PLCε1 regulates SDF-1α–induced lymphocyte adhesion and migration to sites of inflammation

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Regulation of integrins is critical for lymphocyte adhesion to endothelium and migration throughout the body. Inside-out signaling to integrins is mediated by the small GTPase Ras-proximate-1 (Rap1). Using an RNA-mediated interference screen, we identified phospholipase Cε 1 (PLCε1) as a crucial regulator of stromal cell-derived factor 1 alpha (SDF-1α)-induced Rap1 activation. We have shown that SDF-1α-induced activation of Rap1 is transient in comparison with the sustained level following cross-linking of the antigen receptor. We identified that PLCε1 was necessary for SDF-1α-induced adhesion using shear stress, cell morphology alterations, and crawling on intercellular adhesion molecule 1 (ICAM-1)–expressing cells. Structure–function experiments to separate the dual-enzymatic function of PLCε1 uncover necessary contributions of the CDC25, Pleckstrin homology, and Ras-associating domains, but not phospholipase activity, to this pathway. In the mouse model of delayed type hypersensitivity, we have shown an essential role for PLCε1 in T-cell migration to inflamed skin, but not for cytokine secretion and proliferation in regional lymph nodes. Our results reveal a signaling pathway where SDF-1α induces T-cell adhesion through activation of PLCε1, suggesting that PLCε1 is a specific potential target in treating conditions involving migration of T cells to inflamed organs.

Rap1 | adhesion | T cells | PLCε1 | SDF-1α

T-cell adhesion contributes to routine immune surveillance and generating inflammatory responses. The interaction between a T cell and an antigen presenting cell (APC) requires an adhesion complex that is stable long term; relative to each other, this interaction is static (1). Here, the signaling cascade leading to integrin activation is initiated through the T-cell receptor (TCR) and coreceptors (2). Alternatively, T-cell trafficking and extravasation require a more dynamic, firm adhesion between T cells and endothelial cells of blood vessel walls. Chemokines are largely responsible for orchestrating T-cell trafficking throughout the body. The localized expression of chemokines on endothelia, and chemokine receptor expression patterns on T-cell populations, give rise to a network of T-cell recruitment with signatures for anatomical compartments or disease states (3). Whereas much work has been aimed at establishing these signatures, the same cannot be said for elucidating the signaling cascades downstream of chemokine receptors. The small GTPase Ras-proximate-1 or Ras-related protein 1 (Rap1) and its effectors are necessary for integrin activation and, therefore, T-cell adhesion (4). Rap1 is active in the GTP-bound form following interaction with a guanine exchange factor (GEF) and inactive in the GDP-bound form following interaction with a GTPase activating protein (GAP). Phospholipase Cε 1 (PLCε1) is a protein with dual enzymatic functions of a lipase and a GEF (5, 6). Here we show that T-cell adhesion induced by the stromal cell-derived factor 1 alpha (SDF-1α)/C-X-C chemokine receptor type 4 (CXCR4) axis involves Rap1 activation, exclusively mediated by PLCε1. We provide evidence that the lipase function of PLCε1 does not contribute to the activation of adhesion in this pathway. Through expanding these studies to a mouse model of contact sensitivity, we conclude that PLCε1 plays an essential role in T-cell recruitment to the site of inflammation. These findings suggest that PLCε1 is a potential target in treating inflammatory conditions.

Results

PLCε1 Is Required for SDF-1α–Induced Rap1 Activation. To analyze Rap1 activation, we transfected Jurkat T cells with a GFP-tagged version of the Ras-binding domain of Ral guanine nucleotide dissociation stimulator (GFP–Ral–GDS–RBD). When Rap1 is GDP bound (inactive), GFP–Ral–GDS–RBD is cytosolic; however, when Rap1 is GTP bound (active) it binds to the sensor, mainly at plasma membrane. Therefore, GFP–Ral–GDS–RBD acts as an indicator of Rap1 activation that can be analyzed microscopically and quantified by fluorescent line analysis (Fig. S1*A*). Upon stimulation with anti-CD3 or SDF-1α, Rap1 was activated at the plasma membrane (Fig. 1*A*). Similar results were also obtained with a cherry-tagged version of the Ras-associating and Pleckstrin homology domains of Rap1-GTP–interacting adaptor molecule (cherry–RIAM–RAPH), an alternative biosensor (Fig. S1*B*) (7). In primary human T cells, Rap1 GST pull-down assay shows that Rap1–GTP is increased nearly twofold (Fig. 1*B*) upon stimulation with SDF-1α. We next questioned whether the kinetics of Rap1 activation downstream of the TCR and CXCR4 were distinct. Following anti-CD3 stimulation Rap1-GTP remains elevated throughout the 30 min (Fig. 1*C* and Fig. S2*A*). In contrast, Rap1-GTP levels following SDF-1α have two distinct peaks (Fig. 1*C* and Fig. S2*A*). The differences in kinetics in these two cascades suggest differential regulation at the level of

Significance

We performed a screen to identify unique proteins regulating T-cell adhesion downstream of the chemokine receptor CXCR4. We identified that the guanine exchange factor mediating stromal cell-derived factor 1 alpha (SDF-1α)/C-X-C chemokine receptor type 4 (CXCR4) activation is phospholipase Cε 1 (PLCε1). Experiments to separate the dual-enzymatic function of PLCε1 uncover necessary contributions of the CDC25, Pleckstrin homology, and Ras-associating domains, but not phospholipase activity, to its guanine exchange activity. In the mouse model of contact sensitivity, we have shown a necessary role for PLCε1 in T-cell recruitment to the site of secondary challenge, but not for priming after antigen sensitization. We uncover a signaling pathway that mediates SDF-1α–induced T-cell adhesion and suggest that PLCε1 is a potential specific target for future antiinflammatory drugs.

