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Vav Activation and Function as a Rac Guanine Nucleotide Exchange Factor in Macrophage Colony-Stimulating Factor-Induced Macrophage Chemotaxis

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Signal transduction mediated by phosphatidylinositol 3-kinase (PI 3-kinase) is regulated by hydrolysis of its products, a function performed by the 145-kDa SH2 domain-containing inositol phosphatase (SHIP). Here, we show that bone marrow macrophages of SHIP$^{-/-}$ animals have elevated levels of phosphatidylinositol 3,4,5-trisphosphate [PI (3,4,5)P_3] and displayed higher and more prolonged chemotactic responses to macrophage colony-stimulating factor (M-CSF) and elevated levels of F-actin relative to wild-type macrophages. We also found that the small GTPase Rac was constitutively active and its upstream activator Vav was constitutively phosphorylated in SHIP$^{-/-}$ macrophages. Furthermore, we show that Vav in wild-type macrophages is recruited to the membrane in a PI 3-kinase-dependent manner through the Vav pleckstrin homology domain upon M-CSF stimulation. Dominant inhibitory mutants of both Rac and Vav blocked chemotaxis. We conclude that Vav acts as a PI 3-kinase-dependent activator for Rac activation in macrophages stimulated with M-CSF and that SHIP regulates macrophage M-CSF-triggered chemotaxis by hydrolysis of PI (3,4,5)P_3.

Macrophages act as an essential component of the innate immune system by eliminating opsonized pathogens through a variety of surface receptors and through antigen presentation to cells of the adaptive immune system. Accordingly, the migration of macrophages to sites of infection is an important biological event. The migration of macrophages occurs in response to soluble chemokines elicited at the site of inflammation (51). Chemotaxis is mediated by coordinated reorganization of the actin cytoskeleton, which is initiated by a signal transduction process that emerges from the chemokine receptor.

Macrophage colony-stimulating factor (M-CSF) is a cytokine that controls the growth, survival, and differentiation of monocyte-macrophage lineage cells (65). M-CSF also acts as a chemokine to recruit macrophages to the site of inflammation. Cells transfected with c-fms, the receptor for M-CSF, show potent chemotaxis upon M-CSF stimulation (46). Rat models of allergic encephalomyelitis show increased M-CSF and c-fms expression and an increased presence of macrophages immediately prior to disease onset, and these levels decline upon resolution of the disease (27). Macrophages stimulated with M-CSF show immediate cell polarization, actin reorganization, and migration to the M-CSF source (63). Macrophage accumulation at sites of renal injury is abrogated for animals treated with antibodies to M-CSF or to the c-fms receptor (37). Together, these studies show that M-CSF is a biologically relevant chemokine for macrophages in the inflammatory response.

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Chemotactic migration requires phosphatidylinositol 3-kinases (PI 3-kinases) (8), which phosphorylate the 3 position of the inositol ring of phosphoinositides. The class I PI 3-kinase enzymes are heterodimers, composed of a p110 catalytic subunit (α, β, and γ) and a p85α, p85β, or p55γ SH2 domain-containing regulatory subunit. The lipid products of PI 3-kinase include phosphatidylinositol 3,4-bisphosphate [PI (3,4)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PI (3,4,5)P_3]. These 3-phosphoinositide lipids recruit enzymes containing a pleckstrin homology (PH) domain to further the response (54, 62). M-CSF chemotaxis is abolished in macrophages that have been microinjected with neutralizing antibodies to p110β or p110γ but not p110α (58). Furthermore, neutrophils (20, 39) or macrophages (31) of mice lacking p110γ do not migrate in response to chemokines. Thus, PI 3-kinase, particularly the gamma isoform, is required for M-CSF-mediated macrophage migration (50).

The PI 3-kinase requirement reflects, at least in part, the activation of the small GTPases Rac and cdc42. Studies using chemical inhibitors of PI 3-kinase have established that GTP nucleotide binding to Rac (21, 48) and cdc42 (34) requires the activation of PI 3-kinase (reviewed in reference 50). Microinjection of the p85 adapter protein of PI 3-kinase blocks receptor recruitment of the p110 catalytic subunit and prevents growth factor-induced Rac activation (23). Furthermore, membrane targeting of the catalytic p110 subunit triggered Rac-dependent actin reorganization in a model of neutrophil responses to chemokines (3).

Rac and cdc42 are involved in chemotactic responses of myeloid cells. Transfection of macrophage cell lines with dominant-interfering mutants of cdc42 (1) and Rac (1, 16) blocks chemotactic responses to many agents. M-CSF chemotaxis in particular requires Rac but not cdc42, as shown by sensitivities

to dominant-interfering mutants of these proteins (52). Neutrophils (38), macrophages (47), and hematopoietic stem cells (66) of animals deficient in Rac2, the hematopoietic-cell-restricted Rac isoform (55), show reduced chemotactic responses. Rac and PI 3-kinase are required for chemotactic responses of cells upon stimulation of tyrosine kinase-linked growth factors (21), including M-CSF (1), or of G-protein-coupled receptors (4, 13). Lastly, patients with mutations in Rac (17) exhibit recurring bacterial infections and impaired in vitro chemotactic responses (2, 35).

While it is clear that Rac is important in chemotactic responses to M-CSF and that PI 3-kinase regulates Rac, the means by which Rac is activated is not known. GTP binding of small GTPases is catalyzed by a guanine nucleotide exchange factor (GEF) which mediates the release of bound GDP. The Rho family, Rac in particular, has numerous GEFs identified through biochemical, enzymatic, and genetic approaches. An important issue that remains unsolved is how receptors stimulate the GEFs to initiate the activation of the small GTPases.

The Vav family of proteins act as GEFs for Rac and cdc42 (57). The Vav1 isoform is restricted to hematopoietic cells (33) and is tyrosine phosphorylated in response to M-CSF stimulation (67). In vitro, the GEF activity of Vav towards Rac is regulated by both tyrosine phosphorylation and the PI 3-kinase product PI (3,4,5)P_3 (18), and Vav contains a PH domain with high affinity towards PI (3,4,5)P_3 (11). However, the requirement for PI 3-kinase in the activation of Vav is controversial. Experiments using the avian DT40 B-lymphocyte model suggest that Vav acts upstream of PI 3-kinase rather than downstream (29), and similar findings have been reported for T cells (49). However, a recent report indicated that this is not the case for mammalian B cells stimulated through their antigen receptors (59). Vav is recruited to the antigen receptor in T cells (60, 61) and to c-fms in M-CSF-stimulated macrophages (67), but the means of its recruitment in any cell type is not understood.

