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Atypical PKC-ζ regulates SDF-1–mediated migration and development of human CD34+ progenitor cells

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The chemokine stromal cell–derived factor–1 (SDF-1) and its receptor, CXCR4, play a major role in migration, retention, and development of hematopoietic progenitors in the bone marrow. We report the direct involvement of atypical PKC-ζ in SDF-1 signaling in immature human CD34+-enriched cells and in leukemic pre-B acute lymphocytic leukemia (ALL) G2 cells. Chemotaxis, cell polarization, and adhesion of CD34+ cells to bone marrow stromal cells were found to be PKC-ζ dependent. Overexpression of PKC-ζ in G2 and U937 cells led to increased directional motility to SDF-1. Interestingly, impaired SDF-1–induced migration of the pre-B ALL cell line B1 correlated with reduced PKC-ζ expression. SDF-1 triggered PKC-ζ phosphorylation, translocation to the plasma membrane, and kinase activity. Furthermore we identified PI3K as an activator of PKC-ζ, and Pyk-2 and ERK1/2 as downstream targets of PKC-ζ. SDF-1–induced proliferation and MMP-9 secretion also required PKC-ζ activation. Finally, we showed that in vivo engraftment, but not homing, of human CD34+-enriched cells to the bone marrow of NOD/SCID mice was PKC-ζ dependent and that injection of mice with inhibitory PKC-ζ pseudosubstrate peptides resulted in mobilization of murine progenitors. Our results demonstrate a central role for PKC-ζ in SDF-1–dependent regulation of hematopoietic stem and progenitor cell motility and development.

Introduction

Chemokines are involved in a great number of developmental and homeostatic events as well as tumor progression and metastasis in adult life (1). The chemokine stromal cell–derived factor–1 (SDF-1, also named CXCL12) is the only powerful chemoattractant for human $CD34^+CD38^{-/low}$ and murine $Sca\text{-}1^+Thy\text{-}1^{low}kit^+Lin^{-/low}$ hematopoietic stem cells (2–4). It is constitutively expressed in several tissues, including human and murine BM endothelium, endosteum, and stromal cells (5–8). In addition, SDF-1 is also secreted by the more committed human $CD34^+CD38^+$ progenitors (9). The sole SDF-1 receptor, CXCR4, is a 7-transmembrane receptor coupled to G proteins expressed on a variety of immature and mature hematopoietic cells as well as neuronal, endothelial, and epithelial cells (10). Mice lacking either SDF-1 or CXCR4 exhibit many lethal defects, including impaired hematopoiesis and stem cell seeding of the fetal BM, which can be partially corrected by forced expression of SDF-1 in the BM endothelium of SDF-1–null murine embryos (8, 11, 12). We and others have demonstrated a major role for SDF-1/CXCR4 interactions in human stem cell migration in vivo. We have shown that homing and engraftment of human $CD34^+CD38^{-/low}$ stem cells in transplanted NOD/SCID and β2-null NOD/SCID mice are regulated by cell surface human CXCR4 and SDF-1 produced within the murine BM (3, 7, 13, 14). Furthermore, release of stem and progenitor cells from the BM into the circulation during granulocyte colony-stimulating factor–induced mobilization is also dependent on BM SDF-1 degradation and CXCR4 oscillation and inactivation (15, 16). Yet it has been observed that hematopoietic progenitors from fetal liver of $CXCR4^{-/-}$ embryos can engraft BM of irradiated mice; however, impaired retention of immature myeloid and lymphoid cells and increased release to the circulation was also observed (17). Moreover, Tokoyoda et al. recently showed that most primitive murine BM $Sca\text{-}1^+c\text{-}kit^+Lin^-$ cells, as well as pre-pro-B progenitors, are located in close contact with BM SDF-1–expressing stromal cells, implicating SDF-1/CXCR4 interactions in progenitor retention and development within the BM microenvironment (18).

The signal transduction pathways initiated by the binding of SDF-1 to CXCR4 are not fully understood. In human T and immature $CD34^+$ cells, SDF-1 stimulates the activation of PI3K, the phospholipase C/PKC (PLC/PKC) cascade, and MAPK p42/44 (ERK1/2) (19, 20). In immature human $CD34^+$ cells, SDF-1 induces actin polymerization (2) and the phosphorylation of several focal adhesion components, including paxillin, the related adhesion focal tyrosine kinase (RAFTK/pyk2), p130 CAS, Crk-II, and Crk-L (20, 21). The superfamily of PKC serine-threonine kinase enzymes is divided into 3 groups of isoenzymes, according to their mode of activation. The conventional PKCs (α, β, and γ) are activated by diacylglycerol (DAG), calcium, or phosphatidylserine; the novel PKCs (δ, ε, η, and θ) are activated only by DAG or phosphatidylserine; while atypical PKCs (ζ and λ/ι) are calcium and DAG independent (22). Activation of PLC, calcium mobilization, and activation

Nonstandard abbreviations used: ALL, acute lymphocytic leukemia; CB, cord blood; DAG, diacylglycerol; PI, phosphatidylinositol; PLC, phospholipase C; PS, pseudosubstrate; PtdIns-3,4-5P3, phosphatidylinositol triphosphate; SDF-1, stromal cell–derived factor–1; wbc, white blood cell.

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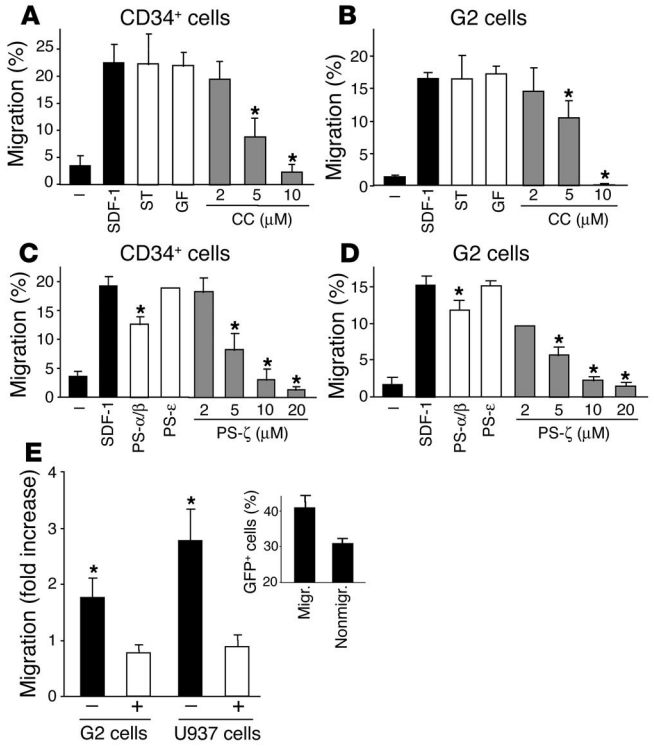

