PKCα Regulates T Cell Motility via Ezrin-Radixin-Moesin Localization to the Uropod

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Abstract

Cell motility is a fundamental process crucial for function in many cell types, including T cells. T cell motility is critical for T cell-mediated immune responses, including initiation, activation, and effector function. While many extracellular receptors and cytoskeletal regulators have been shown to control T cell migration, relatively few signaling mediators have been identified that can modulate T cell motility. In this study, we find a previously unknown role for PKCα in regulating T cell migration to lymph nodes. PKCα localizes to the migrating T cell uropod and regulates localization of the MTOC, CD43 and ERM proteins to the uropod. Furthermore, PKCα-deficient T cells are less responsive to chemokine induced migration and are defective in migration to lymph nodes. Our results reveal a novel role for PKCα in regulating T cell migration and demonstrate that PKCα signals downstream of CCR7 to regulate protein localization and uropod formation.

Introduction

T cells comprise the major effectors of immune responses: T cells assist B cells in antibody production and are critical to cell-mediated immune responses, including initiation, activation, and effector function. While many extracellular receptors and cytoskeletal regulators have been shown to control T cell migration, relatively few signaling mediators have been identified that can modulate T cell motility. In this study, we find a previously unknown role for PKCα in regulating T cell migration to lymph nodes. PKCα localizes to the migrating T cell uropod and regulates localization of the MTOC, CD43 and ERM proteins to the uropod. Furthermore, PKCα-deficient T cells are less responsive to chemokine induced migration and are defective in migration to lymph nodes. Our results reveal a novel role for PKCα in regulating T cell migration and demonstrate that PKCα signals downstream of CCR7 to regulate protein localization and uropod formation.

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null.
that PKC\(h\) may regulate T cell migration downstream of CCR7. CCR7 is required for T cell entry into lymph nodes via the high endothelial venules (HEV) as well as T cell motility within lymph nodes [5]. We determined whether T cell migration to lymph nodes was affected by the absence of PKC\(h\) using a competitive in vivo homing assay. We differentially labeled purified primary T cells from wild type or PKC\(h\)\(^{2/2}\) mice with fluorescent vital dyes CFSE or PKH26, then combined the two populations and adoptively transferred the combined WT and PKC\(h\)\(^{2/2}\) T cells into recipient mice expressing the congenic Ly5.1 marker. We allowed for migration into lymph nodes, then harvested the blood and lymph nodes of recipient mice and assessed the ratio of WT versus PKC\(h\)\(^{2/2}\) T cells in each organ by CFSE or PKH26 staining with anti-Ly5.2 (donor T cells) and anti-CD4 antibodies to identify transferred T cells using flow cytometry. We first identified the percentage of each cell population in both blood and lymph nodes (Fig. 2A). We also calculated the ratio of WT to PKC\(h\)\(^{2/2}\) T cells after we normalized the percentage of each population to the injected population (Fig. 2B, see materials and methods for more details).

We assessed migration at 1, 4, and 16 hours post transfer. We found that in the blood, the percentage of WT and PKC\(h\)\(^{2/2}\) T cells recovered was approximately 50%–50% for all time points observed, resulting in a ratio of close to 1 for WT: PKC\(h\)\(^{2/2}\) T cells at each time point assayed (Fig. 2B,C). Interestingly, we found that at 1 hour post injection, more PKC\(h\)\(^{2/2}\) T cells were found in lymph nodes than WT T cells. In contrast, at 4 and 16 hours, we saw fewer PKC\(h\)\(^{2/2}\) T cells in lymph nodes compared to WT T cells (Fig. 2C). At 1 hour, the ratio of WT: PKC\(h\)\(^{2/2}\) T cells was 1 for the blood and 0.8 for lymph node, indicating a 20% increase in PKC\(h\)\(^{2/2}\) T cells in the lymph node relative to WT T cells. However, at 4 hours and 16 hours, we saw approximately 10–20% increase in the number WT cells compared to PKC\(h\)\(^{2/2}\) T cells (Fig. 2C). We found a similar migration effect whether we assayed total T cells, CD4 or CD8 populations, or naïve CD62Lhi populations (data not shown).

Because in vivo migration to lymph nodes combines effects on multiple aspects of T cell motility, we wanted to determine whether PKC\(h\) had direct effects on T cell migration downstream of CCR7. To isolate CCR7 induced migration, we used an in vitro transwell assay. The transwell filter contains an upper