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The authors declare no conflict of interest.

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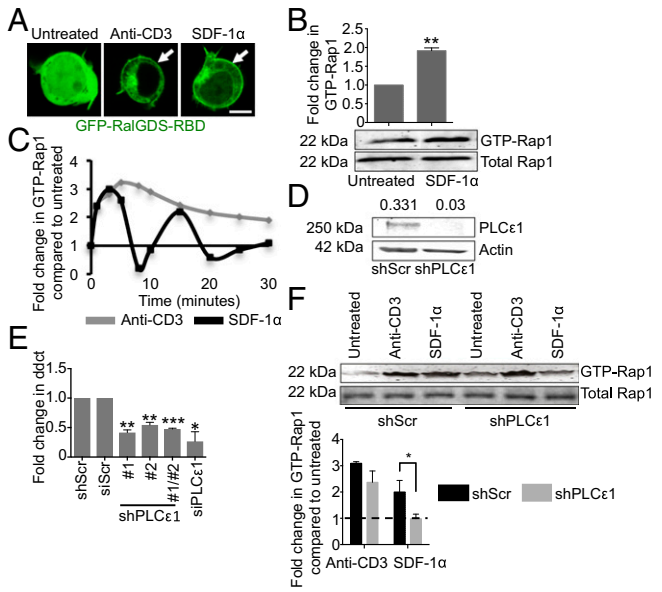

Fig. 1. PLCε1 is required for SDF-1α–induced Rap1 activation. (*A*) Jurkat T cells transfected with GFP–Ral–GDS–RBD; stimulation with soluble anti-CD3 (5 μg/mL) or SDF-1α (100 ng/mL). (*B*) GTP–Rap1 in primary human T cells following SDF-1α treatment for 2 min quantified by pull-down using the GST–Ral–GDS–RBD. (*C*) Primary human T cells treated with soluble anti-CD3 or SDF-1α for indicated amounts of time before GST pull-down assay; $n = 3$. (*D*) Western blot of PLCε1 in Jurkat T cells, shScr, or shPLCε1. (*E*) RT-PCR of cDNA from primary human and Jurkat T cells expressing scramble (si/shScr) or PLCε1 (si/shPLCε1) interfering RNA. Two shPLCε1 constructs were used (#1 and #2). (*F*) Jurkat T cells stimulated with soluble anti-CD3 or SDF-1α followed by GST pull-down. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $n = 3$.

GEFs or GAPs. To identify the GEFs in each cascade, individual GEF expression was targeted in Jurkat T cells. GEFs included in this screen were chosen based on previously shown GEF activity toward Rap1 and expression in T cells. To identify the most essential GEFs for each stimulation, the magnitude of fold change in Rap1–GTP was compared (Fig. S2*B*). Whereas more than one GEF contributed to anti–CD3-mediated Rap1 activation, phospholipase Cε 1 (PLCε1) was the only GEF that resulted in a drop below twofold activation following SDF-1α stimulation. Notably, C3G was one of four potential GEF contributors to anti–CD3-mediated Rap1 activation. Whereas other studies have shown that C3G is the main GEF responsible (8), additional studies with combined knockdowns should be done to rule out potential compensation from other GEFs when C3G levels are decreased.

Although the expression of PLCε1 has previously been shown in lymphocytes (www.proteinatlas.org/ENSG00000138193-PLCE1/tissue; www.proteinatlas.org/ENSG00000138193-PLCE1/cell; medicalgenomics.org/details_view_limited?gene_id=51196), we confirmed expression and reduction at the protein level in Jurkat T cells (Fig. 1*D*). We further confirmed expression in primary human and Jurkat T cells (Fig. 1*E*) by quantifying mRNA expression and showing efficient knockdown by si/shRNA. We confirmed the necessary role for PLCε1 in SDF-1α, but not anti-CD3, induced Rap1 activation, by GFP–Ral–GDS–RBD recruitment (Fig. S2*C*) and GST pull-down (Fig. 1*F*). These studies suggest a role for PLCε1 in Rap1 activation downstream of SDF-1α.

