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Evidence that the Lipid Phosphatase SHIP-1 Regulates T Lymphocyte Morphology and Motility

Stephanie J. Harris,* Richard V. Parry,* John G. Foster,* Matthew D. Blunt,* Amu Wang, ‡ Federica Marelli-Berg, ‡ John Westwick, ‡ and Stephen G. Ward*

SHIP-1 negatively regulates the PI3K pathway in hematopoietic cells and has an emerging role in T lymphocyte biology. PI3K and SHIP can regulate cell migration in leukocytes, particularly in neutrophils, although their role in T cell migration has been less clear. Therefore, we sought to explore the role of SHIP-1 in human CD4+ T lymphocyte cell migration responses to chemottractants using a lentiviral-mediated expression system and a short hairpin RNA approach. Silencing of SHIP-1 leads to increased basal phosphorylation of protein kinase B/Akt and its substrate GSK3β, as well as an increase in basal levels of polymerized actin, suggesting that SHIP-1 might regulate changes in the cytoskeleton. Accordingly, silencing of SHIP-1 led to loss of microvilli and ezrin/radixin/moesin phosphorylation, which could not be rescued by the PI3K inhibitor Ly294002. There were striking morphological changes, including a loss of microvilli projections, which mirrored changes in wild type cells after stimulation with the chemokine CXCL11. There was no defect in directional T cell migration toward CXCL11 in the SHIP-1–silenced cells but, importantly, there was a defect in the overall basal motility of SHIP-1 knockdown cells. Taken together, these results implicate SHIP-1 as a key regulator of basal PI3K signaling in human CD4+ T lymphocytes with important phosphatase-independent actions, which together are key for maintaining normal morphology and basal motility. The Journal of Immunology, 2011, 186: 000–000.

*Inflammatory Cell Biology Laboratory, Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, United Kingdom; ‡Division of Medicine, Inflammatory Cell Biology Laboratory, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K. E-mail address: S.G.Ward@bath.ac.uk

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Address correspondence and reprint requests to Prof. Stephen G. Ward, Inflammatory Cell Biology Laboratory, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, U.K. E-mail address: S.G.Ward@bath.ac.uk

Abbreviations used in this article: ERM, ezrin/radixin/moesin; HMDS, hexamethyldisilazane; pERM, phospho-Thr567ezrin/Thr564radixin/Thr558moesin; PI(3,4,5)P3, phosphatidylinositol-3,4,5-trisphosphate; PKB, protein kinase B; shRNA, short hairpin RNA; Treg, regulatory T cell; TRITC, tetramethyl rhodamine isothiocyanate.
ample structural domains that facilitate protein–protein interactions and cellular relocation upon receptor stimulation. An SH2 site within SHIP-1 interacts with proteins via the amino acid sequence L/I/V/S x-Y-x-x-L/W, which defines an ITIM; a proline-rich region of SHIP-1 enables interaction with Grb2 and phospholipase Cγ via their SH3 domains; and two NPXY motifs in SHIP-1 can become tyrosine phosphorylated and bind the phosphotyrosine-binding domain motif in Src. These structural domains of SHIP-1 are able to support the relocation of SHIP-1 from the cytosol to the plasma membrane, where its catalytic activity regulates PI(3,4,5)P3 accumulation. Thus, there are two aspects to SHIP-1’s functional role: its catalytic activity and its interaction with other proteins (24, 25). The interaction of SHIP-1 with other proteins can also indirectly abrogate PI3K signaling, because SH2 domain-mediated interactions with ITAM-containing adaptor proteins was demonstrated to dislodge or prevent further recruitment of PI3K via the p85 subunit (4, 26).

Polarization and migration of T cells are requisite to their ability to traffic to secondary lymphoid organs and peripheral tissue during routine immunosurveillance and immune responses. Polarized plasma membrane accumulation of PI3Ks at the leading edge of migrating cells leads to localized production of PI(3,4,5)P3 in several models (27, 28). PI3K is activated by most chemokine receptors expressed on T cells, yet paradoxically it is now clear that activation of PI3K by chemokines can be a dispensable signal for directional migration of T cells (27–29). Interestingly, recent studies performed in SHIP-1–null neutrophils revealed that PI(3,4,5)P3 failed to localize to the front of the cell, suggesting that SHIP-1 governs polarization and the formation of the leading edge (30). Introduction of constitutively active SHIP-1 into leukemic cell lines normally deficient in SHIP-1 abrogates CXCL12-mediated chemotaxis (31), possibly as a result of the overexpression and unregulated cellular localization of this mutant that overrides normal polarization mechanisms.

