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L-Plastin Regulates Polarization and Migration in Chemokine-Stimulated Human T Lymphocytes

Michael Freeley,* Francis O’Dowd,† Toby Paul,† Dmitry Kashanin,† Anthony Davies,* Dermot Kelleher,*†,1 and Aideen Long*†,1

Chemokines such as SDF-1α play a crucial role in orchestrating T lymphocyte polarity and migration via polymerization and reorganization of the F-actin cytoskeleton, but the role of actin-associated proteins in this process is not well characterized. In this study, we have investigated a role for L-plastin, a leukocyte-specific F-actin–bundling protein, in SDF-1α–stimulated human T lymphocyte polarization and migration. We found that L-plastin colocalized with F-actin at the leading edge of SDF-1α–stimulated T lymphocytes and was also phosphorylated at Ser5, a site that when phosphorylated regulates the ability of L-plastin to bundle F-actin. L-plastin phosphorylation was sensitive to pharmacological inhibitors of protein kinase C (PKC), and several PKC isoforms colocalized with L-plastin at the leading edge of SDF-1α–stimulated lymphocytes. However, PKC ζ, an established regulator of cell polarity, was the only isoform that regulated L-plastin phosphorylation. Knockdown of L-plastin expression with small interfering RNAs demonstrated that this protein regulated the localization of F-actin at the leading edge of chemokine-stimulated cells and was also required for polarization, lamellipodia formation, and chemotaxis. Knockdown of L-plastin expression also impaired the Rac1 activation cycle and Akt phosphorylation in response to SDF-1α stimulation. Furthermore, L-plastin also regulated SDF-1α–mediated lymphocyte migration on the integrin ligand ICAM-1 by influencing velocity and persistence, but in a manner that was independent of LFA-1 integrin activation or adhesion. This study, therefore, demonstrates an important role for L-plastin and the signaling pathways that regulate its phosphorylation in response to chemokines and adds L-plastin to a growing list of proteins implicated in T lymphocyte polarity and migration. The Journal of Immunology, 2012, 188: 6357–6370.

*Department of Clinical Medicine, Institute of Molecular Medicine, Trinity College Dublin, Dublin 2, Ireland; † Cellix Limited, Dublin 12, Ireland; 1 D.K. and A.L. are coprincipal investigators in this study.

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Address correspondence and reprint requests to Dr. Michael Freeley, Department of Clinical Medicine, Institute of Molecular Medicine, Trinity College Dublin, Dublin, Ireland. E-mail address: freelym@tcd.ie

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Abbreviations used in this article: ABD, actin-binding domain; CV, coefficient of variance; GAP, GTPase-activating protein; HCA, High Content Analysis; LPL, L-plastin null murine T lymphocyte; PKC, protein kinase C; PS, pseudosubstrate; PTX, pertussis toxin; siRNA, small interfering RNA; SLO, secondary lymphoid organ; TEM, transendothelial migration; TRITC, tetramethyl rhodamine iso-thiocyanate.

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Although primary T lymphocytes express L-plastin, but not L-plastin or T-plastin (19), there is a paucity of information regarding its precise function in these cells, particularly in human lymphocytes. In this study, we have characterized the signaling pathways that regulate L-plastin phosphorylation and its role in chemokine-mediated human T lymphocyte polarization and migration. We demonstrate that L-plastin phosphorylation is mediated by the established cell polarity protein kinase C (PKC) ζ, and that L-plastin is required for T lymphocyte polarization, lamellipodia formation, and chemotaxis in response to SDF-1α stimulation. L-plastin is also required for SDF-1α-mediated lymphocyte migration on the integrin ligand ICAM-1 by influencing velocity and directionality, but in a manner that is independent of LFA-1 integrin activation or adhesion.

**Materials and Methods**

**Cell culture**

Peripheral human T lymphocyte blasts (in this article, referred to as T lymphocytes) were expanded from buffy coat blood packs obtained from the Irish Blood Transfusion Service in St. James’s Hospital Dublin using PHA and IL-2, as described previously (20). This expansion protocol resulted in >98% CD3+ T lymphocytes, with the proportion of T lymphocytes typically being 83% CD4+, 15% CD8+, and <1% CD4+/CD8+ (20). T lymphocytes were used between days 5 and 12 after culturing in IL-2–supplemented RPMI 1640 containing 10% FCS and antibiotics. Unstimulated CD3+ human T cells were purified from buffy donors using CD3+ T cell isolation columns (R&D Systems, Oxford, U.K.). Where indicated, CD3+ T cells were further stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) for 3 d, followed by culturing in IL-2 for 5 d to produce effector T cells as described previously (21). Primary HUVECs were purchased from Lonza Biologics (Cambridge, U.K.) and cultured as outlined by the supplier.

**Abs and reagents**

The Ab that recognizes L-plastin when phosphorylated at Ser9 was generously provided by Dr. Eric Brown (Genentech, San Francisco, CA). Anti-plastin L-plastin Ab (clone 4A.1) and FITC-conjugated rabbit anti-human IgG were obtained from Thermo Fisher Scientific (Freemont, CA). Anti-PKC ζ (sc-68049), PKC δ (sc-937), PKC ζ (sc-212), PKC ε (sc-214), PKC η (sc-215), PKC ιζ (sc-216), and PKC τ (sc-17837) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CD3+ T cell isolation columns, recombinant human ICAM-1–Fc, recombinant human VCAM-1–Fc, and PE-labeled anti-CXCR4 Ab (clone 12G5) were obtained from R&D Systems. Hoechst 33258, Hoechst 33342, and Alexa Fluor 488/568-conjugated mouse or rabbit IgG Abs were obtained from Molecular Probes-Invitrogen (Paisley, U.K.). Anti-CD28 was obtained from Ancell Corporation (Bayport, MN). Abs to p-ERK1/2 (#9101), total ERK1/2 (#9102), HRP-conjugated goat anti-mouse IgG, and anti-CD3, Rac1, and LFA-1 were obtained from BD Biosciences (Oxford, U.K.). Anti-CD28 was obtained from Alexis Biochemicals (Nottingham, U.K.). Abs to p-ERK1/2, total ERK1/2, total Akt, p-Akt, p-PI3K, p-STAT3, p-STAT5, p-JNK, p-p38, p-ERK1/2, p-AKT, p-STAT3, p-STAT5, p-JNK, p-p38, and p-ERK1/2; Abs to PKC ζ, PKC δ, PKC ε, PKC η, PKC ζ, PKC ιζ, and control nontargeting siRNAs were obtained from Thermo Scientific Dharmacon (Lafayette, CO).

**siRNA transfection**

Human T lymphocytes were expanded for 3 d with 2 μg/ml PHA, washed three times in complete cell culture medium, and then incubated for 5 d in cell culture medium supplemented with 20 ng/ml IL-2, as described previously (20). The cells were harvested, and viable cells were isolated using density gradient centrifugation on Lymphoprep solution (Axis Shield, Dundee, U.K.). The cells were enumerated and washed in sterile PBS. A total of 5 × 10⁶ T lymphocytes were pelleted and resuspended in 100 μl Luminogen Electroporation solution (Merz Bio, Madison, WA), and siRNA was added to a final concentration of 1000 nM. The cell suspension was transferred to an Ingenio electroporation cuvette and electroporated with the Amxaca nucleofector device (Lonza, Cologne, Germany) using program T-07. The cells were removed from the cuvette and incubated in RPMI 1640 containing 10% FCS without antibiotics. IL-2 (20 ng/ml) was added after 4 h and the cells were cultured for 72 h. After this time, the cells were retracted as outlined earlier and incubated with IL-2 for a further 48 h. Anti-CD3/CD28–stimulated effector CD3+ T cells were electroporated with Amxaca program T-07 in an identical manner as outlined earlier. T lymphocytes and effector T cells were serum-starved by incubation in RPMI 1640 containing 0.5% BSA for 2 h at 37˚C before performing all experiments. Unstimulated CD3+ T cells were electroporated with Amxaca program U-14 as outlined by the manufacturer and harvested after 72 h.

