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Phospholipase Cβ Is Critical for T Cell Chemotaxis[1]

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Chemokines acting through G protein-coupled receptors play an essential role in the immune response. PI3K and phospholipase C (PLC) 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 PI3K in $G_{\alpha i}$ protein-coupled receptor-mediated neutrophil and lymphocyte chemotaxis. Although PLCβ is not essential for the chemotactic response of neutrophils, its role in lymphocyte migration has not been clearly defined. We compared the chemotactic response of peripheral T cells derived from wild-type mice with mice containing loss-of-function mutations in both of the two predominant lymphocyte PLCβ isoforms (PLCβ2 and PLCβ3), and demonstrate that loss of PLCβ2 and PLCβ3 significantly impaired T cell migration. Because second messengers generated by PLCβ lead to a rise in intracellular calcium and activation of PKC, we analyzed which of these responses was critical for the PLCβ-mediated chemotaxis. Intracellular calcium chelation decreased the chemotactic response of wild-type lymphocytes, but pharmacologic inhibition of several PKC isoforms had no effect. Furthermore, calcium efflux induced by stromal cell-derived factor-1α 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 play a critical role in T lymphocyte chemotaxis. *The Journal of Immunology,* **2007, 179: 2223–2227.**

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 Ags are initiated. Many of the cues needed for the trafficking of lymphocytes toward Ag are provided by members of the chemokine family (1). Lymphocyte chemotaxis plays a central role in immune surveillance, acute immune defense, and chronic inflammation as evidenced by the increasing number of animal models highlighting chemokine-related immunological defects (2–5). 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 G_i proteins (6). Stromal cell-derived factor-1α (SDF-1α)[3] 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 (7–9). The signal transduction pathways initiated by the binding of SDF-1α to CXCR4 are not fully understood. In human T cells, SDF-1α stimulates the activation of PI3K, the phospholipase C/protein kinase C (PLC/PKC) cascade, and MAPK p42/44 (ERK1/2) (10, 11).

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 (12). By contrast, we are only recently beginning to appreciate the subtle network of homeostatic chemokines that 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 PI3K and PLC (13, 14). PI3K and PLC are widely expressed enzymes that modify the membrane-bound phosphoinositol, phosphatidylinositol 4,5-bisphosphate (PIP_2). PI3Kγ phosphorylates PIP_2 to generate the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3). This second messenger, in turn, has been demonstrated to play a vital role in actin organization, proliferation, and survival within a variety of different cell types (15), and has a clear role in lymphocyte and neutrophil chemotaxis (1, 16–18). PLC hydrolyzes PIP_2 to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (19). IP_3 leads to a rise in intracellular calcium, whereas DAG activates some isoforms of PKC (20). PLCβ2 and PLCβ3 are the most abundant isoforms of PLC in hemopoietic cells (21). 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 (17).

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[3] Abbreviations used in this paper: SDF-1α, stromal cell-derived factor-1α; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; WT, wild type.

In this study, we analyzed the contribution of PLCβ-synthesized second messengers in T cell migration. This analysis was accomplished using peripheral node lymphocytes derived from genetically altered mice lacking the predominant isoforms of these enzymes (17), in conjunction with a variety of pharmacologic inhibitors. We find that in contrast to granulocytes, PLCβ is fundamental for T cell chemotaxis that appears to be dependent upon the generation of IP_3 and the resultant increase of intracellular calcium, but independent of PKC activity.

Materials and Methods

Materials

Cell culture media and supplements were obtained from Invitrogen Life Technologies. SDF-1α and fMLP were obtained from Sigma-Aldrich. The calcium chelators BAPTA-AM and quin-2/AM, the PKC inhibitor GF109203x, and 7-aminoactinomycin D were obtained from EMD Biosciences. Fluorescence-conjugated Abs were obtained from BD Biosciences. PLC$\beta2\beta3$ knockout mouse lines were previously described (17).

Cell isolation

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) for 30 min at 4°C, and B cells were removed using a Bio-Mag magnetic stand (Polysciences). T cell purity was generally >98%. T cells were activated with 5 nM PMA and 0.1 μM ionomycin.

