 \times 10^4$ cells/well were grown for 1–2 days in the presence of 20 ng/ml M-CSF and labeled with BrdU for the last 2 or 4 h of culture. The BrdU ELISA was conducted, as recommended, using the cell proliferation Biotrak ELISA system (Amersham Biosciences). Cell death was analyzed in quadruplicate using cell death detection ELISAPLUS kit (Roche Applied Science), which detects cytoplasmic histone-associated DNA fragmentation. All experiments were repeated two to three times.

**RT-PCR**

RNA was isolated using RNasy kits (Qiagen). First-strand cDNA was generated from 1 μg of total RNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies), as recommended by the manufacturer. One-fifth of the reverse-transcriptase reaction product, 45 μl of PCR SuperMix (Invitrogen Life Technologies), and 1 μM primers for the genes to be tested were amplified in a PCR Express Thermal Cycler (Thermo Hybaid). The cDNA was denatured at 94°C for 5 min and subsequently subjected for various amplification cycles comprised of 94°C for 40 s, 60°C for 45 s, and 72°C for 45 s. The primers used were as follows: 1) cyclin D1, ATGAACTACCTGGACCGG and AGGCTTGACTCCA GAAGG; 2) cyclin D2, TTACAGAGGATGAGTGAAGTGA and GAG AAGGGGCTAGCAGATGA; 2) cyclin D3, AGGCTTGCGAGGAG ATGTCTT and GCCAGGAAGTCGTGCGCAATC; and 3) GAPDH, ACT TTGTCAAGCTCATTTCC and TGCAGCGAACTTTATTGATG.

**Bone marrow transplantation**

Eight-week-old C57BL/6 mice that had received 10 Gy of total body gamma irradiation were injected via the tail vein with 100 μl of PBS containing $2 \times 10^9$ bone marrow cells from either SHIP1 knockout mice or their WT littermates. These mice were used at 3–4 wk after bone marrow transplantation.

**Serum transfer arthritis and histological analysis**

Arthrogenic serum was obtained from K/B × N mice (6–12 wk old) (24, 25). A single dose of 200 μl of serum per mouse was used to induce arthritis in one group, whereas 200 μl of PBS was injected in the control group of mice. Five or more mice were used for each group. Paw thickness was measured at days 0, 2, 4, and 6 using a dial thickness gauge (Mitutoyo). All mice were sacrificed at day 6. Paws were stripped of soft tissue, fixed in 10% buffered formalin for 24 h, decalcified in 14% EDTA (pH 7.2) for 7 days, dehydrated in graded alcohol, cleared through xylene, and embedded in paraffin. Paraffin sections were stained histochemically for TRAP to visualize osteoclasts. Histomorphometric quantitation for osteoclast-covered bone surface and periarticular inflammation was performed using the Biosoft System (BIOQUANT Image Analysis), as previously described (26).

All animal experimentation was approved by the Animal Studies Committee of Washington University School of Medicine.

**Results**

**SHIP1$^{-/-}$ macrophages are hyperproliferative in response to M-CSF, but not RANKL**

We established previously that SHIP1$^{-/-}$ BMMs undergo enhanced osteoclastogenesis in response to M-CSF and RANKL, and the osteoclasts so formed are resistant to apoptosis (15). Because the number of osteoclasts is reflective of the proliferative and apoptotic rates of their precursors, we examined these parameters in M-CSF-treated BMMs. At all times during a 6-day culture period, we found that the numbers of SHIP1$^{-/-}$ BMMs are enhanced relative to WT cells (Fig. 1A). In contrast to mature osteoclasts, this increase in SHIP1$^{-/-}$ osteoclast precursors is not due to decreased cell death as assessed by trypan blue exclusion (Fig. 1B) or cell death ELISA (data not shown). In contrast, BrdU incorporation into M-CSF-treated mutant BMMs is significantly increased over a range of M-CSF doses (Fig. 1C). The enhanced prolifera-

