**Article**

Protein kinase C-δ and -β coordinate flow-induced directionality and deformation of migratory human blood T-lymphocytes

Shu-Yi Wei¹, Ting-Er Lin¹, Wei-Li Wang¹, Pei-Ling Lee¹, Min-Chien Tsai², and Jeng-Jiann Chiu¹,3,4,*

1 Institute of Cellular and System Medicine, ‘National’ Health Research Institutes, Miaoli 350
2 Department of Physiology and Biophysics, ‘National’ Defense Medical Center, Taipei 114
3 Institute of Biomedical Engineering, ‘National’ Tsing Hua University, Hsinchu 300
4 * Correspondence to: Jeng-Jiann Chiu, E-mail: jjchiu@nhri.org.tw

T-lymphocyte migration under flow is critical for immune responses, but the mechanisms by which flow modulates the migratory behaviors of T-lymphocytes remain unclear. Human peripheral blood T-lymphocytes (PBTLs), when stimulated with phorbol 12-myristate 13-acetate (PMA), stretched their cell bodies dramatically and moved along the flow direction. In contrast, stromal cell-derived factor-1α-stimulated PBTLs deformed and migrated in a random manner. Here we elucidated the molecular mechanisms underlying flow-induced directionality and deformation of PMA-stimulated PBTLs. PMA primed PBTLs for polarization under flow, with protein kinase C (PKC)-δ enriched in the leading edge, PKC-βI in the microtubule organizing center, and PKC-βII in the uropod and peripheral region. PKC-δ regulated cell protrusions in the leading edge through Tiam1/Rac1/calmodulin, whereas PKC-β regulated RhoA/Rho-associated kinase activity and microtubule stability to modulate uropod contractility and detachment. Our findings indicate that PKC-δ and -β coordinate in the cell leading edge and uropod, respectively, to modulate the directionality and deformability of migratory T-lymphocytes under flow.

**Keywords:** deformability, directionality, migration, PKCs, T-lymphocyte

**Introduction**

Migration of circulating T-lymphocytes requires the initiation and maintenance of their polarized process, with the formation of leading edge and uropod, which are mediated by various regulatory molecules and signals dispersing along their anterior–posterior axis (Krummel and Macara, 2006). In the leading edge of migratory cells, the filamentous actins (F-actins) are plentifully formed within the filopodia and lamellipodia, providing a pushing force for cell protrusion and spreading (Burnette et al., 2011). Myosin II within the lamella contributes to the condensation of F-actin into an actin arc to drive the retraction of protrusion (Burnette et al., 2011). Repeated cycles of protrusion, pause, and retraction of the cell lead to its forward movement (Burnette et al., 2011), which requires intracellular signals, such as small GTPases Rho, Rac, and Cdc42, to mediate the assembly of actin and myosin (Nobes and Hall, 1995; Mitchison and Cramer, 1996; Stewart et al., 1996). Previous studies demonstrated that dynamic changes of actomyosin in the leading edge of migratory T-lymphocytes are regulated by calmodulin (CaM) and its downstream molecule myosin light chain kinase (MLCK) (Smith et al., 2003). In the uropod of T-lymphocytes, microtubule depolymerization induced by RhoA/Rho-associated kinase (ROCK) results in the detachment of the tail, which is followed by the actomyosin-mediated uropod retraction, leading to cell migration (Takesono et al., 2010). Actin filaments can be coupled to extracellular substrate through integrins, facilitating cell adhesion, protrusion formation, and front and rear retractions (Le Clainche and Carlier, 2008). Thus, cell migration is a net result of coordination of attachment in the leading edge and detachment in the tail of the cell, with morphological alterations (Krummel and Macara, 2006).

Phorbol 12-myristate 13-acetate (PMA) and stromal cell-derived factor 1α (SDF-1α) are two common molecules that can activate T-lymphocytes (Chen et al., 2006; Shulman et al., 2009). PMA is an analogue of diacylglycerol (DAG) that is generated upon the activation of T-lymphocytes to modulate the re-orientation of their...
Results

PMA and SDF-1α differentially modulate the migratory behaviors of PBTLs along the EC monolayers under flow