Negative regulation of PI 3-kinase signal transduction occurs by hydrolysis of PI (3,4,5)P_3. In hematopoietic cells, the 145-kDa SH2 domain-containing inositol phosphates (SHIP) hydrolyses the 5-phosphate of the inositol ring to generate PI (3,4)P_2 (45). SHIP negatively regulates many cytokine (28, 40) and antigen receptors (44, 56), which are linked to protein tyrosine kinases. Likewise, phosphatase and tensin homolog deleted from chromosome 10 (PTEN) hydrolyzes PI (3,4,5)P_3 to PI (4,5)P_2. Lymphocytes from PTEN-deficient mice (14) or PTEN$^{-/-}$ *Dictyostelium* cells (15, 25) show enhanced migration in G-protein-coupled signaling pathways of chemotaxis. Thus, it is clear that chemotaxis in response to G-protein-coupled receptors is PI 3-kinase dependent. However, PTEN is regulated solely by its own expression levels, while SHIP is regulated by SH2 domain recruitment to phosphotyrosines on receptors (reviewed in reference 7).

Since M-CSF is a receptor tyrosine kinase, we explored the role of SHIP in regulating M-CSF-triggered chemotaxis of primary macrophages. We found that bone marrow-derived macrophages (BMMs) of SHIP$^{-/-}$ animals exhibited higher levels of spontaneous migration and higher levels of M-CSF-induced migration than their wild-type counterparts. In macrophages from wild-type mice, M-CSF-induced chemotaxis required both Vav and Rac, as shown by transduction with interfering or activating mutations. However, macrophages of SHIP$^{-/-}$ mice showed constitutively phosphorylated Vav and constitutively active, GTP-bound Rac. Both these biochemical features and the spontaneous and M-CSF-induced chemotaxis were abrogated by inhibitors of PI 3-kinase. Confocal microscopy showed Vav recruitment in a PI 3-kinase-dependent manner, and recruitment was mediated by the PH domain of Vav. These findings suggest a model in which Vav is recruited to membrane-bound Rac by PI (3,4,5)P_3 formed through the activity of PI 3-kinase. Vav recruitment leads to Vav tyrosine phosphorylation and activation. Vav activation promotes GTP binding on Rac to initiate chemotaxis.

MATERIALS AND METHODS

Animals. The SHIP$^{-/-}$ mice (C57BL/6 background) were provided by G. Krystal. Heterozygous mice were bred, and animals were genotyped at day 10 after birth. Littermate wild-type C57BL/6 mice were used as controls.

Reagents. Rabbit polyclonal antibody to Vav was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Rac activation kit was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant mouse M-CSF was purchased from R&D systems (Minneapolis, MN). Migration assay plates were purchased from Costar (Corning, NY).

Bone marrow-derived macrophages. Bone marrow-derived macrophages were prepared as previously described (44) from wild-type and SHIP$^{-/-}$ mice. Briefly, the bone marrow cells were isolated by flushing femurs and tibias. The isolated cells were cultured overnight in 10-cm dishes with complete medium containing 20% L-cell-conditioned medium at 37°C in 5% CO_2. Nonadherent cells were transferred to new dishes and cultured for an additional 5 days at 37°C in 5% CO_2 before experiments. After the additional 5 days, the cells were uniformly positive for mature macrophage markers F4/80, Mac-1, and Fc receptors I and II/III by flow cytometry. For experiments, bone marrow macrophage cells were depleted of both serum and cytokines by overnight incubation. The cells were collected from the plates and stimulated in suspension by recombinant M-CSF for immunoprecipitations and pull-down experiments.

Immunoprecipitation. All immunoprecipitations were done as previously described (30). Briefly, cells were lysed in TN-1 buffer (50 mM Tris-HCl, pH 8.0, 125 mM NaCl, 10 mM EDTA, 1% Triton, 10 mM NaF, 3 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 μg/ml aprotinin, 10 μg/ml leupeptin) and centrifuged at 16,000 × *g* for 10 min at 4°C to remove the insoluble material. The resulting supernatant was subjected to immunoprecipitation using the indicated antibodies and protein A agarose (Invitrogen, Carlsbad, California). The agarose beads were washed with TN-1 buffer, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoretically transferred to nitrocellulose membranes, probed with appropriate antibodies, and visualized by an enhanced-chemiluminescence system (Pierce, Rockford, IL). The bands were quantitated by measuring individual chemiluminescence by using a Lumi-Imager F1 workstation (Roche Molecular Biochemicals, Indianapolis, IN). The appropriate dose of the PI 3-kinase inhibitor LY294002 was determined by observing the phosphorylation of AKT by Western blotting (not shown).

Rac assay. The Rac assay was performed using a Rac activation kit, following the protocol described by the manufacturer (Upstate Biotechnology, Lake Placid, NY). The protocol is based on a Rac-GTP pull-down method using GST–p21-activated kinase (PAK), as described previously (4). Briefly, cytokine-starved cells were stimulated with M-CSF, lysed, and treated with GST-PAK prebound to glutathione-agarose beads. After being washed, the beads were incubated with Laemmli SDS-PAGE sample buffer, and the eluted proteins were subjected to SDS-PAGE. After the transfer of proteins to a nitrocellulose membrane, Western blotting was performed using pan monoclonal antibody to Rac1 and Rac2. The amount of GTP-loaded Rac was quantitated by measuring the mass of GTP-Rac in comparison to the mass of total Rac. The value was then normalized to the GTP-Rac/total Rac ratio of unstimulated, wild-type macrophages.

Chemotaxis assay. The chemotaxis assay was performed using 5-μm-size transwell migration plates. Cytokine-starved cells were harvested and resuspended at a concentration of 1.25×10^6 cells/ml. Cells (250×10^3) were incubated with inhibitors as required and as described in the figure legends. Eight hundred microliters of medium containing the chemoattractant was added to the lower chamber of each transwell plate. The insert was placed in the well

containing the chemoattractant, and the cells were added to the insert. The migration plates were placed in a 37°C incubator for 2 h. The transwell insert was then washed to remove unbound cells, and cells were fixed with methanol and stained with Giemsa. The membrane was then cut, and cells that did not migrate, from the top, were removed using a cotton swab. The membrane was then mounted onto a coverslip, and the migrated cells from three separate fields were counted. The individual experiments were done in triplicate, and each experiment was repeated identically for a minimum of three trials.