Given that most of our understanding of the function of SHIP-1 in T lymphocyte biology stems from the use of mouse models, this study sought to explore the role of SHIP-1 in primary human T lymphocyte biology (including during cell-migration responses to chemotactants). We adopted a lentiviral-delivery system to introduce SHIP-1–targeting short hairpin RNA (shRNA). Silencing of SHIP-1 leads to increased basal phosphorylation of protein kinase B (PKB)/Akt and its substrate GSK3β, as well as an increase in basal levels of polymerized actin. There were striking morphological changes, including a loss of microvilli projections, which mirrored changes in wild type cells after stimulation with the chemokine CXCL11. There was no defect in directional T cell migration toward CXCL11 in the SHIP-1–silenced cells but, importantly, there was a defect in the overall basal motility of SHIP-1–silenced cells.

Materials and Methods

Lentiviral plasmids

A third-generation system was used to construct replication-incompetent lentiviral particles to deliver shRNAs to T lymphocytes (32). pRSV.Rev (Addgene No. 12255) and pMDL.gmrRE (Addgene No. 12251) were from Addgene, and pVSVG was a gift from David Baltimore (California Institute of Technology, Pasadena, CA). Scrambled shRNA control (SHC002) and SHIP-1 shRNA (TRCN0000039895 Clone ID: NM_005541.2-3413s1c1 sequence 5′-CCCCGCCGCAATTACCCCAAGAAGTCTTGGAGAATCTTC-TCGGGTGATATGGGCTTTTG-3′) were purchased from Sigma.

Cell isolation and culture

CD4+ T cells were isolated from samples of peripheral blood from healthy volunteer donors using a CD4+ T cell isolation kit II human (Miltenyi Biotec MACS). Procedures using human blood were carried out under University of Bath and Departmental safety and ethical guidelines for the use of human tissue. Cells were cultured in RPMI 1640, 10% FCS, 10 μg/ml penicillin, 10 μg/ml streptomycin. After isolation, cells were activated by incubation with anti-CD3/CD28 Ab-coated Dynabeads (Invitrogen 111.31D), at a ratio of 3 beads:1 cell, and IL-2 at 36 U/ml. Cells were infected with lentivirus 24 h after activation, as described elsewhere (33). After 72 h, the cells were plated under selection with puromycin (0.3 μg/ml) for an additional 72 h in the presence of anti-CD3/CD28 Ab-coated Dynabeads and IL-2, after which the cells were ready for use. Cells were removed from IL-2 and anti-CD3/CD28 Ab-coated Dynabeads and maintained in RPMI 1640 + 10% FCS for 16 h before use in experiments. For signaling and chemotaxis experiments, cells were incubated in RPMI 1640 without FCS for an additional hour prior to experiments.

Immunoblotting

Cell stimulations, lysis, and Western blotting were performed as described previously (34). Cells were treated as described in the figure legends, centrifuged, and lysed by addition of 100 μl solubilization buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM sodium vanadate, sodium molybdate, 10 mM sodium fluoride, 40 μg/ml PMSF, 0.7 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor). The samples were mixed and gently rotated at 4°C for 20 min and then centrifuged at 600 × g for 10 min. The supernatant was transferred to fresh tubes and diluted 1:10 with 10% SDS containing 2× sample buffer. Before loading onto the gel, samples were boiled for 5 min at 100°C. The samples were separated by electrophoresis on 10% SDS-PAGE. Proteins were then electrotransferred onto nitrocellulose membrane, blocked in 5% milk, and incubated with anti–SHIP-1 (1/ 500 dilution; Santa Cruz #sc-6244) as primary Ab and anti-rabbit or anti-goat HRP (1/10,000 dilution) as secondary Ab. Immune complexes were visualized using ECL (ECL Western blotting system; Amersham Bioscience, Little Chalfont, U.K.). To confirm equal loading, membranes were stripped by incubation in stripping buffer (100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]) at 60°C for 20 min and reprobed with anti-ERK1/2 (1/1,000 dilution; Santa Cruz #sc-94) Ab.

Phosphoinositide lipid extraction and quantification

To extract lipids, 5 × 10⁶ cells/wash were incubated with 0.5% TCA and then 5% TCA/mi EDTA. Neutral lipids were extracted with MeOH/CHCl₃ (2:1). Acidic lipids were extracted with MeOH/CHCl₃/12 N HCl (80:40:1), followed by centrifugation. The pellet was discarded, and the supernatant was phase separated by addition of CHCl₃ and 0.1 M HCl. After centrifugation, the lower, organic phase was dried in a vacuum dryer. Dried lipids were resuspended in 60 μl PI(3,4,5)P3 buffer, and ELISA was performed according to the manufacturer’s instructions (Echelon Bioscience).

Detection of phospho-Akt, phospho–GSK-3β, and phospho-p70S6K using Mesoscale Discovery multiaarray system

Cells were stimulated, as required, and then 1 × 10⁶ cells/point were lysed in the supplied Tris-based lysis buffer. The Mesoscale multiaarray plate assay was then performed, according to the manufacturer’s instructions, with phospho-Ser⁴⁷³ Akt, phospho-Thr³⁸⁴/Thr³⁸⁷ p70S6K, and phospho-Ser³⁴ GSK-3β (K1111D-1), and total protein plates (K1133D-1) were performed in parallel. Plates were analyzed using a FACS Canto flow cytometer (BD Bioscience, Oxford, U.K.) and DIVA software.