**Immunofluorescence staining**

Serum-starved T lymphocytes were resuspended at 0.5 × 10⁶ cells/ml and were stimulated with 100 ng/ml soluble SDF-1α or 250 ng/ml soluble CCL19 at 37˚C for the indicated times. The cells were then fixed at 3000 rpm for 3 min and permeabilized with 0.3% Triton X-100 in PBS at room temperature. The cells were centrifuged and blocked in PBS for 30 min at room temperature. The cells were incubated with the appropriate primary Abs diluted in 3% BSA/PBS for 1 h at room temperature, followed by three washes in PBS. The cells were then incubated with the appropriate Alexa Fluor-labeled secondary Abs (also containing, where appropriate, phalloidin-TRITC and Hoechst 33258 to label F-actin and nucleus, respectively) for 30 min at room temperature. After 3 washes in PBS, the cells were added into Nunc 96-well plates previously coated with poly-lysine and imaged under 20× magnification using the IN Cell Analyzer High Content Analysis (HCA) Imaging System (GE Healthcare, Little Chalfont, U.K.) for cell population analysis (60 fields/well were imaged and analyzed, which corresponds to ~10,000–15,000 cells per treatment). Image analysis was performed using IN Cell Investigator Morphology 1 analysis software. This software detects cells for morphology analysis by nuclear uptake, with quantification of fluorescent intensities determined from one or more intracellular stains (i.e., in our experiments, F-actin staining). In other experiments, cells were stimulated with soluble chemokines and fixed and stained as described earlier, incubated in 96-well glass chamber plates (GE Healthcare) coated with poly-L-lysine and imaged under 63× oil immersion with a Zeiss laser-scanning confocal 510 microscope (Carl Zeiss, Hertfordshire, U.K.).

**SDF-1α and SDF-1α/ICAM-1 live cell assays**

For SDF-1α live cell assays, serum-starved lymphocytes at a density of 1 × 10⁶ cells/ml were incubated with 100 ng/ml soluble SDF-1α before being immediately placed in Greiner half-well μClear 96-well plates and centrifuged for 15 s at 10 × g. Live cell imaging was performed at 37˚C using the IN Cell Analyzer platform under 40× magnification. Bright-field images were acquired every 10 s for 10 min. The images were then imported into the ImageJ software program, and individual cells were manually scored in terms of forming either broad or transient lamellipodia. For SDF-1α/ICAM-1 live cell migration assays, Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4˚C with 3 μg/ml ICAM-1–Fc and 1 μg/ml SDF-1α (preliminary experiments determined that these concentrations of ICAM-1 and SDF-1α were optimal for human T-lymphocyte migration in our solution with IL-2) and electroporated with Amaxa 0.375 T07. The cells were removed from the cuvette and incubated in RPMI 1640 containing 0.5% BSA for 30 min at 37˚C. Serum-starved T lymphocytes were resuspended at 1 × 10⁵ cells/ml and incubated in the plates. Live cell imaging was performed at 37˚C using the IN Cell Analyzer platform under 20× magnification (images acquired every 45 s for 90 frames) or 40× magnification (images acquired every 10 s for 60 frames). The images were then imported into the ImageJ software program, and manual cell tracking was performed using the MTTrack plugin (developed by Erik Meijering at the University Medical Center Rotterdam, Rotterdam, The Netherlands; http://www.imagescience.org/meijering/software/mtrackj/). The average velocity was calculated for each cell by dividing the total distance traveled by the cell by the total number of frames. Persistence was calculated by dividing the net distance traveled (deceleration) by the total distance traveled. Wind Rose plots were generated in Microtrack Extra and manual cell tracking was performed using the MTTrack plug-in (developed by Erik Meijering at the University Medical Center Rotterdam, Rotterdam, The Netherlands; http://www.imagescience.org/meijering/software/mtrackj/).
mobilized with SDF-1α overnight before live cell migrations were carried out as described earlier.

**SDS-PAGE and Western blotting**

Serum-starved T lymphocytes were resuspended at 0.5×10^6 cells/ml and were stimulated with 100 ng/ml soluble SDF-1α at 37°C for the indicated times. Where indicated, T lymphocytes were preincubated with pharmacological inhibitors for 30 min at 37°C before stimulation (or overnight pretreatment in the case of pertussin toxin experiments). T lymphocytes were centrifuged at 3000 rpm for 30 s and lysed directly into SDS-PAGE sample buffer. The samples were sonicated for 5 s to shear the DNA, followed by incubation for 5 min at 100°C. The samples were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane by Western blotting. After blocking in 5% Marvel milk powder in TBS-0.05% Tween for 1 h at room temperature, the membranes were washed three times in TBS-0.05% Tween. The membranes were then incubated overnight at 4°C with diluted primary Abs in 5% BSA/TBS-0.05% Tween with gentle rocking. After three washes in TBS-0.05% Tween, the membranes were incubated with the appropriate HRP-conjugated secondary Abs for 2 h at room temperature. After three further washes in TBS-0.05% Tween, the membranes were incubated with HRP substrate solution (22) and exposed to light-sensitive film for various times. Rac1-GTP levels were assessed according to the manufacturers’ protocol (Millipore). In brief, serum-starved T lymphocytes (1×10^6 cells per time point) were left unstimulated or stimulated with 100 ng/ml soluble SDF-1α for various times and centrifuged for 15 s at 5000 rpm. Cell pellets were lysed in 1% Nonidet P-40 containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 25 mM NaF, 10 mM MgCl₂, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 1 mM PMSF. Insoluble material was removed by centrifugation at 10,000 rpm for 2 min at 4°C. A portion of the supernatant was retained and mixed with an equal volume of 2× SDS-PAGE sample buffer to assay for total Rac1 levels. The remainder of the lysate was incubated with 10 μg GST-PAK1 agarose beads to precipitate Rac1-GTP and incubated for 1 h at 4°C with end-over-end mixing. The beads were recovered by centrifugation and washed three times in lysis buffer. The beads were incubated with SDS-PAGE sample buffer and heated to 100°C for 5 min. The beads were removed by centrifugation, and the supernatants were resolved by SDS-PAGE and probed for Rac1-GTP by Western blotting with an anti-Rac1 Ab. Densitometry was used to quantify the bands on the Western blot films using Kodak 1D Image Analysis Software (New Haven, CT).

**Transwell chemotaxis assays**

The chemotaxis of T lymphocytes toward SDF-1α was performed using Transwell assays containing 9-μm pore polycarbonate membrane inserts (Corning Life Sciences, Lowell, MA). A total of 100 μl serum-free cells at a density of 2.5×10^5 cells was plated on the upper side of the filter, whereas the lower chamber contained 600 μl RPMI 1640 with 0.5% BSA and the indicated concentration of chemokines. The Transwell chambers were incubated at 37°C for 2 h, after which time cells that had migrated through the pores into the lower chambers were recovered. The cells in the lower chamber were then incubated in Nunc 96-well plates that had been previously coated with poly-l-lysine and incubated for 1 h at 37°C. One tenth of the volume of input cells was also incubated in 96-well plates to control for cell loading. Half of the volume of medium was then removed from the wells, and an equal volume of 8% paraformaldehyde containing 2 μg/ml cell-permeable Hoechst 33342 was then added. Cell enumeration was quantified using the IN Cell Analyzer HCA Imaging System under 4× magnification. We imaged 7 fields/well under 4× magnification, which achieved ~85% well coverage. Cell counts were quantified using IN Cell Investigator Morphology 1 analysis software based on nuclear dye staining.