Bone marrow-derived neutrophils were isolated as previously described (22). Briefly, bone marrow was flushed from the femur and tibia of mice with HBSS Prep (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 137 mM NaCl, 5.6 mM dextrose, 20 mM HEPES, 0.5% FBS). RBC were lysed in hypotonic sodium chloride, followed by addition of an equal volume of hypertonic sodium chloride solution to restore normal tonicity. Cells were resuspended in 5 ml of HBSS Prep, layered over a 62.5% Percoll gradient, and centrifuged at 2200 rpm for 30 min. Mature neutrophils were harvested from the bottom layer of the gradient. The purity of the neutrophil preparation was assessed using flow cytometry; standard preparations yielded neutrophils of >90% purity.

Flow cytometry

Expression of TCR and BCR (CD4, CD8, CD3, CD45R/B220, CD25, CD69, CD44, CD62L, CD45RB, CXCR4) on peripheral lymphocytes and Gr-1 on bone marrow-derived neutrophils was determined by direct immunofluorescence. Cells were incubated with murine fluorescence-conjugated mAbs in cell-appropriate medium for 30 min at 37°C and washed three times. Cell-bound fluorescence was determined in a FACSCalibur flow cytometer (BD Biosciences) using the CellQuest software.

Chemotaxis

Assays for T lymphocyte and neutrophil chemotaxis were performed in polycarbonate membrane (6.5-mm diameter, 10-μm thickness, 5-μm diameter pore size) Transwell cell culture chambers (Costar). T cells (100 μl at 2×10^6/ml) suspended in RPMI 1640 containing 0.5% BSA (fraction V; Sigma-Aldrich) and 25 mM HEPES, and neutrophils (100 μl at 1×10^6/ml) suspended in serum-free HBSS with 0.5% BSA, were added to the upper chamber; SDF-1α as a T cell chemokine or fMLP as a neutrophil chemokine was added to the lower chamber. Pharmacologic inhibitors, calcium chelators, or DMSO (<1%) were preincubated with the cells 15–30 min before their addition to the upper Transwell chamber. Cells were allowed to migrate for 3 h at 37°C in 5% CO_2 atmosphere. T cell migration was quantified by collecting T cells in both the upper and lower chambers and counting by a FACSCalibur flow cytometer. For neutrophil migration, filters were removed and at least 10 fields of cells adherent to the bottom part of the polycarbonate membrane were counted. Visual inspection of the membranes in the lymphocyte assay verified that few lymphocytes were adherent to the filter. The percentage of migration was determined for T cells by the number 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. The chemotaxis index for neutrophils was determined by dividing the migration in the presence of fMLP by migration in the absence of fMLP. Cell viability was verified by staining with 7-aminoactinomycin D and trypan blue.

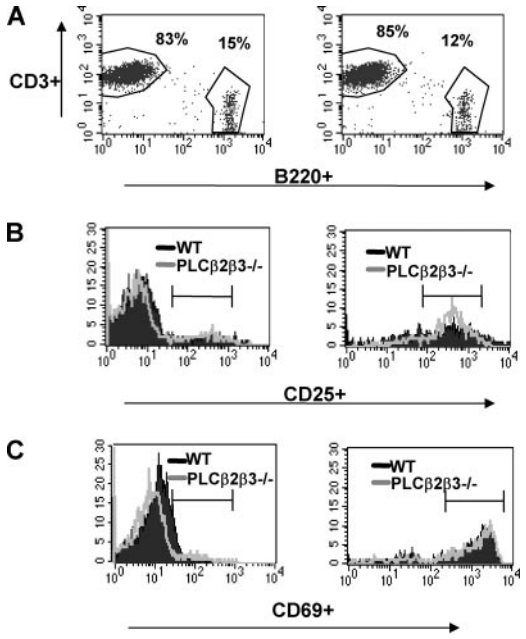

FIGURE 1. PLC$\beta2\beta3$-null lymphocytes are phenotypically similar to WT lymphocytes. *A*, Lymphocytes were isolated from peripheral lymph nodes of WT and PLC$\beta2\beta3^{-/-}$ mice and submitted to flow cytometric analysis using anti-CD3 allophycocyanin or anti-B220 CyChrome. A representative example of seven separate experiments is shown. Isolated lymphocytes expressing CD3-allophycocyanin were gated and analyzed with anti-CD25 FITC (*B*) or anti-CD69 PE (*C*) Abs. Resting lymphocytes (*left*) and lymphocytes activated with PMA (5 nM) and ionomycin (0.1 μM) (*right*) are represented. A representative example of four separate experiments is shown.