Figure 1
Effect of PKC inhibitors on SDF-1–induced migration. CD34+ cells (**A** and **C**) or G2 cells (**B** and **D**) were preincubated for 30 minutes with PKC inhibitors staurosporine (ST), GF 109203X (GF), or indicated concentrations of chelerythrine chloride (CC) (**A** and **B**) or for 1 hour with 10 μM of either PS-α/β, PS-ε, or indicated concentrations of PS-ζ peptides (**C** and **D**). SDF-1–induced migration was determined by the transwell assay. Migration of control cells to medium only (–) and to SDF-1 is indicated. Data show average ± SD of at least 3 experiments; **P* < 0.05. (**E**) G2 and U937 cells transfected with GFP–PKC-ζ–expressing plasmid were subjected to transwell migration to SDF-1. Where indicated (white bars), transfected cells were preincubated for 1 hour with PS-ζ peptides (10 μM). Fold increase migration represents the ratio of the number of GFP–PKC-ζ migrating cells to the number of migrating mock-transfected cells. Insert indicates the percentage of GFP+ G2 cells in the migrating (Migr.) and nonmigrating (Nonmigr.) cell populations. Results are average ± SD of 2 independent experiments for each cell line; **P* < 0.05.

of DAG-dependent PKCs by chemokines have been proposed as regulators of cell adhesion and migration (23). Activation of the PLC pathway and rise in intracellular calcium in response to SDF-1 indeed suggest a role for PKC in SDF-1 chemotaxis (20); however, no study has addressed the involvement of specific PKC isoforms in this process. We investigated the role of PKC in SDF-1–mediated motility of human CD34+ progenitor cells and leukemic pre-B acute lymphocytic leukemia (ALL) cells and identified the PKC-ζ isoform as a key regulator of SDF-1 signaling.

Results

The atypical PKC-ζ regulates in vitro migration toward SDF-1. In order to test the role of PKC in the migration process of human CD34+ progenitors, we used a panel of PKC inhibitors. Pretreatment of enriched cord blood (CB) CD34+ cells with staurosporine or GF 109203X, both inhibitors of calcium- and DAG-dependent PKC isoforms (24, 25), did not affect SDF-1–induced chemotaxis (Figure 1A). However, treatment with chelerythrine chloride, a broad-range inhibitor of calcium- and DAG-dependent as well as -independent PKC isoforms, including atypical PKCs, decreased the migration of CD34+ cells to SDF-1 in a dose-dependent manner (Figure 1A). Similar effects on human G2 pre-B ALL cell migration were observed (Figure 1B). These results suggest that an atypical PKC isoform might be involved in SDF-1–induced motility. Since PKC-ζ is essential for migration of neutrophils to another CXC chemokine, IL-8 (26), we assessed the role of this atypical isoform in SDF-1–induced migration of human CD34+ progenitors. We used isoform-specific inhibitory myristoylated peptides derived from the pseudosubstrate (PS) region of PKC-ζ, -α/β, and -ε, which mimic the substrate and maintain PKC in its nonactive form. PS-ζ peptides significantly inhibited, in a dose-dependent manner, the migration of human CD34+ cells (Figure 1C) and G2 cells (Figure 1D) to SDF-1. In contrast, PS-ε peptides had no effect on SDF-1–induced motility, while PS-α/β peptides moderately reduced migration of both CD34+ cells and G2 cells (Figure 1, C and D). Next, we transiently overexpressed PKC-ζ in G2 cells and in the promyelocytic U937 cell line. Cells transfected with GFP–PKC-ζ plasmid had significant increased migration toward a gradient of SDF-1 (1.7 ± 0.3–fold for G2 cells and 2.7 ± 0.5–fold for U937) (Figure 1E). Increased motility induced by PKC-ζ overexpression was abrogated by pretreatment with PS-ζ peptides (Figure 1E). In addition, transfected G2 cells were more frequent among migrating cells compared with nonmigrating cells (Figure 1E, insert). These results show the involvement of atypical PKC-ζ isoform in SDF-1–induced migration of hematopoietic progenitors.

Adhesion of CD34+ cells to BM stromal cells is PKC-ζ dependent. Adhesion of hematopoietic progenitors to their microenvironment niches is crucial for their retention within the BM. SDF-1 activates integrins and subsequent adhesion of human CD34+ and B cell progenitors (27, 28, 29). We tested the role of PKC-ζ in SDF-1–triggered adhesion of CB CD34+ cells to a monolayer of the murine BM-derived stromal cell line MS-5, which secretes high levels of SDF-1 (30). We found that CB CD34+ cells adhered to MS-5 monolayer (Figure 2A, control) in a CXCR4/SDF-1–dependent manner (data not shown). Adhesion of CD34+ cells was partially mediated by VLA-4/VCAM-1 interactions, since blocking VLA-4 by neutralizing antibody reduced by 35% the number of adhered cells (Figure 2A). Inhibition of LFA-1/ICAM-1 interactions by an allosteric LFA-1 inhibitor had no effect (Figure 2A). Adhesion of CD34+ cells was decreased by 45% when the cells were pretreated with PS-ζ peptides, while PS-α/β had no effect (Figure 2A). These results suggest that SDF-1–induced adhesion of progenitor cells to BM stromal cells is mediated by PKC-ζ.

SDF-1–induced actin rearrangements require PKC-ζ activation. We next tested the effect of chelerythrine chloride as well as PS-ζ and PS-α/β peptides on SDF-1–induced actin polymerization. SDF-1 induced a maximal increase in polymerized actin content after a 15-second stimulation (Figure 2B), which was totally abrogated by pretreatment of CD34+ cells with chelerythrine chloride or PS-ζ peptides, while PS-α/β peptides had no effect (Figure 2B). Polarization and membranal protrusions are classical hallmarks of motile cells. We found that SDF-1–induced polarization was accompanied by membranal protrusions in CB CD34+ cells adhered to hyaluronic acid (Figure 2, D and G).