**Soluble ICAM-1 binding assay**

Soluble ICAM-1–binding assays were carried out essentially as described previously (23). In brief, ICAM-1–Fc (3 μg/ml) was first conjugated to FITC-labeled rabbit anti-human IgG Fc Ab (20 μg/ml) in RPMI 1640 for 30 min at room temperature in the dark. Serum-starved T lymphocytes (1×10^6 cells per time point) were resuspended in 50 μl FITC-conjugated ICAM-1–Fc and left unstimulated or stimulated with 100 ng/ml SDF-1α or 5 mM MgCl₂/1.5 mM EGTA for 10 min at 37°C. Stimulation was performed in duplicate. Staining control experiments were performed with 5 mM MgCl₂/1.5 mM EGTA-treated cells that were resuspended in RPMI 1640 and stained with labeled rabbit anti-human IgG Fc Ab (20 μg/ml) and immobilized anti-CXCR4 Ab (clone 12G5). The samples were centrifuged and washed twice with RPMI 1640 containing 0.5% BSA and incubated on ice for 30 min at room temperature. The samples were incubated with 10 μg/ml PE-labeled anti-CXCR4 Ab (clone 12G5) and resuspended in 0.5% BSA in PBS. Thereafter, 10 μl PE-labeled anti-CXCR4 Ab (clone 12G5) was added to the tubes and incubated on ice for 45 min. After one wash in ice-cold PBS, the cells were resuspended in 400 μl cold PBS and analyzed immediately by flow cytometry. Surface levels of CXCR4 were determined using mean fluorescence intensity values. Where indicated, total CXCR4 levels (surface levels and intracellular levels) were determined by first fixing the cells in 2% PFA, before washing the cells in PBS and subsequently resuspending the cells in 0.1% saponin in PBS on ice. Thereafter, 10 μl PE-labeled anti-CXCR4 Ab was added and the samples were processed as outlined earlier.

**Adhesion to ICAM-1 under continuous shear flow and transendothelial migration under shear flow assays**

Cellix Vena8 Fluoro- biochips (Cellix Ltd., Dublin, Ireland) were coated overnight at 4°C with 20 μg/ml ICAM-1–Fc in PBS. Where indicated, the channels were also coated with 2 μg/ml SDF-1α overnight at 4°C. The channels were then washed with PBS using Cellix’s Mirus Nanopump (Cellix Ltd.). Serum-starved T lymphocytes were resuspended in cell-counting buffer (HBSS containing 2 mg/ml BSA, 10 mM HEPES pH 7.4, 1 mM CaCl₂, 1 mM MgCl₂) at a density of 5×10^6 cells/ml and introduced into the channels at a shear stress of 0.5 dyn/cm² using the automated pump. The cells were perfused through the channels for 2 min, after which time images were acquired at defined points along the channels. Cell adhesion to ICAM-1 was quantified by scoring the number of “in-focus” cells (by adjusting the exposure levels; flowing or nonadherent cells remain out of focus). Transendothelial migration (TEM) under continuous shear flow assays was conducted as follows: HUVECs were stimulated overnight with TNF-α to induce ICAM-1 expression before being removed from the culture dishes by treating with EDTA solution. ICAM-1 expression on the HUVECs was analyzed by flow cytometry (data not shown). The HUVECs were seeded in Cellix Vena8 Endothelial+ chips and incubated for a minimum of 2 h at 37°C until confluent. Where indicated, the HUVECs were incubated with 2 μg/ml SDF-1α for 5 min and then washed with PBS before being perfused with T lymphocytes under shear flow as described earlier. T lymphocyte adhesion and TEM through the endothelial layer were recorded by capturing a time-lapse sequence of one frame per second over a period of 4 min. T lymphocyte adhesion to the HUVEC layer and TEM were quantified manually using the MtrackJ plug-in from ImageJ. Control experiments were conducted without SDF-1α where the majority of T lymphocytes rolled on the endothelial surface and did not undergo TEM (data not shown). Lymphocytes that accumulated on the surface of the HUVECs were quantified as either undergoing: 1) transient arrest (adhesion for <5 s), 2) stable arrest (adhesion for >5 s with no migration or TEM), 3) detached, 4) migration (cells that migrated on the endothelial surface and did not undergo TEM), 5) coalescence (cells that tended to accumulate on specific clusters of HUVECs), and 6) TEM (cells that migrated through the HUVEC layer).

**Statistical analysis**

Statistical analysis was performed using the two-tailed Student t test using GraphPad Prism 5. Effects were deemed to be significant at p values <0.05.

**Results**

**Stimulation of human T lymphocytes with SDF-1α results in translocation of L-plastin to the leading edge and phosphorylation on Ser9**

SDF-1α is a potent chemokine that promotes T lymphocyte polarization and migration via CXCR4 receptor signaling. This change in cellular morphology at the leading edge is regulated by cytoskeletal rearrangement, with an important contribution of the F-actin cytoskeleton (3, 11). In chemokine-stimulated T lymphocytes, the F-actin cytoskeleton localizes to the leading edge of the cell (3). We investigated a potential role for the leukocyte-specific actin-bundling protein, L-plastin, in SDF-1α–mediated human T lymphocyte polarization and migration. We observed that L-plastin was distributed toward the periphery of the cell in unstimulated T lymphocytes but was not colocalized with F-actin.
(Fig. 1A). Stimulation of the cells with SDF-1α resulted in a striking redistribution of L-plastin to the leading edge of the cell where it strongly colocalized with F-actin (Fig. 1A). This redistribution of L-plastin to the leading edge was observed as early as 2.5 min after SDF-1α stimulation. Colocalization of L-plastin with F-actin at the leading edge was observed for up to an hour after SDF-1α stimulation (Fig. 1A, and data not shown).

Phosphorylation of L-plastin at Ser5 increases its actin bundling activity (16, 17). We therefore investigated whether L-plastin was phosphorylated in response to SDF-1α. Initial experiments revealed that L-plastin was phosphorylated at Ser5 in IL-2–cultured human T lymphocytes (Supplemental Fig. 1A), which is consistent with reports that IL-2 induces L-plastin phosphorylation in T lymphocytes (24). Culturing the cells overnight in the absence of IL-2 or serum-starving the cells for 2 h in RPMI 1640 containing 0.5% BSA was sufficient to reduce the high basal levels of phosphorylation of L-plastin and ERK (Supplemental Fig. 1A). Using serum-starved T lymphocytes, we determined that SDF-1α stimulation of human T lymphocytes resulted in L-plastin phosphorylation at Ser5 (Fig. 1B). Phosphorylation was detected as early as 2.5 min, peaked between 5 and 30 min, and decreased thereafter (Fig. 1B). Interestingly, phosphorylation of L-plastin was sustained in comparison with ERK 1/2 phosphorylation, a pathway that is also activated downstream of SDF-1α/CXCR4 signaling in T lymphocytes (25) (Fig. 1B). L-plastin phosphorylation was not maximal after SDF-1α stimulation, however, by comparison with the phorbol ester PMA (an activator of PKC isoforms and positive control of L-plastin phosphorylation) (26, 27).