Firm Adhesion Under Shear Stress Downstream of SDF-1α Requires PLCε1. To establish a necessary role for PLCε1 in Rap1-mediated cellular functions, a chemokinesis assay was performed. We observed that control cells rapidly arrest on the CHO–intercellular adhesion molecule 1 (ICAM-1) monolayer following SDF-1α addition, with average distance traveled per cell dropping from 7.6 ± 0.63 to 3.4 ± 0.42 μm (Fig. 2*A*). In contrast, PLCε1 knockdown cells fail to arrest, with average distance traveled per cell decreasing only slightly from 11.0 ± 0.91 to 8.2 ± 1.14 μm (Fig. 2*A*). This result suggests that PLCε1 may play a role in adhesion and crawling. To further explore this functional defect, an assay of firm adhesion to ICAM-1 using shear stress conditions was employed (9–11). While SDF-1α induces an increase in adhesion of control T cells, there is no observed adhesion in PLCε1 knockdown T cells (Fig. S3*A* and Fig. 2*B*), providing evidence of an essential role for PLCε1 in SDF-1α–induced firm adhesion. Calculating the circularity index before and after SDF-1α stimulation provides evidence that PLCε1 knockdown cells are defective in adopting a "crawling morphology" and maintain a circularity index (Fig. 2*C*) and ratio of Ferets near 1 (Fig. S3*B*). PLCε1-deficient primary human T cells are also less responsive to SDF-1α (Fig. 2*D*). PLCε1 knockdown did not induce any alteration in the level of surface CXCR4 expression (Fig. 2*E*). Collectively, these results suggest that PLCε1 plays an essential role in T-cell firm adhesion induced by SDF-1α.

The Lipase Function of PLCε1 Is Not Required to Activate Rap1 downstream of SDF-1α. PLCε1 functions as a phospholipase and a GEF. To determine whether the phospholipase activity of PLCε1 is induced by SDF-1α stimulation, we examined diacylglycerol (DAG) accumulation. Baseline expression of this biosensor is cytosolic, but following stimulation with phorbol myristate acetate (PMA) or anti-CD3 it localizes to the plasma membrane (Fig. 3*A*). Strikingly, following SDF-1α stimulation there was no recruitment of the biosensor, indicating no observable accumulation of DAG at that compartment (Fig. 3*A*). Following stimulation of primary human T cells with anti-CD3, intracellular calcium levels increase and peak after the addition of 2 mM calcium to the buffer (Fig. 3*B*). Following SDF-1α stimulation there is no initial increase in

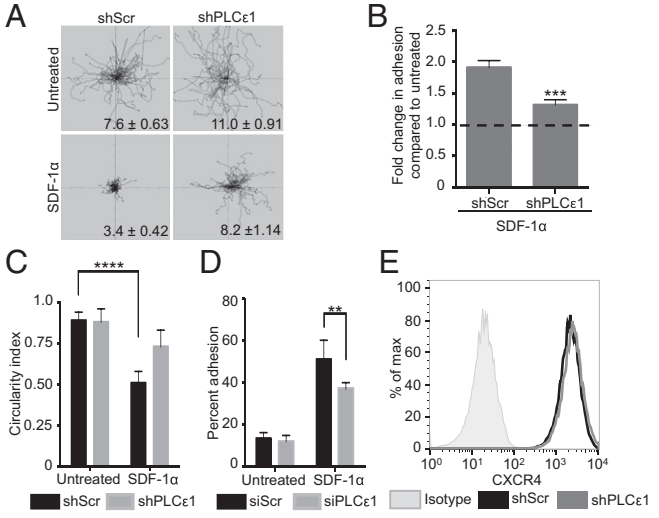

Fig. 2. Firm adhesion using shear stress downstream of SDF-1α requires PLCε1. (*A*) Carboxyfluorescin succinimidyl ester (CFSE)-labeled Jurkat T cells expressing shScr or shPLCε1 plated onto a CHO–ICAM-1 monolayer. A series of 30 images was captured over 10 min following SDF-1α stimulation, analyzed by Volocity 3D Image Analysis Software. Each vector indicates an individual cell. *Inset* number is average vector length (in micrometers). (*B* and *D*) Flow adhesion assay with CFSE-labeled T cells expressing shScr or shPLCε1, plated onto a CHO–ICAM-1 monolayer on μ-flow VI chambers. Images taken before shear force and after 5 min. Quantification of Jurkat T cells (*B*) and primary human T cells (*D*) using ImageJ. (*C*) Circularity index of individual cells was calculated using ImageJ. Experimental conditions as described. (*E*) Jurkat T cells expressing shScr or shPLCε1 were stained for surface expression of CXCR4 and analyzed by flow cytometry. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P < 0.0001$; $n = 3$.