PMA and SDF-1α are widely used to activate T-lymphocytes for migration study (Chen et al., 2006; Shulman et al., 2009). In this study, PBTLs were treated with PMA (20 nM) or SDF-1α (125 nM) for 10 min, 2, 6, and 24 h, and then perfused over tumor necrosis factor-α (TNF-α)-activated EC monolayers. The migratory paths of arrested PBTLs were traced for 10 min under flow with a shear stress at 7 dynes/cm² (Figure 1A). PBTLs stimulated with PMA for 24 h (PMA-PBTLs) stretched their cell bodies dramatically and moved along the flow direction (Figure 1A and Supplementary Video S1). In contrast, SDF-1α-stimulated PBTLs (SDF-PBTLs) showed winding and random migrations along the EC monolayers, regardless of the SDF-1α treatment time (Figure 1A and Supplementary Video S2). Quantitative analyses of migration paths and morphological changes of arrested PBTLs showed that PMA-PBTLs exhibited lower levels of randomness of movements (Figure 1B), and higher migratory speed (Figure 1C) and deformation index (DI) (Figure 1D) than SDF-PBTLs. Immunostaining of migratory PMA-PBTLs with an antibody against CD3, which is a common antigen on the cell surfaces of T-lymphocytes, confirmed their elongation, with a prominent head-and-tail structure along the flow direction (83.6% ± 6.8% of total cells, P < 0.05, n = 80; Figure 1E). Conversely, SDF-PBTLs exhibited more rounded shape under flow (99.1% ± 0.7% of total cells, P < 0.05, n = 80). Time-lapse microscopic observations revealed that PMA-PBTLs exhibit abundant protrusions in the leading edge, with obvious uropod in the rear (Figure 1F and Supplementary Video S1). In contrast, SDF-PBTLs migrated with no prominent flow-directionality of leading edge and uropod; instead, they exhibited random distributions of lamellipodia/filopodia around the cell periphery (Figure 1F and Supplementary Video S2). These results indicate that PMA and SDF-1α play differential roles in modulating the migratory behaviors of PBTLs along the EC monolayers under flow.

To investigate whether PMA and SDF-1α change the T-lymphocyte subsets, PBTLs were immunostained for CD3, CD4 (a glycoprotein found on the surface of helper T-lymphocytes), and CD8 (a glycoprotein found on the surface of cytotoxic T-lymphocytes). Flow cytometric analysis of immunostained cells showed that PMA stimulation resulted in decreased expression of CD4 on PBTLs after stimulation for 6 and 24 h, but it had no effect on CD3 and CD8 expressions (Supplementary Figure S1). SDF-1α stimulation of PBTLs did not effect on their CD3, CD4, and CD8 expressions, when compared with unstimulated control cells. These are in agreement with the previous results, which showed that PMA reduces the surface expression of CD4 and T cell receptor (TcR) complex, but not CD8, on T cells (Kaldjian et al., 1988; Yoshida et al., 1992). PMA-stimulated PBTLs were subjected over the TNF-α-treated ECs to flow at a shear stress of 7 dynes/cm² for 10 min, and then immunostained with antibodies against CD3, CD4, and CD8. The results showed that all CD3-, CD4-, and CD8-positive stained cells exhibited the elongation with a head-and-tail structure along the flow direction (Supplementary Figure S2), indicating that PMA has the same effect on all subtypes of PBTLs, indicating their directionality and deformation along the flow direction under flow.

PMA, but not SDF-1α, modulates LFA-1 affinity, association, and activation on PBTLs

LFA-1 (αβ) is the most abundant integrin in T-lymphocytes and plays important roles in modulating T-lymphocyte tethering, adhesion, and migration on the activated ECs (Stewart et al., 1996; Shulman et al., 2009). Flow cytometric analysis showed that PMA increased HA-LFA-1 expression on PBTLs (43.1% ± 7.9% vs. 20.5% ± 1.9%, P < 0.05), while SDF-1α did not (23.1% ± 6.2% vs. 20.5% ± 1.9%) (Figure 2A). Native polyacrylamide gel electrophoresis (PAGE) analysis using anti-β2 integrin antibody demonstrated that PMA, but not SDF-1α, induced the association of α1 and β2 integrins to form LFA-1 in PBTLs (Figure 2B). The longer the PMA treatment time, the higher were the levels of LFA-1 (Figure 2C). PMA induced rapid (within 10 min) and sustained (24 h) increases in β2 integrin phosphorylation in PBTLs.
The levels of αL integrin were also increased by PMA stimulation within 2 h. However, SDF-1α stimulation had no effect on these signaling events in PBTLs over the 24-h test period. Pre-treating PBTLs with anti-β2 integrin neutralizing antibody (1:100 w/v) before PMA and SDF-1α stimuli inhibited the adhesion of these PBTLs to TNF-α-activated ECs (Figure 2E). However, for the arrested PBTLs on ECs under flow, anti-β2 integrin antibody treatment only inhibited the migration (Figure 2F and G) and deformation (Figure 2F–H) of PMA-PBTLs, but not those of SDF-PBTLs. These results indicate that PMA, but not SDF-1α, modulates migration and deformation of PBTLs under flow due to the modulation of LFA-1 affinity, association, and activation on these cells.