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**Figure 1. PKC\(h\) is activated by CCR7 signaling and shows specific localization within migrating T cells.** (A) WT T cells from C57Bl/6 or C57Bl/6 Ly5.1 mice were activated with 300 ng/ml CCL21 for 10 minutes, adhered onto Poly-L-lysine coated coverslips, fixed, and processed for immunofluorescence. Cells were stained with anti-\(\alpha\)-tubulin antibody (in red) to mark the uropod and anti-PKC\(h\) (green). The scale bar indicates 5 \(\mu\)m. Quantitation of percentage of migrating cells showing PKC\(h\) localization to the uropod is shown on the right. 3 experiments with at least 50 cells in each experiment were quantitated and the average shown. (B and C) Purified T cells were activated with either 50 ng/ml PMA and 500 ng/ml ionomycin (P+I) or 300 ng/ml CCL21 as marked for the indicated time points. Cells were lysed and analyzed on SDS–PAGE, transferred onto PVDF membranes and blotted with anti-actin and anti-phospho-PKC\(h\) S676 (B) or anti-phospho-PKC\(h\) T538 (C) antibodies. Signals were quantified using the Licor Odyssey and data shown is representative of at least 3 independent experiments. Fold increase in phosphorylated PKC\(h\) was normalized to the level in the unstimulated WT condition. (D) WT T cells were activated with 300 ng/ml CCL21, adhered onto Poly-L-lysine coated coverslips, fixed, and processed for immunofluorescence. Cells were stained with anti-tubulin antibody (in red) to mark the uropod, and also phospho-PKC\(h\) S676 (top), or phospho-PKC\(h\) T538 (bottom) (both in green). The scale bar indicates 5 \(\mu\)m. doi:10.1371/journal.pone.0078940.g001
PKCδ in T Cell Migration

PKCδ Effect on T Cell Motility within Lymph Nodes

In addition to migration into lymph nodes, CCL21-CCR7 signaling also regulates T cell migration within lymph nodes [5]. To determine whether PKCδ also has an effect on CCR7 induced intra-lymph node motility, we utilized 2-photon microscopy to visualize T cell migration in intact explanted lymph nodes. We isolated WT and PKCδ−/− T cells, labeling each population with either CFSE or CMTMR, then injected the mixed population into recipient animals. After 12–18 hours, we removed LNs from recipient mice, placed them in a chamber with 95% O2/5% CO2, then captured images of T cell movement within lymph nodes (Movie S1). We quantified T cell motility and found that PKCδ−/− T cells showed slightly slower speed of movement within lymph nodes compared to WT T cells (Fig. 3A). WT T cells moved at a mean speed of 9.03 μm/minute while PKCδ−/− T cells moved at 8.75 μm/minute. In addition, we analyzed the turning angles taken by WT and PKCδ−/− T cells but found no significant difference in the mean turning angle (WT T cells: 51.8°; PKCδ−/− T cells 52.4°) or the distribution of the angles taken by WT and PKCδ−/− T cell populations (Fig. 3B).

PKCδ Regulates Protein Localization to the Uropod and Uropod Length

To understand the mechanism that might underlie the effect of PKCδ on migration to lymph nodes, we determined the effect of PKCδ on uropod formation. Migrating T cell uropods have recently been shown to be crucial for migration into lymph nodes via transendothelial migration, likely through a role in force generation [6,30]. Uropod formation is regulated in part by specific cytoskeletal proteins that can control both uropod formation and migration [6]. The microtubule organizing center (MTOC), or centrosome, localizes to the T cell uropod to facilitate uropod retraction and T cell motility [27]. Other proteins, including the transmembrane protein CD43, also localize to the uropod [31,32]. To determine whether PKCδ might affect overall uropod protein localization, we assayed CD43 and MTOC polarization to the uropod in WT and PKCδ−/− T cells activated with CCL21. We found that in the absence of PKCδ, there was a significant decrease in the number of T cells showing MTOC polarization to the uropod.

Figure 2. PKCδ is required for T cell migration to lymph nodes.

T cells were isolated from C57Bl/6 or B6.PKCδ−/− mice and each population stained with different concentrations of CFSE (D), or 0.5 μM CFSE and 0.5 μM PKH26 (A,B,C), combined, and adoptively transferred into recipient C57Bl/6 Ly5.1 mice (A,B,C) or added to the top of a Costar 3 μm Transwell insert (D). (A,B) Cells were allowed to migrate for 4 hours, and the ratio of migrated CD4+ cells was analyzed using flow cytometry. Percentage of each population in each organ (A) or as a ratio (B) is shown. (B) Data shown are the average of 3 independent experiments with 6 mice each, each dot represents an individual mouse. Significance was determined by a paired student's t-test. (C) Adoptively transferred cells were allowed to migrate for 1, 4, and 16 hours, then blood and lymph nodes were harvested and percentage of cells in each organ analyzed by flow cytometry and the ratio of CD4+ WT: PKCδ−/− T cells calculated. Data are the average of 3 independent experiments and error is the SEM. Significance was determined using the unpaired student's t-test with * indicating p<0.05. The *** p = 0.0007 indicates a 2-way ANOVA analysis of the difference between the ratio of WT and PKCδ−/− T cells in the blood vs LN. (D) The bottom chamber which separates cells by pores of 3 μm size from the lower chamber which holds chemokines and adhesion ligands. We differentially labeled wild type and PKCδ-deficient T cells with different concentrations of the fluorescent dye CFSE, then combined the differentially labeled populations and added them in approximately equal ratio (1:1) to the upper chamber. We allowed the cells to migrate to CCL21, then analyzed the ratio of migrated cells in the bottom of the transwell compared with the ratio of input cells. In agreement with our hypothesis, PKCδ-deficient T cells showed approximately 2-fold defect in migration to CCL21 compared to wild type T cells (Fig. 2D). We also determined whether PKCδ might have effects on migration to LFA-1. We found that PKCδ−/− T cells migrated less to ICAM-1 compared with WT T cells (Fig. 2D). We found no additional defect when we combined CCL21 with ICAM-1 (data not shown). Our in vivo and in vitro migration data show that PKCδ can affect T cell migration directly in response to CCL21 via CCR7 signaling.