![FIGURE 3. The PI3K/Akt pathway mediates BMM proliferation. A, WT BMMs were grown in the absence of serum and M-CSF for 6 h and subsequently treated with or without LY294002 (10 μM) or wortmannin (0.2 μM) 1 h before stimulation with 20 ng/ml M-CSF for 5 min. Cell lysates were analyzed by immunoblotting with indicated Abs. B and C, Growth-arrested WT or SHIP1$^{-/-}$ BMMs were grown for 24 h in the presence of 20 ng/ml M-CSF and the indicated concentration of LY294002 or wortmannin. Proliferation was determined by BrdU incorporation ($+, p < 0.001$ vs cells not treated with inhibitors). D, WT BMMs were grown for 24 h in the presence of 20 ng/ml M-CSF with indicated concentrations of LY294002 or wortmannin. Cell death was assayed by cell death ELISA, which quantitates DNA fragmentation. E, WT BMMs were grown in the presence of 10 ng/ml M-CSF, and WT osteoclasts were generated with 10 ng/ml M-CSF and 100 ng/ml RANKL. Cells were treated with LY294002 for 8 h at the indicated concentration and assayed for DNA fragmentation by cell death ELISA ($+, p < 0.001$ vs cells not treated with LY294002).
FIGURE 4. Cell cycle progression in the absence of SHIP1 or by inhibition of PI3K correlates with alterations in levels of cyclin D1, D2, D3, and p27. A, D, and F, WT (+/+) and SHIP1-deficient (−/−) BMMs were grown in the absence of M-CSF for 16–24 h, followed by stimulation with 20 ng/ml M-CSF. Cell lysates were collected at the indicated times and subjected to immunoblotting with Abs, as indicated in the figure (A and D). Cells were also assayed for BrdU incorporation at the indicated time points (F) (p < 0.05 vs +/+ from 6 to 14 h). B, C, and E, WT BMMs were grown in the absence of M-CSF for 20 h. A total of 10 μM LY294002 or 10 μM U0126 was added for 1 h, as indicated, followed by 20 ng/ml M-CSF for 12 h. B and E, Cell lysates were subjected to immunoblotting, as indicated, or C, RNA expression was analyzed by RT-PCR for cyclin D1, D2, and D3. GAPDH serves as loading control.

Loss of SHIP1 decreases M-CSF-stimulated activation of Akt, but not MAPKs

On binding to its receptor c-Fms, M-CSF rapidly activates MAPKs and PI3K/Akt, each of which may influence cell number (27, 28). To determine whether the hyperproliferative state of SHIP1−/− osteoclast precursors is mediated by these signaling pathways, we assessed their activation by M-CSF. Although phosphorylation of the MAPKs ERK, JNK, and p38 is unaltered in SHIP1−/− BMMs (Fig. 2A), the cytokine enhances Akt activation in a time- and dose-dependent manner (Fig. 2, B and C). Similar to the proliferative response (Fig. 1C), the relative enhancement of Akt phosphorylation is most evident at lower concentrations of M-CSF (Fig. 2C).

Consistent with our preceding data, which suggest that hyperproliferation of SHIP1 null macrophages is independent of RANKL (Fig. 1D), we find that absence of SHIP1 fails to alter RANKL-induced signaling events, including phosphorylation of Akt, ERK, JNK, p38, and IκBα (Fig. 2D). Moreover, expression of protein markers for osteoclast differentiation, such as NFAT2 or cathepsin K, is not accelerated in SHIP1-deficient cells during osteoclastogenesis (data not shown), demonstrating that lack of SHIP1 does not impact RANKL-driven differentiation of osteoclasts from macrophages.

Inhibition of PI3K alters M-CSF-induced proliferation

The data presented to date suggest that M-CSF-stimulated hyperproliferation of SHIP1−/− BMMS reflects activation of the PI3K/Akt pathway. If such is the case, one would expect that inhibition of PI3K/Akt signaling leads to growth arrest. To address this issue, we used the PI3K inhibitors, LY294002 and wortmannin, which prevent M-CSF-induced activation of Akt, while not affecting that of ERK (Fig. 3A). The same inhibitors dose dependently reduce the capacity of the cytokine to stimulate BrdU incorporation in BMMs (Fig. 3, B and C); in contrast, they have no impact on cell death at concentrations that diminish proliferation (Fig. 3D), excluding the possibility that the reduced BrdU incorporation in their presence is secondary to cell death.

In contrast to BMMs, osteoclasts derived from SHIP1−/− BMMs exhibit enhanced survival (15). To explain this apparent inconsistency between osteoclasts and their precursors, we compared cell death induced by LY294002 in WT BMMs and mature bone-resorbing osteoclasts. LY294002 induces marked cell death in osteoclasts even at the concentrations as low as 2.5 or 5 μM (Fig. 3E). This result suggests that survival of osteoclasts is more sensitive to the changes of PI3K/Akt activation than are BMMs. The molecular basis for this difference remains to be determined.