**PMA primes PBTLs for polarization under flow, with PKC-δ enriched in the leading edge, PKC-βI in the MTOC, and PKC-βII in the uropod and peripheral region**

PMA stimulation induced rapid (5 min) and sustained (24 h) increases in PKC-δ phosphorylation and decreases in PKC-βI and -βII expressions and phosphorylations in PBTLs (Figure 3A and Supplementary Figure S4A). The expression of microtubule motor protein kinesin was transiently increased within 2 h, whereas the
Figure 2 PMA, but not SDF-1α, induces HA-LFA-1 and LFA-1 expressions to modulate PBTL adhesion and migration. PBTLs were kept as controls or stimulated with PMA or SDF-1α for 24 h (A and B) or the indicated times (C and D), and their HA-LFA-1 expression (A), α₅β₃ integrin association (B and C), α₃ integrin expression, and β₂ integrin expression and phosphorylation (D) were examined by flow cytometry, native PAGE, and western blot, respectively. (E–H) PMA-PBTLs and SDF-PBTLs were incubated with control IgG or anti-β₂ integrin neutralizing antibody (1:100 w/v) before exposure to the flow. The adhesion and migration assays were performed to determine the number of adherent cells (E), the migration paths (F), migratory speed (G), and DI (H) of migratory cells. Data are mean ± SEM from 3–5 independent experiments. *P < 0.05 vs. control cells. Statistical analysis of the results in D is shown in Supplementary Figure S3.
Figure 3 PMA- and SDF-1α-stimulated PBTLs exhibit differential signaling events. PBTLs were kept as controls or stimulated with PMA and SDF-1α for the indicated times (A) or 24 h (B–G). (A) The expression and phosphorylation of indicated proteins were analyzed by western blot. (B) Cell fractionation assay was performed to examine the expression and phosphorylation of PKC-δ, -βI, and -βII in different cellular compartments. Suspended PBTLs were co-immunostained for PKC-δ (C), -βI (D), and -βII (E) with γ-tubulin and F-actin, and counterstained with DAPI. Dashed arrows indicate MTOC. The arrow indicates PKC-βI staining. In parallel experiments, PBTLs were subjected over the TNF-α-treated EC monolayers to flow for 10 min, and the expression of HA-LFA-1 (F), γ-tubulin, and RhoA (G) were co-immunostained. Arrows indicate the leading edge of a migratory PBTL. Arrowheads indicate cell uropod. Dashed arrows indicate MTOC. Statistical analysis of the results in A is shown in Supplementary Figure S4. Results and images in B–G are representative of the triplicates with similar results.
PKC-δ and PKC-β regulate PMA-induced expression, activation, and affinity change of LFA-1 on PBTLs, respectively

We next investigated whether PKC-δ and -β play a role in the modulations of LFA-1 expression and affinity in the migratory PBTLs under flow. Stimulating PBTLs with PMA (20 nM) for 6 h resulted in increases inα1 integrin expression (Figure 4A), β2 integrin phosphorylation (Figure 4B), and the association of αL and β2 integrins (Figure 4C). Since there is no available specific PKC-δ inhibitor, PKC-δ-specific small interfering ribonucleic acid (siRNA) was used to block its function. Transfecting PBTLs with PKC-δ-specific siRNA (50 nM), which caused ~60% reduction in PKC-δ protein expression compared with control siRNA, inhibited PMA-induced αL integrin and LFA-1 expression, but not β2 integrin phosphorylation. In contrast, pre-treating PBTLs with a specific PKC-β inhibitor LY317615 (1 μM) inhibited PMA-induced β2 integrin phosphorylation, but not αL integrin or LFA-1 expression. Immunoprecipitation assays with anti-phospho-β2 integrin antibody demonstrated that PMA stimulation induced associations of phospho-β2 integrin with αL integrin, phospho-PKC-β1, and β-actin, but not with phospho-PKC-δ1, in PBTLs (Figure 4D). This PKA-induction of PKC-β1–integrin–actin associations was inhibited by pre-treating the cells with LY317615, indicating that phospho-PKC-β1 may target cell membrane to form complexes with actin and β2 integrin to phosphorylate β2 integrin. In addition, LY317615 pre-treatment also inhibited PMA-induced HA-LFA-1 expression on PBTLs (51.4% ± 3.8% vs. 42.6% ± 1.9%, P < 0.05) (Figure 4E). These results indicate that PMA induces LFA-1 formation and affinity change on PBTLs through PKC-δ and PKC-β, respectively.

PKC-δ regulates the formation of Rac1-GTP–actin complexes and cell protrusions in the leading edge of migratory PMA-PBTLs under flow through Tiam1

Given our findings that PKC-δ is mainly located in the leading edge of migratory PMA-PBTLs under flow, we investigated whether PKC-δ can regulate Rac1, an important molecule regulating actin polymerization in the leading edge of migratory cells (Nobes and Hall, 1995; Krummel and Macara, 2006). Immunostaining data showed that Rac1 was co-localized with PKC-δ in the leading edge of migratory PMA-PBTLs under flow (70.6 ± 6.1% of total cells, P < 0.05, n = 40), but not in SDF-PBTLs (Figure 5A). Transfecting PMA-PBTLs with PKC-δ-specific siRNA or pre-treating with a specific Rac1 inhibitor NSC23766 (100 μM) inhibited flow-induced migration (Figure 5B) and deformation (Figure 5C) of these cells, which became more rounded without a clear head-and-tail structure and lost their migration directionality under flow (72.5% ± 6.6% and 83.8% ± 7.2% of total cells for siRNA and NSC23766 treatments, respectively, P < 0.05, n = 80 for each; Figure 5D and Supplementary Videos S3–S5). The human p21-activated kinase (PAK)-GST pull-down assays demonstrated that PMA stimulation induced PBTL Rac1 activity and its binding to actin (Figure 5E), as well as the mRNA (Figure 5F) and protein (Figure 5G) expressions of Tiam1, which is a guanine nucleotide exchange factor (GEF) that activates Rac1 (Leeuwen et al., 1997). These PMA-induced responses were inhibited by transfecting PBTLs with PKC-δ-specific siRNA. As negative controls, SDF-1α did not induce these signaling events in PBTLs. These results indicate that PKC-δ can regulate the formation of Rac1-GTP–actin complexes and hence cell protrusions in the leading edge of migratory PMA-PBTLs under flow through Tiam1.