**L-plastin phosphorylation on Ser5 is regulated in a PKC-dependent manner**

We investigated the signaling pathways that regulate L-plastin phosphorylation at Ser5 in SDF-1α–stimulated human T lymphocytes. As the PTX-sensitive Gαi heterotrimeric G protein is activated downstream of SDF-1α/CXCR4 signaling in T lymphocytes (28), we determined whether Gαi influenced L-plastin phosphorylation. As shown in Fig. 2A, pretreatment of T lymphocytes with various concentrations of PTX completely inhibited SDF-1α–mediated L-plastin phosphorylation. We next undertook a pharmacological approach to determine the signaling pathways that regulate L-plastin phosphorylation. L-plastin phosphorylation at Ser5 was originally reported as being catalyzed via protein kinase A after FcγRIIA stimulation of neutrophils (29). We determined that pretreating T lymphocytes with H-89, an inhibitor of protein kinase A signaling, had no effect on SDF-1α–mediated L-plastin phosphorylation (Fig. 2B). In contrast, inhibitors...
FIGURE 2. L-plastin phosphorylation on Ser5 is regulated in a PKC-dependent manner. (A) T lymphocytes were pretreated with the indicated concentrations of PTX overnight before being stimulated with SDF-1α for 10 min. Cell lysates were resolved by SDS-PAGE and probed by Western blotting with the indicated Abs. (B) T lymphocytes were pretreated with the indicated concentrations of pharmacological inhibitors for 30 min before being stimulated with SDF-1α for 10 min. Lysates were resolved by SDS-PAGE and probed by Western blotting with the indicated Abs. (C) T lymphocytes were transfected with siRNAs to the indicated PKC isoforms. Lysates were resolved by SDS-PAGE and probed by Western blotting with Abs to the indicated PKC isoforms. (D) Lymphocytes transfected with siRNAs to individual PKC isoforms (top and middle panels) or to two PKC isoforms (bottom panel) were stimulated with SDF-1α for 10 min, and lysates were resolved by SDS-PAGE and probed by Western blotting with the indicated Abs. (E) T lymphocytes were pretreated with indicated concentrations of a cell-permeable PS peptide inhibitor of PKC ζ for 60 min before being stimulated with SDF-1α for 10 min. Lysates were resolved by SDS-PAGE and probed by Western blotting with the indicated Abs. Data are representative of at least two independent experiments. Densitometry was used to estimate the intensity of the bands and is expressed as p-Ser5 (pSer5) L-plastin/total L-plastin or PKC isoforms/actin.
of PKC signaling including Go6976, Bisindolylmaleimide I, and Ro-31-8220 blocked L-plastin phosphorylation in SDF-1α–treated T lymphocytes (Fig. 2B). Inhibitors of other signaling pathways such as tyrosine kinases (Genistein), the PI3K pathway (LY294002 and Wortmannin), p38 pathway (SB203580), or the ERK pathway (PD98059) had little or no effect on L-plastin phosphorylation (Fig. 2B).

PKC is a family of nine serine/threonine kinases that are sub-grouped into three distinct families based on their mechanism of activation by lipid cofactors and structure (30). These subgroups are known as conventional PKCs (α, β, β2, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (ζ, η). We thus investigated the individual PKC isoforms involved in L-plastin phosphorylation in human T lymphocytes. We determined by Western blotting that primary human T lymphocytes and Jurkat T cells express PKC α, PKC β, PKC δ, PKC ε, PKC η, PKC θ, and PKC ζ (data not shown). The Ab we used to detect PKC ζ also cross-reacts with PKC α; however, we failed to detect PKC ζ using an Ab specific to this isofrom (data not shown). In addition, PKC γ expression is restricted to cells of the CNS, and thus not expressed in T lymphocytes (32). Immunofluorescence staining experiments showed that L-plastin or F-actin colocalized with PKC β, PKC δ, PKC θ, and PKC ζ at the leading edge of SDF-1α–stimulated primary T lymphocytes (Supplemental Fig. 2). F-actin staining was used instead of the mouse anti-human L-plastin Ab in cases where the PKC Ab was also a mouse Ab). In contrast, L-plastin/F-actin did not colocalize with PKC α, PKC ε, and PKC η in SDF-1α–stimulated cells (Supplemental Fig. 2). To determine the PKC isoforms potentially involved in L-plastin phosphorylation, we knocked down the expression of the individual PKC isoforms in primary T lymphocytes using siRNAs specific to each family member and determined the level of L-plastin phosphorylation after SDF-1α stimulation (Fig. 2C). Satisfactory knockdown was achieved for all T lymphocyte–expressed PKCs except for PKC ζ (Fig. 2C). However, knockdown of these PKC isoforms individually did not appreciably affect L-plastin phosphorylation (Fig. 2D, top and middle panels). Because redundancy and/or synergy has been reported for PKCs in different cell types (including T lymphocytes), and thus may require inhibition of more than one isoform (33–35), we transfected cells with a combination of siRNAs to PKC isoforms (i.e., PKC α/β, PKC δ/θ, PKC ε/θ, and PKC η/θ), but again found no obvious role for these PKCs in L-plastin phosphorylation (Fig. 2D, bottom panel). Although we failed to knockdown the expression of PKC ζ by siRNA in T lymphocytes, inhibition of PKC ζ activity with a cell-permeable PS peptide inhibitor of this isoform did inhibit L-plastin phosphorylation in a dose-dependent manner (Fig. 2E). These experiments collectively demonstrate that SDF-1α–stimulated L-plastin phosphorylation in human T lymphocytes is mediated via PKC signaling and is dependent on PKC ζ.

L-plastin regulates the distribution of F-actin at the leading edge and cellular polarization in chemokine-stimulated T lymphocytes

Because L-plastin colocalizes with F-actin at the leading edge of SDF-1α–treated T lymphocytes (Fig. 1A), we investigated whether L-plastin expression influences the localization of this cytoskeletal protein and cellular polarization in response to chemokine stimulation. We used a combination of imaging systems to address this question in fixed cells. We first used the IN Cell Analyzer HCA Imaging System (GE Healthcare) to quantify the effect of knocking down L-plastin expression on cell polarization and F-actin distribution in a cell population-based manner (10,000–15,000 cells were analyzed per treatment in a typical experiment) using a software “toolbox” of defined morphological and fluorescence intensity parameters (36). High content analysis revealed that SDF-1α stimulation of T lymphocytes resulted in a significant increase in cellular morphology parameters including cell area, nuclear displacement (the position of the nucleus relative to the center of the cell body), I/ (form factor) (form factor measures cell roundness), and cell gyration radius (a measure of the spread of the cell; Fig. 3A). SDF-1α stimulation also resulted in a significant increase in the fluorescence intensity of the F-actin cytoskeleton, as measured by phalloidin–TRITC binding (1.6 ± 0.27-fold increase in F-actin intensity; a finding that was also confirmed by flow cytometry of phalloidin-stained cells; data not shown) and an increase in the cell intensity coefficient of variance (CV) parameter (Fig. 3A). Cells that display homogenous staining of a particular protein or stain have low cell intensity CV values, whereas cells with localized or polarized distribution of a particular protein or stain have higher cell intensity CV values. As shown in Fig. 3B, the cell intensity CV values for F-actin in nonpolarized T lymphocytes were typically 0.4–0.6, whereas polarized cells with F-actin distributed at the leading edge typically had cell intensity CV values in the range of 0.8–1.3.

We used this toolbox of morphological and fluorescence intensity parameters to determine the effect of knockdown of L-plastin expression on SDF-1α–stimulated T lymphocyte polarization and F-actin localization. Knockdown of L-plastin expression was achieved by transfecting siRNAs specific to this target in primary T lymphocytes. We consistently attained 75–95% knockdown of this protein in primary T lymphocytes for all experiments (Fig. 3C). We determined that knockdown of L-plastin expression resulted in a significant decrease in cellular morphology parameters including nuclear displacement and I/ (form factor) in SDF-1α–stimulated cells, implying that L-plastin is required for chemokine-induced cellular polarization (Fig. 3D). Other parameters such as cell area and cell gyration radius were reduced after knockdown of L-plastin expression but did not reach statistical significance (Fig. 3D). Interestingly, knockdown of L-plastin expression also resulted in a small but significant reduction in the fluorescence intensity of F-actin as measured by phalloidin–TRITC binding (14 ± 7% decrease in F-actin intensity; again, this result was confirmed by flow cytometry; data not shown; Fig. 3D). Using the cell intensity CV parameter as a readout of F-actin localization in the cell, we determined that SDF-1α–stimulated T lymphocytes transfected with L-plastin siRNAs had significantly reduced F-actin distribution at the leading edge of the cell in comparison with SDF-1α–stimulated T lymphocytes transfected with control siRNAs (Fig. 3E). These experiments thus reveal that L-plastin is required for SDF-1α–mediated cellular polarization and for the localization of F-actin at the leading edge of the cell. Confocal imaging analysis of the same cells also revealed that after knockdown of L-plastin expression, the F-actin cytoskeleton was not polarized at the leading edge of many cells and was diffusely localized throughout the cell body (Fig. 3F). A similar role for L-plastin was also observed in anti-CD3/CD28–stimulated effector T cells (Supplemental Fig. 3A) and unstimulated CD3+ T cells (Supplemental Fig. 3B) in response to SDF-1α stimulation. Furthermore, we also found that L-plastin was phosphorylated in response to CCL19 stimulation in human T lymphocytes (Supplemental Fig. 3C), and that the expression of L-plastin was also required for CCL19-mediated F-actin localization and cell polarization (Supplemental Fig. 3D).