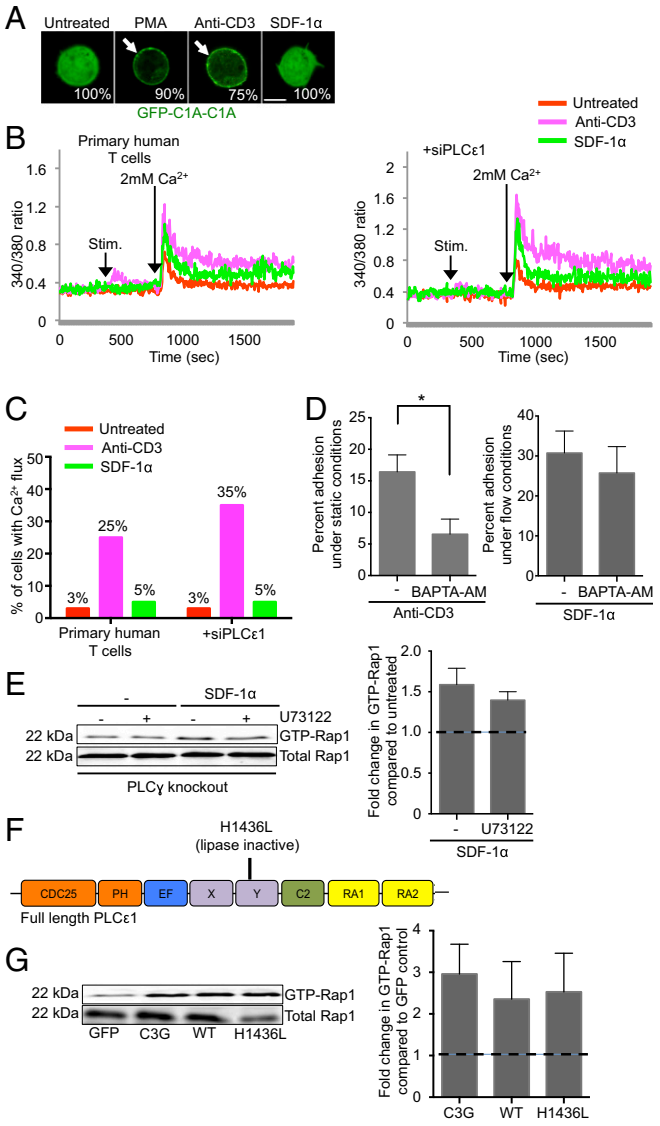

Fig. 3. The lipase function of PLCε1 is not required to activate Rap1 downstream of SDF-1α. (*A*) Jurkat T cells transfected with GFP–C1A–C1A. Images represent majority; live cells were imaged 20 min following PMA (10 ng/mL), anti-CD3, or SDF-1α. *Inset* percentage from 20 cells counted over two independent experiments. (*B*) Intracellular calcium by fura-2 fluorescence in primary human T cells. (*C*) Percent of cells counted exhibiting calcium flux in response to stimulation. (*D*) Adhesion under static conditions (*Left*), primary T cells were treated with BAPTA-AM (50 μM) before anti-CD3. Adhesion using flow conditions (*Right*), primary human T cells were treated with BAPTA-AM before SDF-1α. (*E*) Jurkat T cells deficient in PLCγ were treated with U73122 (1 μM) before SDF-1α followed by GST pull-down assay. Quantification at *Right*. (*F*) Schematic of PLCε1 containing the H1436L point mutation. (*G*) HEK 293T cells transfected with GFP alone, C3G, full-length PLCε1–GFP (FL), or the lipase dead mutant PLCε1–GFP H1436L. GTP–Rap1 levels were quantified by GST pull-down assay. Quantification at *Right*.

calcium-free buffer, and the peak following the addition of 2 mM calcium is smaller (Fig. 3*B*). Knockdown of PLCε1 by siRNA does not alter the calcium response to either stimulus (Fig. 3*B*), suggesting that this protein is not a significant contributor to intracellular calcium levels in these pathways. Also of note, a mere 5% of cells showed any flux in intracellular calcium following SDF-1α stimulation (compared with 25–35% following anti-CD3) (Fig. 3*C*). Together these studies suggest that there is likely a limited contribution of the activation of phospholipase, in this specific pathway, and that further studies should be done to prove independent regulation of the GEF and lipase functions of PLCε1. Pretreatment of T cells with the intracellular calcium chelator BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid]-AM abrogated T-cell adhesion induced by anti-CD3; in contrast SDF-1α–induced T-cell adhesion was not disrupted (Fig. 3*D*). Further, we used the PLC inhibitor U73122 in PLCγ1 knockout Jurkat T cells (to promote a preference of the inhibitor