PKC-β regulates RhoA activity and microtubule stability, and hence uropod detachment of migratory PBTLs under flow

Since PKC-β was enriched in the MTOC and PKC-β was co-localized with RhoA in the uropod and peripheral region of PMA-PBTLs under flow, we investigated whether PKC-β regulates uropod stability of migratory PBTLs under flow through modulating RhoA/ROCK activity and microtubule stability. Treatment with PMA (20 nM) for 2 h or SDF-1α (125 nM) for 10 min (control) facilitated the initial adhesion of PBTLs to the activated ECs, but had no effect on PKC-β (Figure 3) or cell elongation under flow (Figure 6A, Supplementary Figure S5 and Video S6). Inhibition of PKC-β or RhoA/ROCK activity in these PBTLs by LY317615 (1 μM) or Y27632 (10 μM), respectively, impaired uropod detachment, which led to the cell elongation along the flow direction and retarded cell migration (88.4% ± 7.6% and 91.2% ± 4.3% of total cells for LY317615 and Y27632 treatment, respectively, P < 0.05, n = 80 for each; Figure 6A and B; Supplementary Figure S5, Videos S7 and S8). Stimulating PBTLs with PMA for 24 h resulted in RhoA activity reduction (Figure 6C) and acetylated α-tubulin induction (Figure 6D), indicating an increased microtubule stability in these cells. Treatment with LY317615 or a microtubule stabilizing reagent Taxol (10 μM) on PMA (2 h)- or SDF-1α (10 min)-pretreated (control) PBTLs can mimic the effects of 24-h PMA treatment to reduce RhoA activity (Figure 6E) and induce acetylated α-tubulin

expressions of microtubule motor protein dynein and MTOC marker γ-tubulin were not affected by PMA. SDF-1α stimulation did not have effects on these examined molecules over the 24-h test period. Cellular fractionation assays showed that PMA induced translocations of PKC-δ, -β1, and -β1 from cell cytosol to membranes (Figure 3B). Co-immunostaining PKC-δ (Figure 3C), -β1 (Figure 3D), and -β1 (Figure 3E) with γ-tubulin and F-actin showed that PMA, but not SDF-1α, induced polarization of PBTLs under static condition (90.4 ± 5.2%, 92.5 ± 2.7%, and 89.6 ± 6.2% of total cells for Figure 3C–E, respectively, P < 0.05, n = 80 for each figure). In PMA-PBTLs, PKC-β1 was enriched and co-localized with γ-tubulin in MTOC, whereas PKC-β1 and PKC-δ were mainly localized in membrane protrusions in cell peripheries. SDF-PBTLs showed similar distribution patterns of these molecules as in control PBTLs. Subjecting PMA-PBTLs over the TNF-α-activated ECs to flow resulted in re-distribution of PKC-δ to the leading edge of migratory cells, where it was co-localized with HA-LFA-1 and F-actin (85.5% ± 6.6% of total cells, P < 0.05, n = 40; Figure 3F). While PKC-β1 was enriched and co-localized with γ-tubulin in MTOC (77.9% ± 4.7% of total cells, P < 0.05, n = 40), PKC-β1 was co-localized with RhoA in the uropod and peripheral region of migratory cells (70.8% ± 8.3% of total cells, P < 0.05, n = 40; Figure 3G). These molecules in different cellular compartments may coordinate the migration and deformation of PBTLs under flow.
PKC-δ and PKC-β regulate cell contractility in the leading edge and uropod of migratory PBTLs under flow through Rac1/CaM and RhoA/ROCK, respectively.

PMA, but not SDF-1α, induced PBTL phosphorylation of myosin light chain 2 (MLC2), a readout of myosin activity and cell contractility (Figure 7A). This PMA-stimulated MLC2 activation was inhibited by pre-treating PBTLs with LY317615 (1 μM) for 4 h, and NSC23766 (Figure 7C) or by transfecting with PKC-δ-specific siRNA (Figure 7C). In addition, PKC-δ-specific siRNA and NSC23766 treatment both inhibited PMA-induced CaM expression (Figure 7D), indicating that PKC-δ/Rac1 regulates myosin activity through CaM in these PBTLs. As controls, SDF-PBTLs showed rounded shapes on TNF-α-activated ECs under flow, with phospho-MLC2 uniformly distributed in the cell periphery (Figure 4).

