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PLCβ is Critical for T-cell Chemotaxis

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Abstract

Chemokines acting through G-protein-coupled receptors play an essential role in the immune response. Phosphatidylinositol-3-kinase and phospholipase-C are distinct signaling molecules that have been proposed in the regulation of chemokine-mediated cell migration. Studies with knockout mice have demonstrated a critical role for PI3Kγ, but not PLCβ, in Gαi-coupled receptor-mediated neutrophil chemotaxis. We compared the chemotactic response of peripheral T-cells derived from wild type mice with mice containing loss-of-function mutations of either PI3Kγ, or both of the two predominant lymphocyte PLCβ isoforms (PLCβ2 and PLCβ3). Loss of PI3Kγ did not significantly impair T-cell migration, whereas loss of PLCβ2β3 did. PI3K pharmacologic inhibition suggests that an isoform other than PI3Kγ contributes to T-cell migration. Intracellular calcium chelation decreased the chemotactic response of wild type lymphocytes, which was not impaired by pharmacologic inhibition of PKC isoforms. SDF1α-induced calcium efflux was undetectable in PLCβ2β3-null lymphocytes suggesting that the migration defect is due to the impaired ability to increase intracellular calcium. This study demonstrates that, in contrast to neutrophils, phospholipid second messengers generated by PLCβ and isoforms of PI3K, other than PI3Kγ, play a critical role in T-lymphocyte chemotaxis.

Keywords

T-cells; chemotaxis; signal transduction

INTRODUCTION

Leukocyte traffic represents a key element in the regulation of the primary immune response. Secondary lymphoid organs (lymph nodes, spleen, tonsils, and Peyer's patches), are the sites where immune responses against foreign antigens are initiated. Many of the cues needed for the trafficking of lymphocytes toward antigen are provided by members of the chemokine family (1). Lymphocyte chemotaxis plays a central role in immune

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surveillance, acute immune defense, and chronic inflammation as evidenced by the increasing number of animal models highlighting chemokine-related immunological defects (2–5). Recent evidence indicates that the selectivity necessary to regulate cell traffic under homeostatic and inflammatory conditions is provided by a differential tissue distribution of chemokines and a regulated expression of chemokine receptors on varied leukocyte subsets (1).

Chemokines are chemotactic cytokines that signal through seven transmembrane receptors coupled to pertussis toxin-sensitive Gi-proteins (6). They are divided into two functional subfamilies: (a) homeostatic or lymphoid chemokines, which are constitutively produced at discrete locations in lymphoid and extra-lymphoid tissues and regulate basal traffic of resting B- and T-cells, and (b) inflammatory chemokines, which are produced locally during inflammation and recruit circulating effector leukocytes, including granulocytes, monocytes and effector T-cells (7). Examples of homeostatic chemokines are stromal derived factor-1 alpha (SDF1α; CXCL12), constitutively expressed by stromal cells in the bone marrow and secondary lymphoid organs and found on the endothelium of bone marrow microvessels (7, 8) and secondary lymphoid chemokine (SLC; CCL21), which is produced by endothelial cells of lymphatics and high endothelial venules (HEVs) and by stromal cells in the T-cell area of lymph nodes (9). SDF1α is one of the most extensively investigated chemokines, with regard to signal transduction mechanisms, and binds exclusively to its receptor, CXCR4. CXCR4 is uniformly expressed in all types of mature blood cells, including monocytes, granulocytes, T-and B-cells, and platelets (10, 11). Both the SDF1α and CXCR4 knockout mice die perinatally and their phenotypes show the same defects (12). Examples of inflammatory chemokines are CXCL9, CXCL10, CXCL11 which are ligands for the chemokine receptor CXCR3 that is expressed on effector T-cells after activation by antigen in lymph nodes. Several cell types such as endothelial, epithelial, and stromal cells, as well as leukocytes produce inflammatory chemokines. These cells express high levels of chemokines after exposure to inflammatory stimuli such as LPS, IL-1, and TNF-α (13).

Publications over the past several years have greatly advanced our understanding of the contribution of inflammatory chemokines in the control of inflammation and autoimmune diseases (14). By contrast, we are only recently beginning to appreciate the subtle network of homeostatic chemokines which orchestrates the basal, inflammation-unrelated leukocyte traffic. Furthermore, although it is well established that chemokine-induced signaling is mediated by G-protein-coupled cell surface receptors, the mechanisms underlying lymphocyte chemotaxis are largely unknown.

Two of the pathways that culminate in intracellular calcium fluxes, cytoskeletal reorganization, directional movement, and activation of specialized leukocyte functions depend on lipid-derived second messengers produced by phosphoinositide-3-kinase (PI3K) and phospholipase C (PLC) (15, 16). PI3K and PLC are widely expressed enzymes that modify membrane-bound the phosphoinositol, phosphatidylinositol-4, 5-bisphosphate (PIP2.) PI3Kγ phosphorylates PIP2 to generate the second messenger, phosphatidylinositol-3, 4, 5-trisphosphate (PIP3.) In turn, PIP3 has been demonstrated to play a vital role in actin organization, proliferation, and survival within a variety of different cell types (19), and has a clear role in neutrophil chemotaxis (20–22). PLC hydrolyzes PIP2 to produce inositol trisphosphate (IP3) and diacylglycerol (DAG) (17). IP3 leads to a rise in intracellular calcium, whereas DAG activates some isoforms of protein kinase C (PKC) (18). PLCβ2 and PLCβ3 are the most abundant isoforms of PLC in hematopoietic cells (23). Together, PLCβ2 and PLCβ3 are critical to chemoattractant-induced responses in neutrophils, such as calcium efflux, superoxide production and regulation of protein kinases, but are not required for chemotaxis of these cells (20).

In this paper, we analyzed the relative contribution of PI3Kγ and PLCβ-synthesized second messengers in T-cell migration. This was accomplished using peripheral node lymphocytes derived from genetically altered mice lacking either of these enzymes (20), in conjunction with a variety of pharmacologic inhibitors. We find that (a) in contrast to granulocytes, PI3Kγ is not necessary for the early signaling events of T-lymphocyte chemotaxis, and (b) PLCβ is fundamental for SDF1α-induced T-cell chemotaxis which appears to be dependent upon the generation of IP3 and the resultant increase of intracellular calcium, but independent of PKC activity or actin polymerization.

MATERIALS and METHODS

Materials

Cell culture media and supplements were obtained from Invitrogen Life Technologies (Carlsbad, CA). SDF1α was obtained from Sigma-Aldrich (St. Louis, MO). The phosphatidylinositol kinase inhibitors (wortmannin and LY294002), the calcium chelators (BAPTA-AM and Quin-2 AM), the PKC inhibitor (GF109203x), and 7-amino-actinomycin D (7-AAD) were obtained from EMD Biosciences (San Diego, CA). Alexa 633 phalloidin, Fura-2 AM, and Cell Tracker™ were obtained from Molecular Probes (Eugene, OR). Fluorescence-conjugated antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA). The phospho-PKCα, -βI, -βII, -γ, -δ, and −ε (pan-PKC) antibody was purchased from Cell Signaling Technology (Beverly, MA). PLCβ2β3 and PI3Kγ knockout mouse lines were previously described (20).