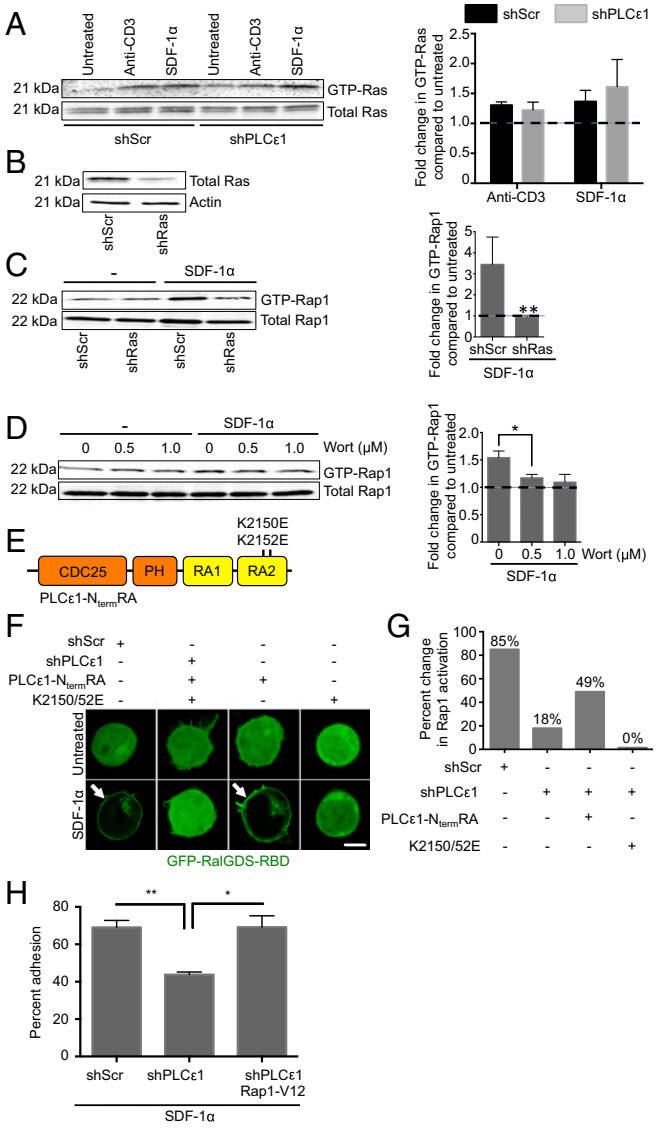

Fig. 4. The RA1/2 domains of PLCε1 contribute to SDF-1α signaling. (*A*) Jurkat T cells (shScr or shPLCε1) treated with anti-CD3 or SDF-1α. GTP–Ras levels were quantified by GST-Raf1 pull-down assay. (*B*) Western blot of Ras in Jurkat T cells shScr or N-Ras and K-Ras targeting shRNA (shRas). (*C*) Jurkat T cells (shScr or shRas) treated with SDF-1α followed by GST pull-down assay. (*D*) Jurkat T cells treated with wortmannin before SDF-1α stimulation followed by GST pull-down assay. (*E*) Schematic of PLCε1–N_{term}RA constructs. (*F*) Jurkat T cells (shScr or shPLCε1) transfected with GFP–Ral–GDS–RBD ± PLCε1–N_{term}RA or K2150E/K2152E (K2150/52E) or PLCε1$_{NRA\Delta PH}$ mutant constructs, and SDF-1α stimulated. Images represent majority phenotype for each condition. (*G*) Percent of cells imaged displaying an activated Rap1 phenotype based on probe recruitment to the plasma membrane and fluorescent line analysis; $n = 3$; more than 200 cells counted per condition. (*H*) Flow adhesion assay with Jurkat T cells (shScr or shPLCε1) transfected with GFP alone or GFP–Rap1–V12, on a CHO–ICAM-1 monolayer on μ-flow VI chambers. $^*P \leq 0.05$; $^{**}P \leq 0.01$; $n = 3$.

for PLCε1) and show that Rap1 activation following SDF-1α stimulation is unaffected by PLCγ1 deficiency and U73122 pretreatment (Fig. 3*E*). As this inhibitor also inhibits PLCβ, these results suggest no contribution of any phospholipase C family member to this pathway. To confirm that the GEF activity of PLCε1 is not dependent on the lipase activity, we generated GFP-tagged full-length, wild-type PLCε1 or containing H1436L point mutation (Fig. 3*F*) shown to result in inactive lipase (12). Through expression of GFP alone, the established Rap1 GEF C3G, wild-type PLCε1, or the lipase-dead PLCε1 mutant, we show that there is no defect in Rap1 activation with the loss of lipase activity (Fig. 3*G*). Together, these results show that the lipase activity of PLCε1 does not contribute to the Rap1 GEF activity of the protein.