Knockdown of L-plastin expression perturbs the Rac1 activation cycle, Akt phosphorylation, lamellipodia formation, and chemotaxis

We investigated whether L-plastin expression regulated signaling through the CXCR4 receptor. Consistent with reports that che-
mokine receptors localize to the leading edge of chemokine-stimulated lymphocytes (37), we found that L-plastin colocalized with the CXCR4 receptor in SDF-1α–stimulated T lymphocytes (data not shown). Cellular polarization and the formation of the leading edge/lamellipodia in response to chemokine stimulation is driven by activation of GTPases such as Rac1 (10–12), whereas the PI3K/Akt and ERK pathways are also activated downstream of SDF-1α/CXCR4 signaling in human T lymphocytes (25, 28). We therefore investigated the impact of knockdown of L-plastin expression on Rac1, Akt, and ERK activation in response to SDF-1α.
stimulation. As shown in Fig. 4A, Rac1, Akt, and ERK pathways were rapidly activated (15 s to 5 min) in response to SDF-1α stimulation in T lymphocytes transfected with control siRNAs, with time scales that are consistent with published reports (38). Although L-plastin phosphorylation at Ser5 was also detectable at these early time points, maximal L-plastin phosphorylation did not occur until later time points (5–15 min; Fig. 4A). Interestingly, we found that knockdown of L-plastin expression did not affect the kinetics of Rac1 activation (which peaked at 15–30 s), but that the loss of L-plastin expression resulted in sustained activation of Rac1, as a low but detectable level of Rac1 activation was still observed after 15 min of SDF-1α stimulation (Fig. 4A). In addition, the activation of Akt (as measured by phosphorylation on its activation-loop residue Thr308) was impaired in response to SDF-1α stimulation after knockdown of L-plastin (Fig. 4A). Knockdown of L-plastin expression, however, did not perturb ERK activation in response to SDF-1α stimulation (Fig. 4A). Furthermore, although knockdown of L-plastin expression slightly affected the surface levels of expression of CXCR4 (Supplemental Fig. 4A), the rate of internalization of the CXCR4 receptor from the cell surface after SDF-1α stimulation was similar in T lymphocytes transfected with control siRNAs or L-plastin siRNAs (Supplemental Fig. 4B). These results imply that L-plastin regulates a subset of signaling pathways downstream of the CXCR4 receptor in response to SDF-1α stimulation in human T lymphocytes including Rac1 and Akt, whereas other signaling pathways such as ERK activation and CXCR4 internalization are unaffected.

Because Rac1-GTP signaling was perturbed after knockdown of L-plastin expression (Fig. 4A), and it is well-known that this GTPase regulates lamellipodia formation at the leading edge of chemokine-stimulated cells (10, 11), we also investigated the impact of knockdown of L-plastin expression on lamellipodia formation in response to SDF-1α stimulation using live cell imaging. A high proportion of T lymphocytes (>50%) transfected with control siRNAs and subsequently stimulated with SDF-1α underwent rapid polarization with the formation of a single broad lamellipodium at the leading edge of the cell (Supplementary Video 1). Such structures were highly dynamic, and in many cells, the lamellipodia underwent rapid changes in direction. The lamellipodia also often retracted back into the cell, which was followed by the formation of a new lamellipodia in a different direction. In contrast, T lymphocytes transfected with L-plastin siRNAs and subsequently stimulated with SDF-1α made attempts at polarizing

FIGURE 4. Knockdown of L-plastin expression perturbs the Rac1 activation cycle, Akt phosphorylation, lamellipodia formation, and chemotaxis. (A) T lymphocytes transfected with control siRNA or L-plastin siRNA were stimulated with 100 ng/ml SDF-1α for the indicated times. Lysates were prepared and probed by Western blotting with the indicated Abs. Rac1-GTP levels were assessed as outlined in Materials and Methods. Densitometry was used to estimate the intensity of the bands and is expressed as p-Ser5 L-plastin/total L-plastin, Rac1-GTP/total Rac1, p-Thr308 Akt/total Akt, and p-ERK 1/2/total ERK 1/2. (B) T lymphocytes were transfected with control or L-plastin siRNAs and subsequently stimulated with SDF-1α. Bright-field images were acquired every 10 s over a period of 10 min, and lamellipodia formation was quantified for individual cells. See Supplementary Videos 1 and 2. (C) T lymphocytes were transfected with control siRNA or L-plastin siRNA and subsequently analyzed for their ability to undergo chemotaxis toward the indicated concentrations of SDF-1α using Transwell chambers. Data are representative of at least two independent experiments.
but generated poorly formed and less pronounced lamellipodia (Supplementary Video 2). We quantified lamellipodia formation in both control and L-plastin siRNA transfected cells in response to SDF-1α stimulation. As shown in Fig. 4B, knockdown of L-plastin expression resulted in a lower number of cells forming broad lamellipodia in comparison with cells transfected with control siRNA. This reduction in lamellipodia formation was primarily due to an increase in the number of cells forming poorly formed lamellipodia, as well as an increase in the number of nonresponsive cells (Fig. 4B). Consistent with the role for L-plastin in lamellipodia formation was the observation that L-plastin was also required for chemotaxis toward a directed source of SDF-1α using Transwell chambers, albeit this role for L-plastin in chemotaxis was more easily observed at greater concentrations of SDF-1α (i.e., 300 ng/ml; Fig. 4C). The expression of L-plastin was also required for chemotaxis in response to SDF-1α in anti-CD3/CD28–stimulated effecter T cells (data not shown).

L-plastin regulates lymphocyte velocity and migratory persistence in response to SDF-1α and ICAM-1 stimulation but is dispensable for LFA-1 integrin activation and TEM

Chemokines also contribute to lymphocyte migration by activating intracellular signaling pathways that result in conformational changes in integrins such as LFA-1 (a β2 integrin) that permits cell adhesion to vascular barriers via interactions with its corresponding ligand ICAM-1 (6, 7). In addition, ligation of chemokine–dependent manner, and undergo polarization and migration in an apparently random fashion (20). Immunofluorescence staining showed that L-plastin was distributed at the leading edge of T lymphocytes migrate in response to immobilized SDF-1α in the presence of ICAM-1 (20). In this system, the lymphocytes adhere to ICAM-1 in an SDF-1α–dependent manner, and undergo polarization and migration in an apparently random fashion (20). Immunofluorescence staining showed that L-plastin was distributed at the leading edge of T lymphocytes that were actively migrating on SDF-1α and ICAM-1, and that this bundling protein colocalized with F-actin at the leading edge of these cells (Fig. 5A). When we knocked down the expression of L-plastin by siRNA and analyzed the speed of the cells migrating on SDF-1α/ICAM-1 over a period of 45 min, we noted that the loss of L-plastin resulted in a small but significant reduction in cell velocity (p = 0.03, n = 4 experiments; Fig. 5B). In addition, migrating T lymphocytes with reduced levels of L-plastin expression exhibited increased migratory persistence (the net displacement of the cell/total distance divided by the cell; p = 0.004, n = 4 experiments; Fig. 5B). The trajectories taken by the individual cells over the course of the experiment are shown in Fig. 5C. Persistence is a measure of the directionality of a cell as it migrates: cells that move randomly or meander (such as lymphocytes migrating on immobilized SDF-1α/ICAM-1) tend to display low persistence values (<1), because the total distance traveled by the cell is always greater than its net displacement. In contrast, cells that move from one point to another in a perfectly straight line have a persistence value of 1, because the total distance traveled will equal the net displacement of the cell. T lymphocytes transfected with L-plastin siRNAs also displayed a lack of polarity compared with their control siRNA counterparts when analyzed under higher magnification (40×) on SDF-1α/ICAM-1 (data not shown), which likely contributes to the phenotype of reduced cell velocity and increased persistence. Our finding that L-plastin regulates lymphocyte speed and migratory persistence on SDF-1α/ICAM-1 suggests that this actin-bundling protein may influence cell turning and meandering. A similar role for L-plastin was observed with anti-CD3/CD28–stimulated effecter T cells and with unstimulated CD3+ T cells migrating on SDF-1α/ICAM-1 (Supplementary Fig. 4C, 4D). We also investigated whether L-plastin influenced cell migration on other integrin ligands including fibronectin and VCAM-1 (which bind to β3 integrins) in the presence of SDF-1α. Although we found that knockdown of L-plastin expression in T lymphocytes consistently reduced cell velocity on SDF-1α/fibronectin and SDF-1α/VCAM-1, increases in migratory persistence on β3 integrin ligands were not always observed, perhaps suggesting that L-plastin may perform distinct roles in the migratory responses of T lymphocytes to different integrin ligands (Supplemental Fig. 4E).