Confluent microscopy

Cells were prepared as for flow cytometry, pERM was stained as for flow cytometry (1/50; Cell Signal Technology #3141) for 3 h (all incubations at 4°C). Cells were then washed three times in BD Perm/Wash buffer and then, for pERM, incubated with goat anti-rabbit FITC (1/100 Sigma). After 2 h, cells were washed twice in BD Perm/Wash buffer, then once in FACS buffer (PBS/1% BSA), and analyzed using a FACS Canto flow cytometer (BD Bioscience, Oxford, U.K.) and DIVA software.

Flow cytometry

A total of 1 × 10⁶ CD4+ T cells were stimulated as required, washed, and fixed in BD Cytofix (containing 4% paraformaldehyde) for 1 h. Cells were washed twice in BD Perm/Wash Buffer (containing FCS and saponin) and incubated with phallolidin-tetramethyl rhodamine isothiocyanate (TRITC) (50 ng/ml; Sigma) or anti-phospho-Thr²³⁷/Thr²³²/Thr²⁴² p70S6K, and phallolidin–TRITC (50 ng/ml; Sigma). After 2 h, cells were washed twice in BD Perm/Wash Buffer, then once in FACS buffer (PBS/1% BSA), and analyzed using a FACS Canto flow cytometer (BD Bioscience, Oxford, U.K.) and DIVA software.

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The blot was quantified using ImageJ software (lower panel). Upper panel immunoblotted with anti–SHIP-1 Ab (samples were diluted to equal concentrations of protein before being verified by stripping and reprobing with anti-ERK1/2 Abs (middle panel). Knockdown of SHIP-1 does not affect viability of human primary T cells. CD4+ T cells were left uninfected (Control) or were infected with a scrambled short hairpin lentivirus (shRNA Control) or SHIP-1 shRNA and were used day 9–10 postisolation and activation with anti-CD3/CD28 beads. A total of 1 × 10⁶ cells was lysed, and samples were diluted to equal concentrations of protein before being immunoblotted with anti–SHIP-1 Ab (upper panel). Equal loading was verified by stripping and reprobing with anti-ERK1/2 Abs (middle panel). The blot was quantified using ImageJ software (lower panel).

**FIGURE 1.** Knockdown of SHIP-1 does not affect viability of human primary T cells. CD4+ T cells were left uninfected (Control) or were infected with a scrambled short hairpin lentivirus (shRNA Control) or SHIP-1 shRNA and were used day 9–10 postisolation and activation with anti-CD3/CD28 Ab-coated Dynabeads. A, A total of 1 × 10⁶ cells was lysed, and samples were diluted to equal concentrations of protein before being immunoblotted with anti–SHIP-1 Ab (upper panel). Equal loading was verified by stripping and reprobing with anti-ERK1/2 Abs (middle panel). The blot was quantified using ImageJ software (lower panel). B, Cells were stained with Annexin-Cy5 and propidium iodide, and viability was assessed by flow cytometry. All data shown are from a single experiment representative of three independent experiments using cells from different donors.

**Scanning electron microscopy**

A total of 2 × 10⁶ CD4+ T cells was removed from anti-CD3/CD28 beads and cultured in RPMI 1640/10% FCS. They were incubated in RPMI 1640 without FCS for 1 h, transferred to 24-well plates with Thermosax coverslips at the bottom of each well, and incubated for an additional hour in the absence or presence of inhibitors, as necessary. Cells were fixed by replacing the media with RPMI 1640/2.5% glutaraldehyde/1% tannic acid. Plates were incubated for 2 h at 37 °C. Coverslips were then washed with RPMI 1640 without FCS. Cells were postfixed in aqueous 1% osmium tetroxide for 1 h at room temperature and washed twice in distilled water, each time incubating for 5 min. Coverslips were transferred to glass petri dishes, and cells were stained with aqueous 2% uranyl acetate for 1 h in the dark. Cells were dehydrated in acetone by washing twice in 50, 70, 90, and 100% acetone, each time incubating for 5 min. They were then incubated in 1:1 acetone/hexamethyldisilazane (HMDS) for 15 min and then twice in 100% HMDS for 15 min. HMDS was pipetted off, and coverslips were dried in a fume hood overnight. Coverslips with cells were coated in gold, and cells were visualized on a JEOL JSM6480LV scanning electron microscope.