Measurement of cytoplasmic calcium concentration

Purified T cells were suspended in RPMI 1640 containing 1% FBS at a concentration of 1×10^7/ml. After loading with 5 μM fura 2-AM (Molecular Probes) for 40 min at 37°C in the dark, T cells were sedimented at $833 \times g$ for 15 min 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 SDF-1α. 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 and 380 nm, and emission spectra measured at 510 nm.

Results

PLC$\beta2\beta3$-null lymphocytes are phenotypically similar to wild-type (WT) lymphocytes

Mice lacking PLC$\beta2$ and PLC$\beta3$ develop spontaneous multifocal skin ulcers starting around 6 mo of age (17). In view of this ulcerative phenotype, our studies to evaluate resting T cell migration have been performed with mice younger than 6 mo, before the development of skin ulceration. At this age, no gross phenotypic differences are seen between the PLC$\beta2\beta3$-null (PLC$\beta2\beta3^{-/-}$) and WT mice.

Peripheral lymph nodes from WT and PLC$\beta2\beta3^{-/-}$ mice were of comparable size and produced a similar total number of lymphocytes ranging from 15 to 20 million cells per mouse. As shown in Figs. 1*A* and 2*A*, the ratios of $CD3^+$ and $B220^+$ lymphocytes were similar between WT and PLC$\beta2\beta3^{-/-}$ mice ($p < 0.5$). Furthermore, markers of T cell activation (CD25 and CD69) were comparable in both WT and PLC$\beta2\beta3^{-/-}$ mice ($p < 0.5$), as shown in Figs. 1, *B* and *C*, and 2*A*. Additionally, the ratio of $CD4^+$ to $CD8^+$ cells, and the percentage of the naive and memory T cell

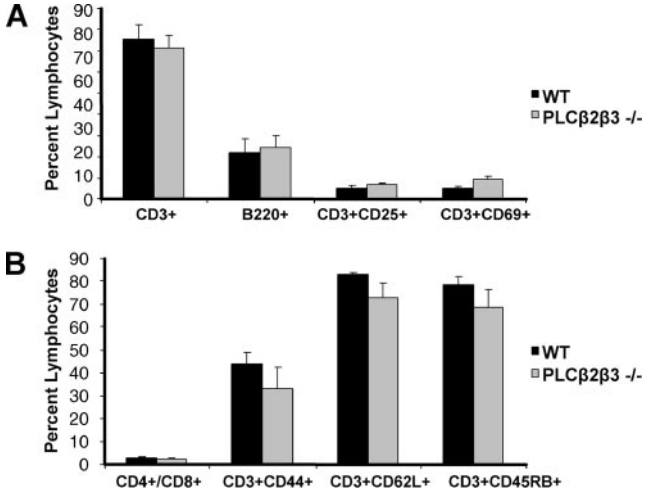

FIGURE 2. Quantitation of lymphocyte phenotypic markers. *A*, Lymphocytes were stained and analyzed with anti-CD3 allophycocyanin and anti-B220 CyChrome Abs. Isolated lymphocytes expressing CD3-allophycocyanin were gated and analyzed for staining with anti-CD25 FITC or anti-CD69 PE Abs. *B*, Lymphocytes were stained and analyzed with anti-CD4 FITC and anti-CD8 PE Abs. Isolated lymphocytes expressing CD3-allophycocyanin were gated and analyzed for staining with anti-CD44 FITC, anti-CD62L CyChrome, and anti-CD45RB PE Abs. Data were expressed as a percentage of total lymphocytes isolated from peripheral lymph nodes of WT and PLC$\beta2\beta3^{-/-}$ mice. The mean and SE of the paired Student's *t* test reflect data from four separate experiments.

markers CD44, CD45RB, and CD62L was the same between WT and PLC$\beta2\beta3$-null lymphocytes ($p < 0.1$) as shown in Fig. 2*B*. These data demonstrate that the lymphocytes from WT and PLC$\beta2\beta3^{-/-}$ 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 SDF-1α