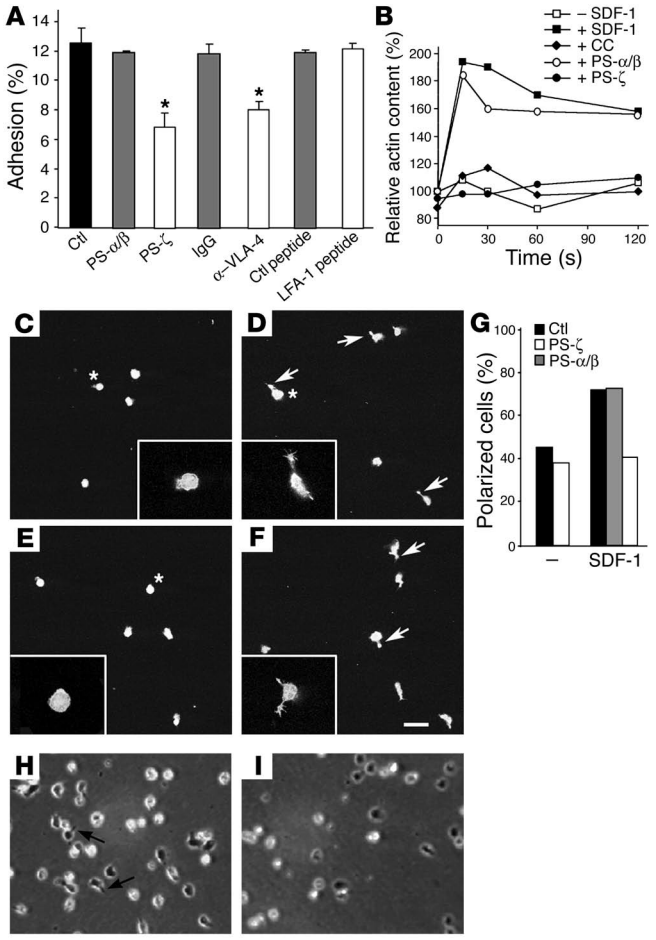

Figure 2
Inhibition of PKC-ζ impairs cytoskeletal rearrangements and adhesion of CD34⁺ cells. (**A**) CB CD34⁺ cells, either untreated (Ctl) or preincubated with 10 μM PS-α/β or -ζ peptides, 10 μg/ml anti–VLA-4 or nonrelevant IgG, or 10 μM LFA-1 inhibitory or control peptides were subjected to adhesion assay to MS-5 cells. Data show average ± SD of 3 independent experiments; **P* < 0.05. (**B**) Actin polymerization assay. CD34⁺ cells pretreated with chelerythrine chloride or with PS-α/β or -ζ peptides were stimulated with SDF-1 for the indicated times, and phalloidin-FITC fluorescence intensity was measured by flow cytometry. (**C**–**G**) SDF-1–induced cell polarization of CD34⁺ cells: untreated cells (**C**), SDF-1–treated cells (**D**), SDF-1/PS-ζ (**E**), and SDF-1/PS-α/β (**F**). Cell morphology and polymerized actin distribution were examined by phalloidin staining. Scale bar: 10 μm. Arrows indicate cells displaying highly polarized morphology in response to SDF-1. Cells similar to ones indicated by asterisks are shown in insets (original magnification, ×100). Quantification of the number of cells with elongated morphology (**C**, inset) and highly polarized morphology (**D** and **F**) from 1 representative experiment is shown (**G**). (**H** and **I**) Spreading and motility under laminar shear flow. G2 cells, control (**H**) or pretreated with PS-ζ peptides (10 μM) (**I**), were perfused on VCAM-1/SDF-1–coated plates under shear flow. Percentage of motile cells was determined by video analysis (Supplemental Videos 1 and 2). Original magnification, ×20. Arrows indicate protrusions formed in control cells (**H**), which are absent in cells treated with PS-ζ peptides (**I**).

Pretreatment with PS-ζ peptides completely abrogated SDF-1–induced polarization (Figure 2, E and G), while PS-α/β peptides had no effect (Figure 2, F and G).

Finally, we examined the role of PKC-ζ in G2 cell adhesion and motility under shear flow on VCAM-1/SDF-1–bearing surfaces. G2 cells spread, formed protrusions, and were motile (Figure 2H, arrows, and Supplemental Video 1; supplemental material available online with this article; doi:10.1172/JCI200521773DS1). However, after treatment with PS-ζ peptides, the cells appeared rounded and only partially spread, and no protrusions were formed in response to SDF-1 (Figure 2I, and Supplemental Video 2; doi:10.1172/JCI200521773DS2).

Impaired migration of pre-B ALL B1 cells correlates with the absence of PKC-ζ. B1 cells, another human pre-B ALL cell line, expressed levels of cell surface CXCR4 similar to those of G2 cells (Figure 3A), which was determined to be functional by measurement of calcium influx in response to SDF-1 (Figure 3B). However, B1 cells did not migrate toward SDF-1 in vitro (Figure 3C). We observed that despite the ability to adhere to the VCAM-1/ SDF-1–coated plates, B1 cells were not able to spread and extend protrusions under shear flow unlike G2 cells (Figure 3, D and E, and Supplemental Videos 3 and 4; doi:10.1172/JCI200521773DS3 and doi:10.1172/JCI200521773DS4. Video legends also available online with this

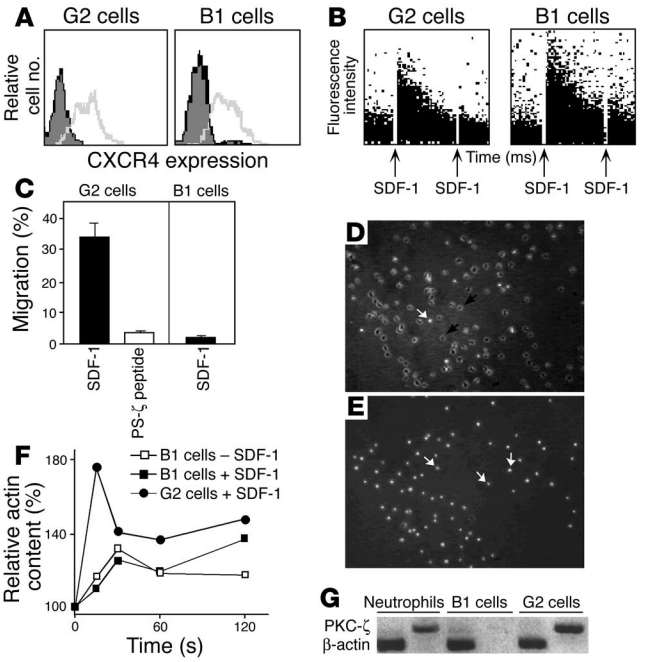

Figure 3
Impaired response of pre-B ALL B1 cells to SDF-1. (**A**) CXCR4 expression on G2 and B1 cells was assayed by flow cytometry. (**B**) SDF-1–induced calcium mobilization. Cells were loaded with Fluo-3 and stimulated twice with SDF-1 (0.5 μg/ml) and analyzed by flow cytometry. (**C**) Transwell migration toward SDF-1 (125 ng/ml). (**D** and **E**) Spreading and motility under laminar shear flow of G2 (**D**) and B1 cells (**E**) on VCAM-1/SDF-1–coated surfaces. Original magnification, ×10. Black arrows indicate spread and thus dark cells; white arrows indicate nonspread rounded cells that appear bright in phase light. (**F**) Actin polymerization following SDF-1 (300 ng/ml) stimulation in G2 and B1 cells was measured by phalloidin-FITC staining. (**G**) Detection of PKC-ζ mRNA expression by RT-PCR in neutrophils, B1 cells, and G2 cells.