**Cell-migration assays**

Cell-migration assays were performed using a 96-well plate-based chemotaxis system, as described previously (34). Briefly, CD4+ T cells (day 9–10 postisolation and activation) were loaded with 29 10⁶ cells/ml in RPMI 1640 media containing 0.1% BSA. Lower chambers were loaded with 29 μl chemokine and overlaid with a 3-μm pore-size filter. The cell suspension (25 μl) was placed on the hydrophobic surface surrounding each well on the filter (upper chamber), and the plate was incubated in a humidified incubator at 37 °C, 5% CO2 for 3 h. Cells migrating to the lower chamber were counted using flow cytometry, as previously described (34). Alternatively, migration was assessed using μ-slides (Ibidi 80301, Thistle Scientific) in conjunction with videomicroscopy. Hydrophobic μ-slides comprising left- and right-hand chambers linked by a bridge were coated with fibronectin at 45 μg/ml in PBS. The slide was filled with RPMI 1640/0.1% BSA; chemokine was added to one chamber, if required. Cells (prepared as above, but to a concentration of 5 × 10⁶/ml) were added to the bridge between the chambers. Images were captured every 15 s for 15 min at 37 °C using a Zeiss LSM 510 Meta microscope. Paths of individual cells were tracked using the ImageJ manual tracker and analyzed using the ImageJ chemotaxis tool.

**Separation and culture of HUVECs**

HUVECs were isolated from human umbilical cord veins and serially subcultured at 37 °C with 5% CO2 in Medium 199 (Sigma) supplemented with 20% heat-inactivated FCS (Sera Laboratories), 2 mM glutamine (Invitrogen), 150 μg/ml Endothelial Cell Growth Supplement (Sigma), 12 U/ml heparin (Sigma), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 2.5 μg/ml Fungizone (Sigma) in gelatin (Sigma)-coated tissue-culture flasks (WWR), as previously described (35). At confluence, HUVECs were detached from the culture flasks using a solution of 0.125% trypsin in 0.2% EDTA (Sigma) and passaged. For functional assays, HUVECs were used at passage 4–6.

**Lymphocyte-transmigration assays**

The transmigration experiments were carried out using HUVEC monolayers grown on Transwell tissue-culture well inserts (diameter 24.5 mm), which contained polycarbonate membranes with a 3-μm pore size (Costar, High Wycombe, U.K.). A total of 3 × 10⁶ endothelial cells was seeded onto gelatin-coated Transwells overnight to form a monolayer. T cells in RPMI 1640 supplemented with 2% FCS were added to each insert (5 × 10⁶/well) and left to migrate through the monolayer. The number of migrated T cells was determined by counting the lymphocytes present in the lower cham-
Results

Silencing of SHIP-1 expression alters PI(3,4,5)P3 accumulation and phosphorylation status of PI3K effectors

SHIP-1–targeting shRNA reduced SHIP expression at the protein level in human CD4+ T cells (Fig. 1A). Because SHIP-1 function potentially impacts on the key survival molecule PKB/Akt, we examined the viability of SHIP-1–deficient cells. The shRNA silencing of SHIP-1 had no detrimental effect on cell viability, as assessed by propidium iodide and Annexin staining (Fig. 1B).

Having verified loss of protein expression using SHIP-1 shRNA, the impact on PI3K-dependent signaling was investigated. First, we monitored the levels of PI(3,4,5)P3, which is regarded as one of the primary products of receptor-activated PI3K. Levels of PI(3,4,5)P3 in control cells were low but increased upon stimulation with UCHT1, an anti-CD3 Ab (Fig. 2A). The nontargeting shRNA control exhibited little or no effect on basal and stimulated levels of PI(3,4,5)P3 accumulation. In marked contrast, cells treated with SHIP-1–targeting shRNA exhibited significantly ($p < 0.05$) increased levels of PI(3,4,5)P3 under basal conditions or UCHT1 Ab-stimulated conditions. UCHT1 was still able to elicit significant elevation of PI(3,4,5)P3 above basal levels in SHIP-silenced cells, although there was a small reduction in the fold increase above basal levels compared with nontargeting shRNA-treated cells. This indicated that although SHIP-1 silencing impacts basal levels of PI(3,4,5)P3, there is only modest impact upon receptor-stimulated events.

We next investigated the impact of SHIP-1 silencing on PI3K-dependent signaling by using phospho-specific Abs that recognize phosphorylated downstream effectors of the PI3K-signaling cascade. Cells expressing nontargeting shRNA control exhibited no change in basal phosphorylation of Akt or its downstream targets p70S6K and GSK3β compared with untreated control cells (Fig. 2B). However, levels of basal phosphorylation of Akt, p70S6K, and GSK-3β were markedly increased in cells infected with SHIP-1–targeting shRNA (Fig. 2B). These results correlated well with the observed changes in PI(3,4,5)P3 levels. Interestingly, UCHT1 was unable to elicit further increases in phosphorylation of Akt/p70S6K/GSK3β above basal levels in the SHIP-1–silenced cells, although it was able to stimulate phosphorylation of these molecules above basal levels in noninfected and control shRNA-infected cells (Fig. 2B).