Retroviral transfection. For retroviral infections, plasmids containing DH6A Vav (32), N-17 Rac, onco-Vav (12), and full-length SHIP (43) were cloned into the retroviral expression vector MIGR1, which contains an internal ribosomal entry site and green fluorescent protein (GFP) expression cassette and a puromycin resistance gene. Full-length Vav, DH6A-Vav, a Vav PH domain deletion mutant, and the isolated PH domain of Vav were cloned into the retroviral vector pLEGFP-C1. Ecopack-2 cells were transfected by using Fugene-6 (Roche Molecular Biochemicals, Indianapolis, IN) as described by the manufacturer. Forty-eight hours after transfection, supernatants containing retrovirus were harvested. For retroviral infection, BMMs were incubated with virus-containing supernatants supplemented with 10 μg/ml Polybrene (Sigma Chemical Company, St. Louis, MO) and subjected to spin infection by centrifuging at 2,000 × *g* for 2 h at 32°C. After 48 h, bone marrow cells expressing high levels of GFP were enriched by fluorescence-activated cell sorting using a MoFlo cell sorter (Cytomation Inc., Fort Collins, CO). Sorted cells were depleted of cytokine for 12 h prior to chemotaxis experiments.

Confocal microscopy. Transfected cells were seated on sterile 22-mm glass coverslips overnight at 37°C. The cells were then stimulated with M-CSF for 30 min. The cells on the coverslips were washed twice with Hanks balanced salt solution, fixed with 2% formaldehyde, mounted using antifade solution, and viewed under a Zeiss LSM 510 laser scanning microscope.

Intracellular staining and flow cytometry. A total of 2×10^6 cells per treatment were pretreated with the inhibitors or dimethyl sulfoxide (DMSO) for 15 min at 37°C. The cells were then stimulated with M-CSF and fixed with 1 ml of 3.7% paraformaldehyde at 4°C for 30 min. The cells were then permeabilized by the use of 0.05% saponin-containing buffer. For F-actin staining, permeabilized cells were stained with fluorescein-conjugated phalloidin (Molecular Probes, Eugene, OR). For PI (3,4,5)P_3 staining, the macrophages were incubated with a neutralizing FcγR antibody (2.4G2) overnight at 4°C. The cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-PI (3,4,5)P_3 antibody (Echelon Biosciences Inc., Salt Lake City, UT) for 1 h at 4°C. The cells were then washed thrice and analyzed by flow cytometry. The mean fluorescence intensity was calculated for every sample and was plotted as an average of three independent experiments.

RESULTS

Macrophages lacking SHIP display elevated chemotactic responses. To test the involvement of SHIP in a PI 3-kinase-dependent chemotactic process, we measured the responses to M-CSF of primary wild-type and SHIP-deficient macrophages. We found that macrophages from wild-type mice exhibited a dose-dependent chemotactic response to M-CSF (Fig. 1A), and the response was maximal at 1 μg/ml of M-CSF. The macrophages of SHIP-deficient mice showed a much higher chemotactic response (Fig. 1A). Interestingly, the SHIP-deficient macrophages also exhibited migration in the absence of any chemotactic gradient (0 ng M-CSF). We called the unstimulated macrophage movement "spontaneous migration." Thus, macrophages of SHIP$^{-/-}$ animals showed spontaneous migration and elevated chemotactic responses to M-CSF.

We also tested the responses of wild-type and SHIP$^{-/-}$ macrophages to other chemokines that use G-protein-coupled receptors. We found that chemotactic responses of macrophages from SHIP-deficient mice were elevated in response to both formylated peptide methionine-leucine-proline (Fig. 1B) and stromal cell-derived factor-1 (Fig. 1C). Consistent with the data shown above, the macrophages of SHIP$^{-/-}$ mice showed elevated spontaneous migration in the absence of either chemokine. Thus, like the responses to stimulation of the protein tyrosine kinase receptor c-fms, the responses of macrophages of SHIP$^{-/-}$ mice to G-protein-coupled receptors were elevated.

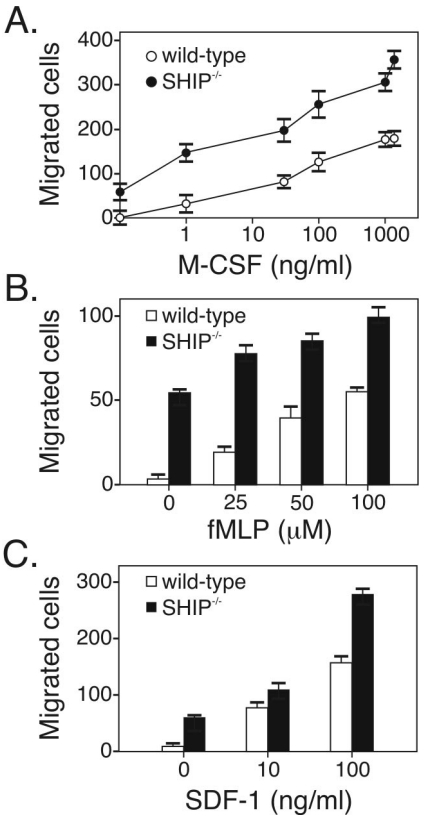

FIG. 1. SHIP$^{-/-}$ macrophages exhibit spontaneous and enhanced migration. (A) Cytokine-starved BMMs were subjected to a chemotaxis assay in response to M-CSF as described in Materials and Methods. The cells that have migrated across the membrane were counted manually from three separate fields. The data shown are the averages (± standard deviations [error bars]) from five independent experiments. (B and C) The assays mentioned above were performed using stromal cell-derived factor-1 (SDF-1) and formylated peptide methionine-leucine-proline (fMLP) as chemoattractants. The results represent averages (plus standard deviations [error bars]) from two independent experiments.