T-cell isolation, activation, and immunoblotting

Peripheral inguinal, axillary, and cervical lymph nodes were removed from donor mice and pressed through a cell strainer to generate single cell suspensions. T-cells were isolated using a negative-selection magnetic-sorting approach. In brief, single cell suspensions were incubated with anti-B220-conjugated Dynabeads (Dynal Biotech, Brown Deer, WI) for 30 minutes at 4°C, and B-cells were removed utilizing a Bio-Mag magnetic stand (Polysciences, Warrington, PA). T-cell purity was generally greater than 98%. T cells were activated with 5 nM PMA and 0.1 μM ionomycin. For immunoblotting, the cells were lysed in boiling 1% SDS with β-mercaptoethanol, normalized for their protein concentration, fractionated by SDS-PAGE, and immunoblotted with anti-phospho-PKC (pan) antibody.

Flow cytometry

Expression of T- and B-cell receptors (CD4, CD8, CD3, CD45R/B220, CD25, CD69, CD44, CD62L, CD45RB, CXCR4) on peripheral lymphocytes was determined by direct immunofluorescence. Lymphocytes were incubated with murine fluorescence-conjugated monoclonal anibodies in PBS, containing 2% FBS, for 30 min at 37 °C and washed three times with PBS. Cell-bound fluorescence was determined in a FACSCalibur® flow cytometer (Becton Dickinson, Mountain View, CA) using the CellQuest software.

Chemotaxis

Assays for T-lymphocyte chemotaxis were performed in polycarbonate membrane (6.5-mm diameter, 10-μm thickness, 5-μm-diameter pore size) Transwell cell culture chambers (Costar Corp., Cambridge, MA). Cells (100 μl at 2×10^6/ml) suspended in RPMI 1640, containing 0.5% BSA (fraction V; Sigma) and 25 mM Hepes, were added to the upper chamber; SDF1α at 300 ng/ml was added to the lower chamber. Pharmacologic inhibitors, calcium chelators, or DMSO (<1%) were pre-incubated with the cells 15–30 minutes prior to their addition to the upper Transwell chamber. Cells were allowed to migrate for 3 hours at 37 °C in 5% CO_2 atmosphere. Cell migration was quantified by collecting T-cells in both

the upper and lower chambers and counting by a FACSCalibur® flow cytometer. Filters were removed and at least ten fields of cells adherent to the bottom part of the polycarbonate membrane were counted. Visual inspection of the membranes verified that few lymphocytes were adherent to the filter. Percent migration was determined by the quantity of cells collected from the lower chamber divided by the sum of the number of cells collected in lower and upper chambers, multiplied by 100. Viability was verified by staining with 7-AAD and trypan blue. All conditions were tested in duplicate.

PKC phosphorylation assay and immunoblotting

To determine whether SDF1α impacted PKC activation, and if this activation could be inhibited with GF109203x, immunoblotting was performed using a phospho-PKC antibody to isoforms α, -βI, -βII, -γ, -δ, and −ε. T-lymphocytes were preincubated for 15 minutes at 25 °C with or without 5 μM GF109203x, and then stimulated with 300 ng/ml SDF1α for one minute. PMA at 160 nM for 5 minutes was used as a positive control of PKC activation. Immunoblotting was performed as described above with a phospho-PKC (pan) antibody.

Measurement of cytoplasmic calcium concentration

Purified T-cells were suspended in RPMI containing 1% FBS at a concentration of 1×10^7/ml. After loading with 5 μM Fura-2 AM for 40 min at 37°C in the dark, T-cells were sedimented at 833 × g for 15 minutes at room temperature, and the pellet resuspended in RPMI containing 0.5% BSA at a final cell concentration of 1×10^6/ml. Aliquots of Fura-2-loaded T-cells were transferred to a 10 × 10 mm cuvette and prewarmed to 37°C. The cells were stimulated with 300 ng/ml SDF1α. Subsequent measurements of Fura-2 fluorescence were performed under continuous stirring using an SLM/Aminco model AB2 fluorescence spectrophotometer with excitation at dual wavelengths of 340 nm and 380 nm, and emission spectra measured at 510 nm.

Actin assembly

Cells were suspended in RPMI 1640 with 0.5% BSA in a concentration of 1×10^6 cells/mL. SDF1α was added to the cell suspension (150 ng/ml) and at indicated time points, 100 μL of cell suspension was transferred to 100 μL of fixation solution (4% paraformaldehyde). Cells were incubated in the fixation solution for at least 15 minutes. Thereafter, the cells were centrifuged and resuspended in 100 μL of permeabilization reagent (0.1% Triton-X 100) for 10 minutes. Alexa 633 phalloidin (0.5 μM) was added to the cell suspension for 30 minutes. Mean fluorescence intensity was measured by a FACSCalibur® flow cytometer. All conditions were tested in duplicate.

RESULTS

PI3Kγ is not essential for T-cell migration

Studies of murine granulocytes containing a loss-of-function mutation within PI3Kγ, or studies with pharmacologic inhibition of all PI3K isoforms, have demonstrated an essential role for D3-phosphoinositides in neutrophil chemotaxis (21, 22, 24, 25). To begin to determine whether PI3Kγ signals are required for chemokine-directed T-lymphocyte migration, murine peripheral node lymphocytes were isolated from wild type mice and mice containing a null mutation for PI3Kγ. Using a Transwell assay, migration of lymphocytes toward SDF1α was quantitated after 3 hours, the time point at which migration was maximal for both wild type and knockout T-cells (data not shown). Over this period we found that T-cells isolated from wild type mice exhibited a twenty-fold increase in migration with SDF1α stimulation compared to baseline. During this same time period, PI3Kγ-deficient T-cells migrated by only 85% ± 9% as efficiently as wild type T-cells, a difference which does not

reach statistical significance (p=0.11) (Figure 1A). These results demonstrate that, in contrast to the findings with neutrophils, PI3Kγ does not significantly contribute to T-lymphocyte migration in response to SDF1α.

Distinct isoforms of PI3K have been shown to be critical for chemotaxis depending upon the cell type studied (20, 21, 26–28). We next evaluated the role of other PI3K isozymes in SDF1α-mediated T-cell migration using the isoform non-selective PI3K inhibitors, wortmanin and LY294002 (Figure 1B). Both inhibitors impaired SDF1α-induced chemotaxis of wild type and PI3Kγ-null T-cells ($p<0.05$), while no impairment was seen with DMSO used as a carrier control (data not shown). This demonstrates that although PI3Kγ is not essential for SDF1α-mediated migration of murine primary T-cells, other PI3K isoforms contribute significantly to this process.

PLCβ2β3-null lymphocytes are phenotypically similar to wild type lymphocytes

In contrast to PI3Kγ-null mice, mice lacking PLCβ2 and PLCβ3 develop spontaneous multifocal skin ulcers starting around 6 months of age. Histological examination of the lesions revealed hyperinfiltration of leukocytes in the tissues shown in Figure 2A. The dermis is characterized by a mixed inflammatory infiltrate, noticeably abundant in neutrophils and lacking in lymphocytes. This ulcerative phenotype is consistently and specifically observed in the PLCβ2β3-null mice (20). No ulcerative lesions were observed in wild-type mice that were housed in the same rooms under the same conditions (Figure 2B). In view of this ulcerative phenotype, our studies to evaluate resting T-cell migration have been performed with mice younger than 6 months, prior to the development of skin ulceration. At this age, no gross phenotypic differences are seen between the PLCβ2β3-null and wild type mice.