Granulocytes from mice lacking the two dominant isoforms of PLCβ, PLC$\beta2$, and PLC$\beta3$ migrate at least as well as WT cells in response to fMLP or MIP-1α (17) (Fig. 3*A*). To determine whether migration of T cells was dependent on the PLCβ isoform, we analyzed T cell migration in response to the CXCR4 ligand, SDF-1α. Using a Transwell assay, migration of lymphocytes toward SDF-1α was quantitated after 3 h (the time demonstrated by previous studies to be optimal for migration of both WT and PLC$\beta2\beta3$-null T cells). We found that after 3 h, T cells isolated from WT mice exhibited a 20-fold increase in migration with SDF-1α stimulation compared with baseline. The maximal response concentration to SDF-1α (300 ng/ml) was determined by a titrated dose response for both WT and PLC$\beta2\beta3$-null T cells ($p < 0.05$) (Fig. 3*B*). The loss of PLC$\beta2\beta3$ decreased chemokine-stimulated migration over 3 h in T cells by 60 ± 7% ($p < 0.0001$) (Fig. 3*C*). The impaired response in PLC$\beta2\beta3$-null T cells was not attributable to impaired surface expression of CXCR4, the SDF-1α receptor, as demonstrated in Fig. 3*D* ($p < 0.5$). These results demonstrate that, in contrast to the findings with neutrophils, loss of