Loss of SHIP alters expression of cell cycle proteins that regulate proliferation

Virtually all growth factors regulate proliferation during the G1 to S phase of the cell cycle. Formation of active complexes of cyclins
and cyclin-dependent kinases (CDKs) and subsequent phosphorylation of Rb by these complexes determine advancement from G₁ into S phase (29, 30). Because M-CSF induces expression of cyclin D1 and cyclin D2 in other cells (7, 8), we examined whether accelerated proliferation of SHIP1−/− BMMs reflects enhanced D-type cyclin expression in response to the cytokine. Following growth arrest, cyclin D1 is detectable in WT BMMs 6 h after M-CSF stimulation and maximizes by 12 h, whereas in the absence of SHIP1 it appears as early as 3 h and reaches a higher steady state level at 6 h (Fig. 4A). Similarly, cyclins D2 and D3 exhibit earlier and are more responsive to M-CSF (Fig. 4A). We next examined the phosphorylation state of Rb in SHIP1−/− BMMs as a functional index of the altered expression of D-type cyclins. Mirroring cyclin expression, M-CSF induces phosphorylation of Rb more rapidly and to a greater extent in the mutant cells than in their WT counterparts (Fig. 4A). Although c-Fos and c-Myc have been reported to contribute to M-CSF-induced proliferation (5, 31), neither is enhanced by the cytokine in SHIP1−/− BMMs (data not shown).

Because SHIP1−/− BMMs exhibit elevated Akt activation in response to M-CSF, we next investigated the mechanism by which PI3K pathway regulates D-type cyclins. We found that the PI3K inhibitor LY294002 attenuates M-CSF-induced protein levels of all three D-type cyclins (Fig. 4B) without changing their mRNA levels (Fig. 4C), suggesting that PI3K-mediated regulation of D-type cyclins is posttranscriptional. Consistent with reduced levels of D-type cyclins in cells treated with LY294002, phosphorylation of Rb is abolished in the same circumstance (Fig. 4B). Suppression of the ERK pathway by U0126 significantly blunts both the protein and mRNA levels of cyclin D1 and D2 (Fig. 4, B and C). In contrast, U0126 does not reduce M-CSF-stimulated cyclin D3 at either the mRNA or protein level (Fig. 4, B and C).

The activity of the cyclin/CDK2 complex is inhibited by Cip/Kip proteins, including p27, and, therefore, down-regulation on p27 will facilitate entry of cells into S phase (32, 33). M-CSF has been reported to decrease p27 expression in human monocytes (34), and we find the same to be true in WT and SHIP1−/− BMMs (Fig. 4D). In keeping with their greater rate of proliferation, SHIP1−/− BMMs exhibit lower basal expression of p27 than their WT counterparts, and the cytokine suppresses this molecule more effectively in the null cells (Fig. 4D). Furthermore, inhibition of PI3K by LY294002 prevents M-CSF-mediated down-regulation of p27 (Fig. 4E), again reflecting the importance of the PI3K/Akt pathway in mediating signals that control G₁ to S transition in BMMs in response to M-CSF.

The earlier appearance of D-type cyclins and hyperphosphorylated Rb as well as the low levels of p27 in SHIP1−/− BMMs suggest accelerated S phase entry. In fact, measurement of BrdU incorporation into synchronized cells stimulated with M-CSF establishes that more SHIP1−/− than WT macrophages are in S phase from 6 to 14 h, which is beyond the restriction point for BMMs, namely 11 h (S. Takeshita, unpublished data) (Fig. 4F). Thus, induction by M-CSF of D-type cyclin expression and loss of p27 are most likely responsible for stimulated proliferation of SHIP1−/− osteoclast precursors.

Reconstitution of SHIP1 expression in SHIP1−/− BMMs reverses enhanced proliferation and osteoclastogenesis in response to M-CSF