Peripheral lymph nodes from wild type and PLCβ2β3-null mice were of comparable size and produced similar total numbers of lymphocytes ranging from 15–20 million cells per mouse. As shown in Figure 3, A and D, the ratios of CD3+ and B220+ lymphocytes were similar between wild type and PLCβ2β3-null mice ($p>0.1$). Furthermore, markers of T-cell activation (CD25 and CD69), were comparable in both wild type and PLCβ2β3-null mice ($p>0.05$) as shown in Figure 3, B–D. Additionally, the ratio of CD4+ to CD8+ cells, and the percentages of the naïve and memory T-cell markers, CD44, CD45RB, and CD62L, were the same between wild type and PLCβ2β3-null lymphocytes ($p>0.05$) as shown in Figure 3E. These data demonstrate that the lymphocytes from wild type and PLCβ2β3-null mice are similar with respect to their subtype distribution and level of activation, and appear to display a predominantly naive phenotype.

Loss of function mutations in both PLCβ2 and PLCβ3 impair T-cell migration to SDF1α

Granulocytes from mice lacking the two dominant isoforms of PLCβ, PLCβ2 and PLCβ3, migrate at least as well as wild type cells in response to fMLP or MIP-1α (20). To determine whether migration of T-cells was dependent on the PLCβ isoform, we analyzed T-cell migration in response to the CXCR4 ligand, SDF1α. T-lymphocytes extracted from wild type mice or mice lacking PLCβ2β3 demonstrate similar surface expression of CXCR4, the SDF1α receptor (not shown). Using a Transwell assay, migration of lymphocytes toward SDF1α was quantitated after 3 hours (the time demonstrated by previous studies to be optimal for migration of both WT and PLCβ2β3-null T-cells). We found that T-cells isolated from wild type mice exhibited a twenty-fold increase in migration with SDF1α stimulation compared to baseline. An overlap at the maximal response concentration of 300 ng/ml was determined by a titrated dose response between wild type and PLCβ2β3-null T-cells ($p<0.05$) (Figure 4A). Interestingly, loss of PLCβ2β3 decreased chemokine-stimulated migration over three hours in T-cells by 60% ± 7% ($p<0.0001$) (Figure 4B). These results

demonstrate that, in contrast to the findings with neutrophils, loss of both PLCβ2 and PLCβ3 significantly impairs the migration of T-cells.

SDF1α -induced T-cell migration is largely chemotactic

Chemokinesis is the process of random cell migration, while chemotaxis is defined as directional cell migration in response to a gradient of chemoattractant. To evaluate whether the results demonstrated in Figure 4B were due to an effect on chemokinesis or chemotaxis, we examined the effect of PLCβ on chemokinesis, when the same concentration of chemoattractant was placed above and below the filter. If the migration of the T-cells between the two Transwell chambers is due solely to chemotaxis, elimination of the chemoattractant gradient should ablate this migration. However, if the cell migration is due to chemokinesis, the cell migration should be unaffected by elimination of the chemoattractant gradient. The results shown in Figure 4C demonstrate that most of the wild type T-cell migration depends on the gradient, and only a small component of their movement is due to random migration. The migration of PLCβ2β3-null T-cells was too small to accurately determine whether it was predominantly chemotactic or chemokinetic.

SDF1α -induced T-cell migration through PLCβ2β3 is calcium-dependent

Hydrolysis of PIP2 by PLCβ liberates IP3 which contributes to signaling required for release of cytoplasmic calcium stores, and DAG which binds to and activates both classical and novel isoforms of PKC. We investigated whether one or both second messengers generated by PLCβ contributed to T-cell migration. Pharmacologic chelation of intracellular calcium release by BAPTA-AM ($p<0.05$) and Quin-2 AM ($p<0.05$) eliminated the chemotactic response of wild type lymphocytes suggesting that the release of IP3 from PIP2 is critical for T-lymphocyte migration ($p>0.05$)(Figure 5A). No impairment was seen with DMSO used as a carrier control (data not shown). Consistent with these studies, we found that SDF1α-induced calcium efflux was deficient in the PLCβ2β3-null T-cells when compared to those of wild type (Figure 5B). Together, these data suggest that the T-cell migration defect seen in the PLCβ2β3 knockout T-cells is likely due to an impaired ability to increase intracellular calcium.

To evaluate the contribution of PKC to T-cell chemotaxis, T-cells were pre-incubated with a pharmacologic inhibitor of PKC, GF109203x. Using doses of GF109203x that completely eliminated phosphorylation of the PKC activation loop (Figure 5C), we did not find any impairment of T-cell chemotaxis (Figure 5A). This latter observation demonstrates that the impaired migration observed in the PLCβ2β3-null T-cells cannot be attributed to defective activation of PKC. In fact, T-cell migration was enhanced in the presence of PKC inhibition, perhaps due to the known ability of PKC to promote cell adhesion (29).

PLCβ2β3 T-cells assemble actin normally in response to SDF1α

Chemotaxis occurs through the alignment of cell polarity along a gradient of chemoattractant. Migrating granulocytes and lymphocytes develop polarity with the leading edge having F-actin rich lamellipodia and the trailing edge having a uropod rich in F-actin and actin binding proteins, including moesin, ICAM-3 and myosin (30). We measured the SDF1α-induced increase in F-actin in PLCβ2β3 knockout mice and compared this to the increase in wild type mice. As shown in Figure 6, basal amounts of F-actin were higher in the PLCβ2β3-null T-cells compared to control ($p<0.05$). However, upon SDF1α stimulation, both PLCβ2β3-null and wild type T-cells increased F-actin from baseline upon stimulation at 15 seconds. Furthermore, there was no statistically significant difference between PLCβ2β3-null and wild type T-cell F-actin at peak stimulation ($p=0.21$). This suggests that, similar to neutrophils, signals generated by PLCβ are not required for chemokine-induced actin polymerization.

DISCUSSION

T-cell migration is a process essential to the normal physiology of the immune system and contributes to lymphocyte recruitment in pathologic processes, such as rheumatoids arthritis, psoriasis, and multiple sclerosis (31). Growing evidence indicates that chemokines and their receptors control the recruitment and positioning of leukocytes as well as their organization into node-like lymphoid structures. Homing chemokines, such as SDF1α, are constitutively expressed in discrete areas within lymphoid and non-lymphoid tissues, and control the physiological traffic and homing of leukocytes (6). Our experiments indicate that both PLCβ2β3 and PI3K are required for SDF1α-initiated signals in murine T-lymphocytes. These enzymes have previously been shown to contribute to several aspects of lymphocyte function including (a) cell proliferation, (b) rescue from apoptosis, and (c) CD4+ and CD8+ T-cell differentiation (22, 32). Thus, PLC and PI3K play a role in both early and late signaling events in T-cells. This study highlights the important differences between the activation of lymphocytes and neutrophils by chemokines, in terms of receptor regulation and signaling cascades.

PI3K has been shown to be critical for chemotaxis of cells in general, but the particular isoform required appears to depend on the specific cell type. Neutrophils undergoing chemotaxis require PI3Kγ (20, 21, 26), and monocytes use both PI3Kβ and PI3Kδ for chemotaxis (27). Our findings with pharmacologic inhibitors of PI3K have corroborated reports (33–35) that PIP3 production is required for T-cell migration. However, in this study we have demonstrated that PI3Kγ is not essential in the process of SDF1α-induced T-cell chemotaxis, demonstrating that another isoform of PI3K plays a role in this process.