Interestingly, knockdown of L-plastin expression did not affect LFA-1 activation in response to SDF-1α, as assessed by a soluble ICAM-1–binding assay (Fig. 5E), whereas clustering of the LFA-1R at the leading edge of the cell in response to SDF-1α stimulation was not substantially perturbed in comparison with the F-actin cytoskeleton (data not shown). Furthermore, SDF-1α–mediated adhesion to ICAM-1 under static conditions or under continuous shear flow was not affected after depletion of L-plastin levels (Fig. 5E, and data not shown). Knockdown of L-plastin expression also did not affect the ability of these cells to adhere to and migrate through an ICAM-1–expressing endothelial cell layer under shear flow (Supplemental Fig. 4F). These experiments collectively reveal that L-plastin influences chemokine-induced lymphocyte migration on integrin ligands in a manner that is independent of LFA-1 activation and adhesion (Fig. 6).

Discussion

Signaling through cell surface receptors such as the TCR and chemokine receptors result in dynamic polymerization and rearrangement of the F-actin cytoskeleton. Polymerization and remodeling of F-actin facilitates reorganization of the plasma membrane and the formation of membrane protrusions such as lamellipodia and filopodia that enable T lymphocyte scanning of APCs and migration along chemokine-enriched surfaces and tissues. Although it is well established that the F-actin cytoskeleton localizes to the immune synapse after Ag stimulation (39) and also localizes to the leading edge of migrating lymphocytes (13), how cytoskeletal-associated/regulatory proteins contribute to immune synapse formation and migration in T lymphocytes is not fully characterized. In this study, we have demonstrated a key role for the actin-bundling protein L-plastin in regulating cellular polarization, lamellipodia formation, and migration in response to chemokines in primary human T lymphocytes. Our results strongly suggest that L-plastin regulates lymphocyte polarity and migration by enabling cells to establish an axis of asymmetry in response to chemokine signaling, in particular, the localization of the F-actin cytoskeleton to the leading edge of the cell. We also uncovered a novel mechanistic link between PKC ζ, an established regulator of lymphocyte polarity (40–43), and L-plastin in human T lymphocytes, specifically via PKC ζ-mediated phosphorylation of L-plastin on its Ser2 residue. L-plastin expression also influenced the Rac1 activation cycle, Akt phosphorylation, and migration on the integrin ligand ICAM-1, but in a manner that was independent of LFA-1 activation or adhesion. This study, therefore, reveals new and important mechanistic insights into the signaling pathways that regulate T lymphocyte polarity and migration.

Although the expression of L-plastin is restricted to leukocytes, relatively little has been reported on its role or function in T lymphocytes. L-plastin contains a pair of EF-hand domains at its N terminus that bind intracellular Ca2+ and two actin-binding domains (ABDs; termed ABD1 and ABD2) situated on the C
FIGURE 5. L-plastin regulates lymphocyte velocity and migratory persistence in response to SDF-1α and ICAM-1 stimulation but is dispensable for LFA-1 integrin activation and TEM. (A) T lymphocytes were incubated on immobilized SDF-1α/ICAM-1 for 30 min at 37°C before being fixed and processed for immunofluorescence for L-plastin (green), F-actin (red), and the nucleus (blue). The cells were imaged by confocal microscopy (original magnification ×63). (B) T lymphocytes were transfected with control siRNA or L-plastin siRNA and subsequently incubated on immobilized SDF-1α/ICAM-1. Bright-field images of the cells were acquired every 45 s over a period of 90 frames on the IN Cell Analyzer HCA Imaging System at 37°C under 20× magnification. Cell tracking and calculation of average speed and persistence for each individual cell were performed as outlined in Materials and Methods. Densitometry was used to estimate the intensity of the bands from the Western blot and is expressed as L-plastin/Talin. (C) Wind Rose plots of the trajectories taken by the individual cells over the course of the experiment in (B) are shown. (D) T lymphocytes transfected with control siRNA or L-plastin siRNA were analyzed for their ability to bind to fluorescently labeled soluble ICAM-1 in the absence or presence of indicated stimuli. Control stimuli consisted of MgCl2/EGTA-treated cells incubated in the absence of ICAM-1 but containing the fluorescently labeled secondary Ab. (E) T lymphocytes transfected with control or L-plastin siRNAs were analyzed for adhesion to ICAM-1 under continuous shear flow in the absence or presence of immobilized SDF-1α. Stimulation of the cells with MgCl2/EGTA served as a positive control to induce the high-affinity form of LFA-1. Densitometry was used to estimate the intensity of the bands from the Western blots in (D) and (E), and is expressed as L-plastin/Talin.
FIGURE 6. Schematic model depicting the proposed role of L-plastin in response to SDF-1α stimulation in human T lymphocytes. SDF-1α stimulation induces rapid activation of a multiprotein cascade (Rap1, Cdc42-GTP, Par3/6, PKC z, and Tiam1) that promotes Rac activation (Rac-GTP) and F-actin polymerization and lamellipodia formation. PKC z also regulates L-plastin phosphorylation on Ser5, which regulates F-actin bundling. In the absence of L-plastin expression, the F-actin cytoskeleton is mislocalized in the cell, resulting in poorly formed lamellipodia and a defect in cell polarity and chemotaxis. In addition, the Rac1 activation/inactivation cycle and Akt phosphorylation is perturbed in cells deficient in L-plastin expression.