Silencing of SHIP-1 alters actin polymerization

PI3K signaling has been implicated in the control of actin reorganization that occurs during Ag-induced T cell activation, as well as migratory responses to chemokines (28, 36, 37). Given that SHIP-1 was also reported to play a key role in the polarization of cells during neutrophil responses to chemoattractants (30), we investigated the impact of SHIP-1 silencing on actin polymerization in human T cells. Under basal conditions, polymerized actin existed in concentrated pockets around the cell. Upon silencing of SHIP-1, staining of polymerized actin became more intense, and the polymerized actin had a uniform distribution around the circumference of the cell (Fig. 3). Upon stimulation with CXCL11, previously activated CD4+ T cells (10 d postisolation) displayed intense and polarized actin staining mainly associated with areas of membrane ruffling. Silencing of SHIP-1 led to a significant increase in levels of polymerized actin, as assessed by immunofluorescent staining or flow cytometry using phalloidin-TRITC (Fig. 3), under basal conditions compared with control cells.
Silencing of SHIP-1 leads to microvilli disassembly

Having observed altered basal actin polymerization following SHIP-1 silencing, we next investigated the impact on cell morphology using scanning electron microscopy. Control cells exhibit multiple microvilli, which are actin-rich projections from the body of the cell that express adhesion molecules and are believed to be important in the initial adhesion of the T cell to the endothelium (38). These microvilli are rapidly lost (within 2 min) upon treatment with the chemokine CXCL11 as the cells flatten and polarize with membrane ruffling (Fig. 4). After a longer exposure to chemokine, cells exhibited a rounded morphology and lacked microvilli that were observed at 15 min but also maintained for ≥1 h post-chemokine stimulation (Fig. 4), consistent with previous findings (39). Remarkably, SHIP-1–silenced cells exhibited a loss of microvilli, reminiscent of the morphology observed following prolonged chemokine stimulation. We reasoned that if the loss of microvilli was due to increased levels of PI(3,4,5)P₃ as a consequence of loss of SHIP-1 expression, then a PI3K inhibitor should be able to prevent their loss. However, preincubation with Ly294002 was unable to rescue the loss of microvilli observed in resting SHIP-1–silenced cells (Fig. 4), whereas treatment with Ly294002 alone did not affect expression of microvilli in control cells.

Silencing of SHIP-1 leads to loss of pERM proteins

Formation of microvilli involves the ezrin/radixin/moesin (ERM) proteins that link the actin cytoskeleton to the surface membrane (39). It was reported that microvilli are rapidly lost upon activation of the cell as a result of Rac1-mediated ERM dephosphorylation (39–42). Therefore, we examined the impact of SHIP-1 silencing on phosphorylation of ERM proteins. When control cells were stained for phosphorylated ERM and examined by confocal microscopy, pERM exhibited a distinct punctate distribution that correlated with localized expression at the tips of microvilli. However, SHIP-1–silenced cells exhibited very little detectable pERM (Fig. 5A), although total levels of ERM proteins were unaffected by silencing of SHIP-1 (data not shown). To investigate the involvement of Rac1 in this loss of ERM phosphorylation, we examined the effect of a Rac inhibitor NSC23766 (43, 44) on levels of pERM in the presence and absence of SHIP-1 silencing, using flow cytometry to allow quantification of the data (Fig. 5B). Silencing of SHIP-1 reduced basal phosphorylation of ERM. It was observed that the Rac inhibitor NSC23766 (43, 44) was sufficient to abrogate CXCL11-stimulated ERM dephosphorylation in control cells expressing nontargeting

**FIGURE 3.** Silencing of SHIP-1 expression increases actin polymerization. CD4⁺ human T lymphocytes (10 d postisolation and activation) that had been infected with lentivirus to deliver scrambled or SHIP-1–targeting shRNA were stimulated with 10 nM CXCL11 for 2 min, as indicated, and then fixed with BD Cytofix/Cytoperm using 1 × 10⁶ cells/sample. A, Polymerized actin was stained with phalloidin-TRITC, and cells were mounted on coverslips and examined by confocal microscopy. Data are from a single experiment representative of three independent experiments. Scale bar, 10 μm. B, Cells were infected, fixed, and stained for polymerized actin under basal conditions, as above, and analyzed by flow cytometry of 1 × 10⁵ cells/sample. Data are the mean of five independent experiments using cells from different donors, normalized to polymerized actin in control (uninfected) cells ± SEM. *p < 0.05, Student paired t test.