of the inserts contained 300 ng/ml CCL21 or coated with 6 μg/ml ICAM-1. Cells were allowed to migrate for 4 hours, and the ratio of migrated cells was analyzed using flow cytometry. Data shown are the average of 3 independent experiments and error is the SEM. Significance was determined using the unpaired student's t-test with p value shown. doi:10.1371/journal.pone.0078940.g002
PKC<sub>h</sub> regulates MTOC and CD43 localization to uropods. T cells were activated with 300 ng/ml CCL21, fixed, and processed for immunofluorescence. Cells were stained with anti-tubulin (A) in red or anti-CD43 (B). (A) MTOC polarization to the uropod was determined by the presence of the MTOC in the proximal third of the cell including the uropod. For quantitation, at least 50 cells showing T cell uropod morphology were counted for 3 independent experiments, totaling at least 150 cells. Error bars show SEM. Significance was determined using the unpaired student’s t-test.

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PKC<sub>h</sub> Affects ERM Protein Localization within Uropods

The Ezrin-radixin-moesin (ERM) family of actin regulatory proteins regulate membrane tension and have been hypothesized to be important in uropod formation [6]. ERM proteins do so by specific localization of proteins to the uropods of migrating T cells [33]. ERM proteins are also responsible for localizing transmembrane proteins including CD43 to this region [8,10]. PKC<sub>h</sub> has been shown to interact with and phosphorylate moesin [34], and as we found a defect in CD43 localization to the uropod, we hypothesized that one potential mechanism by which PKC<sub>h</sub> affects T cell uropod formation may be via effects on ERM localization. We asked whether ERM protein localization to the T cell uropod is perturbed in the absence of PKC<sub>h</sub>. We purified primary T cells from wild type and PKC<sub>h</sub><sup>−/−</sup> mice, treated the T cells with CCL21, then fixed and processed the migrating T cells for immunofluorescence. We detected ERM localization in T cell uropods by staining for the ERM family member moesin. In agreement with previously published results, we found that a majority of wild type migrating T cells localized moesin to the uropod (Fig. 6A). In contrast, the percentage of T cells showing moesin localization to the uropod was significantly decreased in PKC<sub>h</sub><sup>−/−</sup> T cells compared to WT T cells (Fig. 6A; WT: 55%; PKC<sub>h</sub><sup>−/−</sup>: 31%). Because PKC<sub>h</sub> has also been shown to phosphorylate moesin, we also determined the phosphorylation state of ERM proteins in the absence of PKC<sub>h</sub>. Using an antibody that detects phosphorylation of all members of the ERM family, total level of ERM phosphorylation was not significantly changed in T cells lacking PKC<sub>h</sub> in resting T cells (Fig. 6C). Upon treatment of T cells with CCL21, we found that WT cells showed a slight increase in total ERM phosphorylation while PKC<sub>h</sub><sup>−/−</sup> T cells showed a slightly smaller increase in ERM phosphorylation.
(Fig. 6D). We also observed cases in which PKC<sup>h</sup>2/2 T cells showed a slight decrease rather than increase in pERM (gel shown in Fig. 6D). While we saw differences between WT and PKC<sup>h</sup>-deficient T cells in phosphorylated ERM levels in response to CCL21, the differences were not statistically significant.

Phospho-ERM also localizes to the uropod, so we assessed phospho-ERM localization in migrating T cells. We found that in WT T cells, p-ERM localized to the uropod in a majority of migrating T cells similar to moesin (WT: 70% Fig. 6B). In contrast, PKC<sup>h</sup>2/2 T cells were unable to properly localize phosphorylated ERM proteins to the uropod (PKC<sup>h</sup>2/2: 32% Fig. 6B). These results show that PKC<sup>h</sup> specifically regulates ERM protein localization to the uropod and phosphorylation of ERM proteins at the uropod.

**Discussion**

T cell migration is a coordinated process beginning with extracellular chemokine signals leading to integrin activation and T cell motility. In this study, we identify a novel role for PKC<sup>h</sup> in regulating T cell motility. Our findings show for the first time that PKC<sup>h</sup> can be activated downstream of CCR7 signaling. We find that PKC<sup>h</sup> affects uropod length and protein localization to the uropod, specifically ERM proteins. As therapeutics targeting PKC<sup>h</sup> are being investigated as a treatment for autoimmune diseases, our results shed light on the additional