PKC-δ and PKC-β modulate the amount of α1 integrin and the affinity of β2 integrin in PMA-PBTLs, respectively. Before stimulating with PMA for 6 h, PBTLs were transfected with control (siC) or PKC-δ-specific (siδ) siRNAs (50 nM for each) for 48 h, or pre-treated with LY317615 (1 μM) for 4 h. The α1 integrin expression (A), β2 integrin phosphorylation (B), and α1β2 integrin association (C) were examined. (D) Immunoprecipitation with anti-phospho-β2 integrin antibody, followed by western blot analysis for the indicated proteins. (E) Flow cytometric analysis of HA-LFA-1 expression on PBTLs. Data are mean ± SEM from three independent experiments. *P < 0.05 vs. control cells. **P < 0.05 vs. PMA-simulated cells.
(Figure 7E-a). Inhibition of PKC-β activity in SDF-PBTLs by LY317615 resulted in cell elongation along the flow direction, with higher levels of phospho-MLC2 in the leading edge than the tail (90.1% ± 3.2% of total cells, P < 0.05, n = 40; Figure 7E-b). As expected, PMA stimulation induced the enrichment of phospho-MLC2 in the leading edge of migratory PBTLs under flow (Figure 7E-c). Transfecting PBTLs with PKC-δ-specific siRNA inhibited PMA-induced phospho-MLC2 enrichment in the leading edge and hence cell elongation under flow (92.3% ± 3.5% of total cells, P < 0.05, n = 40; Figure 7E-d). These results indicate that PKC-δ and PKC-β may regulate cell contractility in the leading edge and tail of migratory PBTLs under flow through Rac1/CaM and RhoA/ROCK, respectively.

Discussion

PKC-δ is required for the migration of T-lymphocytes on lamellipodia and filopodia in the front of migratory cells through its downstream effectors Wiskott–Aldrich syndrome protein and the Scar family, which act on Arp2/3 (Nobes and Hall, 1995; Stewart et al., 1996; Machesky and Insall, 1998). PKC-δ is activated in response to SDF-1α, which can regulate the polymerization of actin in the protrusion of lamellipodia and filopodia in the front of migratory cells (Figure 5B–D). These results indicate that PKC-δ plays important roles in modulating the formation of actin-based lamellipodia/filopodia and hence cell attachment in the leading edge of migratory PBTLs under flow through Rac1-mediated activation of PKC-δ.

ROCK activities are required for uropod retraction and tail detachment of migratory cells (Smith et al., 2003; Takesono et al., 2006; Kurokawa et al., 2003; Wei et al., 2006; Shulman et al., 2009). In the present study, we demonstrate for the first time that PMA and SDF-1α stimuli induce differential behaviors of PBTLs migrating along the EC monolayers under flow. PMA-stimulated PBTLs stretch their cell bodies dramatically and move along the flow direction. In contrast, SDF-stimulated PBTLs have rounded cell shape and show winding and random migration under flow. These may be attributed to the differential regulations of PKC-δ and PKC-β, which modulate downstream Tiam1/Rac1 and RhoA/ROCK activities, respectively, and consequently cell contractility and lamellipodia/filopodia formation (Figure 8). Our findings indicate that PKC-δ and PKC-β can coordinate the directionalities and deformation of PBTLs during their migration along the EC monolayers under flow.

The polarization of T-lymphocytes is an initial step leading to their migration, which requires the coordination of intracellular signaling events in the front and rear of the cell to regulate cytoskeletal reorganization (Krummel and Macara, 2006). As the cell polarity is formed, abundant F-actin appears in the front of the cell, whereas the MTOC is repositioned behind the cell nucleus in the rear to regulate microtubule assembly (Hogg et al., 2003). An interesting finding of our study is that only PMA, but not SDF-1α, can induce polarization of PBTLs before their adherence to the activated ECs (Figure 3C–E). Once adherence to the activated ECs, only PMA-PBTLs can move along the flow direction, showing their persistent directional migration under flow (Figure 1 and Supplementary Video S1). These results suggest that the mechanisms by which PMA and SDF-1α stimulate PBTL migration under flow are different. Shulman et al. (2009) showed that SDF-1α induces shear-resistant migration of T-lymphocytes on and across the activated ECs. EC-bound SDF-1α can induce the formation of numerous adhesive filopodia, which are dispersed underneath T-lymphocytes to strengthen their adhesion to ECs. These results may account for the phenomenon that SDF-PBTLs can frequently turn their front despite the direction of flow (Figure 1 and Supplementary Video S2). On the other hand, PMA can induce re-localizations of PKC-δ and PKC-β to the leading edge and the MTOC and uropod of the cell, respectively. While PKC-δ in the leading edge can modulate the actin-dependent protrusion (attachment) through Tiam1/Rac1, PKC-β in the MTOC and cell uropod can modulate the dynamics of microtubules and detachment of uropod through RhoA/ROCK-mediated microtubule disruption. In addition, PMA, but not SDF-1α, up-regulates PKC-δ and down-regulates PKC-β signaling in PBTLs (Figure 3A), which consequently promotes the attachment of cell leading edge and impairs the retraction and detachment of cell uropod under flow. These findings may explain why PMA-PBTLs, but not SDF-PBTLs, can stretch their cell bodies dramatically and move along the flow direction under flow.