**FIGURE 4.** Microvilli are disassembled upon SHIP-1 silencing. Human CD4⁺ T lymphocytes (2 × 10⁵, 9 d postisolation and CD3/CD28 activation) that had been infected with lentivirus to deliver scrambled or SHIP-1–targeting shRNA were incubated in RPMI 1640 on Theranox coverslips in 24-well plates for 1 h in the absence or presence of Ly294002 (20 μM 1 h). They were then stimulated with CXCL11 (10 nM; 2 min or 1 h), fixed and dehydrated, and mounted for scanning electron microscopy, as described in Materials and Methods. Images are from a single experiment, representative of three independent experiments using cells from different donors.
Because of the observed morphological changes in SHIP-1–silenced T cells, coupled with evidence from SHIP-1−/− murine neutrophils and T cells, which have altered basal or directional migration responses to chemoattractants, respectively (30, 45), we investigated the impact of SHIP-1 silencing on T cell migration. Expression of CXCR3, the receptor for CXCL11, was unchanged by silencing of SHIP-1 (data not shown). In addition, the expression of adhesion molecules CD11a and CD49d were examined because it was thought that the absence of microvilli might reduce the expression of adhesion molecules, because some are enriched upon the tips of microvilli, whereas others are expressed upon the planar body of the cell (38, 46). Although the expression of CD11a was found to be reduced by ~50%, the cells were equally able to adhere to fibronectin or ICAM-coated surfaces (data not shown). Despite no impact on adhesion, silencing of SHIP-1 significantly reduced the number of cells that basally migrated in Neuroprobe chemotaxis assays, as well as transmigrated across endothelial cell layers (Fig. 6). The nontargeting shRNA had no effect on basal migration responses in the Neuroprobe (data not shown) or the transendothelial cell-migration assays (Fig. 6C). This reduction in basal motility reduced the number of cells that migrated to the chemokine CXCL11, although the chemotactic index (the ratio of the number of cells migrated to the chemokine/number of cells that migrated basally) was unaffected (Fig. 6A, 6B). To better assess the impact of SHIP-1 silencing on the directional migration in response to CXCL11, we next used video microscopy and Ibidi μ-slides. This assay also revealed that silencing of SHIP-1 expression led to impaired basal motility, with reduced straight-line (Euclidean) movement from start to end point (Fig. 6C, 6D). Given the impact of SHIP-1 silencing on basal motility, the impact on chemokine-stimulated migration is better assessed by plotting the data as a ratio (or index) of migration to CXCL11 versus basal migration in the absence or presence of targeting and nontargeting shRNA (Fig 6D, right panels). This revealed that SHIP-1 silencing has no impact on the overall directionality of this response (Euclidean distance traveled). Together, these results showed the importance of SHIP in facilitating basal, rather than directional, motility. (Fig. 7).

Discussion

In this study, we examined the role of SHIP-1 in regulating levels of PI(3,4,5)P3, in human peripheral blood-derived CD4+ T lymphocytes and its influence on T cell migratory responses. Using a lentiviral-mediated delivery system, we introduced shRNA-targeting SHIP-1 into freshly isolated human CD4+ T lymphocytes, which successfully silenced SHIP-1 protein expression without adversely interfering with cell viability. The silencing of SHIP-1 expression correlated with elevated basal PI(3,4,5)P3, which can be partially rescued by inhibition of Rac1. A, CD4+ human T lymphocytes (1 × 10^6 cells, 9 d postisolation and activation) expressing scrambled or SHIP-1–targeting shRNA were incubated in RPMI 1640 for 1 h and then fixed with BD Cytofix/Cytoperm. Cells were stained for pERM or rabbit IgG control, phallolidin-TRITC, and DAPI, as described in Materials and Methods, mounted on coverslips, and imaged on a confocal microscope. Images are from a single experiment, representative of three independent experiments using cells from different donors. Scale bar, 10 μm. B, CD4+ human T lymphocytes (9–10 d postisolation and activation) that had been infected with lentivirus to deliver scrambled or SHIP-1–targeting shRNA were incubated in RPMI 1640 with a 1-h preincubation of the Rac inhibitor NSC23766 at 100 μM, followed by application of 100 nM CXCL11 for 2 min, where indicated. Cells were fixed and stained for pERM, as described in Materials and Methods, and 1 × 10^6 cells/sample were assessed by flow cytometry. Data are mean ± SEM (n = 4). *p < 0.05, **p < 0.005.
As such, the levels of \( \text{PI}(3,4,5)P_3 \) are tightly regulated by SHIP-1 and by the 3’ lipid phosphatase PTEN (24). The importance of these lipid phosphatases is underscored by the fact that PTEN is frequently lost in many leukemias and immortalized leukemic cell lines (49). SHIP-1 is also involved in various leukemias and has been implicated as a negative regulator in chronic myelogenous leukemia and other leukemias (50–53). In addition, elevated levels of microRNA-155 and consequent diminished SHIP-1 expression have been linked to B cell lymphomas (12). However, the role of SHIP in leukemia seems to be more complex than initially thought. For example, a recent study revealed that PTEN and SHIP act cooperatively to suppress B cell lymphoma (54), whereas there is evidence that SHIP can actually support cancer cell survival because a small molecule inhibitor of SHIP-1 induced apoptosis of multiple myeloma cells (55). This is consistent with its production of phosphatidylinositol-3,4–bisphosphate \( \text{PI}(3,4)P_2 \), which is known to facilitate Akt activation and, hence, cell proliferation, survival, and tumorigenesis (56). Indeed, inositol polyphosphate 4-phosphatases, which degrade \( \text{PI}(3,4)P_2 \), were implicated as tumor suppressors in epithelial cell models (57, 58).
Other studies showed that SHIP-1 inhibits CD95/APO-1/Fas-induced apoptosis in T cells by promoting CD95 glycosylation, independently of its phosphatase activity (59). Therefore, our finding that silencing of SHIP-1 in primary human T cells did not affect cell viability was surprising, although it was in line with reports of SHIP-1 knockout in mice, whereby the number of T cells is unaffected (23).