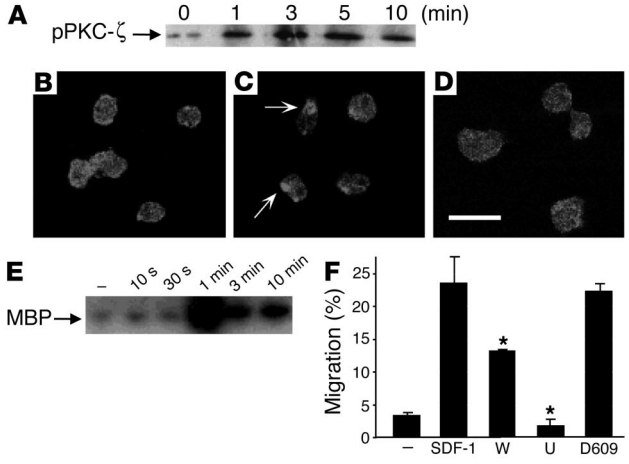

Figure 4
SDF-1 induces PKC-ζ activation. (**A**) G2 cells were stimulated with SDF-1, and a phosphorylated form of PKC-ζ (pPKC-ζ) was detected by immunoblot assay. (**B**–**D**) CD34⁺ cells, untreated (**B**) or stimulated with SDF-1 (200 ng/ml) either pretreated with wortmannin (**D**) or not (**C**), were stained by indirect immunolabeling with anti–PKC-ζ antibody. Arrows indicate membranal PKC-ζ accumulation. Scale bar: 5 μm. (**E**) SDF-1–induced PKC-ζ kinase activity was assayed in vitro in the presence of myelin basic protein (MBP) and ^{32}P-ATP. (**F**) Cells, either untreated or pretreated with wortmannin (W; 50 nM), U7322 (U; 10 μM), or D609 (10 μM), were subjected to SDF-1–induced transwell migration assay. Migration of control cells to medium only (–) and to SDF-1 is indicated. Data shown in **D** are average ± SD of 3 experiments; $^*P < 0.05$.

article; doi:10.1172/JCI200521773DS5.). Furthermore, no increase in polymerized actin in response to SDF-1 was detected in B1 cells (Figure 3F). These data suggest that the lack of chemotactic response to SDF-1 of B1 cells is due to impaired transduction of the signal located downstream to CXCR4. We therefore examined the presence of PKC-ζ mRNA in these cells. Neutrophils, known to express PKC-ζ (26), were used as a positive control. The presence of PKC-ζ mRNA was detected in G2 cells but not in B1 cells (Figure 3G). Thus the reduced PKC-ζ expression in B1 progenitor cells correlates with their unresponsiveness to SDF-1 chemoattraction.

SDF-1 activates PKC-ζ via the PI3K pathway. Activation of PKC requires phosphorylation by an upstream kinase resulting in translocation to the plasma membrane, where PKC can phosphorylate its substrate. First, we detected by immunoblot analysis of G2 cells that SDF-1 increased the relative content of the phosphorylated, i.e., activated, form of PKC-ζ (Figure 4A). Immunocytochemical analysis revealed that SDF-1 induced translocation of PKC-ζ from the cytoplasm to the cell membrane in immature CD34⁺ cells (Figure 4C). Finally, PKC-ζ activity was significantly increased by SDF-1 stimulation of G2 cells as measured by in vitro protein kinase assay (Figure 4E).

In an attempt to decipher the pathway leading to PKC-ζ activation, we investigated the role of PI3K and PLC pathways, both of which have been described as activating PKC-ζ (31, 32). We found that D609, an inhibitor of phosphatidylcholine-specific PLC, had no effect on in vitro migration of CB CD34⁺ cells toward SDF-1, while the specific phosphatidylinositol-PLC (PI-PLC) inhibitor U73122 totally prevented their migration (Figure 4F). G2 cell chemotaxis was similarly impaired (data not shown). Moreover, SDF-1–induced migration of CD34⁺ cells was partially blocked by wortmannin, a specific inhibitor of PI3K (Figure 4F). We also found that pretreatment with wortmannin prevented SDF-1–induced translocation of PKC-ζ to the plasma membrane (Figure 4D), demonstrating that PKC-ζ activation is dependent on PI3K activity.

Characterization of the downstream constituents in PKC-ζ activated pathway. SDF-1 activates MAPKs ERK1 and ERK2 in various cell types including hematopoietic progenitor cells (20, 33). We found by blocking the ERK cascade with PD98059 that ERK activation was not involved in SDF-1–induced cell motility (data not shown). Still, we found that SDF-1–induced ERK activation in G2 cells was PKC-ζ dependent, since inhibition by PS-ζ peptides significantly decreased ERK activation, while PS-α/β and PS-ε peptides had no effect (Figure 5A). Pyk2 is a kinase regulating turnover of focal adhesion of cells to the ECM (34) and therefore might be involved in cell motility. SDF-1 activates Pyk2 in both T and CD34⁺ cells (21). Inhibition of PKC-ζ by PS-ζ peptides also prevented Pyk2 activation in G2 cells, whereas PS-α/β and PS-ε peptides had no effect (Figure 5B).

PKC-ζ is involved in SDF-1–induced gene activation. In addition to regulating cell polarization, adhesion, and motility, SDF-1 also induces cell proliferation and secretion of proteolytic MMP enzymes involved in cell invasion and motility. Migrating hematopoietic progenitors in the blood express MMP-2 and MMP-9 that facilitate their migration to SDF-1, and SDF-1 induces their secretion by human CD34⁺ cells (35, 36). We hypothesized that PKC-ζ might be required for MMP gene activation induced by SDF-1. CB CD34⁺ cells were incubated with SDF-1 and PKC PS peptides for 40 hours, and the levels of MMP-2 and MMP-9 secretion were assayed by zymography. We found that while blocking both PKC-α and PKC-β (PKC-α/β) or PKC-ε had no effect on MMP-2/9 levels, inhibition of PKC-ζ decreased MMP-9 expression (Figure 6A, left). Moreover, treatment with wortmannin, U73122, or PD98059 significantly reduced MMP-9 levels (Figure 6, left), while having no effect on MMP-2 secretion (Figure 6, right). Of note, PD98059 slightly decreased MMP-2 secretion.

SDF-1 regulates cell proliferation by inducing cell cycle progression and acts as a survival factor for human and murine progenitors (37, 38). Four-day culture of G2 cells with SDF-1 led to increased cell proliferation, which was inhibited by the addition of PS-ζ peptides (Figure 6B), demonstrating the involvement of PKC-ζ activation in SDF-1–induced cell proliferation. The proliferation rate of G2 cells cultured with PS-ζ peptides in the absence

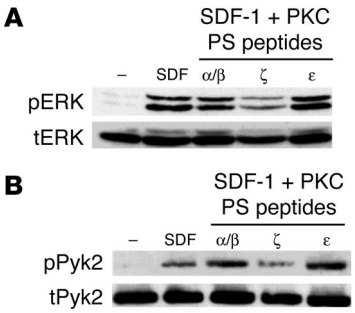

Figure 5
SDF-1–induced ERK and Pyk2 activation is PKC-ζ dependent. G2 cells were stimulated with SDF-1 (200 ng/ml) for 5 minutes after pretreatment with PS-α/β, -ε or, -ζ peptides (10 μM, 60 minutes). Immunoblot assays were performed using specific antibodies for (**A**) total ERK (tERK) and its phosphorylated form (pERK) and for (**B**) total Pyk2 (tPyk2) and its phosphorylated form (pPyk2). Representative experiments are shown.