**Figure 5. PKC<sup>h</sup> regulates uropod length.** Isolated T cells were activated with 300 ng/ml CCL21 for 10 minutes and placed onto Poly-L-lysine coated coverslips and fixed. All measurements and quantitation were done using images captured in DIC. (A) Cells were scored as containing a “uropod” if the cell demonstrated the presence of a smaller portion of the cell extending from the main body of the cell. (B) Length of the uropod was determined by measuring the distance from the end of the “uropod” shape to the point where the uropod budded from the main body of the cell using Slidebook. Length (C) and width (D) of the full cell were calculated using Slidebook. The data are from at least 3 independent experiments with at least 50 cells counted from each experiment, totaling at least 150 cells. Significance was determined using the paired student’s t-test. doi:10.1371/journal.pone.0078940.g005

**Figure 6. PKC<sup>h</sup> is required for ERM phosphorylation and localization to the uropod.** T cells were activated with 300 ng/ml CCL21 for 10 minutes, fixed, and processed for immunofluorescence. Cells were stained for moesin (A) or phosphorylated ERM (B). Moesin and p-ERM localization in the uropod was determined measuring intensity in at least 2 spots in and out of the uropod and assessed as “polarized” if the signal in the uropod was at least 50 units or 2-fold higher than that seen outside the uropod. For quantitation, at least 50 cells showing T cell uropod morphology were counted and the localization of MTOC, moesin, or p-ERM assessed in 3 independent experiments, totaling at least 150 cells. Error bars show SEM. Significance was determined using the unpaired student’s t-test. Scale bar indicates 5 μm. (C,D) T cells from C57Bl/6 or C57Bl/6 Ly5.1 (WT) or B6.PKC<sup>h</sup>2/2 were not activated (C) or activated with 300 ng/ml CCL21 for the indicated time points (D). Cells were lysed and analyzed on SDS–PAGE, transferred onto PVDF membranes and blotted with anti-actin and anti-phospho-ERM antibodies. Signals were quantified using the Licor Odyssey and data shown is representative of at least 3 independent experiments. (C) Fold change in phosphorylated ERM in PKC<sup>h</sup> in T Cell Migration
impact that PKCδ may have on T cell migration in addition to the well-documented effects on T cell activation [35].

While many previous studies have implicated the PKC family proteins in the control of cell migration, including T cells, most of these studies used broad non-specific inhibition of PKC family proteins [25,36,37]. PKCδ and PKCδ have both been shown to play a role in regulating T cell shape during migration through effects on integrins [26,29]. Our study provides the first direct evidence for the specific role of PKCδ in regulating T cell motility in response to CCR7 using PKCδ-deficient T cells. We show that PKCδ is activated downstream of CCR7 as demonstrated by increased PKCδ phosphorylation (Fig. 1). While naive T cells express several chemokine receptors, including CXCR4 and CCR7, CCR7 is uniquely important in driving the homing of cells to lymph nodes, including naive T cells, dendritic cells, and cancer cells. In T cells and dendritic cells, CCR7 is absolutely required for lymph node trafficking, as defects in either CCR7 or its ligands, CCL21 and CCL19, abolish T cell migration [38,39]. In addition to our findings, other studies have also shown that PKCδ is also phosphorylated downstream of the chemokine CXCL12, or SDF1α [40]. Thus, PKCδ activation may be a general feature of chemokine induced motility in T cells.

We find that PKCδ plays a role in regulating in vivo migration of T cells to lymph nodes as well as within lymph nodes. Transendothelial migration requires activation of LFA-1 downstream of CCR7 signaling. In the absence of PKCδ, we saw a defect in homing to lymph nodes (Fig. 2). Our results showing that PKCδ−/− T cells migrate less to LFA-1 suggest that PKCδ acts to regulate entry of T cells into lymph nodes, possibly via LFA-1. In addition, we also find that PKCδ plays a role in intra-lymph node motility (Fig. 3). We saw a small, but statistically significant defect in PKCδ−/− T cell motility within lymph nodes. A previous study had commented that they observed no defect in naive PKCδ−/− T cell motility in lymph nodes [23], suggesting that the T cell motility defect in PKCδ−/− T cells is not easy to observe in vivo. The magnitude of the defect in PKCδ-deficient T cell migration in vivo reflects the likelihood that multiple PKC proteins including PKCδ, PKCδ, or other PKC family members act in concert to mediate normal T cell migration.

We show for the first time distinct localization of PKCδ and phosphorylated PKCδ within the migrating T cell uropod. Although several PKC family members are expressed in T cells, only PKCδ showed specific localization to the immunological synapse and is the only PKC known to be essential for IL-2 expression [21]. PKCδ function is tightly associated with its localization: PKCδ was the original marker for the immunological synapse (IS) [22]. PKCδ also moves away from the IS between a regulatory T cell and a target cell, suggesting that differential PKCδ localization may be important in controlling effector versus regulatory T cell function [24]. Our results add to the connection between PKCδ localization and function, demonstrating that specific localization of PKCδ within migrating T cells results in effects on T cell uropods and motility.