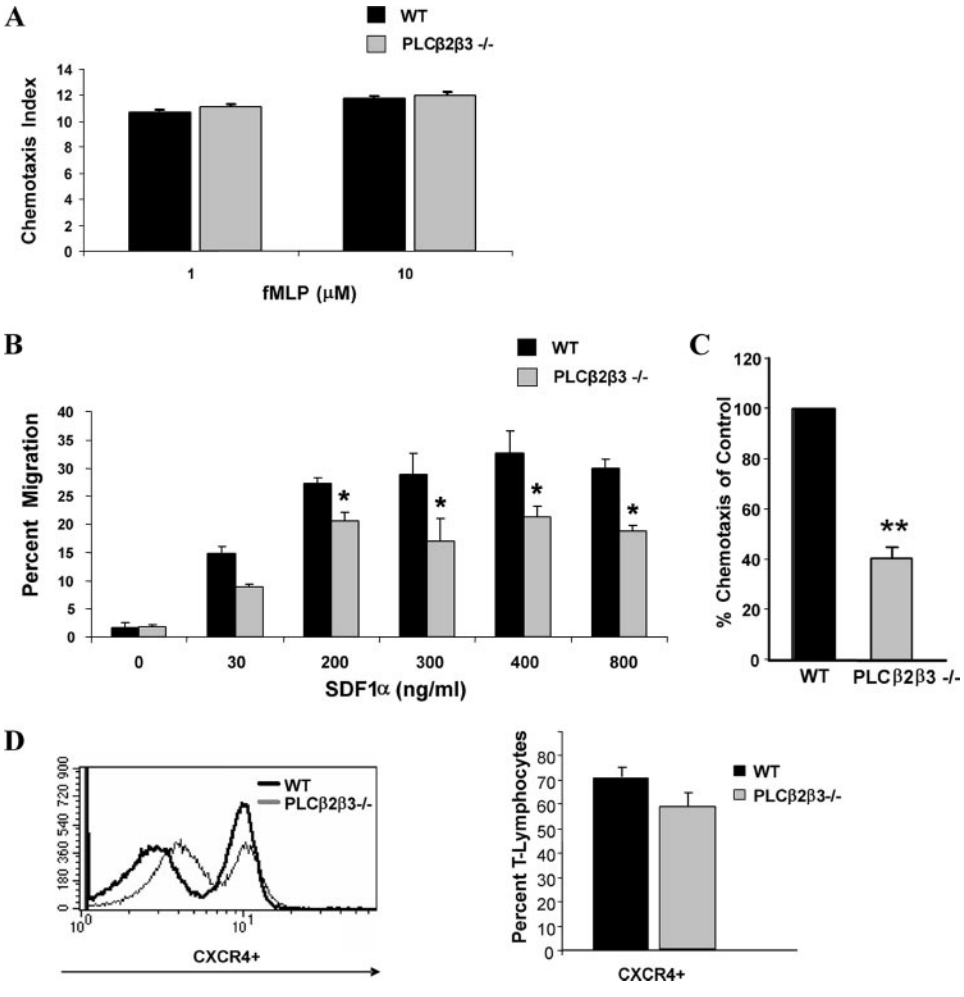

FIGURE 3. Loss-of-function mutations in PLC$\beta2\beta3$ impair T cell migration to SDF-1α. *A*, Neutrophils isolated from the bone marrow of WT and PLC$\beta2\beta3^{-/-}$ mice were analyzed for their migration toward fMLP using a Transwell assay. For this dose response, the mean and SE and the paired Student's *t* test reflect data from three separate experiments. *B*, To determine the optimal concentration at which to measure T cell chemotaxis, peripheral T lymphocytes were isolated from lymph nodes of WT and PLC$\beta2\beta3^{-/-}$ mice and placed into the upper Transwell chamber. Varying concentrations of SDF-1α were placed into the lower chamber. The T cells that migrated toward the resultant SDF-1α gradient were quantitated by flow cytometry. For this dose response, the mean and SE and the paired Student's *t* test reflect data from three separate experiments. *, $p < 0.05$. *C*, The migrating T lymphocytes to 300 ng/ml SDF-1α were expressed as the percentage of chemotaxis of the WT cells. The mean and SE and the paired Student's *t* test reflect data from 12 separate experiments. **, $p < 0.0001$. *D*, T lymphocytes were isolated from WT and PLC$\beta2\beta3^{-/-}$ mice and stained with anti-CXCR4 PE. The mean and SE, and the paired Student's *t* test reflect data from three separate experiments.

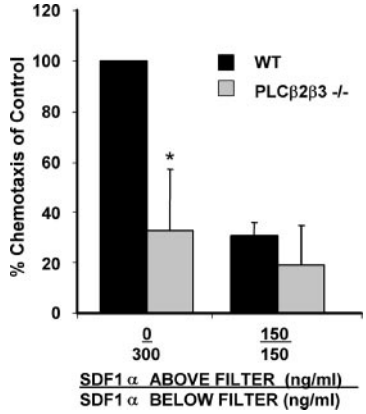

FIGURE 4. SDF-1α-induced T cell migration is largely chemotactic. To evaluate whether SDF-1α-induced T cell movement in the Transwell assay is chemotactic or chemokinetic, WT and PLCβ2β3$^{-/-}$ T cells were isolated and placed in a Transwell chamber. The cells were incubated with 150 ng/ml SDF-1α placed above and below the filter to abolish the chemotactic gradient. The cells were recovered from both the upper and lower chambers and counted by flow cytometry. The migrating T lymphocytes were expressed as the percentage of chemotaxis of the WT cells. The mean and SE, and the paired Student's *t* test reflect data from three separate experiments. *, $p < 0.05$.

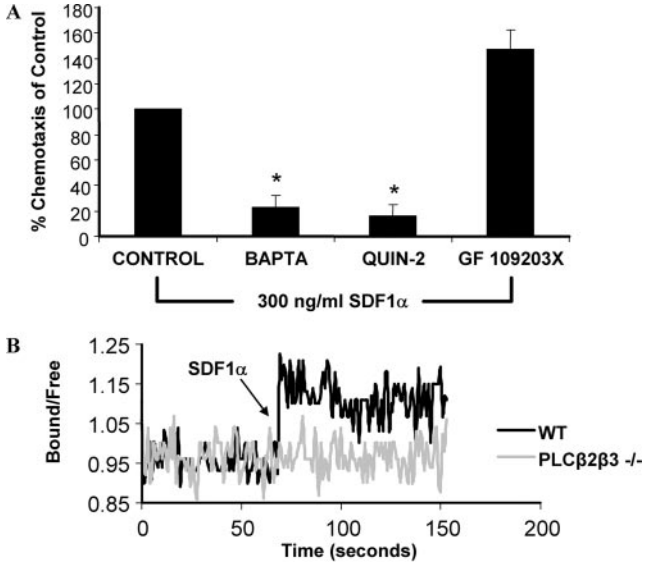

FIGURE 5. SDF-1α-induced T cell migration requires intracellular calcium release. *A*, To determine whether specific second messengers generated by PLCβ contribute to cell migration, WT T cells were placed in a Transwell chamber in the presence of intracellular calcium chelators BAPTA-AM (20 nM) or quin-2/AM (25 μM), or a PKC inhibitor, GF109203x (5 μM). All but control cells were exposed to 300 ng/ml SDF-1α. The migrating T lymphocytes were expressed as the percentage of chemotaxis of the WT cells. The mean and SE reflect data from four separate experiments. *, $p < 0.05$. *B*, PLCβ2β3$^{-/-}$ T cells have impaired changes in the concentrations of cytosolic calcium in response to SDF-1α, as shown by fluorescence spectrophotometry. The data are the representative example of three separate experiments.