Mice deficient in PLCβ2 and PLCβ3 have neutrophils that migrate efficiently in response to chemokine, but are defective in their ability to produce superoxide (20). Our work demonstrates that T-lymphocytes deficient in PLCβ2 and PLCβ3 have impaired migration in response to chemokines. Our findings are consistent with a recent publication by Smit *et al* that showed a sensitivity of T-cell migration through an inflammatory chemokine receptor, CXCR3, using a relatively non-specific inhibitor of PLC (33). These results taken together with our data demonstrate an important role for PLC in T-cell migration in response to homeostatic and inflammatory stimuli.

Our studies have focused on the migration of resting T-cells in response to the homeostatic chemokine, SDF1α, which has been demonstrated to play a role in T-cell lymph node homing and chemotaxis. Notably, Smit *et al* suggested a role for PLC in T-cell homing to sites of inflammation as well (33). The PLCβ2β3-null mice develop an ulcerative dermatitis associated with hyperinfiltration of leukocytes into the affected areas (Figure 2) and reference (20). This is curiously similar to the severe dermatitis and conjunctivitis seen in E- and P-Selectin deficient mice, a process probably dependent upon T-cell migration (36). Though the causative trigger of ulcerative dermatitis in the PLCβ2β3-null mice is not known, we speculate that the combination of impaired lymphocyte trafficking and defective neutrophilic generation of reactive oxygen intermediates is central to the ulcerative phenotype exhibited by these mice. We will attempt to further investigate these mechanisms that perhaps link a dysregulated immune response to the dermatologic findings in the PLCβ2β3-null mice.

In this paper we have begun to elucidate the mechanism by which PLCβ contributes to chemotaxis. The T-cell chemotactic defect in the PLCβ2β3-null mice can be mimicked *in vitro* by chelation of cytoplasmic calcium, but not by inhibition of PKC. This suggests that PLCβ contributes to chemotaxis by transiently raising cytoplasmic calcium concentrations. It is not known at this point whether oscillations of cytoplasmic calcium concentration are

important, and it remains to be determined which calcium-dependent signaling moieties of PLC are responsible for the control of the chemotactic process. Due to their known contribution to cytoskeletal dynamics (37) calmodulin kinase, myosin light-chain kinase, SHIP-1, gelsolin, and calpain are some of the potential calcium-dependent candidates currently under investigation.

The regulation of actin polymerization/depolymerization is another important component of cellular motility. The literature suggests that development of the cell polarity in *Dictyostelium* depends on actin polymerization (38, 39). In *Dictyostelium* and neutrophils, chemoattractant stimulation results in a transient accumulation of PIP3 binding proteins at the plasma membrane at the leading edge (26, 40–42). Until recently, its role in the cytoskeletal organization of lymphocytes has been less intensively studied (19). Precisely how PI3Ks regulate chemotaxis and organize the actin cytoskeleton is not known; however, it is likely to involve a polar distribution of PI3K lipid products after cell stimulation. Additional data argue that tyrosine kinases participate in chemokine-induced Rac activation (43–45). We have found that although PLCβ2β3 knockout cells have higher basal levels of F-actin, they are still able to assemble F-actin in response to chemokine, despite their defect in chemotaxis.

In summary, we have found fundamental differences between the signaling pathways required for T-cell chemotaxis and those responsible for the cell migration of the more commonly studied granulocytes. These dissimilarities involve the alternative requirements for PLCβ-, or PI3Kγ-, generated second messengers (Figure 7). Neutrophils, and most other migrating leukocytes, are recruited to a wide variety of extravascular tissues through their ability to sense and polarize toward distinct chemokine gradients. T-cells also directionally migrate toward established chemokine gradients, but this process occurs in more specialized regions, such as seen in peripheral lymph nodes. It is thus conceivable that second messengers generated by PLCβ are uniquely suited for T-cell microenvironmental localization within secondary lymphoid organs.

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Abbreviations

SDF1α	stromal derived factor-1 alpha
SLC	secondary lymphoid chemokine
HEVs	high endothelial venules
PLC	phospholipase C
PI3K	phosphoinositide-3-kinase
PIP2	phosphatidylinositol-4, 5-bisphosphate
IP3	inositol trisphosphate
DAG	diacylglycerol

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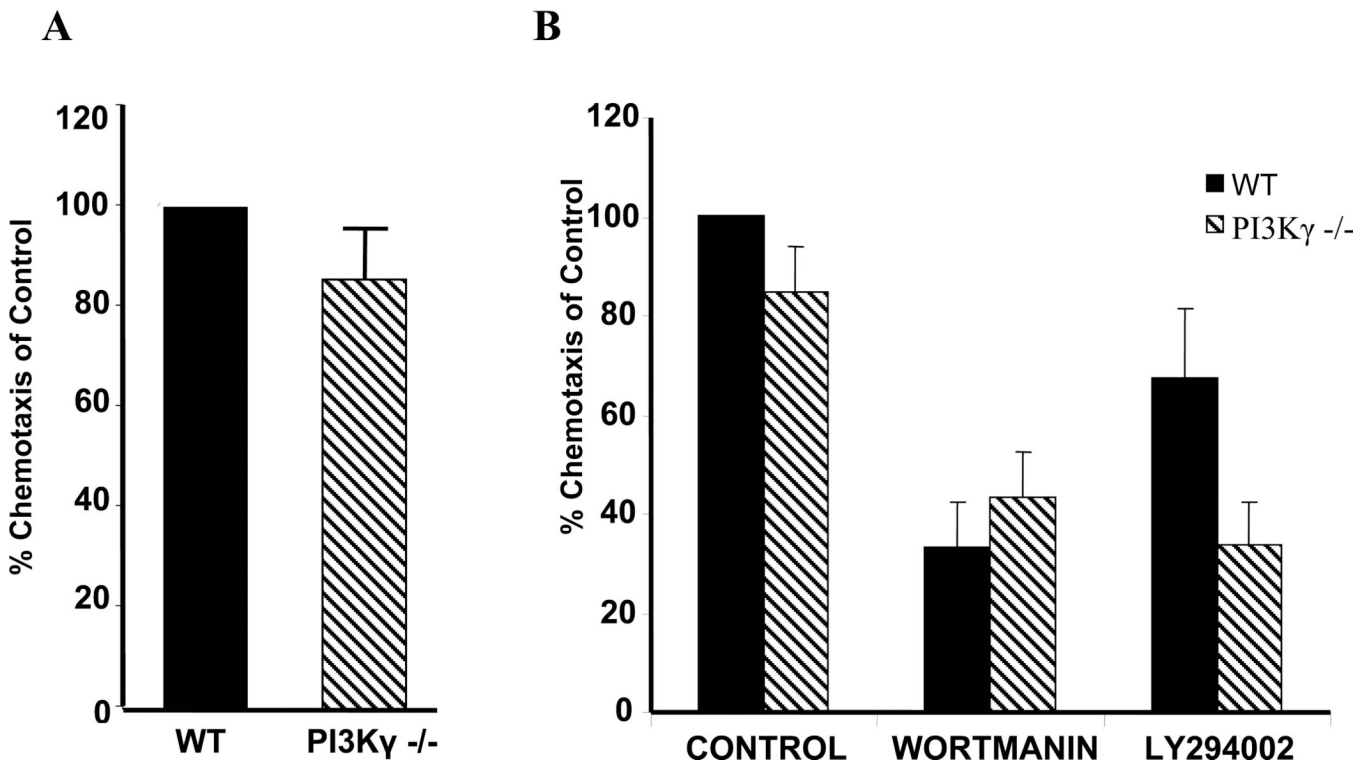