Our findings point to several potential mechanisms by which PKCδ may regulate T cell motility. We find that PKCδ affects MTG localization, which may control T cell retraction, resulting in motility [27]. Defects in MTG localization may be responsible for the shortening of uropods in PKCδ−/− T cells (Fig. 4, 5). We also find that PKCδ also regulates ERM and phospho-ERM localization to the uropod (Fig. 6). PKCδ has been previously shown to interact with ERM proteins [41,42], directly phosphorylating and activating moesin [34]. These studies were done with purified proteins and no direct evidence exists to demonstrate that PKCδ can directly affect ERM phosphorylation in cells. We now show that PKCδ-deficient T cells show a slight decrease in phosphorylated ERM proteins compared to WT cells (Fig. 6). While the difference in pERM levels between WT and PKCδ−/− T cells is not significant, our results suggest that PKCδ may affect total phosphorylation of ERM proteins as well as ERM localization. Our results differ from published results showing pERM levels decrease upon SDF1α stimulation in human PBMCs [43]. Instead of decreasing pERM levels upon SDF1α, we find that WT primary mouse T cells show a slight increase in pERM when stimulated with CCL21. These differences could reflect differences in human and mouse T cells, or signaling downstream of CXCR4 (binding SDF1α) and CCR7 (binding CCL21). Despite these differences, it is clear that chemokine receptor signaling can affect both total pERM levels as well as localization of ERM proteins. Recent evidence shows that ERM proteins directly control integrin function [44] and uropod formation [45] as well as motility within lymph nodes [46]. ERM proteins act on membrane tension in T cells, with constitutively phosphorylated ERM proteins increasing membrane tension [46]. We found that PKCδ−/− T cells showed slightly less ERM phosphorylation, as well as decreased localization of pERM to the uropod, suggesting that PKCδ may affect T cell motility via enhancing ERM phosphorylation and localization to uropods.

We also show PKCδ affects CD43 localization to the migrating T cell uropod (Fig. 4). This is likely to result from defects in ERM protein localization as we have previously shown that ERM proteins are required for CD43 localization [32,47]. We have also shown that PKCδ can phosphorylate the protein CD43 at a key serine that controls T cell migration, however, we find no defects in CD43 phosphorylation in PKCδ−/− T cells [47,48] (data not shown).

Several signaling pathways are important for chemokine induced T cell motility, including activation of Rho family GTPTes Rac1 and Rap1 via the Rac activator DOCK2 [14,49,50]. Another major pathway that regulates signaling from chemokine receptors to cell motility is the PI3K pathway. While the precise contribution of PI3K to neutrophil and T cell migration is still not completely understood [51,52,53], it remains to be determined whether PKCδ may intersect with these pathways. In response to TCR signaling, PKCδ has been shown to interact with Akt, the downstream effector of PI3K activation [54]. PKCδ also regulates Rap1 and LFA-1 upon TCR ligation and controls antigen induced migration [25,55]. Our data demonstrating that T338 and S676 are phosphorylated in response to CCR7 signaling are similar to that seen upon TCR activation [16,17]. These results suggest that while CCR7 signaling via PKCδ to T cell motility may share some pathways in common with TCR signaling, the effects of PKCδ downstream of CCR7 may be separate from the pathways that have already been identified downstream of TCR signaling.

Materials and Methods

Mice
C57BL/6 mice, B6.Ly5.1, and B6.PKCδ-deficient mice were from Jackson Laboratories (Bar Harbor, ME). All mice were bred and/or maintained in a specific pathogen-free condition in barrier facilities (Albuquerque, NM) and conform to the principles
outlined by the Animal Welfare Act and the National Institutes of Health guidelines. The protocol was approved by the IACUC at the University of New Mexico (protocol # 10-100407). All efforts were made to minimize suffering.

Reagents and Antibodies
Antibodies were purchased from the following: α-CD4 from eBiosciences (San Diego, CA); α-CD62L, and α-CD45.2 were from Biolegend (San Diego, CA); α-actin from Sigma Aldrich (St. Louis, MO); α-tubulin was from Thermo Fisher (Lab Vision, Fremont CA); α-PKC0, α-phospho-PKC0 S676 was from Santa Cruz Biotechnology (Santa Cruz, CA); α-moesin and α-phospho-PKC0 T538 were from Cell Signaling Technology (Beverly, MA); and α-CD43 antibody S11 was produced in the laboratory of Dr. Anne Sterling at the University of Chicago. CCL21 was from Peprotech (Rocky Hill, NJ), ICAM–Fc from R&D Systems (Minneapolis, MN), CFSE and Calcine-Are were from Invitrogen (Carlsbad, CA), and PKH26 from Sigma Aldrich (St. Louis, MO). For the Li-Cor Odyssey system, α-rabbit 680 conjugates were from Invitrogen, Molecular Probes (Carlsbad, CA) and α-rat 800 conjugates from Rockland Inc. (Gilbertsville, PA). Secondary fluorescently tagged antibodies for immunofluorescence were purchased from Jackson ImmunoResearch (West Grove, PA).