The Ras-Associating 1/2 and the Pleckstrin Homology Domains of PLCε1 Contribute to SDF-1α Signaling. PLCε1 knockdown does not diminish anti-CD3 or SDF-1α–induced GTP–Ras accumulation (Fig. 4*A*), suggesting that this enzyme is not the GEF-activating Ras in this pathway. After establishing that the lipase activity of PLCε1 is not contributing to SDF-1α–induced adhesion, the contribution of the Ras-associating (RA) domains was left in question (16). In SDF-1α–stimulated Ras knockdown Jurkat T cells (Fig. 4*B*) GTP–Rap1 accumulation was completely abrogated (Fig. 4*C*). To assess a requirement for the Pleckstrin homology (PH) domain in anchoring PLCε1 to the plasma membrane we treated T cells with wortmannin before quantifying Rap1–GTP levels. Pretreatment resulted in decrease in Rap1 activation following SDF-1α stimulation (Fig. 4*D*), suggesting that the PH domain is playing a necessary role. To establish that the CDC25 (GEF), PH, and Ras-associating 1/2 (RA1/2) domains are also sufficient to activate T-cell adhesion, we generated a PLCε1 truncation construct composed of only these four domains (PLCεNtermRA), with two point mutations (K2150E and K2152E) that disrupt Ras binding (14), or with the PH domain excised ($PLC\varepsilon1_{NRA\Delta PH}$) (Fig. 4*E*). Rap1 activation was assessed by microscope and fluorescent line analysis of GFP–Ral–GDS–RBD translocation (Fig. S1*A*). Stimulation with SDF-1α of PLCε1 knockdown T cells results in no translocation of GFP–Ral–GDS–RBD to the plasma membrane in the majority of the cells (Fig. 4*F*). Strikingly, coexpression of the PLCεNtermRA truncation rescues Rap1 activation, whereas rescue with the construct containing the K2150E and K2152E point mutations or with $PLC\varepsilon1_{NRA\Delta\Delta PH}$ does not (Fig. 4*F*). All imaged cells were analyzed individually by fluorescent line analysis and counted as either Rap1 inactive or active (Fig. S1*A*). A comparison of the percent of counted cells exhibiting an active Rap1 localization reveals that SDF-1α stimulation leads to an 85% increase in plasma membrane localization in cells expressing nontargeting shRNA; this amount is decreased to 18% of cells with PLCε1 knockdown, but we observed a 49% increase in Rap1 activation in cells expressing PLCεNtermRA, whereas those expressing K2150E, K2152E mutant PLCεNtermRA did not have any increase in Rap1 activation (Fig. 4*G*). These results suggest that these four domains are sufficient to rescue Rap1 activation in PLCε1-deficient cells, and that the PH domain and Ras binding to PLCε1 play necessary roles in Rap1 activation. Many small GTPases are regulated by the localization of GEFs, and because PLCε1 must be recruited to the plasma membrane, the PH domain and the association between Ras and the RA domains serve as the localization anchor. To rule out a non-Rap1 role for PLCε1, we quantified adhesion in PLCε1 knockdown cells that express a constitutively active form of Rap1 (Rap1–V12). As shown, PLCε1 knockdown T cells are less adhesive than those expressing a nontargeting shRNA, but expression of Rap1–V12 is sufficient to rescue SDF-1α–induced adhesion (Fig. 4*H*). Together, these studies suggest that the role of PLCε1 in SDF-1α–induced T-cell adhesion is solely to activate Rap1, and the CDC25 (GEF), PH, and RA1/2 domains are sufficient.

***PLC*ε1 Is Required for Recruitment of Sensitized T Cells to Site of Inflammation.** To assess the role of PLCε1 in T-cell adhesion in vivo PLCε1 knockout mice were used in a model of contact sensitivity (15) (Fig. S4). PLCε1 knockout mice developed less ear swelling following dinitrofluorobenzene (DNFB) sensitization and challenge than wild-type mice (Fig. 5*A*). A three point scoring system was used to grade each tissue section based on mononuclear cell infiltration and PLCε1 knockout ear tissue demonstrated less infiltration (Fig. 5*B*). In wild-type mice there are more infiltrating $CD3^+$ cells compared with PLCε1 knockout mice (Fig. 5*C*). Further, the infiltrating population is composed of both $CD8^+$ and $CD4^+$ cells (Fig. 5*D*). These results suggest that PLCε1 plays a role in inflammation and T-cell infiltration following DNFB challenge. We found no difference in adhesion of murine T cells from wild-type and PLCε1 knockout mice at baseline; however, T cells isolated from PLCε1 knockout mice were unresponsive to SDF-1α stimulation, whereas those isolated from wild-type mice exhibited a highly significant increased level of adhesion (Fig. 5*E*). These results confirm that PLCε1 knockout T cells are defective in SDF-1α–induced adhesion.

To confirm that PLCε1 plays a role in T-cell infiltration following challenge but not in T-cell activation and proliferation during the sensitization phase of the model, we isolated inguinal lymph nodes

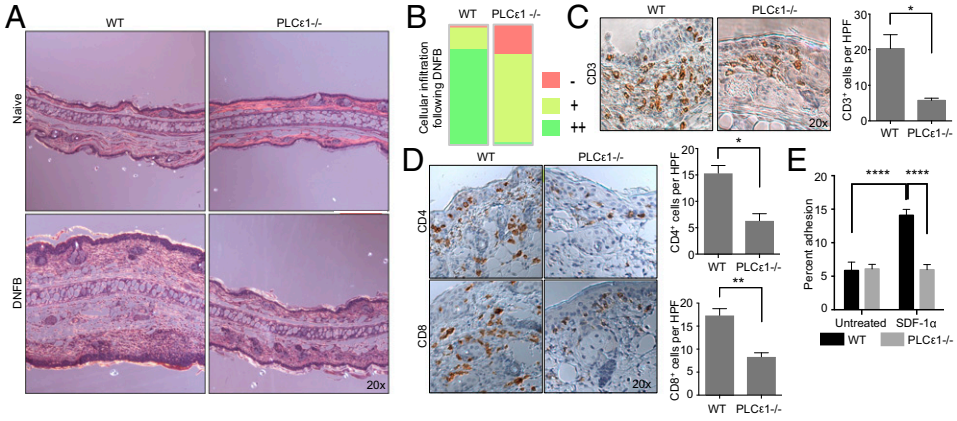

Fig. 5. PLCε1 is required for recruitment of sensitized T cells to site of inflammation. (*A*) Histology of the ears of mice DNFB challenge, collected on day 7; sections were stained with H&E and imaged. (*B*) Grading of mononuclear cell infiltration following DNFB; (–) no infiltration, (+) mild infiltration, (++) major infiltration. (*C* and *D*) Immunohistochemistry of tissue sections with anti-CD3, anti-CD4, or anti-CD8 antibodies were imaged. Quantification per high-power field (HPF) views. (*E*) Flow adhesion assay with CFSE-labeled T cells isolated from spleens. Quantification was performed using ImageJ.