Rac1 regulates the polymerization of actin in the protrusion of lamellipodia and filopodia in the front of migratory cells through its downstream effectors Wiskott–Aldrich syndrome protein and the Scar family, which act on Arp2/3 (Nobes and Hall, 1995; Stewart et al., 1996; Machesky and Insall, 1998). PKC-δ is known to activate Rac1 (Kurokawa et al., 2004). Our present study provides the first demonstration that PMA-activated PKC-δ is highly co-localized with Rac1 in the leading edge of migratory PBTLs under flow (Figure 5A). Moreover, PKC-δ regulates Rac1 activity through Tiam1 in PMA-PBTLs. Blockages of PKC-δ expression and Rac1 activity by the specific inhibitor or siRNA showed similar cellular phenotypes and migratory patterns of PBTLs under flow, with the inhibition of lamellipodia/filopodia formation in the leading edge of migratory cells and reduction of their migratory speed (Figure 5B–D, and Supplementary Videos S3–S5). These results indicate that PKC-δ plays important roles in modulating the formation of actin-based lamellipodia/filopodia and hence cell attachment in the leading edge of migratory PMA-PBTLs through Tiam1-mediated activation of Rac1 under flow.

Figure 5 PKC-δ regulates protrusion formation in the leading edge of migratory PBTLs through Tiam1/Rac1. PBTLs were stimulated with PMA or SDF-1α for 24 h or the indicated times, and then subjected over the TNF-α-treated EC monolayers to flow for 10 min (A–D) or kept suspended (E–G). In some experiments, PBTLs were transfected with control (siC) and PKC-δ-specific (siδ) siRNAs (50 nM for each) for 48 h or treated with NSC23766 (NSC) (100 μM) or vehicle control (1 × PBS) for 4 h before PMA or SDF-1α stimulation. (A) Migratory PBTLs were immunostained for Rac1, PKC-δ, and F-actin. The migratory speed (B) and DI (C) of migratory cells were determined. Data are mean ± SEM of 30 cells from three independent experiments. *P < 0.05 vs. control cells. (D) Sequential views of migratory PMA-PBTLs extracted from Supplementary Videos S3–S5. (E) PAK-GST pull-out assay was performed to examine Rac1 activity and its association with actin. The expressions of Tiam1 mRNA (F) and protein (G) in PMA-PBTLs were examined by RT–PCR and western blot, respectively. Arrows and arrowheads indicate cell leading edge and uropod, respectively. Dashed arrows indicate peripheral filopodium. Data in E–G are mean ± SEM from three independent experiments. *P < 0.05 vs. control cells. **P < 0.05 vs. PMA-stimulated cells. Results in A and D are representative of the triplicates with similar results.
Figure 6 PKC-β regulates the stability of cell uropod via RhoA/ROCK. PBTLs were stimulated with PMA (A–F) or SDF-1α (B, E, and F) for 24 h or the indicated times, and then subjected over the TNF-α-treated EC monolayers to flow for 10 min (A and B) or kept suspended (C–F). In some experiments (A, B, E, and F), PBTLs were pre-treated with LY317615 (LY) (1 μM; 4 h), Y27632 (10 μM; 30 min), Taxol (10 μM; 30 min), or vehicle control. (A) Sequential views of migratory PMA-PBTLs extracted from Supplementary Videos S6–S8. Arrows and arrowheads indicate cell leading edge and uropod, respectively. (B) The randomness, migratory speed, and DI of migratory cells were determined. Data are mean ± SEM of 30 cells from three independent experiments. *P < 0.05 vs. PMA-PBTLs. #P < 0.05 vs. cells without inhibitor treatment. (C and E) Rhotekin-RBD-GST pull-out assay was performed to examine RhoA activity. (D and F) The level of acetylated α-tubulin was examined to evaluate the stability of microtubule. Data in C–F are mean ± SEM from three independent experiments. *P < 0.05 vs. control cells. #P < 0.05 vs. PMA-PBTLs or SDF-PBTLs. Results in A are representative of the triplicates with similar results.
PKC-δ and -β regulate cell contractility in the leading edge and uropod of migratory PBTLs through CaM and RhoA/ROCK, respectively. (A–D) PBTLs were stimulated with PMA or SDF-1α for the indicated times or 24 h. In some experiments (B–E), PBTLs were pre-treated with LY317615 (LY) (1 μM; 4 h), Y27632 (Y) (10 μM; 30 min), NSC23766 (NSC) (100 μM; 4 h), or vehicle control, or transfected with control (siC) or PKC-δ-specific (siδ) siRNAs for 48 h before PMA or SDF-1α stimulation. The level of phospho-MLC2 (A–C) and calmodulin (CaM) (D) was examined by western blot. Data are mean ± SEM from three independent experiments. *P < 0.05 vs. control cells. #P < 0.05 vs. PMA-stimulated cells. (E) Conditioned PBTLs were subjected over the TNF-α-treated EC monolayers to flow for 10 min, and then co-immunostained for phospho-MLC2 and F-actin. White arrows and arrowheads indicate cell leading edge and uropod, respectively. Schematic diagrams in the right panels show morphological changes of the cell treated with different reagents. Right upward arrow, enhanced effect of PMA on the leading edge attachment. Right downward bold arrow, inhibitory effect of PKC-δ-specific siRNA on the leading edge attachment. Left downward arrow, reduced effect of PMA on the uropod detachment. Left downward bold arrow, inhibitory effect of LY317615 on the uropod detachment. Results are representative of the triplicates with similar results.
et al., 2010). They are regulated by the stability of microtubules whose depolymerization causes the release of GEF for Rho (RhoGEF) (Takesono et al., 2010). Our results show that PMA stimulation increases microtubule stability and decreases RhoA activity in PBTLs (Figure 6C and D). SDF-1α stimulation does not show these regulatory effects. Treating SDF-PBTLs with Taxol, a microtubule stabilizing reagent, increases microtubule stability and decreases RhoA activity, similar to that in PMA-PBTLs (Figure 6E and F).