Figure 1. PI3Kγ is not essential for T-cell migration

(A) Peripheral T-lymphocytes were isolated from lymph nodes of wild type (WT) and PI3Kγ-null (PI3Kγ −/− mice). Cells were placed into the upper Transwell chamber and 300 ng/ml SDF1α was placed into the lower chamber. The T-cells that migrated toward SDF1α after 3 hours at 37°C, were quantitated by flow cytometry. The number of migrating T-lymphocytes were expressed as the percent migration of the WT cells. The mean, S.E, and paired student's t-test reflect data from 6 separate experiments. **(B)** The role of PI3K in SDF1α-mediated T-cell migration was studied using the PI3K inhibitors wortmanin and LY294002. WT and PI3Kγ −/− T-cells were isolated and placed in a Transwell chamber as described in part A. Some of the cells were pre-incubated for 10–15 minutes at 25 °C with the PI3K inhibitors wortmanin (100 nM) or LY294002 (10 μM). The cells were exposed to 300 ng/ml SDF1α, placed below the filter, for 3 hours at 37 °C. The migrating T-lymphocytes were expressed as the percent chemotaxis of the wild type control cells. The mean and S.E. reflect data from 5 separate experiments.

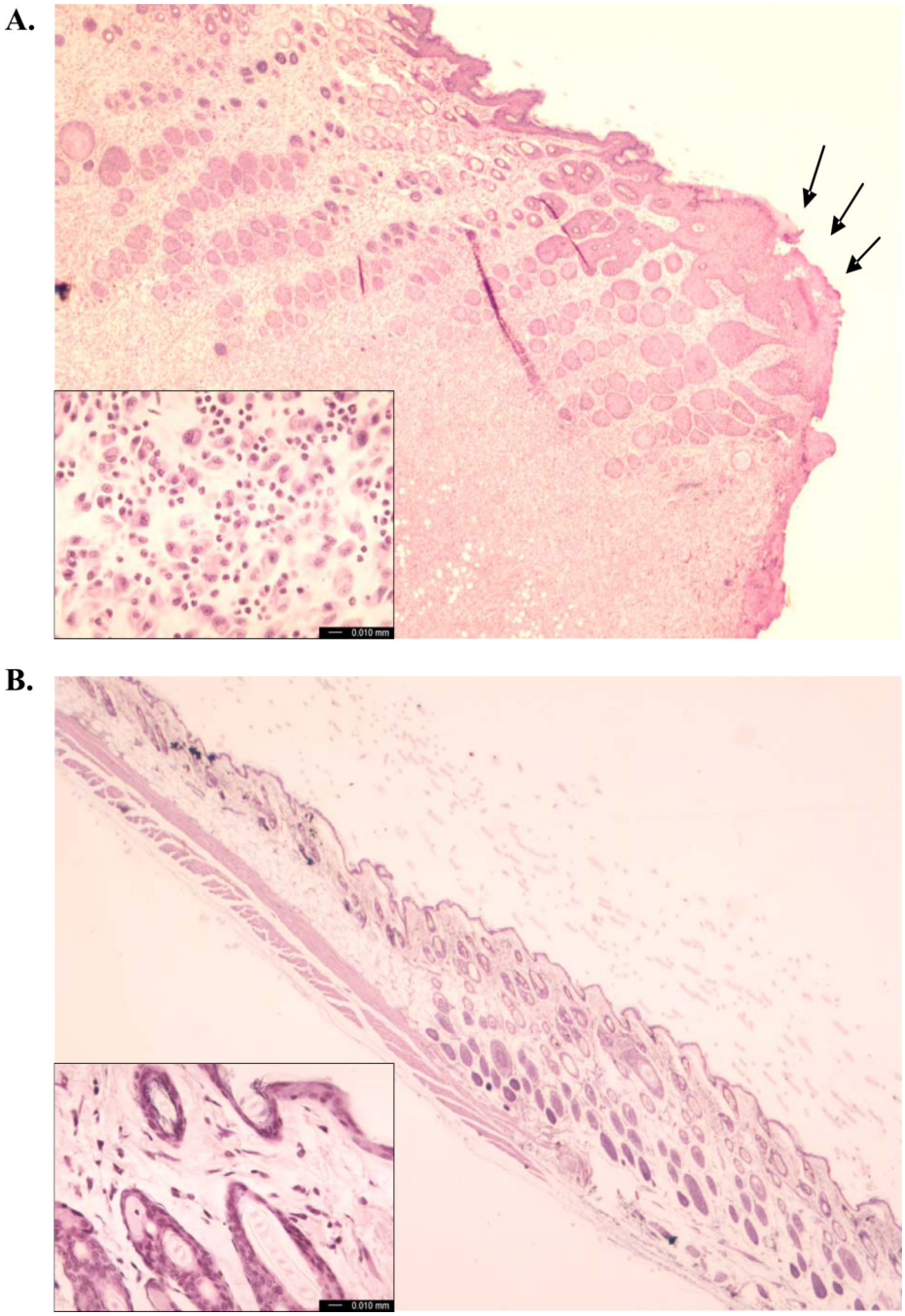

Figure 2. Inflamed skin from PLCβ2β3-null mice demonstrate increased leukocyte infiltration

(A) Ulcerated periorbital skin from PLCβ2β3-null mice demonstrate a chronic hyperplastic epithelial response in areas bordering epithelial denudation (arrows). The dermis is characterized by a mixed inflammatory infiltrate noticeably lacking in lymphocytes, but composed of mononuclear cells, which are predominantly plasma cells, and abundant neutrophils. **(B)** Skin from wild type mice showed none of the changes described in the PLCβ2β3-null mice. The epithelium has a normal uniform thickness, and the dermis contains normal connective and muscular tissue with very few inflammatory cells present. Sebaceous glands and hair follicles also demonstrate normal architecture. Insets represent higher magnification. Original magnification: A and B, X25; insets, X100.