Since SHIP is a negative regulator of PI 3-kinase, our findings imply that M-CSF-induced macrophage chemotaxis is dependent on PI 3-kinase. To test this prediction, we incubated the macrophages in medium containing the PI 3-kinase inhibitor LY294002. The inhibitor-treated cells were applied to the chemotactic measurements as described above with 100 ng/ml M-CSF. We found that the elevated spontaneous migration exhibited by the SHIP$^{-/-}$ macrophages was reduced with the PI 3-kinase inhibitor (Fig. 2A). Likewise, the M-CSF-stimulated migrations (Fig. 2A) of macrophages of both wild-type and SHIP$^{-/-}$ animals were blocked. These data indicate that M-CSF-induced chemotaxis requires PI 3-kinase and that the elevated spontaneous migration of SHIP$^{-/-}$ macrophages is due to a PI 3-kinase-dependent event.

SHIP-deficient macrophages have elevated PI (3,4,5)P_3 and are constitutively active in PI (3,4,5)P_3-dependent signaling events. The data also predicted that SHIP-deficient macrophages would display relatively higher levels of PI (3,4,5)P_3

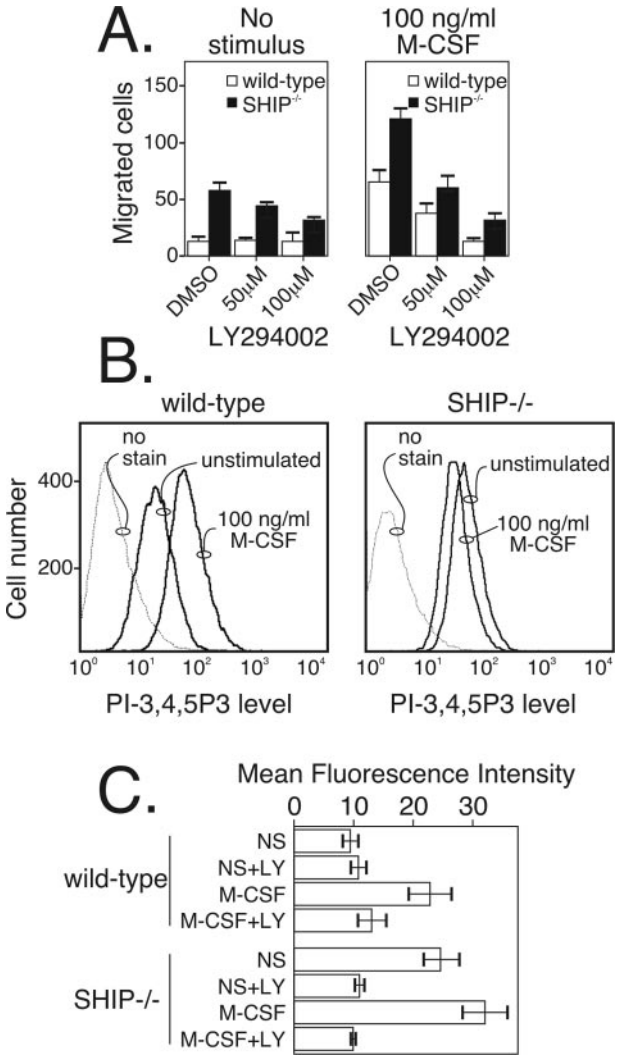

FIG. 2. PI 3-kinase regulates M-CSF-induced chemotaxis. (A) Cytokine-starved BMMs treated with LY294002 or DMSO were subjected to chemotaxis in response to M-CSF. The results are averages (plus standard deviations [error bars]) from three independent experiments. (B) BMMs from wild-type and SHIP$^{-/-}$ were stimulated with 100 ng of M-CSF for 5 min and fixed. Intracellular PI (3,4,5)P_3 staining was performed as described in the Materials and Methods. The results shown are representative of three independent experiments. (C) The average mean fluorescence intensity (± standard deviation [error bar]) for PI (3,4,5)P_3 staining for each treatment from three independent experiments was calculated and is represented here. LY, LY294002; NS, no stimulus.

than wild-type macrophages. To test this hypothesis, we stained macrophages with FITC-labeled antibodies to PI (3,4,5)P_3 before and after stimulation with M-CSF for 5 min and analyzed the relative levels by flow cytometry. We found (Fig. 2B) that resting levels of PI (3,4,5)P_3 in SHIP-deficient macrophages were higher than the levels in unstimulated wild-type macrophages. The average mean fluorescence intensities of three independent measurements are shown in Fig. 2C. We found that M-CSF stimulation elevated PI (3,4,5)P_3 in wild-type macrophages to a level comparable to that in the unstimulated SHIP$^{-/-}$ macrophages. Both resting and M-CSF-stimulated PI (3,4,5)P_3s were reduced by preincubation with the PI 3-kinase inhibitor. Thus, the macrophages of SHIP-deficient mice display elevated PI (3,4,5)P_3 levels, which is consistent with our findings that the cells exhibit constitutive activation of signaling events dependent on PI (3,4,5)P_3.

Chemotaxis requires cytoskeletal reorganization and formation of filamentous actin (F-actin), which is regulated in part by the small GTPase, Rac. We therefore measured Rac activation in macrophages of wild-type or SHIP$^{-/-}$ animals following their stimulation by M-CSF. Rac activation was measured using a PAK pull-down assay, as described in Materials and Methods. Some macrophages were pretreated with the PI 3-kinase inhibitor LY294002 or solvent before a 5-min stimulation with M-CSF to test the requirement for PI 3-kinase. The results (Fig. 3A) showed that Rac is inducibly activated by approximately 20-fold upon M-CSF stimulation in wild-type macrophages. The activation of Rac was completely blocked by the