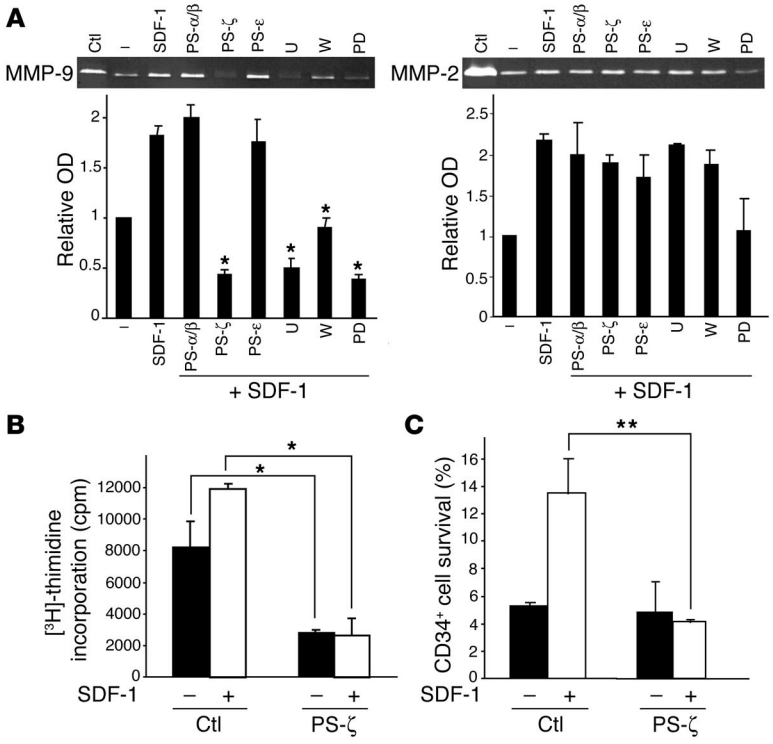

Figure 6
SDF-1–induced MMP-9 secretion and cell proliferation are PKC-ζ dependent. (**A**) MMP-2/9 secretion. CB CD34+ cells were cultured for 40 hours with SDF-1 (500 ng/ml) and either PS-α/β, -ε, or -ζ peptides (10 μM), U73122 (10 μM), wortmannin (50 nM), or PD89069 (PD; 10 μM). MMP-9 (left) and MMP-2 (right) levels in the culture medium were detected by gelatin zymography, using condition medium of HT1080 cells as positive control. Representative gel and densitometry data shown below as an average ± SE of 3 experiments; $^*P < 0.05$. (**B** and **C**) Cell proliferation/survival. (**B**) G2 cells were cultured 4 days in serum-free medium in the presence of SDF-1 (1 ng/ml) and/or PS-ζ peptides (10 μM), and the proliferation rate was measured by [3H]-thymidine incorporation assay. Results shown are the average ± SE of 2 experiments done in triplicate; $^*P < 0.05$. (**C**) CB CD34+ cells were cultured 4 days in serum-free medium in the presence of SDF-1 (5 ng/ml) and/or PS-ζ peptides (10 μM). The number of living cells was determined by trypan blue exclusion. Results shown are the average ± SE of 3 experiments done in triplicate; $^{**}P = 0.01$.

of exogenous SDF-1 was also decreased (Figure 6B), which suggests the involvement of PKC-ζ in the regulation of basal cell proliferation. SDF-1 also increased the survival of CB CD34+ cells cultured for 4 days in a PKC-ζ–dependent manner (Figure 6C).

In vivo engraftment of human CD34+ cells in transplanted NOD/SCID mice requires PKC activation. We next tested the effect of PKC inhibition on the engraftment capacities of human CB CD34+ cells. Cells were treated with chelerythrine chloride prior to injection into NOD/SCID mice, and engraftment in the BM was analyzed 1 month later. PKC inhibition by chelerythrine chloride dramatically impaired the ability of CD34+ progenitor cells to engraft the murine BM (Figure 7A). In line with the inhibitory effect on migration of progenitors in vitro, PS-ζ peptides also significantly reduced in vivo engraftment of CD34+ cells (Figure 7A). Unexpectedly, PS-α/β peptides reduced to a similar extent the number of CD34+ cells in the murine BM, which suggests a role for these isoforms in the engraftment process. A combination of both inhibitors decreased the engraftment level, while control PS-ε peptides had no effect (Figure 7A). In order to assess whether PKC-ζ inhibition affects the initial homing process, we assayed the number of human CD34+ cells in the murine BM 16 hours after injection. Surprisingly, pretreatment of cells with PS-ζ peptides had no effect on homing of CD34+ cells to the BM (control, 48 ± 1.7 human cells per 1×10^6 acquired BM cells; PS-ζ peptides, 53 ± 2.6 human cells per 1×10^6 acquired

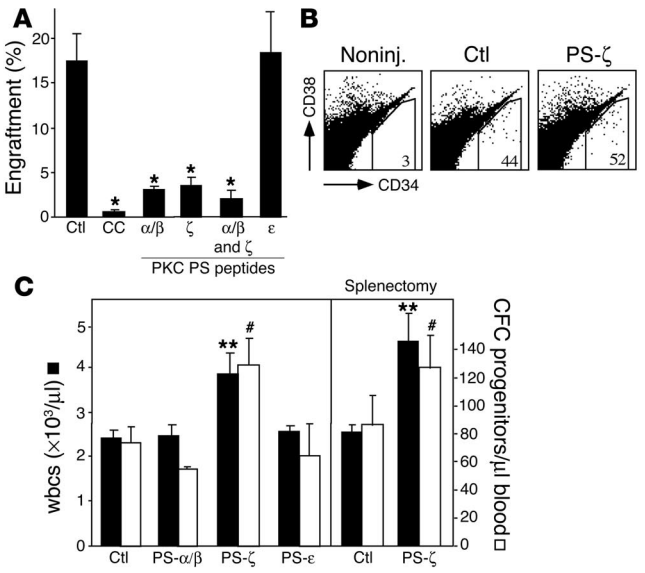

Figure 7
Effect of PKC-ζ inhibition on homing and engraftment of stem cells in NOD/SCID mice. (**A**) CB CD34+ cells were pretreated with chelerythrine chloride, PS-α/β and/or -ζ, or -ε peptides and injected into irradiated NOD/SCID mice. Levels of engraftment were determined 1 month later by FACS analysis and expressed as percentage of human CD45+ cells. Results shown are average ± SE of at least 3 experiments; $^*P < 0.05$. (**B**) Homing assay. CB CD34+ cells were pretreated with PS-ζ peptides (10 μM) and injected into irradiated NOD/SCID mice. Human cells in the BM were detected after 16 hours by flow cytometry using specific anti-human CD34 and anti-CD38 antibodies. One representative experiment (out of 4) shows the number of human cells per 1×10^6 acquired BM cells. Results for a noninjected mouse (Noninj.) are included. (**C**) NOD/SCID mice and previously splenectomized NOD/SCID mice were injected daily with PS-α/β, -ζ, or -ε peptides (50 μg/mouse) for 3 consecutive days. The number of wbcs in the blood was determined 4 hours after the last injection. The number of progenitor cells was determined by CFU-C assay. Results shown are average ± SE of 3 experiments for each type of mice ($n = 5$); $^{**}P = 0.01$ and $^{\#}P = 0.05$.