Immunofluorescence Staining and Microscopy
Primary murine T cells were purified by non-adherence to nylon wool, treated and then fixed for 20 min in 3% paraformaldehyde (PFA) in PBS, quenched with 50 mM NH4Cl/PBS, permeabilized for 1 min with 0.3% Triton-X100, and blocked with a PSG solution (PBS, 0.01% saponin, 0.25% NH4Cl/PBS, permeabilized for 1 min with 0.3% Triton-X100, and blocked with a PSG solution (PBS, 0.01% saponin, 0.25% aqueous cold fish gelatin, and 0.02% NaN3 [all from Sigma, St. Louis, MO]). Fixed cells were incubated with primary antibodies for 1 hour, washed 5 times with PSG, and incubated for 30 minutes with fluorochrome labeled secondary antibodies. Coverslips were washed 5 times with PSG, rinsed with ddH2O, and then mounted on slides with Prolong Gold (Invitrogen, Carlsbad, CA). Cells were visualized using a 63×DIC Oil objective on a Zeiss Axioplan 2 MOT upright LSM510 Confocal microscope. Images were obtained using the Zeiss LSM 510 Image Acquisition software and analyzed with the Zeiss LSM Image Browser.

Two Photon Imaging of Explanted Lymph Nodes
T cells were purified by nylon wool as previously described[32] and purified T cells labeled with either 1 μM CFSE (Invitrogen) or 5 μM CMTMR (Invitrogen). Both WT and PKC0−/− T cells were labeled with both CFSE and CMTMR to account for dye effects. 5 to 10×10^6 labeled T cells were injected i.v. into recipient mice and inguinal lymph nodes were removed 15–18 hours later and imaged using two-photon-imaging.

Imaging experiments were performed using a workstation with a Bio-Rad Radiance 2000 scanner mounted on an Olympus upright microscope with a chamber at 37°C. Explanted lymph nodes were placed on a glass cover-slip in the chamber. The sample is perfused with a 37°C solution of DMEM (phenol red free, Gibco) bubbled with 95% O2 and 5% CO2. T cell behavior within a lymph node was monitored in the T cell area at a minimum of 70 μm below the surface of the node. For 4D analysis of T cell motility, multiple stacks in the z axis (z step = 3 μm) were acquired every 15–20sec (depending on the number of z stacks acquired) for 15–40 min, with an overall field thickness of 40–60 μm. Cell motility was analyzed with Imaris software (version 6; Bitplane). Tracks that lasted fewer than 3 time steps (duration filter in Imaris) were not taken into account in the analysis. Length filter (threshold of 17  μm = 3 times the diameter of the cell) Displace-

Immunoblotting
Nylon non-adherent lymph node T cells were treated with either 50 ng/ml PMA and 500 ng/ml ionomycin or 300 ng/ml CCL21 for the indicated times, and lysed in lysis buffer (0.5% TX-100, 150 mM NaCl, 50 mM Tris pH 7.6, 5 mM EDTA, 1 mM NaF, 1 mM Na3VO4, and protease inhibitors: 10 μg/ml aprotinin, 1 mM Pefabloc [from Roche Applied Sciences, Mannheim, Germany], and 10 μg/ml leupeptin), before being analyzed by SDS-PAGE. All western blotting signal was detected with the Odyssey system (Li-Cor Biosciences, Lincoln, NE). Quantitation was done by drawing a region of interest around the protein of interest, and all levels of proteins of interest were normalized to the level of actin within the same sample through detection of both proteins simultaneously. Statistical significance in changes in phosphorylated PKC0 and ERM proteins was determined by comparing the levels of phosphorylation at each time point with the baseline phosphorylation using the paired student’s t-test.

In Vivo Migration
Competitive migration assays were performed as described[47]. Nylon wool non-adherent primary murine T cells were labeled with either 0.5 μM CFSE or 0.5 μM PKH-26. Differentially dyed populations were mixed in equal numbers, and 5–10×10^6 cells injected into recipient B6.Ly5.1 mice. Ly5.2 /CD45.2+ populations were gated and % CFSE and PKH26 populations determined and compared. Analyses were performed using Flowjo (Treestar Inc. Ashland OR).

Transwell Migration Assay
1×10^5 T cells were labeled with 5 μM CFSE or 0.25 μM CFSE, mixed, and added in a 1:1 ratio to the top of a Costar (Corning Acton, MA) 3.0 μm Transwell permeable support apparatus. For CCL21 conditions, 300 ng/ml CCL21 was added to the bottom of the transwell apparatus. For ICAM conditions, transwell apparatus was coated with 6 μg/ml ICAM overnight, washed in PBS, then blocked with 2.5% BSA for 2 hours, washed, and cells added to the top. At the end of the incubation period, transwell apparatuses and unmigrated cells were discarded, and migrated cells analyzed and normalized to the input population using the LSRII (BD Biosciences, San Jose, CA).

Statistical Analysis
All statistics except for data captured via 2-photon microscopy were done using an unpaired or paired Student two-tailed t test as indicated in figure legends. Error bars represent SEM. For intra lymph node cell motility data, mean speed of individual cells and turning angles taken at each time step were quantified. For statistical analysis, we used nested ANOVA and found that there was no statistically significant effect of the field of cells, the lymph node, the mouse, or the date of experiment performed. We also used nested ANOVA to statistically control the effect of the dye on each cell population. We used the ANOVA test to determine statistical difference in mean speed and turning angle in Figure 3 when controlling for the effect of the dye on WT and PKC0−/− T cell populations.