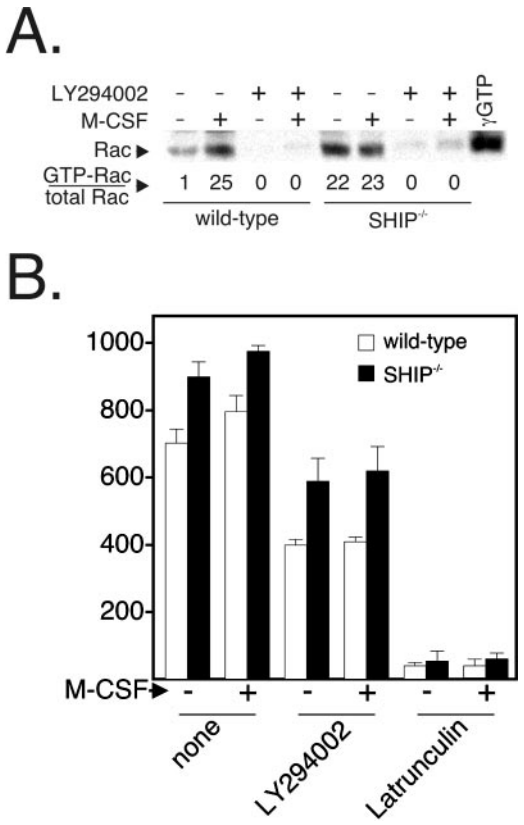

FIG. 3. Rac GTP loading is inducible in wild-type BMMs and constitutive in SHIP$^{-/-}$ BMM. (A) Cytokine-starved BMMs from wild-type and SHIP$^{-/-}$ mice were harvested and treated with LY294002 or DMSO. The cells were unstimulated (−) or stimulated (+) with M-CSF as indicated for 5 min. The Rac assay was performed by a pull-down method with GST-PAK as described in Materials and Methods. This is a blot representative of three independent experiments. The numbers below each lane represent ratios of GTP-Rac (obtained in the PAK pull-down assay) to total Rac (obtained by Rac immunoblotting a portion of the lysate) and normalized to the wild-type unstimulated sample. (B) Cytokine-starved BMMs were treated with inhibitors or DMSO, followed by stimulation with M-CSF. Intracellular F-actin staining was performed as described in Materials and Methods. The data represent the average mean fluorescence intensities per treatment from three independent experiments.

PI 3-kinase inhibitor. In contrast, Rac was constitutively active in unstimulated macrophages of SHIP$^{-/-}$ mice, and the constitutive Rac activation was blocked by inhibition of PI 3-kinase. These data show that Rac activation by M-CSF in normal macrophages requires PI 3-kinase activation. Rac is constitutively active in macrophages of SHIP$^{-/-}$ mice, and the constitutive activity requires PI 3-kinase.

We also measured the level of F-actin in unstimulated and M-CSF-stimulated macrophages of both mouse types by staining the cells with FITC-phalloidin and analyzing the stained cells by flow cytometry. For these experiments, bone marrow-derived macrophages were incubated with LY294002 or medium before a 5-min stimulation with M-CSF stimulation. As a control for the experiment, we also incubated macrophages with latrunculin, a compound that promotes F-actin disassembly (9). Unstimulated SHIP-deficient macrophages displayed about 20%-higher levels of F-actin than their wild-type counterparts (Fig. 3B), and this difference was significant ($P < 0.03$). M-CSF stimulation did not significantly increase F-actin levels in wild-type macrophages, but F-actin levels did rise in M-CSF-stimulated macrophages of SHIP$^{-/-}$ mice ($P < 0.05$). Inhibiting PI 3-kinase reduced both the resting and M-CSF-stimulated increases in F-actin of both cell types, but the levels of F-actin in SHIP$^{-/-}$ macrophages were consistently and significantly ($P < 0.03$) higher than those in the wild-type macrophages.

Vav is a hematopoietic-cell-restricted GEF for Rac that is regulated by both tyrosine phosphorylation and the PI 3-kinase product PI (3,4,5)P_3. The observation that Rac was constitutively GTP loaded and F-actin levels were higher in cells lacking SHIP raised the possibility that Vav might also be constitutively active. To test this possibility, we measured Vav phosphorylation in unstimulated and M-CSF-stimulated macrophages of wild-type and SHIP-deficient animals. The macrophages were pretreated with the PI 3-kinase inhibitor LY294002 or with a Src family kinase inhibitor, PP2 (19). All cells were either unstimulated or stimulated with 100 ng/ml M-CSF for 5 min. Vav was obtained by immunoprecipitation and probed with antiphosphotyrosine antibodies. We found (Fig. 4) that Vav was inducibly phosphorylated in M-CSF-stimulated macrophages of wild-type mice, and the phosphorylation was blocked by inhibition of PI 3-kinase or by inhibition with Src family kinases. These findings suggest that Vav is recruited through its PH domain, by the generation of PI (3,4,5)P_3, to the membrane, where it is phosphorylated by a member of the Src family of kinases. In contrast to macrophages of wild-type mice, SHIP-deficient macrophages showed constitutive Vav phosphorylation. The constitutive Vav phosphorylation in SHIP-deficient macrophages was reduced to background levels upon the inhibition of PI 3-kinase or Src family kinases. Thus, like Rac activation, the phosphorylation and activation of Vav are dependent on PI 3-kinase following stimulation by M-CSF. The fact that the constitutive Vav phosphorylation in SHIP-deficient macrophages was reduced by the inhibition of PI 3-kinase suggests that the constitutive phosphorylation is due to elevated PI (3,4,5)P_3 levels in the macrophages derived from SHIP-deficient mice (Fig. 2). The constitutive Vav phosphorylation may be causally related to the constitutive Rac activation and elevated chemotactic responses seen in these cells.

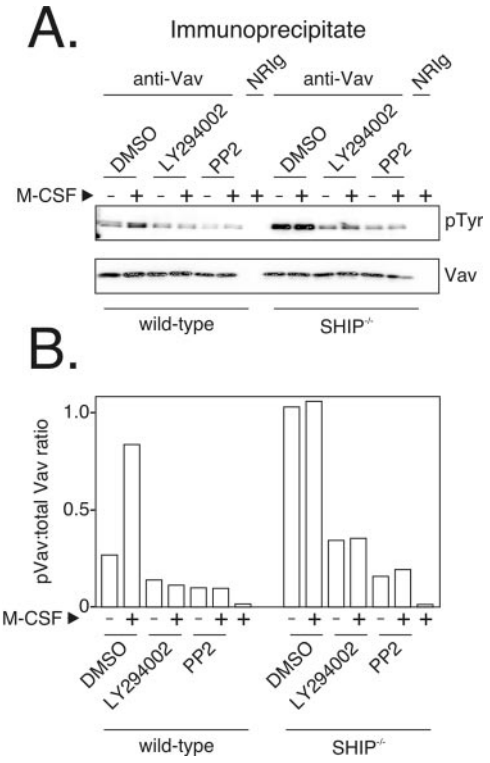

FIG. 4. Vav phosphorylation in response to M-CSF. (A) Cytokine-starved BMMs were treated with LY294002, PP2, or DMSO and stimulated with M-CSF for 5 min. Vav immunoprecipitation and phosphotyrosine (pTyr) immunoblotting were performed as described above. This is a blot representative of five independent experiments. NRIg, normal rabbit immunoglobulin. (B) The ratios of phosphorylated Vav (pVav) to total Vav were quantitated.