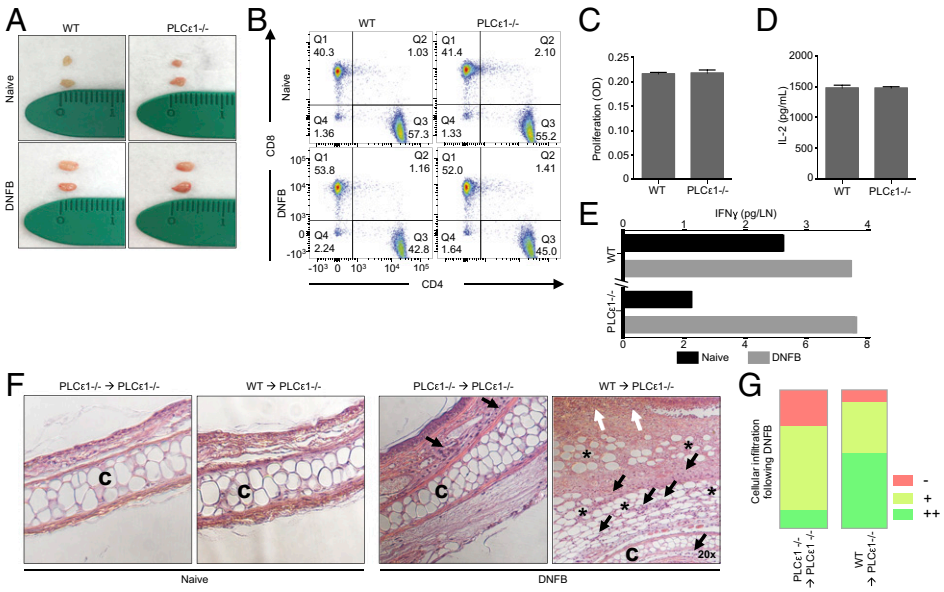

Fig. 6. PLCε1 does not contribute to T-cell sensitization and priming in the lymph node. (*A*) Macroscopic assessment of inguinal lymph nodes collected from naïve or DNFB-sensitized mice on day 5. Images are representative. (*B*) Flow cytometry of cells isolated from lymph nodes following staining for CD3, CD4, and CD8. Scatters show a gated $CD3^+$ population. (*C*) Cell titer proliferation assay on cells isolated from lymph nodes following stimulation with Con A (10 μg/mL) for 48 h. (*D*) Secreted IL-2 levels from cells isolated from lymph nodes following stimulation with Con A were quantified by ELISA. (*E*) Secreted IFN-γ levels from cells isolated from lymph nodes following coculture with DNBS-loaded primary antigen presenting cells for 48 h were quantified by ELISA. $*P \leq 0.05$; $**P \leq 0.01$. (*F*) Adoptive transfer of T cells from wild-type or PLCε1 knockout mice into PLCε1 knockout mice before sensitization. Ear histology of mice after treatments; sections were prepared and analyzed as in *B*. c, cartilage; white arrow, necrosis; black arrow, cellular infiltrate; asterisk, spongiosis necrosis. (*G*) Grading of mononuclear cell infiltration following DNFB; (–) no infiltration, (+) mild infiltration, and (++) major infiltration.

from DNFB naïve and sensitized mice. Inguinal lymph nodes from PLCε1 knockout and wild-type mice were comparably enlarged following sensitization (Fig. 6*A*), suggesting T-cell activation by APC and proliferation took place. Lymphocytes were isolated from these lymph nodes and stained for CD3, CD4, and CD8. Analysis shows that DNFB sensitization results in a shift toward $CD8^+$ T-cell expansion, and importantly, this increase in $CD8^+$ cells was observed in both wild-type and PLCε1 knockout lymphocytes (Fig. 6*B*) (16). These cells were comparably proliferative in response to concanavalin A (conA) (Fig. 6*C*). Likewise, the cells secreted equally high levels of IL-2 in response to conA (Fig. 6*D*). Antigen-specific T-cell response was assessed in vitro by loading primary murine antigen presenting cells with sodium 2,4-dinitrobenzenesulfonate (DNBS) and coculturing with T cells isolated from the inguinal lymph nodes of naïve or sensitized mice of both genotypes. Secreted IFNγ levels were increased comparably by DNFB sensitization (Fig. 6*E*). Together these findings provide evidence that T cells within the inguinal lymph nodes of both wild-type and PLCε1 knockout mice following DNFB sensitization are primed and activated.

Through adoptive transfer before sensitization we were able to assess the role of T cells in the observed inflammatory defect. The transfer of wild-type T cells into PLCε1 knockout mice followed by sensitization and challenge resulted in a rescue of ear swelling (Fig. 6*F*), including more extensive cellular infiltration (Fig. 6*G*), along with areas of spongiosis and necrosis. These results indicate that the role of PLCε1 in generating this response is restricted to the T-cell compartment. Of note, the transfer of PLCε1 knockout T cells into PLCε1 knockout mice followed by sensitization and challenge did not rescue ear swelling and cellular infiltration (Fig. 6 *F* and *G*), confirming that the increase in T-cell number is not responsible for generating the response. Collectively, these studies allow for the conclusion that PLCε1 does not play a role in the sensitization phase of contact sensitivity, but is necessary for lymphocyte trafficking and infiltration to site of challenge.