These results confirm the role of microtubule in modulating RhoA activity in PBTLs. An important finding of this study is that PMA downregulates the expression and phosphorylation of PKC-βI and -βII (Figure 3A) and induces their translocation from cell cytosol to membrane in PBTLs (Figure 3B), with PKC-βI co-localized with γ-tubulin at MTOC (Figure 3D). When PMA-PBTLs over the activated ECs are subjected to flow, PKC-βI remain at the MTOC and PKC-βII is co-localized with RhoA to be enriched in the uropod and peripheral region of migratory PBTLs (Figure 3G). These dynamic changes of PKC-β localization in response to PMA suggest that PKC-βI and PKC-βII may play important roles in modulating PMA-induced migration of PBTLs under flow through modulating their microtubule assembly and RhoA activity, respectively. These results are in agreement with the previous studies (Volkov et al., 2001; Fanning et al., 2005), which showed that PKC-βI may target the microtubule and MTOC to modulate cytoskeletal reorganization. Moreover, inhibition of PKC-β (Supplementary Video S7) and RhoA/ROCK (Supplementary Video S8) activities in control PBTLs had similar effects that impair tail detachment of migratory PBTLs under flow, leading to the elongation of these cells along the flow direction. However, these cells still exhibited abundant protrusions in the cell leading edge, suggesting that PKC-β and RhoA/ROCK signals may affect more in the cell rear than the front. Our results represent the first demonstration that PKC-β can regulate RhoA/ROCK signaling by regulating microtubule stability, with the consequent modulation in the tail detachment of migratory PBTLs under flow.

LFA-1 (αLβ2) is the most abundant integrin in T-lymphocytes and plays important roles in modulating their tethering, adhesion, and migration on activated ECs (Stewart et al., 1996; Shulman et al., 2009). It can be activated through the regulation of conformational changes in extracellular domains of β2 integrin (affinity) and its
clustering in the membrane (avidity) (Valmu et al., 1999; Lu et al., 2001; Shulman et al., 2009). In the present study, we found that PMA, but not SDF-1α, induces α1β2 integrin association and HA-LFA-1 expression in PBTLs (Figure 2), indicating that SDF-1α may regulate PBTL adhesion and migration through different integrins. This notion is supported by a previous study, showing that SDF-1α induces rapid clustering (<0.1 sec) of very late antigen-4 (VLA-4, α4β1), which binds vascular cell adhesion molecule-1 on ECs for T-lymphocyte tethering (Grabovsky et al., 2000). Our recent data also showed that SDF-1α stimulation induces β2 integrin expression in PBTLs within few minutes (Supplementary Figure S6), which may provide a pool for VLA-1 formation and clustering. PKC activation has been shown to be required for LFA-1-mediated adhesion and locomotion of T-lymphocytes through inside-out (i.e. LFA-1 activation) and outside-in (i.e. post-LFA-1 activation) signalings (Stewart et al., 1996; Valmu et al., 1999; Volkov et al., 2001). The involvement of PKC-δ in LFA-1 clustering in pro-B lymphocyte has been reported (Romanova et al., 2010). In addition, PMA has been shown to activate LFA-1 upon PKC-mediated β2 integrin phosphorylation (Peter and O’Toole, 1995; Stewart et al., 1996; Valmu et al., 1999). In the present study, we demonstrated for the first time that PKC-δ is involved in PMA-induced α1 integrin expression and hence α1β2 integrin association in PBTLs (Figure 4A–C). On the other hand, PKC-β may directly modulate β2 integrin phosphorylation in PMA-PBTLs (Figure 4B–D). These findings indicate that PKC-δ and PKC-β play differential roles in modulating different subunits of LFA-1, i.e. α1 and β2, to activate LFA-1 in PMA-PBTLs.