Supporting Information
Movie S1 In vivo motility of WT and PKC0−/− T cells in intact lymph nodes. WT and PKC0−/− T cells were
purified and stained with CFSE (WT) and CMTMR (PKCθ−/−), injected into recipient mice, and 12–18 hours later, inguinal lymph nodes from recipient animals were removed and imaged using 2-photon microscopy as described in the materials and methods.

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Author Contributions

Conceived and designed the experiments: FAB SRO MEM JLC KMB. Performed the experiments: IKB KEK FAB JLC KMB. Analyzed the data: IKB KEK FAB KAL KMB JLC. Wrote the paper: JLC.

References

1. von Andrian UH, Mempel TR (2003) Homing and cellular traffic in lymph nodes. Nat Rev Immunol 3: 867–878.
2. Gajewski TF (2007) Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res 13: 3256–3261.
3. Ley K, Ludewig C, Cybulsky MI, Nourshargh S (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol 7: 670–689.
4. Norman MU, Hickey MJ (2005) Mechanisms of lymphocyte migration in inflammatory disease. Tissue Antigens 66: 163–172.
5. Forster R, Davalos-Misulit AC, Rot A (2006) CCRI and its ligands: balancing immunity and tolerance. Nat Rev Immunol 8: 362–371.
6. Sanchez-Madrid F, Serrador JM (2008) CCR7 and its ligands: balancing the roles of chemokines. Eur J Immunol 38: 3149–3159.
7. Negulescu PA, Krasieva TB, Khan A, Kerschbaum HH, Cahalan MD (1996) Polarity of T cell shape, motility, and sensitivity to antigen. Immunity 4: 421–430.
8. Nieto M, Frade JM, Sanchez D, Mellado M, Martinez AC, et al. (1997) Polarization of chemokine receptors to the leading edge during lymphocyte chemotaxis. J Exp Med 186: 133–138.
9. Sanchez-Madrid F, Serrador JM (1996) Migration of T cells: polarization of chemokine receptors to the leading edge. Immunity 4: 421–430.
10. del Pozo MA, Sanchez-Mateos P, Nieto M, Sanchez-Madrid F (1995) Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of cAMP signaling pathway. J Cell Biol 131: 495–508.
11. Kumar A, Humphreys TD, Kofler K, Gruber T, Tabrizi NG, et al. (2003) PKC-θ activation participates in T cell migration without affecting cell motility, directed migration or uropod elongation. J Immunol 171: 213–224.
12. Forouzi M, Houm O, Zachacza D, Dumont C, Lyck R, et al. (2010) Critical roles for Rac GTPases in T cell migration to and within lymph nodes. Blood 116: 5536–5547.
13. Jacobelli J, Friedman RS, Coti MA, Lennon-Dumenil AM, Piel M, et al. (2010) Confinement-optimized three-dimensional T cell amoeboid motility is modulated via myosin IIα-regulated adhesions. Nat Immunol 11: 935–941.
14. Nombela-Arrieta C, Mempel TR, Soriano SF, Mazo I, Wymann MP, et al. (2000) A central role for PKCθ in the regulation of intracellular calcium concentration in primary T cells. J Mol Biol 302: 533–539.
15. Shahabi NA, Mcallen K, Sharp BM (2008) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent role of PKC-theta. J Leukoc Biol 83: 663–671.
16. Volkov Y, Long A, Kelleher D (2003) Crucial importance of PKC-θ in β2 integrin-mediated locomotion of activated T cells. Nat Immunol 4: 508–514.
17. Ratner S, Sherrod WS, Lichty D (1997) Microtubule retraction into the uropod and its role in T cell polarization and motility. J Immunol 159: 1063–1069.
18. Sun Z, Arendt CW, Ellmeier W, Schaeffer EM, Sunshine MJ, et al. (2000) PKCθ-dependent movement of CD43 from the T cell antigen-presenting cell contact site. J Immunol 161: 6459–6462.
19. Hyun YM, Sumagin R, Sarangi PP, Lomakina E, Overstreet MG, et al. (2012) Uropod elongation is a common final step in leukocyte extravasation through inflamed vessels. J Exp Med 209: 1349–1362.
20. Spirling AI, Sedy JR, Manjunath N, Kupfer A, Arthun B, et al. (1998) TCR signaling induces selective exclusion of CD43 from the T-cell antigen-presenting cell contact site. J Immunol 161: 6459–6462.
21. Allenpach EJ, Collin P, Tong J, Tang Q, Tesicuba AG, et al. (2001) ERMDependent movement of CD43 defines a novel protein complex distal to the intracellular signalling. Immunology 103: 739–740.
22. Lee JH, Kataki T, Hara T, Gonda H, Stagi M, et al. (2004) Roles of p-ERM and Rho-ROCK signaling in lymphocyte polarity and uropod formation. J Cell Biol 167: 327–337.
23. Pietromonaco SF, Simons PC, Altman A, Elia I (1998) Protein kinase C theta phosphorylation of moesin in the actin-binding sequence. J Biol Chem 273: 7594–7603.
24. Skara H, Davidi M, Klein E, Wolf B, Meisinger JG, et al. (2008) The PKC inhibitor AEB071 may be a therapeutic option for psoriasis. J Clin Invest 118: 3151–3159.
25. Southern C, Wilkinson PC, Thor KM, Henderson LK, Nemec M, et al. (1995) Inhibition of protein kinase C results in a switch from a non-motile to a motile phenotype in diverse human lymphocyte populations. Immunology 84: 326–332.
26. Thor KM, Verschueren H, De Baetselier P, Southern C, Matthews N (1998) Protein kinase C isotype expression and regulation of lymphoid cell motility. Immunology 87: 434–438.
27. Gunz MD, Kyuwa S, Tam G, Kakiuchi T, Matsuzawa A, et al. (1999) Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med 189: 451–460.
28. Worts T, Mempel TR, Boettjer J, von Andrian UH, Forster R (2007) CCR7 ligands stimulate the intranodal motility of T lymphocytes in vivo. J Exp Med 204: 409–495.
29. Shahabi NA, Mcallen K, Sharp BM (2007) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent: role of PKC-theta. J Leukoc Biol 81: 639–646.
30. Shahabi NA, Mcallen K, Sharp BM (2008) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent role of PKC-theta. J Leukoc Biol 83: 663–671.
31. Volkov Y, Long A, Kelleher D (2003) Crucial importance of PKC-θ in β2 integrin-mediated locomotion of activated T cells. Nat Immunol 4: 508–514.
32. Shahabi NA, Mcallen K, Sharp BM (2008) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent: role of PKC-theta. J Leukoc Biol 83: 663–671.
33. Volkov Y, Long A, Kelleher D (2003) Crucial importance of PKC-θ in β2 integrin-mediated locomotion of activated T cells. Nat Immunol 4: 508–514.
34. Shahabi NA, Mcallen K, Sharp BM (2008) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent role of PKC-theta. J Leukoc Biol 83: 663–671.
35. Volkov Y, Long A, Kelleher D (2003) Crucial importance of PKC-θ in β2 integrin-mediated locomotion of activated T cells. Nat Immunol 4: 508–514.
36. Shahabi NA, Mcallen K, Sharp BM (2008) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent: role of PKC-theta. J Leukoc Biol 83: 663–671.
37. Volkov Y, Long A, Kelleher D (2003) Crucial importance of PKC-θ in β2 integrin-mediated locomotion of activated T cells. Nat Immunol 4: 508–514.
38. Shahabi NA, Mcallen K, Sharp BM (2008) Stromal cell-derived factor 1-α (SDF1)-induced human T cell chemotaxis becomes phosphoinositide 3-kinase (PI3K)-independent role of PKC-theta. J Leukoc Biol 83: 663–671.
impedes endothelial transmigration of lymphocytes in vivo in mice. Blood 119: 445–453.