Discussion

In this work, we discovered a signaling pathway regulating T-cell adhesion downstream of SDF-1α/CXCR4. We showed that SDF-1α induced transient activation of Rap1 in comparison with the sustained activation following cross-linking the TCR. We identified that the GEF mediating SDF-1α/Rap1 activation was PLCε1. Further studies documented the contribution of the CDC25, PH, and RA domains of PLCε1, but not the enzyme's phospholipase activity, to its function in this particular signaling pathway (Fig. S5). In vivo studies highlighted the specific role of PLCε1 in T-cell recruitment to site of inflammation.

T-cell adhesion plays a critical role not just in normal immune system function but also in the pathogenesis of inflammatory and autoimmune diseases. Due to this phenomenon, adhesion molecules are promising targets for antiinflammatory therapies. Significant achievement has been accomplished with Efalizumab, a monoclonal LFA-1 antibody to treat psoriasis and in the treatment of Crohn's disease with Natalizumab, a monoclonal VLA-4 antibody (17). The adverse effects of these therapeutics often include issues of immune suppression due to the broad effects on T-cell adhesion. With this in mind it is of critical importance to better understand and leverage points of distinction between TCR and chemokine induced T-cell adhesion (3, 18, 19).

PLCε1 functions are not limited to cellular adhesion. PLCε1 has been characterized as a direct effector of Ras (13), and recent work has shown a tumor suppressor role for this protein in Ras-triggered cancers (20, 21). PLCε1 was also shown to modulate adrenergic receptor-dependent cardiac contraction and to inhibit cardiac hypertrophy (22, 23). Patients with focal segmental glomerulosclerosis have been found to have loss-of-function mutations in PLCε1, and PLCε1 itself interacts with transient receptor potential channel 6 (24–27). Even more intriguing is the fact that a genome-wide association study identified the susceptibility loci for dengue shock syndrome at PLCε1 (28). Whether mutations in PLCε1 are directly responsible for this clinical syndrome remains

unclear. The role of PLCε1 in skin inflammation has been questioned before; however, it was suggested that the effects of this enzyme were not mediated exclusively by $CD4^+$ cells and the involvement of other cell types, such as of fibroblasts and keratinocytes, has been suggested (29).

Our work is not free of limitations. Other investigators proposed that additional GEFs, such as C3G, are also important for chemokine-induced migration (8). Further, whether PLCε1 is required for the Rap1 activation downstream of other chemokines is not clear. We showed that Rap1 was activated in cells stimulated with the chemokine RANTES, but this activation failed to result in increase adhesion (Fig. S6). Based on our work we conclude that the GEF activity of PLCε1 is the principal contributing factor to T-cell adhesion. This finding leaves open two intriguing possibilities: that the lipase is activated but not contributing to the regulation of adhesion, or that the GEF and lipase activities of PLCε1 are independently regulated by an unknown mechanism. The lack of DAG accumulation and significant intracellular calcium increase suggests no phospholipase is active. Previous studies provide no evidence of distinct regulation mechanisms and in fact suggest that Rap1 engages in a feedback loop wherein activated Rap1 prolongs PLCε1 lipase activation through binding to the RA domain along with Ras (12, 14). Much of the work aimed at resolving mechanisms of PLCε1 regulation have been done in extracellular assays or in various cell types downstream of receptors with alternate GEFs responsible for Rap1 activation (12). The results presented herein provide a pathway to study PLCε1 regulation in which it is the sole GEF responsible for Rap1 activation, and the lipase activity is not contributing considerably to the resulting function. We believe that understanding the regulation of this unique dual enzyme is a critical next step in PLCε1 biology and targeting.

To summarize, we discovered a signaling pathway downstream of CXCR4 in T cells leading to Rap1 activation and increased adhesion. This phenotype was PLCε1 dependent and therefore targeting this enzyme should offer new approaches to treat inflammatory disease.

Materials and Methods

Cell Culture. Human T cells were isolated from peripheral blood of healthy donors (NY Blood Center). Murine T cells were isolated from spleens of 10- to 12-wk-old mice. Jurkat T cells, Jγ1 cells, HEK-293T, and CHO cells expressing ICAM-1 were obtained from American Type Culture Collection.

Generating PLCε1 T Cells. Individual GEF expression was knocked down in Jurkat T cells using Mission shRNA (Sigma). SMARTpool ON-TARGETplus PLCε1 and control siRNA were obtained from Dharmacon.

Adhesion Assays. Static and shear flow T-cell adhesion assays were performed as reported (1, 11).

Rap1/Ras Activation Assay. Activated Rap1 or Ras was detected by GST pull-down assay (30).

Mice. C57/Bl6 female mice, or PLCε1 knockout (23), at 6–12 wk of age were used. Animal studies were approved by the New York University institutional animal care and use committee.

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