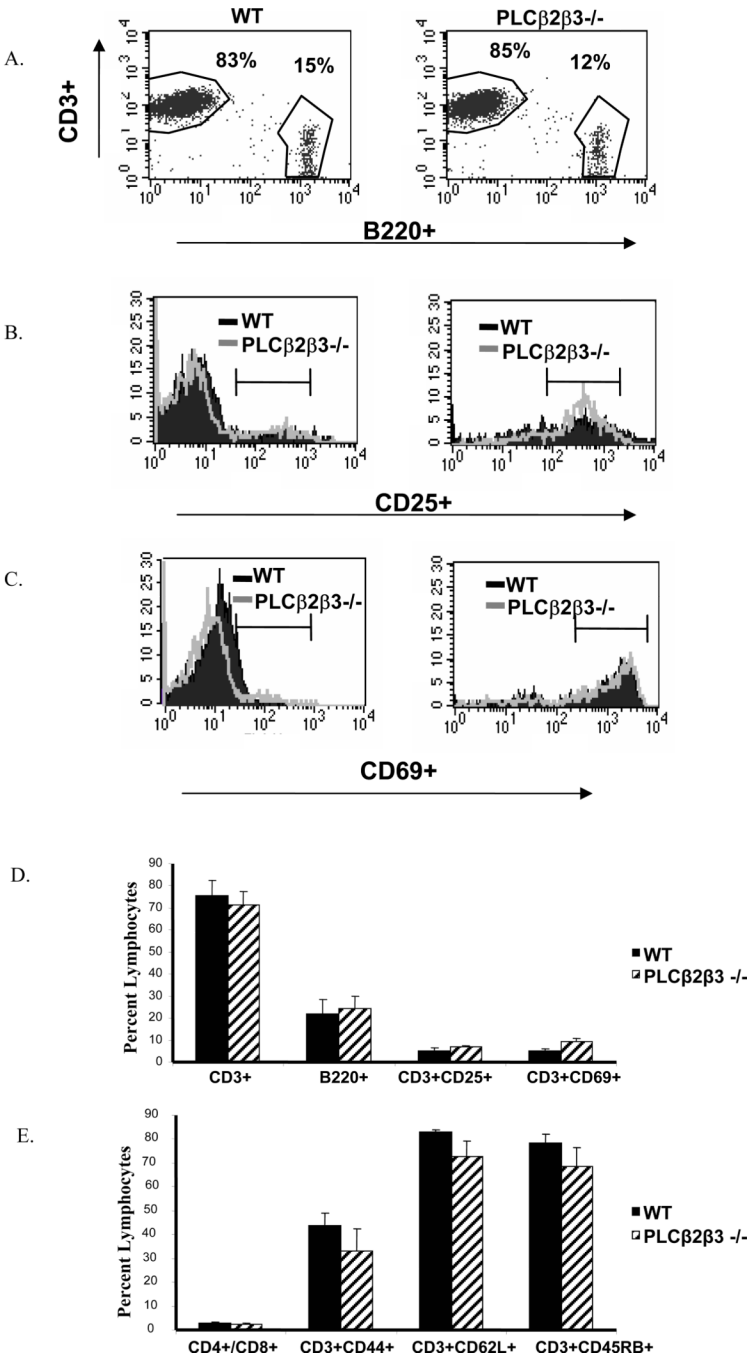

Figure 3. PLCβ2β3-null lymphocytes are phenotypically similar to wild type lymphocytes

(A) Lymphocytes were isolated from peripheral lymph nodes of WT and PLCβ2β3-null (PLCβ2β3 −/−) mice and submitted to flow cytometric analysis using anti-CD3-APC or anti-B220-Cychrome. Shown is a representative example of 7 separate experiments. Isolated lymphocytes expressing CD3 were gated and cells stained with anti-CD25-FITC **(B)** or anti-CD69-PE **(C)** were analyzed. The left panels represent resting lymphocytes and the right panels represent lymphocytes activated with PMA (5 nM) and ionomycin (0.1 μM). Shown is a representative example of 4 separate experiments **(D)** Data from A and B were expressed as a percent of total lymphocytes isolated from peripheral lymph nodes of WT and PLCβ2β3 −/− mice. **(E)** Isolated lymphocytes stained with anti-CD3-APC were gated

and cells stained with anti-CD44-FITC, -CD62L-Cychrome, and –CD45RB-PE were expressed as a percent of total lymphocytes isolated from peripheral lymph nodes of WT and PLCβ2β3 −/− mice. The mean, S.E., and paired student's t-test from D and E reflect data from 4–7 separate experiments.

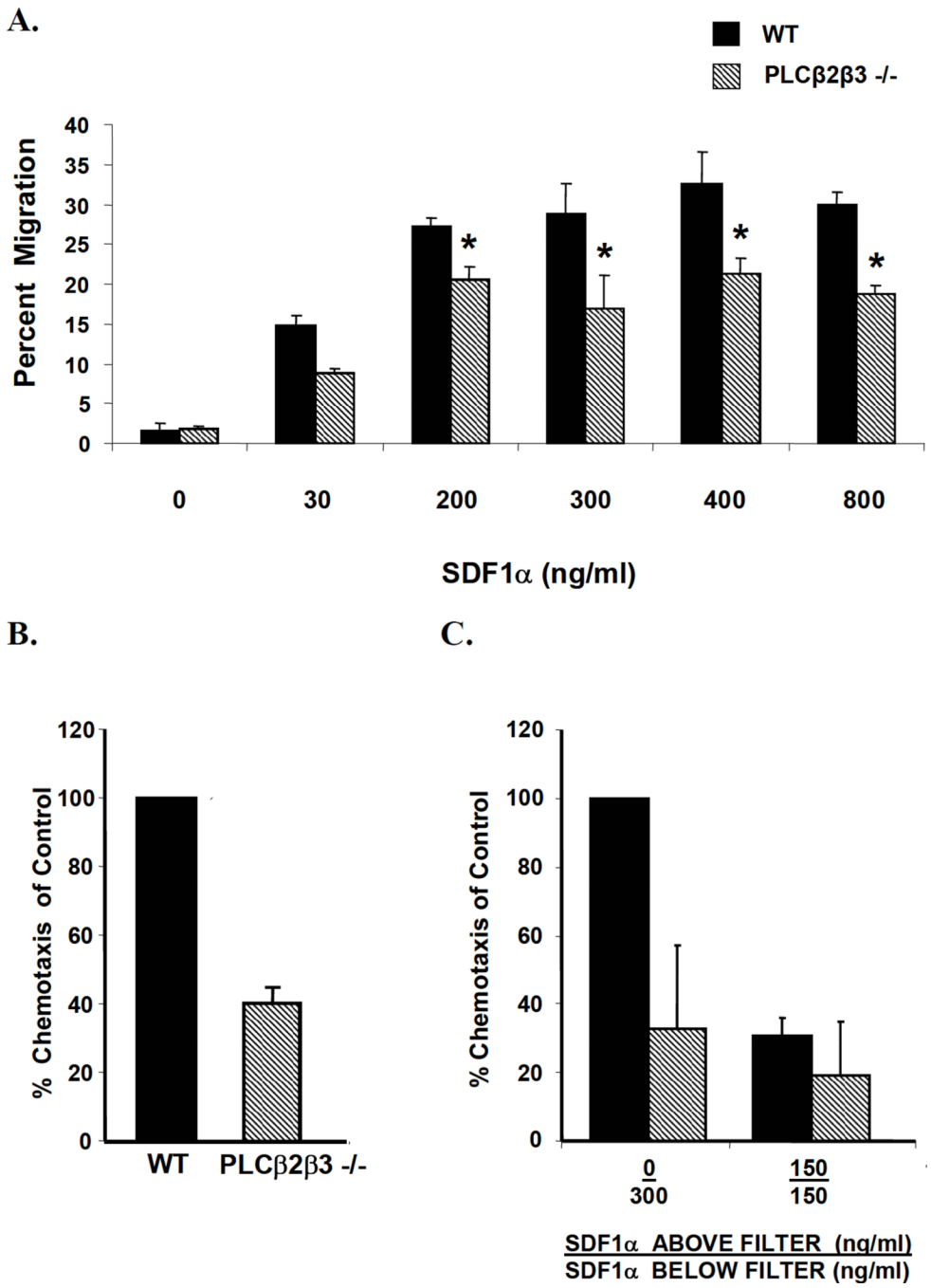

Figure 4. Loss-of-function mutations in PLCβ2β3 impair T cell migration to SDF1α

(A) To determine the optimal concentration at which to measure T-cell chemotaxis, peripheral T-lymphocytes were isolated from lymph nodes of WT and PLCβ2β3 −/− mice and placed into the upper Transwell chamber. Varying concentrations of SDF1α were placed into the lower chamber. The T-cells that migrated toward the resultant SDF1α gradient, after 3 hours at 37°C, were quantitated by flow cytometry. For this dose response, the mean, S.E., and paired student's t-test reflect data from 3 separate experiments.* $p<0.05$. **(B)** The migrating T-lymphocytes to 300 ng/ml SDF1α were expressed as the percent chemotaxis of the wild type cells. The mean, S.E., and paired student's t-test reflect data from 12 separate experiments. **(C)** In order to evaluate whether SDF1α-induced T-cell movement in the

Transwell assay is chemotactic or chemokinetic, WT and PLCβ2β3 −/− T-cells were isolated and placed in a Transwell chamber. The cells were incubated with 150 ng/ml SDF1α placed above and below the filter to abolish the chemotactic gradient. After 3 hours at 37 °C, the cells were recovered from both the upper and lower chambers and counted by flow cytometry. The data are reflective of 3 separate experiments.