The finding that basal levels of PI(3,4,5)P$_3$ and phosphorylation of the PI3K effectors, Akt, GSK3β, and p70S6K, were increased in cells treated with SHIP-1 shRNA suggested a major role for SHIP-1 in regulating basal PI3K activity, in line with observations in murine SHIP-1−/− neutrophils, which had an increase in basal levels of PI(3,4,5)P$_3$ (30). However, evidence from the T cell-specific knockout mouse indicated that SHIP-1−/− T cells had normal Akt phosphorylation and calcium flux when stimulated with anti-CD3 Ab (23). This is consistent with our observation that cells were still responsive to UCHT1 in SHIP-1–silenced cells and there seemed to be no enhancement of receptor-operated increases in PI(3,4,5)P$_3$ levels or Akt phosphorylation levels. UCHT1 was able to stimulate PI(3,4,5)P$_3$ accumulation above the already-elevated basal levels observed in SHIP-1–silenced cells, although the fold increase above basal levels was slightly reduced compared with control or nontargeting shRNA conditions. Remarkably, silencing of SHIP-1 expression did not lead to an amplification in phosphorylation of Akt and downstream-signaling proteins in response to UCHT1. This observation is contradictory to reports that T cells from germline SHIP-1 knockout mice exhibit enhanced calcium flux responses to CXCL12 (45). One explanation for the lack of enhanced Akt phosphorylation in response to UCHT1 stimulation of SHIP-1–silenced cells may be that modest changes in PI(3,4,5)P$_3$ levels can lead to optimal PKB activation (hence the tight regulation by lipid phosphatases), whereas the ceiling for optimal PI(3,4,5)P$_3$ production by PI3K is much higher. Alternatively, insufficient amounts of PI(3,4)P$_2$ may be formed in the absence of SHIP-1 to sustain optimum activation of PKB.

Our observation of increased actin polymerization upon SHIP-1 silencing in human T cells is in agreement with observations made in murine SHIP-1−/− neutrophils (30), splenocytes, and thymocytes (45), in which basal polymerization of actin was found to be increased in the absence of SHIP-1. Furthermore, in our study, the polymerized actin in SHIP-1–silenced cells was not localized to discrete areas of the cell surface membrane as it was in the controls, suggesting that SHIP-1 may be constitutively regulating actin organization through negative regulation of PI(3,4,5)P$_3$ levels and through generation of PI(3,4)P$_2$, which can recruit specific effectors. For example, TAPP1, which is recruited to PI(3,4)P$_2$ via its PH domain, was shown to bind the cytoskeletal protein synotrophin and regulate B cell adhesion (61). TAPP1, which is recruited to PI(3,4)P$_2$ via its PH domain, was shown to bind the cytoskeletal protein synotrophin and control actin polymerization (60), whereas TAP2 was shown to bind synotrophin and utrophin and regulate B cell adhesion (61). Thus, by generation of PI(3,4)P$_2$, SHIP-1 may divert PI3K signaling toward pathways that are essential for the regulation of the actin cytoskeleton and adhesion.

Microvilli collapse was reported in the literature in response to chemokine stimulation (39), a finding replicated in this study using CXCL11. The purpose of this was hypothesized to allow firm adhesion and flattening of the cell during cell migration. Initial adhesion, tethering, and rolling are due to adhesion molecules, selectins, and α4 integrins, such as VLA-4, expressed on the tips of microvilli binding to their ligands (38). Upon stimulation, Rac1-mediated dephosphorylation of ERM proteins occurs, causing the dissociation of actin from the cell membrane (39, 41, 42). Rac1 activation was shown to have PI3K-dependent and -independent components. For example, PI3K was shown to activate Rac and, hence, induce membrane ruffling in T cells (62). However, Tiam1 is a Rac1-specific exchange factor that can be recruited to PI(3,4,5)P$_3$ and PI(3,4)P$_2$, and it can retain some of its activity in the presence of a PI3K inhibitor (63). Therefore, silencing of SHIP-1 could lead to microvilli collapse through an increase in PI3K-dependent Rac1 activation. However, because the morphology of SHIP-1–silenced cells could not be rescued with Ly294002, Rac activity and/or ERM phosphorylation levels may be regulated through noncatalytic actions of SHIP-1, as proposed in Fig. 7. Indeed, SHIP-1 reduces the association of Rac with Akt, independently of its phosphatase activity; hence, loss of SHIP-1 led to increased Rac-mediated activation of Akt (19). Moreover, loss of SHIP-1 in macrophages resulted in increased Rac activation, as well as overactivation of Ras, which could not be reversed with a PI3K inhibitor (64). The noncatalytic domains of SHIP-1 interact with a number of cytoplasmic molecules to inhibit signaling pathways. The best characterized of these is the influence of SHIP-1 on the Ras pathway. At least two models are proposed in this context in B cells: the SHIP-1 SH2 domain competes with Grb2/Sos complex for binding to phosphorylated Shc and, thereby, downregulates Ras activation (65) and SHIP-1 associates with p62dok, which results in hyperphosphorylation of dok, its association with RasGAP, and the subsequent hydrolysis of Ras-GTP (66). Future studies will tell whether there are other SHIP-1–interacting adaptors or GAPs that modulate Rac activity in T cells.