BM cells; $P = 0.2$; Figure 7B), which suggests that PKC-ζ inhibition prevents BM seeding by primitive repopulating cells.

Our in vitro results suggest that PKC-ζ regulates adhesion of CD34$^+$ cells to BM stromal cells and therefore might mediate progenitor cell retention within the BM. To test this hypothesis, we examined the effect of PKC-ζ inhibition in vivo. Three daily injections of 50 μg PS-ζ peptides in NOD/SCID mice resulted in a 60% increase in the number of mature white blood cells (wbcs) in the blood compared with untreated mice (Figure 7C). In addition, the number of murine stem and progenitor cells in the circulation, as determined by CFU-C assay, was also significantly increased (Figure 7C). We did not note differences in the number of mature and immature progenitors in the BM (data not shown), which is similar to what has been observed in G-CSF–induced mobilization (39). Injection of PS-α/β and PS-ε peptides had no substantial effect (Figure 7C). In order to exclude the possibility of mobilization of hematopoietic cells from the spleen, we injected PS-ζ peptides under the same conditions into previously splenectomized NOD/SCID mice. Similar to what was observed in nonoperated mice, both mature and immature cells mobilized after injection with PS-ζ peptides (Figure 7C).

Discussion

While a great number of intracellular molecules that are activated after the binding of SDF-1 to CXCR4 have been discovered in various cell types, the specific pathway that leads to directional cell motility is largely uncharacterized. SDF-1 stimulation results in PLC activation and DAG formation as well as calcium mobilization, 2 pathways known to activate PKC (2, 20). In this study, we show that SDF-1–induced migration of enriched human CD34$^+$ progenitors and human pre-B ALL G2 cells is dependent on PKC activation, and we identify, for the first time to our knowledge, a PKC isoform essential for SDF-1–induced progenitor cell motility: the atypical PKC-ζ isoform. We also found that PKC-ζ is a key molecule that controls several SDF-1–activated pathways regulating progenitor cell development.

The ζ isoform of PKC has previously been implicated in mitogenic signal transduction, cell polarity, and insulin-induced glucose transport (reviewed in ref. 40). However, the involvement of PKC-ζ in cell motility is still poorly documented. Using several PKC inhibitors and specific myristoylated peptides derived from the regulatory region of PKC-ζ molecule, we show here that SDF-1–induced migration of CB CD34$^+$ cells and pre-B ALL cells is PKC-ζ dependent. These results were confirmed by the finding that PKC-ζ–overexpressing cells migrate 2- to 3-fold more efficiently to SDF-1. Moreover, we report here that, despite functional CXCR4 expression, B1 cells are impaired in their ability to migrate to SDF-1, which correlates with reduced levels of PKC-ζ. Laudanna et al. reported the role of PKC-ζ in IL-8–induced actin assembly, adhesion, and in vitro migration of human neutrophils (26). These observations and ours suggest that PKC-ζ may have a central role in chemokine-induced motility of hematopoietic cells. Interestingly, while inhibition of PKC-ε had no effect on SDF-1–induced motility, inhibition of PKC-α/β slightly reduced migration of CD34$^+$ cells and G2 cells toward SDF-1, which implies the possible involvement of PKC-α or PKC-β in SDF-1 chemotaxis.

Uncoupling of G protein subunits after SDF-1 binding results in activation of PI–PLC-β3 and/or PI–PLC-γ and a rise in calcium (2, 20, 41). Using the specific PI-PLC inhibitor U73122, we demonstrate, for the first time to our knowledge, that PI-PLC is essential for SDF-1–induced chemotaxis of human CD34$^+$ progenitor cells. Conflicting studies reported that U7322 either inhibits SDF-1–induced migration of T cells (41) or does not (42). Although atypical PKCs cannot be directly activated by PLC pathway compounds (DAG, calcium), activation of PKC-ζ can occur via secondary messengers downstream of DAG, such as ceramide and phosphatidic acid (31, 32). Moreover, PKC-ζ can be activated in vitro and in vivo by the lipid product of PI3K, phosphatidylinositol triphosphate (PtdIns-3,4-5P3), possibly via the kinase PDK-1 (43). Indeed, PI3K activation by chemokines is involved in cell locomotion and has been well established (reviewed in ref. 44). SDF-1 activates PI3K, although wortmannin only partially blocks chemotaxis (19, 33) and SDF-1 activation in T cell leads to a rapid and transient PtdIns-3,4-5P3 elevation (33). We demonstrate here that SDF-1–induced activation of PKC-ζ is mediated by PI3K, since wortmannin could block translocation of PKC-ζ from the cytoplasm to the plasma membrane.

We also found that blocking PKC-ζ abolished SDF-1–induced actin polymerization and cell polarization in both CD34$^+$ and G2 cells. PKC-ζ is an important regulator of cell polarity (reviewed in ref. 40). Upon cytokine activation, PKC-ζ mediates actin rearrangements by direct association with the cytoskeleton (45). Moreover, PKC-ζ forms a ternary complex with PAR-3 and PAR-6, which, in association with Cdc42, regulates tight junction formation and cell polarization (46). PKC-ζ is essential for cell polarity during migration of astrocytes, and Cdc42 activation induces PKC-ζ activation and relocalization to cell protrusions in the direction of cell movement (47). Our results reveal that PKC-ζ controls SDF-1–induced cell polarization and adhesion of human CD34$^+$ progenitors to BM stromal cells, a process that might be important for the retention and development of stem and progenitor cells within the BM niches. Adhesion of progenitor cells to MS-5 cells was found to be only partially VCAM-1/VLA-4 dependent. However, our data indicate that PKC-ζ does not mediated SDF-1–induced adhesion of progenitors to VCAM-1, since we found that (a) B1 cells adhered to VCAM-1 despite their lack of PKC-ζ; (b) PS-ζ peptide did not prevent adhesion of G2 cells to VCAM-1 in a shear flow assay; and (c) PS-ζ peptide did not prevent adhesion of CB CD34$^+$ cells to VCAM-1 in a static assay

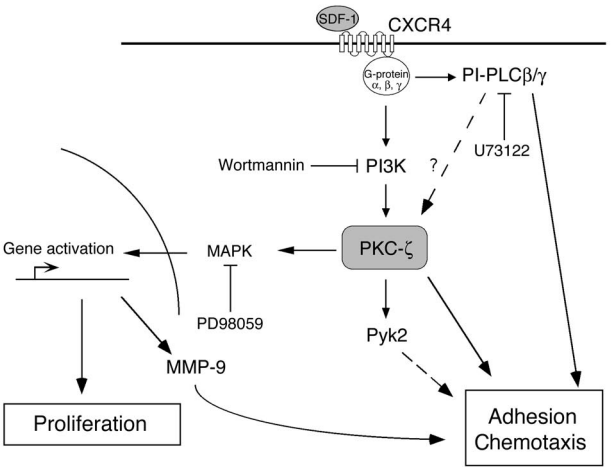

Figure 8
Proposed model for the role of PKC-ζ in SDF-1–induced signaling in human progenitors. Following binding of SDF-1 to CXCR4, G-coupled proteins activate both PI-PLC and PI3K, which activates PKC-ζ. PKC-ζ activation induces Pyk2 and ERK activation, leading to adhesion and chemotaxis as well as gene transcription via MAPK activation. The broken line represents hypothetical links.