47. Cannon JL, Mody PD, Blaine KM, Chen EJ, Nelson AD, et al. (2011) CD43 interaction with ezrin-radixin-moesin (ERM) proteins regulates T-cell trafficking and CD43 phosphorylation. Mol Biol Cell 22: 954–963.

48. Mody PD, Cannon JL, Bandukwala HS, Blaine KM, Schilling AB, et al. (2007) Signaling through CD43 regulates CD4 T-cell trafficking. Blood 110: 2974–2982.

49. Fukui Y, Hashimoto O, Sannai T, Oosu T, Koga H, et al. (2001) Haematopoietic cell-specific CDM family protein DOCK2 is essential for lymphocyte migration. Nature 412: 826–831.

50. Duchniewicz M, Zemojtel T, Kolanczyk M, Grousmann S, Schelle JS, et al. (2006) Rap1A-deficient T and B cells show impaired integrin-mediated cell adhesion. Mol Cell Biol 26: 643–653.

51. Ward SG, Marelli-Berg FM (2009) Mechanisms of chemokine and antigen-dependent T-lymphocyte navigation. Biochem J 418: 13–27.

52. Ferguson GJ, Milne I, Kulkarni S, Sasaki T, Walker S, et al. (2007) PI3/PIgamma has an important context-dependent role in neutrophil chemokinesis. Nat Cell Biol 9: 86–91.

53. Nombela-Arrieta C, Lacalle RA, Montoya MC, Kunisaki Y, Megias D, et al. (2004) Differential requirements for DOCK2 and phosphoinositide-3-kinase gamma during T and B lymphocyte homing. Immunity 21: 429–441.

54. Bauer B, Krumbbeck N, Frese F, Hochholdinger F, Spitaler M, et al. (2001) Complex formation and cooperation of protein kinase C theta and Akt1/protein kinase B alpha in the NF-kappa B transactivation cascade in Jurkat T cells. J Biol Chem 276: 31627–31634.

55. Letschka T, Kollmann V, Pirhofer-Obermair C, Lutz-Nicoladoni C, Obermair GJ, et al. (2008) PKC-theta selectively controls the adhesion-stimulating molecule Rap1. Blood 112: 4617–4627.