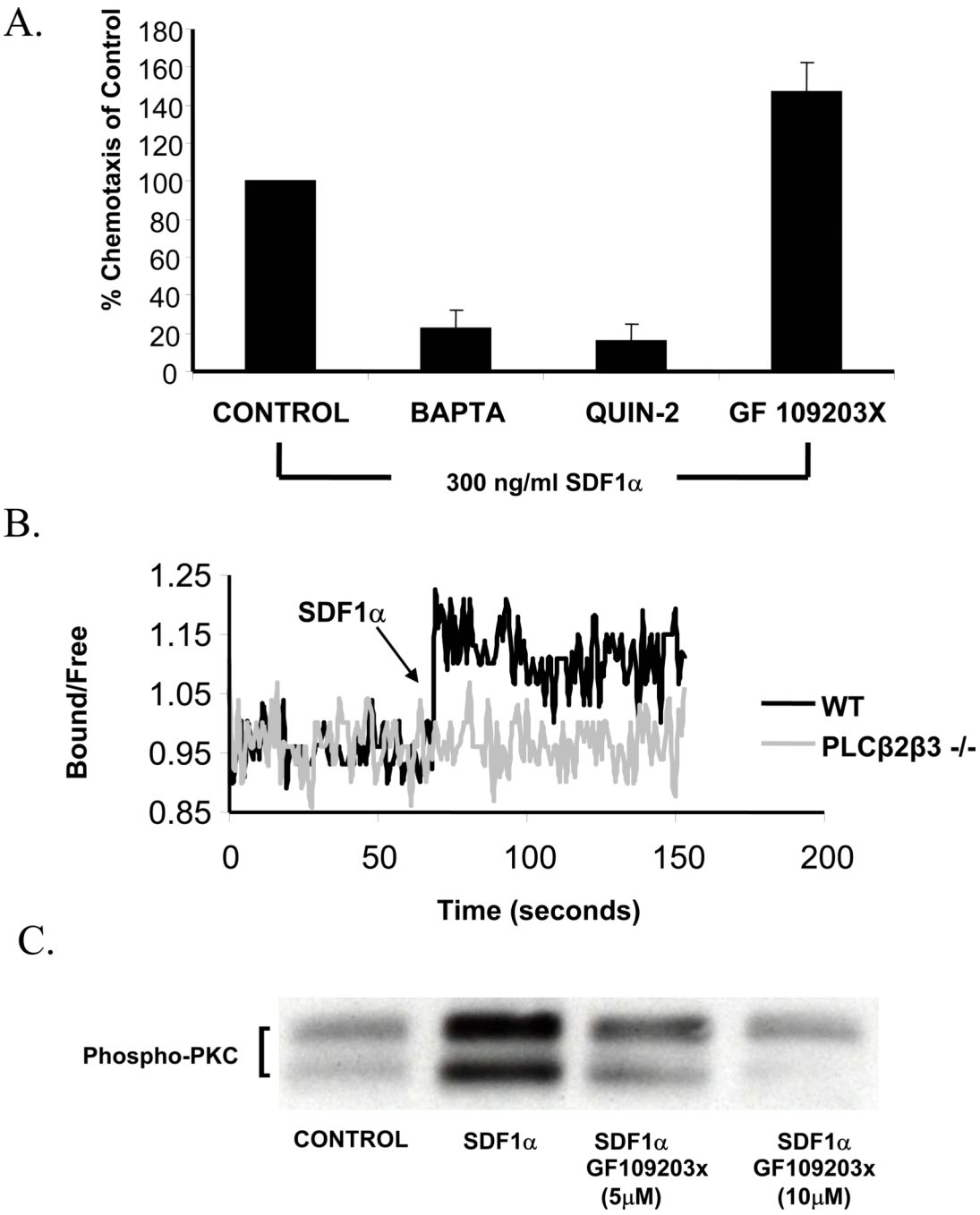

Figure 5. SDF1α -induced T-cell migration requires intracellular calcium release, but not PKC activation

(A) To determine whether specific second messengers generated by PLCβ contribute to cell migration, wild type T-cells were placed in a Transwell chamber. Some of the cells were pre-incubated for 10–15 minutes at 25 °C with the PKC inhibitor GF109203x (10 μM) or the intracellular calcium chelators, either BAPTA-AM (20 nM) or Quin-2 AM (25μM). All but control cells were exposed to 300 ng/ml SDF1α for 3 hours at 37 °C. The migrating T-lymphocytes were expressed as the percent chemotaxis of the WT cells. The mean and S.E. reflect data from 4 separate experiments. **(B)** To determine whether PLCβ2β3 −/− T-cells have impaired changes in the concentrations of cytosolic calcium in response to SDF1α,

peripheral node T-lymphocytes of WT and PLCβ2β3 −/− mice were loaded with 5 μM Fura-2 and placed in a fluorescence spectrophotometer under continuous stirring. SDF1α, 300 ng/ml, was added at the indicated time (arrow), and the ratio of emitted light at 510 nm was recorded when the cells were illuminated by 340 nm and 380 nm excitation light, respectively. The data is a representative example of 3 separate experiments. **(C)** To confirm that GF109203x decreased SDF1α-induced PKC activation, wild type murine T-cells were pre-incubated with GF109203x for 15 minutes at 25 °C. After the addition of 300 ng/ml SDF1α for 1 minute, cells were lysed in boiling 1% SDS with β-mercaptoethanol and immunoblotted with a phospho-pan PKC antibody. PKC activity was inferred by its phosphorylation status.