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 Fig. 3 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 Fig. 4 demonstrate that most of the WT 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.

SDF-1α-induced T cell migration through PLCβ2β3 is calcium-dependent

Hydrolysis of PIP_2 by PLCβ liberates IP_3, 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 of these second messengers generated by PLCβ contributed to T cell migration. Pharmacologic chelation of intracellular calcium release by BAPTA-AM ($p < 0.05$) or quin-2/AM ($p < 0.05$) eliminated the chemotactic response of WT lymphocytes, suggesting that the release of IP_3 from PIP_2 is critical for T lymphocyte migration (Fig. 5*A*). No impairment was seen with DMSO use as a carrier control (data not shown). Consistent with these studies, we found that SDF-1α-induced calcium efflux was deficient in the PLCβ2β3-null T cells when compared with those of WT (Fig. 5*B*). 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 preincubated with a pharmacologic inhibitor of PKC, GF109203x. Use of GF109203x did not impair T cell migration as demonstrated in Fig. 5*A*. We also analyzed WT and PLCβ2β3-null T cells for SDF-1α-mediated PKC activation by quantifying translocation of specific PKC isoforms into the high-speed particulate fraction of T cell lysates. Although SDF-1α induced a barely detectable activation of PKC-α, PKC-β1, and PKC-β2, the effect of this chemokine on PKC activation is quite modest (data not shown). Loss of PLCβ2β3 does not produce a reliable effect on this nearly negligible response. There was no SDF-1α mediated activation of PKC-γ, PKC-δ, or PKC-ε. Additionally, an anti-phospho-ERK1/2 immunoblot demonstrated only a minimal SDF-1α-mediated ERK1/2 activation in both WT and PLCβ2β3-null T cells (data not shown). These data demonstrate that SDF-1α does not induce significant activation of classical or novel PKC isoforms. Furthermore, it shows that PKC is not an essential component of SDF-1α-mediated T cell signaling in general, and it specifically does not contribute to SDF-1α-induced T cell migration.

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 rheumatoid arthritis, psoriasis, and multiple sclerosis (23). 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 SDF-1α, are constitutively expressed in discrete areas within lymphoid and nonlymphoid tissues, and control the physiological traffic and homing of leukocytes (6). Our experiments indicate that PLCβ2β3 is required

for SDF-1α-initiated signals in murine T lymphocytes. Both PI3K and PLCβ have previously been shown to contribute to several aspects of lymphocyte function including 1) cell proliferation, 2) rescue from apoptosis, and 3) $CD4^+$ and $CD8^+$ T cell differentiation (18, 24). 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 and lymphocytes use PI3Kγ during chemotaxis (16, 17, 25, 26). Mice deficient in PLCβ2 and PLCβ3 have neutrophils that migrate efficiently in response to chemokine, but are defective in their ability to produce superoxide (17). 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. (27) that showed a sensitivity of T cell migration through an inflammatory chemokine receptor, CXCR3, using a relatively nonspecific inhibitor of PLC. These results taken together with our data demonstrate an important role for PLC in T cell migration in response to homeostatic and inflammatory stimuli.

In this study, we have begun to elucidate the mechanism by which PLCβ contributes to chemotaxis. The T cell chemotactic defect in the PLC$\beta2\beta3^{-/-}$ mice can be mimicked in vitro by chelation of cytoplasmic calcium, but not by inhibition of PKC. This ability 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 (28) calmodulin kinase, myosin L chain kinase, SHIP-1, gelsolin, and calpain are some of the potential calcium-dependent candidates currently under investigation.

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. The dissimilarities involve the alternative requirements for PLCβ-generated second messengers. 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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Disclosures

The authors have no financial conflict of interest.

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