To confirm that increased Akt activation, higher rate of proliferation, and enhanced osteoclastogenesis in SHIP1−/− BMMs are indeed due to lack of SHIP1 expression, we expressed the protein...
in SHIP1−/− BMMs at levels approximating those of the endogenous molecule using a retroviral vector (Fig. 5A). As expected from our previous data, re-expression of SHIP1 by retroviral transduction attenuates M-CSF-stimulated Akt activation to that approximating WT BMMs transduced with vector (Fig. 5A). In contrast, only minimal difference in ERK activation is apparent in the SHIP−/−-transduced cells, regardless of exogenous SHIP1 expression, confirming the specificity of our earlier findings. In keeping with these observations, the magnitude of BMM proliferation in cells re-expressing SHIP1 is indistinguishable from WT (Fig. 5B). The puromycin-selected transductants were also cultured with these observations, the magnitude of BMM proliferation in inflammatory arthritis are enhanced by the absence of SHIP1. lethally irradiated mice transplanted with either WT (WT > WT) or SHIP1−/− (KO > WT) marrow were injected with PBS or arthrogenic serum (A). Paw thickness was measured with time. B, Six days later, the mice were sacrificed and histological sections of paws were stained for TRAP activity (red reaction product). Arrows indicate inflammatory response. C, Histo- morphometric quantitation of the percentage of bone surface covered by osteoclasts in paws of each group of mice (*, p < 0.005 vs WT > WT PBS; **, p < 0.001 vs WT > WT serum).

SHIP1 dampens osteoclastogenesis and bone destruction in inflammatory arthritis

Our accumulated data indicate that the enhanced osteoclastogenic capacity of SHIP1-deficient BMMs reflects hyperproliferation in response to M-CSF. If this is the case, one would expect accelerated bone loss in a pathological condition accompanied by enhanced levels of the cytokine, namely inflammatory arthritis (35). To assure that the effects of SHIP1 deletion exclusively reflected its absence in marrow cells, we generated chimeric mice consisting of lethally irradiated WT animals subsequently transplanted with WT or SHIP1−/− marrow. In both circumstances, BMMs from transplanted mice assayed for the absence or presence of SHIP1 by immunoblotting verify the success of transplantation (data not shown). Three to 4 wk after transplantation, we injected the chimeric mice with either arthrogenic serum (24, 25) or PBS. Groups of mice bearing WT or SHIP1−/− marrow develop equivalent paw swelling (Fig. 6A) and periarticular inflammation (Fig. 6B) following serum injection. In contrast, whereas osteoclastogenesis and periarticular bone erosion are also evident in both sets of animals, the extent of both events is much more profound in the animals bearing SHIP1−/− marrow than in their WT counterparts (Fig. 6, B and C). Thus, SHIP1 ameliorates bone destruction in inflammatory arthritis.

Discussion

Osteoclasts are derived from macrophage precursors, and, thus, the abundance of hyperresorptive osteoclasts in SHIP1−/− mice almost certainly reflects a combination of the massive and systemic increase in myeloid cells plus the decreased apoptosis of mature osteoclasts found in these animals (13–15). Because M-CSF is central to macrophage propagation and survival, it is a likely candidate to mediate the effect of SHIP1 deletion on these cells. Thus, we investigated whether proliferative signals emanating from c-Fms, the receptor for the cytokine, are altered by the absence of SHIP1. Although M-CSF-stimulated MAPK activity is not modulated by the lipid phosphatase, Akt activation is enhanced in SHIP1−/− BMMs. This observation suggests that SHIP1 arrests M-CSF-induced proliferation by blunting Akt activation, a hypothesis buttressed by the fact that reconstitution of SHIP1 in knockout cells normalizes M-CSF-induced proliferation and Akt activity. Furthermore, the capacity of the PI3K/Akt inhibitor LY294002 to attenuate the proliferative effects of M-CSF in BMMs provides additional support for the proposed mechanism. It was recently reported that re-expression of SHIP1 in Jurkat cells arrests constitutive activation of Akt in these cells and also reduces proliferation without detectable cell death (36). However, our findings are novel in that they represent the first documentation that the PI3K/Akt pathway promotes proliferation and not survival of primary macrophages. Although enhanced Akt activation in SHIP1−/− BMMs has been documented previously (37), these authors did not provide a functional correlation with cell proliferation.

Proliferation of mammalian cells is governed by cyclins and their associated CDKs. D-type cyclins (D1, D2, and D3) are expressed during the G1 to S phase transition, and their levels decline when the mitogens are withdrawn (38, 39). Our data link the PI3K/Akt pathway to D-type cyclins in primary macrophages. All three cyclines are up-regulated more efficiently in response to M-CSF treatment in SHIP1−/− BMMs than in WT cells, and this sensitivity reflects increased Akt, but not MAPK signaling. Although others also find expression of cyclin D1 and cyclin D2 is induced by M-CSF (7, 8), we are the first to demonstrate that the same is true for cyclin D3. The basal level of cyclin D3 is higher in