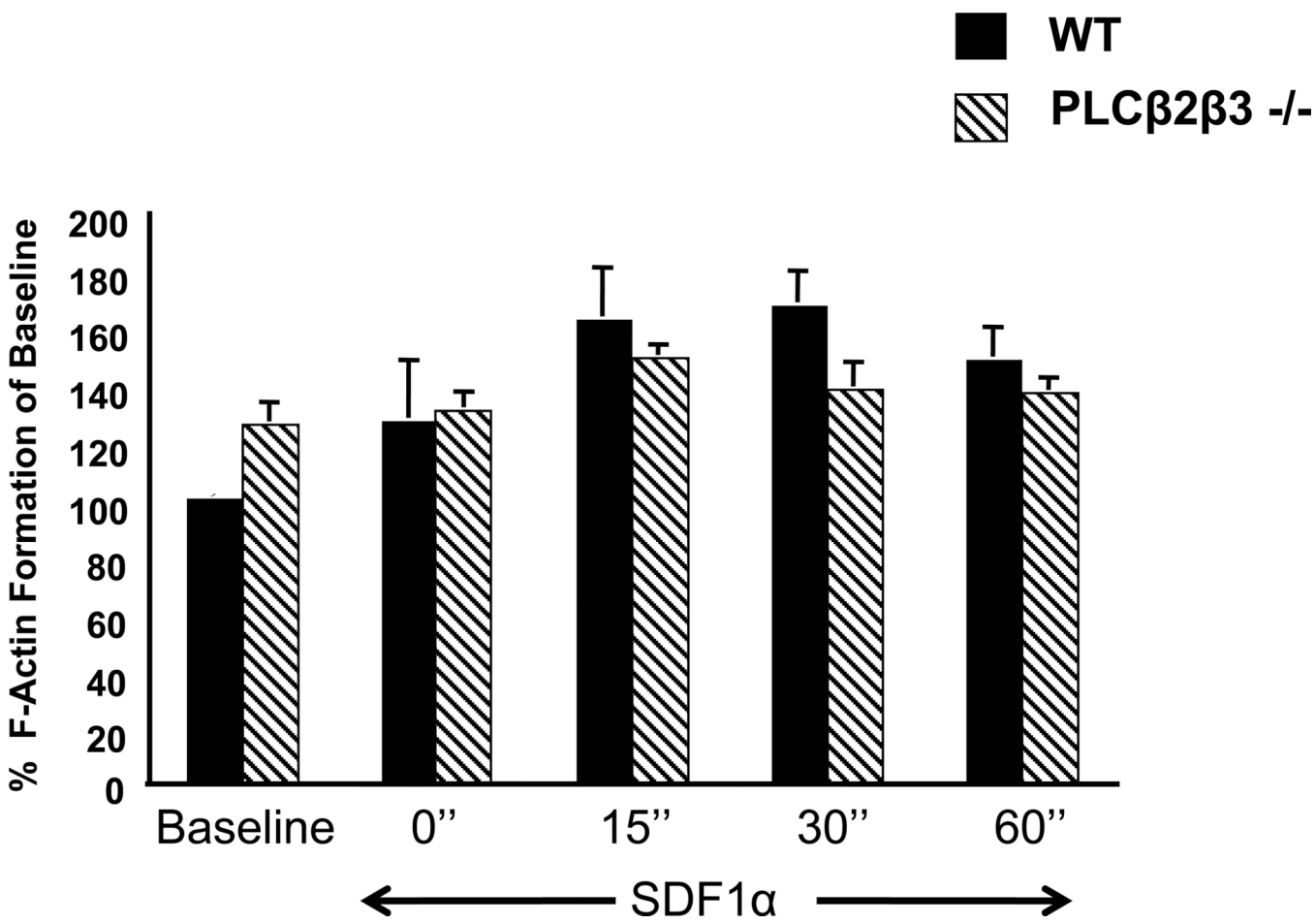

Figure 6. PLCβ2β3 T-cells are able to assemble actin normally in response to SDF1α

Peripheral T-lymphocytes were isolated from lymph nodes of WT and PLCβ2β3 −/−mice. The T-cells were suspended in RPMI 1640 with 0.5% BSA (1×10^6/ml) and treated with 300 ng/ml SDF1α at various time points. The assembly of actin monomers into filaments, or F-actin, was quantitated by staining cells with fluorescently labeled phalloidin followed by flow cytometry. Data are expressed as percent of WT baseline (without SDF1α). The mean and S.E. reflect data from 5 separate experiments.

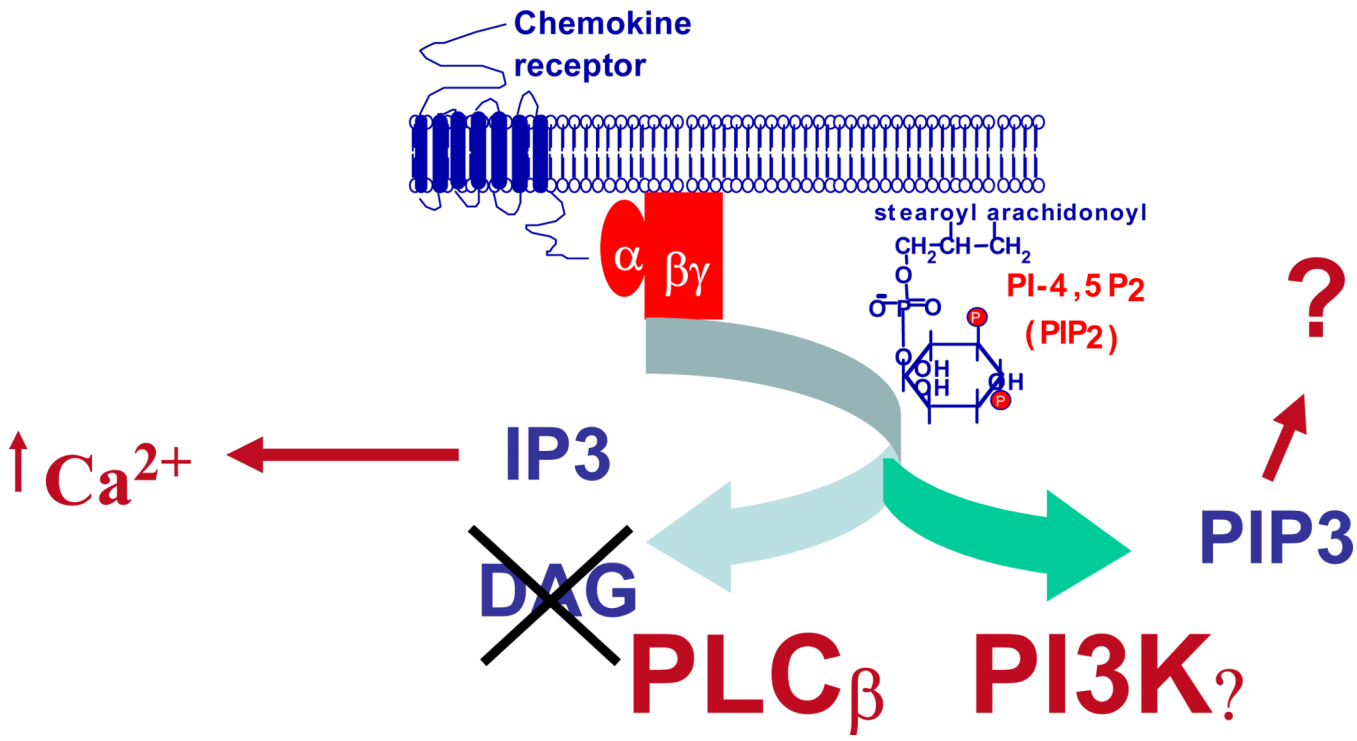

Figure 7. Model of chemokine-stimulated T-cell chemotaxis

In T-lymphocytes, PLCβ and PI3Kγ are activated by Gαi-protein coupled chemokine receptors. Together, they modify phospholipids produced by PIP2. Hydrolysis of PIP2 and generation of IP3 with resultant increase of intracellular calcium has a significant effect on T-cell migration. In contrast, there appears to be little role for the generation of DAG and activation of PKC in this process. Phosphorylation of PIP2 to PIP3 by PI3K is also essential for T-cell migration by a mechanism that is yet unknown.