(data not shown). Interestingly, Giagulli et al. recently demonstrated the critical role of PKC-ζ in LFA-1 clustering and adhesion of T cells to ICAM-1 induced by the secondary lymphoid chemokine (SLC) (48). However, we found that adhesion of CB CD34$^+$ cells to MS-5 cells was ICAM-1/LFA-1 independent. Thus, PKC-ζ might regulate integrin-independent progenitor cell adhesion to BM stromal cells.

In addition to activating the cell locomotion machinery, PKC-ζ also signals toward gene transcription. We show here that PKC-ζ plays a critical role in mediating SDF-1–induced MMP-9 production. PKC-ζ is involved in MMP transcription via activation of the transcription factor NF-κB (49, 50), which is also activated by SDF-1 (19), suggesting that NF-κB could mediate PKC-ζ–dependent MMP-9 gene transcription. MAPKs are not involved in SDF-1–induced motility but rather gene activation, since we found that inhibiting ERK1/2 prevented MMP-9 secretion. PKC-ζ has both activating and inhibiting effects on proliferation, malignant transformation, and survival of various cells (50, 51). So far, no study has investigated the role of PKC-ζ in hematopoietic cell proliferation. Our study indicates that SDF-1–induced proliferation or survival of immature progenitors requires PKC-ζ. Based on these results, we propose the following model for regulation of the SDF-1 signaling by PKC-ζ (Figure 8): SDF-1 binds to CXCR4 on CD34$^+$ cells, activating both PI-PLC and PI3K, which in turn activates PKC-ζ, either directly or indirectly. PKC-ζ then activates both MAPK and Pyk2, leading to adhesion and chemotaxis. MAPK activation leads to transcription of MMP-9 and cell cycle activating genes.

Importantly, we demonstrate that PKC is essential for in vivo repopulation by primitive human CD34$^+$ cells in transplanted NOD/SCID mice. While our results demonstrate that PKC-ζ regulates in vitro migration of progenitor cells, we surprisingly found that homing of progenitors to the BM, a SDF-1/CXCR4–dependent process, was PKC-ζ independent. However, we recently showed that pretreatment of CB CD34$^+$ cells with chelerythrine chloride also prevented their homing to the murine BM, demonstrating that homing is indeed a PKC-dependent process (13). Based on our in vitro results, we propose that adhesion/retention and/or survival of progenitor cells within the BM microenvironment may be severely reduced in vivo due to PKC-ζ inhibition. Moreover, increased numbers of progenitors and mature cells in the circulation were observed after injection of PS-ζ peptides into NOD/SCID mice, while normal levels of CFU-C were detected in the BM, which favors the hypothesis of impaired cell retention over reduced survival and implicates PKC-ζ as a critical molecule for progenitor cell anchorage in the BM niche. PKC-ζ inhibition induces cell mobilization from the BM as it also occurs in mice that underwent splenectomy, excluding cell mobilization from the spleen. However, we cannot rule out the possibility that circulating cells accumulate in the blood due to impaired relocalization following PKC-ζ inhibition.

Of interest, inhibiting the PKC-α and PKC-β isoforms only moderately affected in vitro chemotaxis, while significantly reducing engraftment in vivo. These observations suggest an additional role for PKC-α and/or PKC-β in HSC engraftment, presumably in the proliferation/differentiation of progenitor cells within the BM. Accordingly, it has been shown that PKC-α is involved in erythroid differentiation of human BM CD34$^+$ cells (52). Interestingly, PKC-ζ–deficient mice exhibit severe defects in B cell development and function (53, 54), which have been shown to rely on SDF-1 (11, 17, 55), suggesting that other SDF-1–dependent processes such as stem cell chemotaxis and development are also affected in these mice. The migratory pattern of PKC-ζ$^{-/-}$ progenitor cells and their ability to respond to SDF-1 are currently unknown and are of interest for future studies.

In conclusion, our results point out the important role of the atypical PKC-ζ in the cascade of intracellular events activated by SDF-1/CXCR4 interactions, leading to human progenitor cell motility and proliferation. This study provides a better understanding of human progenitor migratory patterns occurring in a preclinical model with relevance to clinical stem cell transplantation and mobilization procedures. Moreover, recent studies revealed an important role for SDF-1 signaling in metastasis and cancer development (56–58). In addition, Laudanna et al. recently reported that constitutive activation of PKC-ζ is a feature of spontaneous motility in pancreatic adenocarcinoma cells (59). Therefore, a better characterization of the SDF-1–mediated PKC-ζ activation could lead to new therapeutic approaches for these diseases based on specific inhibitory molecules.

Methods

Reagents. Recombinant human SDF-1 was purchased from PeproTech. The PKC inhibitors staurosporine, GF 109203X, and chelerythrine chloride, the PI3K inhibitor wortmannin, the PLC inhibitors U73122 and D609, and the MAPK inhibitor PD98059 were purchased from Calbiochem. Myristoylated PS inhibitor peptides for PKC-ζ, PKC-α/β, and PKC-ε were purchased from BIOMOL International LP.

Human and murine cells. Enrichment of human CB CD34$^+$ cells was performed as previously described (13). Human cells were used in accordance with approved procedures by the Human Experimentation and Ethics Committees of the Weizmann Institute. The pre-B ALL cell lines G2 and B1 were kindly provided by M. Freedman (Hospital for Sick Children, Toronto, Ontario, Canada) (60). Murine MS-5 stromal cell line is a kind gift from J.C. Gutierrez-Ramos (Millennium Pharmaceuticals, Cambridge, Massachusetts, USA). All cells were grown in RPMI supplemented with 10% heat-inactivated FCS.

Chemotaxis assay. Chemotaxis experiments were performed using Costar transwells (6.5 mm/diameter, 5 μm/pore) as previously described (2). Where indicated, cells were pretreated for 30 minutes with nontoxic concentration of staurosporine (1 μM), GF 109203X (1 μM), chelerythrine chloride (2–10 μM), or PKC PS peptides (2–20 μM) before the migration assay.

Adhesion assay. Human CB CD34$^+$ cells (1×10^5/well) were labeled with CFSE (Molecular Probes) and incubated with either PKC PS peptides (10 μM), neutralizing anti–VLA-4 mAb HP1/2 (10 μg/ml; kindly provided by R. Lobb, Biogen Inc., Cambridge, Massachusetts, USA), nonrelevant control IgG2a Ab (IQ Products), the allosteric LFA-1 inhibitor A308925, or its analog, A207412 (10 μM; kindly provided by D. Staunton, ICOS, Bothes, Washington, USA), and allowed to adhere to confluent MS-5 cells for 45 minutes at 37°C (1×10^5 cells/well) in serum-free RPMI plus 0.2% BSA in a 96-well plate. Nonadherent cells were washed twice in FACS buffer (PBS + 1% FCS + 0.02% $NaNO_3$). Adherent cells were collected in 150 μl FACS buffer plus 0.5 mM EDTA. The number of CFSE$^+$ cells was determined by FACS analysis (FACSCalibur; BD).