A major finding from our work was that we observed that L-plastin phosphorylation at Ser5 after SDF-1α stimulation was sensitive to pharmacological inhibitors of PKC. PKC inhibitors have previously been demonstrated to inhibit fMLP-mediated phosphorylation of L-plastin in neutrophils (48), whereas PKC βII and PKC δ have recently been implicated in L-plastin phosphorylation in eosinophils (49) and breast cancer cells (17), respectively. Furthermore, several PKC isoforms such as PKC β, PKC δ, PKC θ, and PKC z colocalized with L-plastin at the leading edge of T lymphocytes after SDF-1α stimulation. Although PKC θ phosphorylation is induced on its activation-loop residue in T lymphocytes in response to SDF-1α stimulation (50) and PKC z colocalized with L-plastin in SDF-1α-stimulated cells (Supplemental Fig. 2), we found no role for this kinase in L-plastin phosphorylation (Fig. 2D). Instead, our studies pointed to a role for PKC z, an established regulator of lymphocyte polarity (40–43), in L-plastin phosphorylation (Fig. 2E). In particular, we found that PKC z colocalized with L-plastin in SDF-1α-stimulated cells, and that inhibition of PKC z activity with a cell-permeable peptide inhibitor blocked L-plastin phosphorylation in response to SDF-1α stimulation (Fig. 2E). PKC z has long been implicated in cellular polarity through its well-defined interactions with members of the Par3/Par6 polarity complex that is conserved from worms and flies to vertebrates (51). PKC z is phosphorylated on its activation-loop residue in response to chemoattractants (42, 43) and Ag stimulation (41) in T lymphocytes, whereas perturbation of PKC z signaling in T lymphocytes using kinase-dead/activation-loop mutants or cell-permeable peptide inhibitors blocks chemokine-induced polarization/chemotaxis (40, 42, 43) and polarization toward APCs (41). We demonstrate in this article for the first time, to our knowledge, a novel mechanistic link between two polarity proteins in T lymphocytes, namely, PKC z and L-plastin, specifically via PKC z-mediated phosphorylation of L-plastin on its Ser5 site. Although we observed that the phosphorylated form of L-plastin was localized at the leading edge in response to SDF-1α stimulation (Supplemental Fig. 1B), it is unlikely that phosphorylation regulates L-plastin distribution in the cell, because mutation of the Ser5 site to a nonphosphorylatable residue does not impair localization of L-plastin at the immune synapse in T lymphocytes (52). Instead, phosphorylation of L-plastin at Ser5 may specifically regulate its ability to bundle actin filaments (16, 17).

Collard and colleagues (42) elegantly demonstrated that PKC z was a crucial component of a multiprotein signaling cascade that
regulated polarization in response to SDF-1α stimulation in T lymphocytes. This cascade involved a Rap1-Cdc42 pathway leading to PKCζ/Par3/Par6 activation, with PKCζ regulating Rac activation and cellular polarization via the Rac-guanine nucleotide exchange factor Tiam-1 (42). Our finding that PKCζ regulates L-plastin phosphorylation suggests that L-plastin could be an intermediary link between PKCζ and Tiam-1; however, we have observed that: 1) L-plastin depletion does not affect the kinetics of Rac1 activation after SDF-1α stimulation; and 2) that maximal L-plastin phosphorylation occurs after Rac1 activation (Fig. 4A), which implies that the PKCζ-Tiam-1-Rac cascade and the PKCζ-L-plastin pathway are mutually exclusive. We therefore propose a model (modified from the model proposed by Collard and colleagues [42]) whereby SDF-1α stimulation induces lymphocyte polarization within seconds via activation of a Rap1-Cdc42-PKCζ/Par3/Par6-Tiam1-Rac cascade, with subsequent recruitment of L-plastin (in its phosphorylated form via PKCζ signaling) to the leading edge enabling stabilization and bundling of F-actin filaments, thus facilitating efficient polarization, lamellipodia formation, and chemotaxis (Fig. 6).

Notably, the phenotypes observed for T lymphocytes lacking Tiam-1 (43) or after inhibition of PKCζ signaling (40, 42, 53) are broadly similar to T lymphocytes with depleted L-plastin protein levels, yet subtle differences in the migratory characteristics of L-plastin-depleted lymphocytes suggest that this bundling protein performs a distinct role in human T lymphocytes. For example, our finding that knockdown of L-plastin expression in T lymphocytes inhibits polarization and chemotaxis, but is dispensable for LFA-1 activation, adhesion to ICAM-1, and TEM, is similar to the phenotypes reported for Tiam-1-deficient T lymphocytes and lymphocytes treated with PKCζ PS inhibitors or over-expressing kinase-inactive/dominant-negative PKCζ mutants (40, 42, 43, 53). Interestingly, Tiam-1 deficiency or inhibition of PKCζ activity does not block TEM per se but results in these lymphocytes switching their route of transmigration from a para-cellular route (between endothelial cells at cell junctions) to a transcellular route (through individual endothelial cells) (43). Because we found that L-plastin, like Tiam-1 or PKCζ, was not required for TEM, it will therefore be interesting to determine whether this bundling protein also influences the route of TEM. L-plastin and Tiam-1 appear to influence lymphocyte migration on the integrin ligand ICAM-1 in different ways, however. For example, although we found that knockdown of L-plastin expression decreased lymphocyte velocity and increased persistence/directionality on ICAM-1 in the presence of SDF-1α, Tiam-1-deficient lymphocytes migrating on ICAM-1 or on top of endothelial monolayers were reported as having decreased migration speed and a corresponding decrease in directionality (43). These studies suggest that L-plastin and Tiam-1 regulate distinct, integrin-independent, membrane remodeling events in the context of lymphocyte polarization and migration. In particular, our data suggest that F-actin bundling may play an important role in directional persistence on integrin ligands, particularly the B2 integrin ligand ICAM-1. We have also shown that loss of polarity is a contributing factor that likely accounts for the phenotype we observe when L-plastin–depleted cells are incubated on SDF-1α/ICAM-1, because these cells are less efficient in forming single, stable protrusive leading edges. Although this phenotype on SDF-1α/ICAM-1 is perhaps more subtle than anticipated, considering the importance of L-plastin in SDF-1α–mediated cell polarization (Fig. 3), our finding that L-plastin does not influence LFA-1 signaling (Fig. 5D, and data not shown) suggests that only the SDF-1α axis, rather than the LFA-1/ICAM-1 axis, is perturbed after knockdown of L-plastin.

It is of interest to note that a role for L-plastin in regulating velocity and directional persistence on integrin ligands in T lymphocytes is similar to what has been reported for Rac1 in other cell types; Yamada and colleagues (54) reported that knockdown of Rac1 expression by siRNA decreased cell velocity and increased directional persistence in migrating fibroblasts and epithelial cells. Knockdown of Rac1 expression selectively reduced the number of peripheral lamellipodia that formed in response to β2 integrin signaling, resulting in a decreased rate in cell turning, and thus an increase in directional persistence (54). Our finding that knockdown of L-plastin expression also perturbs the Rac1 activation cycle (see later) and lamellipodia formation in T lymphocytes strongly suggests that this increase in directional persistence on β2 integrin ligands is likely due to a defect in Rac1-mediated peripheral lamellipodia formation and a decreased rate of cell turning/meandering.

L-plastin knockdown did not affect early Rac1 activation kinetics in response to SDF-1α. L-plastin depletion, however, did result in both a more sustained low-level activation of Rac1 and impaired Akt1 phosphorylation, leaving the ERK pathway unaffected. This defect in the Rac1 activation cycle and Akt phosphorylation in T lymphocytes was associated with a loss in cell polarity, lamellipodia stability, and migration. Although the role of Rac1 in lamellipodia formation and migration is well documented (10–12), the Akt pathway (which is activated downstream of PI3K signaling) (55) has also been implicated in F-actin assembly and migration (56). Although it might seem counterintuitive, elevated or sustained Rac activity has been correlated with a loss of polarity and migration in many cell types, including lymphocytes (57–59). Rac-GTP loading is catalyzed by guanine nucleotide exchange factors, whereas inactivation of Rac-GTP is catalyzed by GTPase-activating proteins (GAPs) (10, 11). Interestingly, in T lymphocytes stimulated through the LFA-1 integrin receptor, the PI3K/Akt pathway is involved in termination of Rac1 signaling (58), presumably via recruitment/activation of a Rac-GAP. It is possible, therefore, that the prolonged Rac1 activation observed after L-plastin knockdown is due to impaired PI3K/Akt signaling, and thus defective recruitment of a Rac1-GAP in response to SDF-1α. It is also possible that these events may be secondary to an unstable F-actin cytoskeleton after knockdown of L-plastin expression.