Confocal microscopy allowed identification of pERM proteins in microvilli tips and confirmed their loss from SHIP-1–silenced cells. However, the Rac1 inhibitor NSC23766 (43, 44) only partially rescued ERM phosphorylation, indicating that other SHIP-1–regulated targets may be required for the regulation of microvilli assembly/disassembly. Although we did not observe a change in the ability of cells to adhere when SHIP-1

![FIGURE 7. Model for role of SHIP-1 in microvilli disassembly, independent of catalytic function. Rac mediates dephosphorylation of ERM proteins that is required for microvilli disassembly. Under basal conditions, noncatalytic structural domains of SHIP-1 suppress dephosphorylation of ERM. One mechanism may involve interaction with Rac-GAP(s) that are responsible for maintaining Rac in its inactive GDP-bound state. Hence, when SHIP expression is silenced under basal conditions, the negative regulation of Rac by SHIP-1 is removed, Rac is activated, and microvilli disassembly occurs. However, the incomplete reversal of ERM dephosphorylation by a Rac inhibitor in the absence of SHIP indicates that SHIP can modulate ERM phosphorylation via Rac-independent routes which have not been determined. Under chemokine receptor-stimulated conditions, the SHIP-1–mediated negative regulatory pathways can be bypassed and/or overridden (larger arrow). See Discussion for further details.](http://www.jimmunol.org/)
expression was silenced, it is likely that the loss of microvilli may have more serious implications for the ability of the cells to undergo initial tethering and rolling during diapedesis, because this stage is reliant upon adhesion molecules localized to the tips of microvilli, and it must be accomplished in the face of shear flow (67).

The ability of T cells to traffic to secondary lymphoid organs and peripheral tissue during routine immunosurveillance and immune responses is contingent upon their proper polarization and migration. It is now clear that activation of PI3K by chemokines can be a dispensable signal for directional migration of T cells (27–29). However, the role of SHIP-1 in T cell migration is less clear, and the loss of microvilli and actin reorganization upon SHIP-1 silencing prompted us to explore the impact of loss of SHIP-1 on migratory responses of T cells. Using two distinct assays, we observed a defect in the basal motility of SHIP-1–silenced cells that, remarkably, did not impinge upon their ability to mount a directional response to a chemokine gradient. The impact on basal motility is consistent with the impact on basal PI3K-dependent signaling but not receptor-mediated PI3K-dependent events. The reduction in basal motility found in SHIP-1–silenced T cells was in agreement with observations made in Dictyostelium deficient in PTEN expression. These single-cell organisms can migrate in response to stimuli, but they lack basal motility because competition between emerging pseudopods cannot be resolved without PI[3,4,5]P3 gradients (68, 69). These findings were also consistent with observations made in SHIP-1–/– neutrophils, which exhibited a higher level of basal actin polymerization and a reduction in basal motility, yet mounted a directional migratory response to fMLP, albeit with impaired velocity (30). Furthermore, basal migration of ex vivo-activated human T cells retains a requirement on PI3K-dependent signaling, although CXCL12-stimulated migration is PI3K independent (34). The lack of impact of SHIP-1 silencing on directional responses to CXCL11 is in contrast to the effect of a constitutively active SHIP-1 mutant introduced into leukemic cell lines normally deficient in SHIP-1, which led to abrogated CXCL12-mediated chemotaxis (31). However, this may be a consequence of unregulated cellular localization of this mutant that overrides normal polarization mechanisms.

The involvement of PI3K in inflammatory and autoimmune conditions has focused intense research efforts toward generating drugs that could inhibit this signaling pathway (70). However, the ubiquitous nature of PI3K signaling means that the use of any successful PI3K inhibitors could be complicated by off-target effects. Activation of lipid phosphatases that regulate levels of PI[3,4,5]P3 offers the same therapeutic benefits of PI3K. Because effects. Activation of lipid phosphatases that regulate levels of ubiquitously, the use of any mechanism.

**References**