M-CSF-triggered chemotaxis requires Vav and Rac. The findings described above suggest a model in which M-CSF triggers PI 3-kinase to produce PI (3,4,5)P_3. The PH domain of Vav is recruited to the membrane by binding the nascent PI (3,4,5)P_3, and Vav is phosphorylated by a membrane-bound Src family kinase. Vav then stimulates the GTP binding of Rac to promote chemotaxis.

The model predicts that M-CSF-induced macrophage chemotaxis would be sensitive to the Src family kinase inhibitor PP2 (19) and the PI 3-kinase inhibitor LY294002 and require Vav and Rac activation. We tested the requirement for Src family kinases by measuring M-CSF chemotactic responses in the presence or absence of PP2. Mature macrophages from wild-type or SHIP$^{-/-}$ mice were preincubated with the amounts of inhibitors that blocked Vav tyrosine phosphorylation (Fig. 4B). We included the actin polymerization inhibitor latrunculin B (9) and the PI 3-kinase inhibitor LY294002 as negative controls. Both latrunculin B and LY294002 completely blocked M-CSF-stimulated migration of macrophages from both mouse strains and blocked spontaneous migration by the SHIP$^{-/-}$ macrophages (Fig. 5A). Likewise, M-CSF-triggered chemotaxis in both wild-type and SHIP$^{-/-}$ macrophages was blocked by PP2. However, while the PI 3-kinase inhibitor LY294002 reduced the spontaneous migration of the SHIP$^{-/-}$ macrophages, the Src family kinase inhibitor PP2 did not. These data indicate that a Src family kinase is

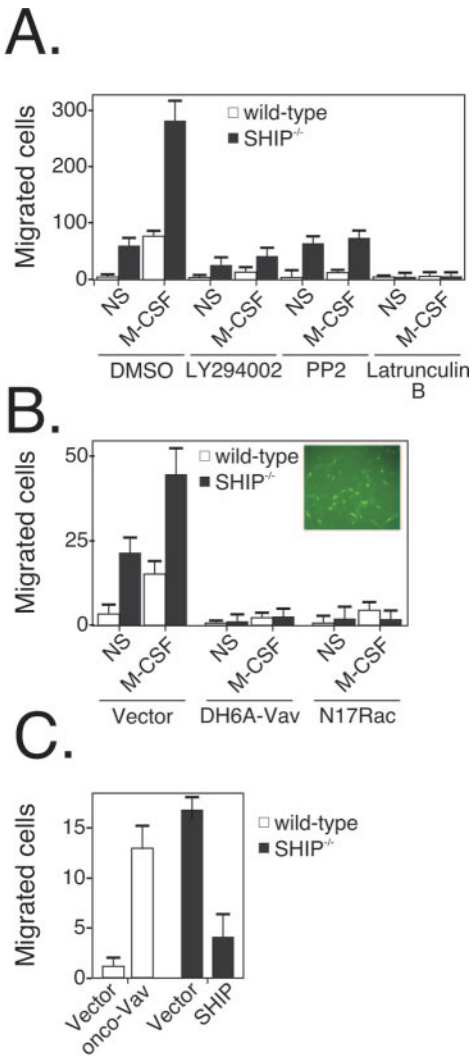

FIG. 5. M-CSF-mediated chemotaxis is regulated by Src, PI 3-kinase, Vav, and Rac. (A) Cytokine-starved cells were harvested and treated with PP2, LY294002, latrunculin, or DMSO as control. These cells were then subjected to a chemotaxis assay as described previously. The results represent an average from three independent experiments. NS, no stimulus. (B) Interfering mutants of Vav or Rac were introduced into wild-type and SHIP$^{-/-}$ BMMs retrovirally by using a GFP vector. GFP-expressing cells were sorted and applied to the chemotaxis assay. The results are averages from three independent experiments. The inset shows GFP-positive cells after sorting. NS, no stimulus. (C) Oncogenic Vav was introduced into wild-type BMMs, and SHIP was reconstituted in SHIP$^{-/-}$ BMMs by retroviral transfection. GFP-expressing cells were sorted and introduced into a transwell in the absence of any chemoattractant. After 2 h at 37°C, the cells which migrated to the lower part of the chamber were fixed and counted. The results shown are averages from four independent experiments.

an essential component of M-CSF-stimulated migration and are consistent with the model mentioned above. The observation that the inhibition of Src kinases did not affect the spontaneous migration of SHIP-deficient macrophages suggests the defect in these cells is distal to the requirement for Src family kinases.

We explored the requirement for Vav and Rac by retroviral transduction of the primary macrophages cultured in M-CSF with cDNAs encoding mutant versions of these proteins. Mature macrophages were infected by retroviruses harboring a bicistronic message encoding GFP and the gene of interest. Two days after retroviral infection, the cells were sorted for GFP expression and the sorted GFP-positive cells were applied to the chemotaxis assay using recombinant M-CSF. The inset of Fig. 5B shows the GFP expression in the GFP-only transduced population and is representative of all transduced cells. The vector-transduced cells (GFP-only) responded to M-CSF (Fig. 5B) like the unmanipulated primary macrophages. The total numbers were less than those from earlier experiments, since fewer cells were applied to the chambers, due to the sorting for GFP-expressing cells. The vector-transduced macrophages from SHIP$^{-/-}$ animals showed elevated spontaneous migration (Fig. 5B), as did their unmanipulated counterparts. Expression of a dominant-interfering mutant of Rac (N17Rac) or of Vav (DH6A, lacking GEF activity) blocked M-CSF-stimulated chemotaxis in macrophages of both mouse strains. Furthermore, both mutants completely blocked the spontaneous migration of the SHIP$^{-/-}$ macrophages. These findings are consistent with the model presented above in which Vav is recruited by its PH domain to activate Rac and promote chemotaxis.