While our experiments described in this study were under way, Morley et al. (60) reported that L-plastin null murine T lymphocytes (LPL−/−) harbored defects in intranodal motility in vivo, and demonstrated defective polarization and chemotaxis toward CCL19 and sphingosine-1-phosphate in vitro. Our results reported in this article with primary human T lymphocytes both complement and contrast with the studies reported for murine LPL−/− lymphocytes. Although both studies are in agreement that L-plastin is required for lymphocyte polarization and chemotaxis, Rac1 activation was reported as being intact in LPL−/− lymphocytes after CCL19 stimulation, albeit this conclusion was based on a single early time point. Furthermore, the Akt pathway was not investigated in LPL−/− lymphocytes. Interestingly, it was also noted that LPL−/− lymphocytes migrating in lymph nodes displayed a decrease in velocity/motility and a slight decrease (albeit nonsignificant) in their meandering index (the degree to which a cells movement varies from a straight line, which is akin to directional persistence). Although the phenotypes of the LPL−/− lymphocytes and human T lymphocytes are largely in agreement, our studies also extend the role of L-plastin in lymphocyte migration by: 1) demonstrating that this protein plays a key role in regulating F-actin localization and lamellipodia formation, 2) demonstrating a key link between PKCζ and L-plastin phos-
phorylation, and 3) showing an association with migratory persistence on the integrin ligand ICAM-1.

In summary, we have demonstrated a role for the F-actin bundling protein L-plastin in regulating chemokine-induced lamellipodia formation, polarization, and migration in human T lymphocytes. Our studies have also implicated the atypical PKCζ isoform, a well-established regulator of cell polarity in mammalian cells (including T lymphocytes) (40–43), in regulating the bundling activity of L-plastin through its Ser1 phosphorylation residue. Because L-plastin localizes to the leading edge of chemokine-stimulated T lymphocytes where it regulates F-actin localization, polarization, and migration, our study therefore adds L-plastin to a growing list (8–10, 61) of proteins implicated in T lymphocyte polarity and migration.

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Disclosures
D.K. is a director of Celllix Ltd. The other authors have no financial conflicts of interest.

References


Supplementary Figure 1: (A) T lymphocytes were cultured overnight in the presence or absence of IL-2 or incubated for 2 hours in serum-free RPMI medium containing 0.5% BSA. The cells were harvested and probed by Western blotting with the indicated antibodies. Densitometry was used to determine the intensity of the bands and is expressed as pSer5 L-plastin/total L-plastin or pERK 1/2/total ERK 1/2 (B) Serum-starved T lymphocytes were left unstimulated or stimulated with SDF-1α for 10 min before being fixed and processed for immunofluorescence staining for phosphorylated L-plastin (pSer5), F-actin and the nucleus. The cells were imaged by confocal microscopy.

Supplementary Figure 2: T lymphocytes were left unstimulated or stimulated with SDF-1α for 10 min before being fixed and processed for immunofluorescence staining for the indicated PKC isoforms (green), F-actin/L-plastin (red) and nucleus (blue). The cells were imaged by confocal microscopy.

Supplementary Figure 3: (A) Anti-CD3/CD28-stimulated effector T cells transfected with control or L-plastin siRNAs and (B) unstimulated CD3⁺ T cells transfected with control or L-plastin siRNAs were stimulated with SDF-1α for 10 min before being fixed and processed for immunofluorescence staining for F-actin and the nucleus. Image acquisition and analysis were carried out using the IN Cell Analyser HCA Imaging System. The Cell Intensity CV parameter was used to determine the distribution of the F-actin cytoskeleton at the leading edge of the cell. Western blotting was performed to determine knockdown of L-plastin expression and is quantified by densitometry as intensity of L-plastin/LFA-1 (C) PHA/IL-2-stimulated T lymphocytes were left unstimulated or stimulated with 100 ng/ml SDF-1α or 250
ng/ml CCL19 for the indicated times before being harvested and probed by Western blotting with the indicated antibodies. Densitometry was used to determine the intensity of the bands and is expressed as pSer5 L-plastin/total L-plastin (D) PHA/IL-2-stimulated T lymphocytes were transfected with control or L-plastin siRNAs and subsequently stimulated with 250 ng/ml CCL19 for 10 min. The cells were fixed and processed for immunofluorescence staining for F-actin and the nucleus. Images were acquired on the IN Cell Analyser HCA Imaging System. The Cell Intensity CV parameter was used to determine the distribution of the F-actin cytoskeleton at the leading edge of the cell.

**Supplementary Figure 4:** (A) T lymphocytes were transfected with control or L-plastin siRNA and were subsequently analysed for surface levels of CXCR4 (surface) using a PE-labelled anti-CXCR4 antibody or were fixed and permeabilised (per) with 0.1% saponin prior to staining to analyse surface and intracellular levels of CXCR4. Mean Fluorescence Intensity (MFI) was used to quantify the levels of fluorescence of CXCR4. Lysates were also prepared and probed by Western blotting for total L-plastin, CXCR4 or Talin (as a loading control). (B) T lymphocytes were transfected with control siRNA or L-plastin siRNA and stimulated for various times with 300 ng/ml SDF-1α before being analysed for surface levels of CXCR4. (C) Anti-CD3/CD28-stimulated effector T cells transfected with control or L-plastin siRNAs and (D) unstimulated CD3+ T cells transfected with control or L-plastin siRNAs were incubated on SDF-1α/ICAM-1 and live cell migrations were performed. Cell tracking and calculation of average speed and persistence for each individual cell were performed as outlined in the Materials and Methods. NS denotes non-significance. (E) Data tables showing the effect of knockdown of L-plastin expression on average cell
speed and persistence on SDF-1α/Fibronectin or SDF-1α/VCAM-1. (F) T lymphocytes transfected with control siRNA or L-plastin siRNA were perfused under shear flow over TNF-α-stimulated HUVEC cells that subsequently had been overlaid with 2 μg/ml SDF-1α. Lymphocytes that accumulated on the surface of the HUVECs were quantified as either undergoing transient arrest (adhesion for less than 5 seconds), stable arrest (adhesion for greater than 5 seconds with no migration on the endothelial cell surface or TEM), detached, migration (cells that migrated on the endothelial surface but did not undergo TEM), coalescence (cells that tended to accumulate around specific clusters of HUVEC cells) and TEM (cells that migrated through the HUVEC layer).

Supplementary Video 1: T lymphocytes transfected with control siRNAs were serum starved and then stimulated with 100 ng/ml SDF-1α. Live cell imaging was recorded using the IN Cell Analyser HCA Imaging System with brightfield images (40X) captured every 10 seconds over a period of 10 minutes.

Supplementary Video 2: T lymphocytes transfected with L-plastin siRNAs were serum starved and then stimulated with 100 ng/ml SDF-1α. Live cell imaging was recorded using the IN Cell Analyser HCA Imaging System with brightfield images (40X) captured every 10 seconds over a period of 10 minutes.
Supplementary Figure 1

(A) Immunoblot analysis showing the expression levels of pSer5 L-plastin, L-plastin, p-ERK 1/2, and ERK 1/2. The intensity of pSer5 L-plastin is 100, 30, and 12, while the intensity of p-ERK 1/2 is 100, 1, and 1.

(B) Non-stimulated vs. SDF-1 stimulation images. The panels show pSer5 L-plastin, F-actin, and merged images.
Supplementary Figure 2
Supplementary Figure 3
Supplementary Figure 4

(A) CXCR4 (Surface) MFI

(B) CXCR4 (Surface)

(C) Effector T cells

(D) Unstimulated CD3+ T cells

(E) Migration on SDF-1α/Fibronectin

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<th>Control siRNA</th>
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<tr>
<td>Speed</td>
<td>3.19 +/- 0.34</td>
<td>2.42 +/- 0.36</td>
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<td>Persistence</td>
<td>0.59 +/- 0.05</td>
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(F) Migration on SDF-1α/VCAM-1

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<th>L-plastin siRNA</th>
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<tr>
<td>Speed</td>
<td>2.63 +/- 0.26</td>
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<td>Persistence</td>
<td>0.52 +/- 0.04</td>
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