PKC-ζ overexpression. G2 and U937 cells were transiently transfected with plasmid encoding GFP-ζ fusion protein (cloned in pEGFP-N1 plasmid; BD Biosciences — Clontech) using the Amaxa method according to manufacturer's protocol (Amaxa Biosystems). GFP expression and chemotaxis were assayed 5 hours after transfection. The efficiency of transfection was 30–50%. Cells that underwent the transfection procedure without plasmid (mock-transfected) served as a control.

Immunofluorescence. Human CB CD34$^+$ cells (2×10^5) were incubated for 60 minutes at 37°C in serum-free RPMI with SDF-1 (200 ng/ml) and inhibitors on 10% poly-L-lysine– or 150 μg/ml hyaluronic acid–coated (Sigma-

Aldrich) coverslips. Indirect immunofluorescence labeling and analysis were performed as previously described (61), using mouse anti–PKC-ζ Ab (H-1) followed by secondary Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) or TRITC-labeled phalloidin (Sigma-Aldrich).

Immunoblot. G2 cells were stimulated with 200 ng/ml SDF-1 for the indicated times, and cell lysates were obtained by 1-hour incubation with lysis buffer (25 mM Tris pH 7.5, 1% Triton, 0.5 mM EDTA, 150 mM NaCl, 50 mM NaF, $NaVO_3$, PMSF, 1% protease inhibitor cocktail [Sigma-Aldrich]). Immunoprecipitation of PKC-ζ was performed by 2-hour incubation at 4°C of cell extracts (300 μg) with rabbit anti–PKC-ζ antibodies (C-20; Santa Cruz Biotechnology Inc.) conjugated overnight at 4°C with protein A beads (Bio-Rad Laboratories). Proteins were resolved by 10% SDS-PAGE. Antibodies used include: anti-Pyk2, anti–phosphorylated PKC-ζ and anti–PKC-ζ from Santa Cruz Biotechnology Inc.; anti–phosphorylated ERK and anti-ERK from Sigma-Aldrich; and anti–phosphorylated Pyk2-402 from Biosource International.

Protein kinase assay. After immunoprecipitation of PKC-ζ performed as indicated above, kinase reaction was performed in 10 μl reaction mixture ×3 (75 mM β-glycerophosphate, pH 7.3, 30 mM $Mg^{2+}Cl$, 100 μM [γ-^{32}P]-ATP, 0.9% BSA, 3 mM DTT, 3 mM EGTA, 0.3 mM $NaVO_3$) and 5 μl 2 mg/ml of substrate myelin basic protein (Sigma-Aldrich) for 30 minutes at 30°C. Samples were run on 10% polyacrylamide gel, which was dried out and the radioactivity was quantified by radiography.

Calcium influx assay. Calcium influx assay with G2 and B1 cells following stimulation with SDF-1 (0.5 μg/ml) was performed as previously described (2).

RT-PCR for PKC-ζ. Total RNA was isolated from human peripheral blood neutrophils, G2 cells, and B1 cells using TRI Reagent (Molecular Research Center Inc.). RT-PCR was performed as previously described (7) using specific primers for PKC-ζ (sense 5′ CCGAGCACCCCTGAGCAGCCTG, antisense 5′ AGACGACAAGAACGAGGACGCCGAC; PCR product 451 bp).

Actin polymerization assay. $CD34^+$ cells were pretreated with chelerythrine chloride (10 μM, 30 min) or the indicated PKC PS peptides (10 μM, 1 h), and actin polymerization assay was performed as previously described (62).

Laminar shear flow assay. Laminar flow assays were performed as previously described (27). Briefly, SDF-1– and VCAM-1–coated (1 or 2 μg/ml and 0.5, 1, or 2 μg/ml, respectively) polystyrene plates were assembled as the lower wall of a parallel wall flow chamber. G2 or B1 cells (2×10^6 cells, untreated or pretreated with PS-ζ peptides; 10 μM) were suspended in cation-free HBSS containing 10 mM HEPES, pH 7.4, and 2 mg/ml BSA; perfused into the chamber; and allowed to settle for 1 minute. The shear flow was increased stepwise from 0.5 to 1 to 2.5 dyn/cm^2 at 5-second intervals. Finally, a shear flow of 5 dyn/cm^2 was maintained for 10 minutes. Cells were visualized by a ×10 or ×20 objective of an inverted phase contrast microscope and photographed with a long integration video camera connected to a time-lapse video recorder. Analysis of motile cells was done by enumerating the number of cells attached to the substrate-coated plates that exhibited spatial movement over time.

Proliferation and survival assay. G2 cells (3×10^5/100 μl) were cultured in serum-free medium with SDF-1 (1 ng/ml) and/or PS-ζ peptides (10 μM). After 3 days, [^{3}H]-thymidine (1 μCi/well; Amersham Biosciences) was added for an additional 16 hours. Cells were harvested onto fiberglass filters, and radioactivity was detected in a Matrix-96 direct β counter (Packard Instrument Co.). For cell survival assay, CB $CD34^+$ cells (5×10^3/500 μl) were cultured in serum-free RPMI with SDF-1 (1 ng/ml) and/or PS-ζ peptides (10 μM). After 4 days, the number of viable cells was determined by trypan blue exclusion.

Gelatin zymography. CB $CD34^+$ cells (2×10^6/ml) were cultured for 40 hours in serum-free RPMI in the presence of SDF-1 (500 ng/ml) and PKC PS peptides (10 μM), wortmannin (50 nM), U73122 (10 μM), or PD98059 (5 μM). Zymography was performed as previously described (35).

Mice. NOD-SCID mice were bred and maintained under defined flora conditions in ventilated sterile micro isolator cages. All the experiments were approved by the Animal Care Committee of the Weizmann Institute. Eight-week-old mice were irradiated with a sublethal dose of 375 cGy from a cobalt source 24 hours prior to transplantation. After 1 hour preincubation with PKC PS peptides (10 μM) or chelerythrine chloride (10 μM) and 2 washings, human CB $CD34^+$-enriched cells (2×10^5 for engraftment or 5×10^5 for homing) were injected into tail vein. Mice were sacrificed after 16 hours (for homing) or 30–45 days (for engraftment). BM cells were flushed from femurs, tibias, and pelvic bones. Percentages of human cells were determined by flow cytometry as described below.

PKC PS peptides (50 μg) were injected i.p. in nonengrafted, nonirradiated NOD/SCID mice daily for 3 consecutive days, and a hematocytometer was used to determine the number of wbcs. The number of CFCs was assayed as previously described (13). Experiments in splenectomized mice were performed 15 days after spleen removal.

Flow cytometry. Expression of CXCR4 was detected with anti-CXCR4 PE (BD Biosciences — Pharmingen). Levels of human cells in the BM of engrafted mice were detected with anti-human CD34 FITC, CD38 PE, or CD45 FITC (IQ Products) as previously described (13). After staining, cells were analyzed by FACSCalibur using CellQuest software (BD Biosciences).

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