In a separate set of studies, we transformed macrophages from SHIP$^{-/-}$ mice with cDNA encoding SHIP to see if restoring SHIP protein levels would reduce the spontaneous migration in the SHIP-deficient macrophages. We also tested a transforming version of Vav (onco-Vav), a mutant lacking the N-terminal ~40 amino acids (33). As described above, the transduced cells were sorted on the basis of GFP expression and applied to the chemotaxis chambers. Here, we tested only spontaneous unstimulated migration that occurs in the absence of M-CSF. We found (Fig. 5C) that onco-Vav increased by four- to fivefold the spontaneous migration of macrophages derived from wild-type mice, to a level comparable to that of the SHIP-deficient macrophages. Likewise, transforming SHIP-deficient macrophages with SHIP cDNA decreased the spontaneous migration to a level similar to that of wild-type macrophages. These findings are consistent with our model described above, in which elevated PI (3,4,5)P_3 levels in SHIP-deficient cells leads to Vav recruitment and activation to promote the elevated spontaneous migration.

Vav is recruited to the plasma membrane through its PH domain, and recruitment requires PI 3-kinase. To directly test the means of Vav recruitment, we transduced GFP fusion protein of Vav or the Vav PH domain into primary macrophages of wild-type animals. The GFP-expressing cells were incubated with recombinant M-CSF for 20 min, fixed, and examined by confocal microscopy. We found (Fig. 6A) that the GFP-Vav fusion protein was recruited to the plasma membrane in M-CSF-stimulated cells but remained diffusely cytoplasmic in unstimulated cells (Fig. 6A). Likewise, the isolated PH domain of Vav fused to GFP was recruited in M-CSF-stimulated cells (Fig. 6B). Thus, the findings demonstrate that recruitment of Vav to the plasma membrane is mediated by the PH domain binding to PI 3-kinase lipid products. To further explore Vav recruitment, we conducted an identical experiment in which primary macrophages were transduced with the Vav PH domain fused to GFP and stimulated with M-CSF for 20 min in the presence or absence of LY294002. The images of unstimulated macrophages are not shown but resembled those

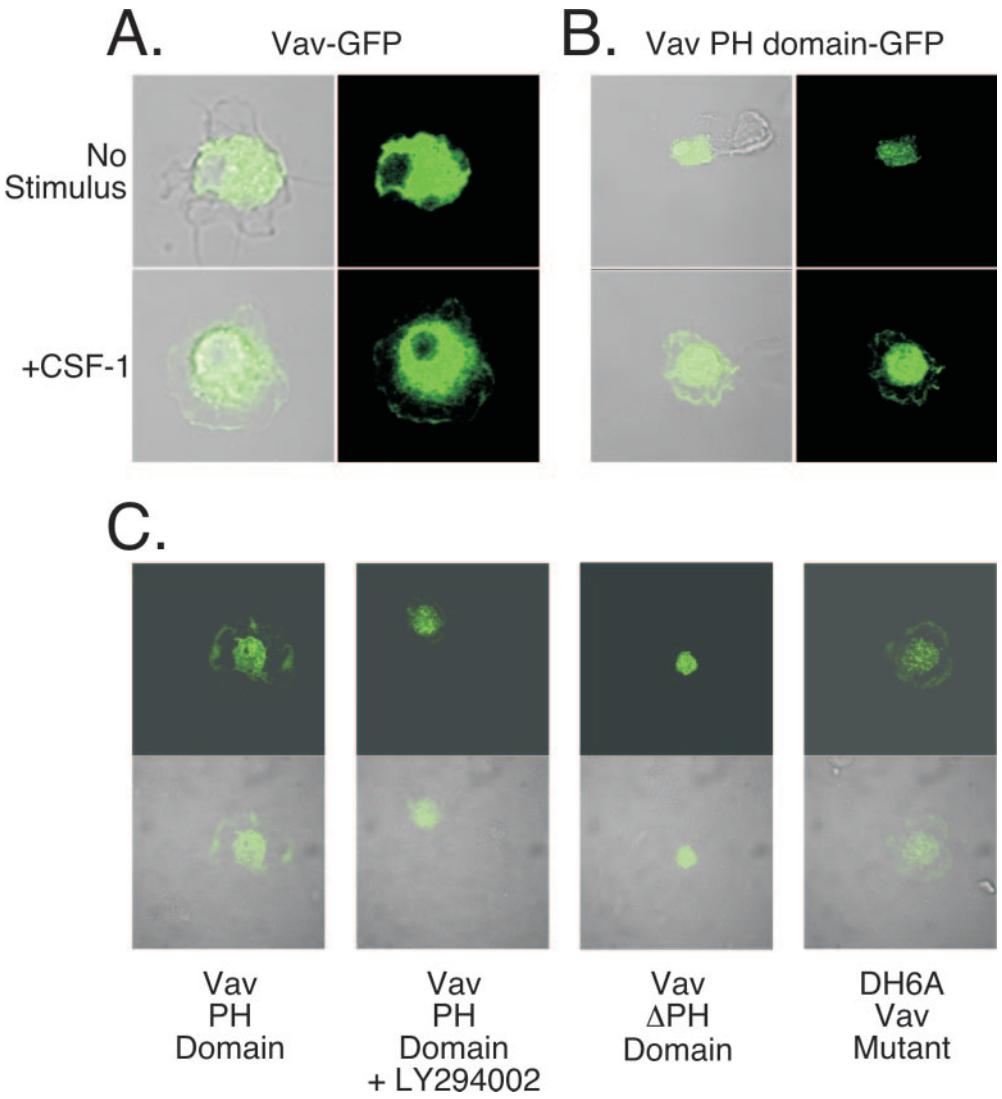

FIG. 6. Vav is recruited through its PH domain by PI 3-kinase activation. (A and B) GFP fusions of full-length Vav and PH domain of Vav were introduced retrovirally into wild-type BMMs. The cells were then seated on glass coverslips and stimulated with M-CSF for 20 min and fixed. Confocal microscopy was performed as described in Materials and Methods. The images are representative of 100 GFP-positive cells counted. Seventy percent of the cells exhibited this representative morphology of Vav recruitment to the membrane. (C) GFP fusions of PH domain of Vav (Vav PH domain), a deletion mutant of Vav lacking the PH domain (Vav ΔPH), and the Vav mutant which lacked GEF activity (DH6A Vav) were transfected into wild-type BMMs retrovirally. Cells were treated with LY294002 or DMSO and stimulated as described above. Confocal microscopy was performed, and 100 GFP-positive cells were counted. All the images are from M-CSF stimulated cells. The images are representative of the majority of the GFP-positive cells.