FIGURE 6. Osteoclastogenesis and bone erosion in inflammatory arthritis are enhanced by the absence of SHIP1. Lethally irradiated mice transplanted with either WT (WT > WT) or SHIP1−/− (KO > WT) marrow were injected with PBS or arthrogenic serum (A). Paw thickness was measured with time. B, Six days later, the mice were sacrificed and histological sections of paws were stained for TRAP activity (red reaction product). Arrows indicate inflammatory response. C, Histo- morphometric quantitation of the percentage of bone surface covered by osteoclasts in paws of each group of mice (*, p < 0.005 vs WT > WT PBS; **, p < 0.001 vs WT > WT serum).
SHIP1\(^{-/-}\) than WT cells, a result that probably involves a posttranscriptional mechanism, based on the fact that amount of cyclin D3 mRNA is unchanged in response to M-CSF or following blockade of the signaling pathways downstream of the cytokine. Interestingly, M-CSF differentially modulates the three cyclins. Cyclins D1 and D2 are induced transcriptionally via the MAPK/ERK pathway, whereas regulation of cyclin D3 is largely posttranscriptional. Furthermore, cyclins D1 and D2 are also altered posttranscriptionally via PI3K/Akt pathway.

In other cell types, cyclin-CDK complexes have been reported to phosphorylate critical cellular substrates, including Rb, resulting in cell cycle progression (29, 30). We demonstrate this mechanism to be present in primary BMMs. We find also that M-CSF downregulates p27, a member of the Cip/Kip family of CDK inhibitors, molecules that arrest entry into S phase. Consistent with their hyperproliferative response to the cytokine, SHIP1\(^{-/-}\) BMMs exhibit enhanced suppression of p27 when treated with M-CSF. Similar to D-type cyclins, the effect of M-CSF on p27 in the absence of SHIP is mediated via the PI3K/Akt pathway, perhaps by direct phosphorylation of the cell cycle inhibitor, an event that leads to its proteosomal degradation (40–43). A previous report indicates that down-regulation of p27 is important for proliferation of human monocytes (34), but these studies involved coculture of the myeloid cells with endothelium, which produced an unidentified mitogen; thus, the mechanisms underlying the observations were not defined.

Akt is classically viewed as an antiapoptotic molecule, and indeed the longevity of SHIP1\(^{-/-}\) osteoclasts is prolonged (15). Although we expected the same to be true in SHIP\(^{-/-}\) BMMs, we were surprised to find this is not the case. The distinction between the two cell types may relate to the fact that osteoclasts are more sensitive to PI3K inhibition elicited by LY294002 compared with macrophages. These findings raise the possibility that macrophages require less Akt for survival than do osteoclasts. In this regard, we consistently find that higher levels of PI3K inhibitor are required to induce BMM death than to block their proliferation, indicating lower levels of active Akt are needed to block macrophage apoptosis. Thus, it is reasonable to suggest that the magnitude of M-CSF-induced Akt activation in WT macrophages is sufficient for their optimal survival. Therefore, increased Akt activity in the absence of SHIP1 enhances only proliferation in the precursor cells.

Interestingly, RANKL can promote proliferation of early osteoclast precursors, although the same molecule arrests cell growth at later times. How this cytokine elicits these opposing effects on proliferation is not known. Nonetheless, RANKL does not impact proliferation in the context of SHIP1. In contrast, lack of SHIP1 alters neither RANKL-induced signals nor its biological effects on osteoclast differentiation, as indicated by expression of osteoclast-specific proteins. Therefore, the enhanced osteoclastogenesis in SHIP1-deficient cells most likely reflects increased proliferation in response to M-CSF, rather than accelerated differentiation in response to RANKL.

Finally, we investigated the therapeutic implications of our observations using a murine model of inflammatory arthritis, a condition complicated by periarticular osteolysis. We chose to study this disorder in the context of SHIP1, as the joints of patients with rheumatoid arthritis contain an abundance of M-CSF (35). Despite equivalent degrees of inflammatory arthritis, osteoclastogenesis and periarticular erosion are substantially more profound in mice lacking the phosphatase than in their WT counterparts, establishing a central role for SHIP1 as a suppressor of pathological bone destruction. The importance of this observation relates to the fact that substantial bone loss persists in rheumatoid arthritis in the face of anti-TNF monotherapy, indicating that a combined approach to this problem may provide more promising outcomes (44). Given that SHIP1 targets M-CSF, another osteoclastogenic cytokine abundant in the disease, induction of the phosphatase represents a possible adjunct therapy in preventing inflammatory osteolysis.

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**Disclosures**

The authors have no financial conflict of interest.

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