shown in panels A and B of Fig. 6. We found (Fig. 6C) that the PH domain fusion protein was recruited to the plasma membrane as described above and that the recruitment was blocked by pretreating the cells with the PI 3-kinase inhibitor LY294002. We also transformed macrophages with a Vav construct in which the PH domain was deleted (Vav ΔPH). This fusion protein failed to be membrane recruited upon M-CSF stimulation. However, the DH6A mutant that is catalytically inactive towards Rac (Fig. 5) but contains an intact PH domain was able to be recruited upon M-CSF stimulation. These findings establish that Vav is recruited to the plasma membrane to PI (3,4,5)P_3 via its PH domain and correlates with GTP loading of Rac.

DISCUSSION

Our data show that M-CSF triggers Vav recruitment through the production of PI (3,4,5)P_3 by PI 3-kinase. Vav is recruited via its PH domain, as shown by confocal microscopy and its sensitivity to inhibitors of PI 3-kinase. Vav is then phosphorylated by a membrane-bound Src family kinase (or a kinase distal to Src family kinases), since phosphorylation was sensitive to the chemical inhibitor PP2. Following its recruitment, Vav stimulates the GTP binding of Rac. We found that both Vav and Rac were essential to M-CSF-triggered chemotaxis. The events involving Vav and Rac are constitutively active in macrophages of SHIP$^{-/-}$

mice, likely due to their elevated levels of PI (3,4,5)P_3 (5, 6, 53).

Earlier studies using a transfected fibroblast model of chemotaxis to formylated peptide showed a requirement for Rac, PI 3-kinase, and Vav acting as a Rac GEF (42). Transfected Vav deletion mutants lacking the PH domain failed to support chemotaxis in this model. Our findings in which primary macrophages responding to a receptor tyrosine kinase were used are in agreement with this data but extend the data in several important ways. First, we identified a functional and mechanistic relationship between PI 3-kinase and Vav by demonstrating direct Vav recruitment to products of PI 3-kinase. Indeed, this is the first chemotaxis model to establish how any Rho family GEF is activated and functions in a native cellular environment. Second, we identified a set of constitutively active events in the macrophages of SHIP-deficient mice that include Vav and Rac and directly support the model described above.

The relationship between PI 3-kinase, Vav, and Rac is controversial. In a chicken B-cell model of DT40, the activation of PI 3-kinase was shown to be dependent on Vav and Rac (29). Similar findings have been reported for the T-cell antigen receptor, for which activation of PI 3-kinase-dependent events are inhibited in Vav-deficient T cells (49). In the mammalian M-CSF system described here, the activation of Vav and Rac was shown to be dependent on PI 3-kinase. The differences are unclear but likely due to the different receptors (c-fms for M-CSF versus lymphocyte antigen receptor) and/or differences between models (mammalian versus avian).

Animals deficient in SHIP (22, 26) show a great increase in peripheral myeloid cells. Beginning when the $SHIP^{-/-}$ mice are 2 weeks of age, their lung tissues show massive infiltration of macrophages and neutrophils without evidence of pathogen involvement. The animals have short life spans, possibly due to severe lung pathology accompanying the increase in peripheral myeloid cells. The increased myeloid cellularity was associated with enhanced Akt activity and reduced apoptosis in response to cytokine withdrawal (41). Our studies on macrophage chemotactic functions indicated that SHIP negatively regulates macrophage migration. Thus, our findings in this study may at least in part account for the lung pathology seen in these mice.

Our experiments with chemical inhibitors indicated that the spontaneous migration of $SHIP^{-/-}$ macrophages showed greater sensitivity to inhibitors of PI 3-kinase than to inhibitors of Src family kinases. Both recruitment via PI 3-kinase products and phosphorylation via a Src family kinase are necessary for Vav function. However, the differential sensitivity with regard to spontaneous migration suggests that the membrane recruitment of Vav by PI (3,4,5)P_3 is rate limiting towards its distal function. It may be that membrane- or receptor-bound kinases other than those of the Src family are able to phosphorylate Vav after PH domain-mediated recruitment. Accordingly, the inhibition of recruitment completely blocks Vav function in migration, while the inhibition of a subset of tyrosine kinases does not. An alternative possibility is that other proteins distal to Src are involved in M-CSF-stimulated migration but are not involved in the spontaneous migration of the SHIP-deficient macrophages. Proteins regulated by Src have been described for M-CSF-stimulated macrophages, including the activation of PI 3-kinase (36).

A number of earlier studies established that Rac is essential for M-CSF-driven biological events in macrophages. The microinjection of constitutively active or dominant-negative Rac mutants into macrophage cell line BAC.1 increases or decreases M-CSF-induced chemotaxis, respectively (1). Expression of similar Rac mutants in murine macrophage cell line RAW-264 alters M-CSF-induced changes in the actin cytoskeleton (10). However, bone marrow-derived macrophages deficient in Rac1 undergo normal chemotaxis in response to M-CSF (64). The authors of the last study concluded that Rac2 compensates for Rac1 deficiency.

While a requirement for Rac activation in M-CSF-stimulated macrophage chemotaxis is clear, the nature of the Rac exchange factor and how it is activated by M-CSF are not known. There are multiple Rac GEFs identified (reviewed in reference 24), and many are expressed in macrophages. Our findings directly implicate Vav in this role. First, the expression of interfering mutants of Vav blocks M-CSF-induced macrophage chemotaxis. Second, Vav and Rac showed constitutive activities in macrophages of SHIP-deficient mice, and those macrophages exhibited increased migration responses to M-CSF and elevated levels of spontaneous migration. Additionally, we found that the phosphorylation of Vav was blocked by conditions which inhibit PI 3-kinase and was constitutive in the SHIP-deficient macrophages that have elevated levels of PI 3-kinase products. Lastly, we showed direct evidence that Vav is recruited to the plasma membrane of M-CSF-stimulated macrophages through its PH domain in a PI 3-kinase-dependent manner.

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