Myosin motor activity has been shown to be required for cell contractility in the front and rear compartments of T-lymphocytes (Krummel and Macara, 2006) and regulated by CaM/MLCK in the cell leading edge and RhoA/ROCK in the cell uropod (Hogg et al., 2003; Smith et al., 2003). In the present study, we further demonstrated that these CaM/MLCK- and RhoA/ROCK-mediated myosin activities in the cell leading edge and uropod are regulated by PKC-δ and PKC-β, respectively. Blocking PKC-β, RhoA/ROCK, and Rac1 activities and knockdown of PKC-δ in PMA-PBTLs inhibited PMA-induced MLC2 phosphorylation in these cells (Figure 7A–D). Moreover, blockage of PKC-β activity in PMA-PBTLs can mimic the PMA effects to cause lower levels of phospho-MLC2 in the cell tail than the front, and hence impair the tail detachment and induce cell elongation under flow (Figure 7E–b). In contrast, knockdown of PKC-δ in PMA-PBTLs can inhibit MLC2 phosphorylation in the cell leading edge, and hence impair the protrusion extension to cause more rounded shape (Figure 7E–d). These results close a loop to indicate that PKC-δ and PKC-β play important roles in modulating myosin activity and hence cell contractility in the leading edge and tail of PMA-PBTLs through Rac1/CaM and RhoA/ROCK, respectively, thus affecting the cellular phenotype and migratory pattern of these cells under flow.

It is known that PMA can mimic the function of second messenger DAG, which is an activator of PKC signaling pathway, to induce cell cycle arrest and macrophage differentiation (Das et al., 2000). T-lymphocytes are involved in the inflammatory responses in the vessel wall, including the development of atherosclerotic plaques (de Boer et al., 1999). PMA stimulation of T-lymphocytes generated from atherosclerotic lesions can induce the production of interferon-gamma (IFN-γ) and interleukin-4 (IL-4) that consequently induce vascular endothelial inflammation, suggesting the important role of T-lymphocytes in modulating the formation and progression of atherosclerotic lesions (de Boer et al., 1999). MTOC polarization of T-lymphocytes toward the antigen-presenting cells was shown to be driven by the accumulation of DAG and recruitment of different PKCs at the immunological synapse, which is crucial for the directional secretion of cytokines and cytotoxic responses in T-lymphocytes (Carrasco and Merida, 2004; Quann et al., 2009, 2011). In addition, PMA can induce the expression of adhesion receptors (integrins) that are crucial for the interaction between circulating leukocytes and vascular endothelium during immune responses (Cotran and Mayadas-Norton, 1998; Jonathan et al., 2004; Shulman et al., 2009). In the present study, we demonstrated that PMA can induce the polarization of T-lymphocytes and lead to their directional, persistent migration along the flow direction. These results suggest that PMA, like DAG, may guide the orientation and directionality of T-lymphocytes under flow to exert their functions in the inflammatory and immune responses. Our results also indicate that PMA can activate α1β2 integrin (LFA-1) through PKC-δ and -β, which may facilitate the interaction between T-lymphocytes and vascular endothelium during inflammatory and immune responses.

In summary, our study provides a new concept that PKC-δ and -β can coordinate the directionality and deformation of PBTLs during their migration under flow (Figure 8). PMA induces PKC-δ activity and hence protrusion formation in the leading edge of migratory PBTLs through the up-regulation of Tiam1/Rac1/CaM/LMLC2 cascade. In contrast, PKC-β is downregulated in PMA-PBTLs, which causes decreased activities of RhoA/ROCK/MLC2 and increased stability of microtubules, thus impairing the uropod retraction and tail detachment of migratory cells. The coordination of these signaling events in the head and tail of migratory PMA-PBTLs controls the directionality and deformation of these cells under flow. Our findings help the understanding of mechanisms that control T-lymphocyte migration under flow, thus benefiting the manipulation of T-lymphocyte behaviors during their extravasation in response to differential microenvironments.

Materials and methods

Materials

PMA was purchased from Sigma Chemicals. SDF-1α was obtained from R&D Systems. Detailed materials used in this study are provided in Supplementary materials and methods.

Cell cultures

Human PBTLs were isolated from citrate-anticoagulated fresh whole blood from healthy volunteers by density-gradient centrifugation method, as described (Boyum, 1968; Shulman et al., 2009). Before adhesion and migration experiments, PBTLs were stimulated with PMA (20 nM) for 24 h or SDF-1α (125 nM) for 10 min, or the designated times. Human umbilical vein ECs were isolated from human umbilical cords by collagenase perfusion method (Gimbrone, 1976). Secondary cultures stimulated with TNF-α...
(200 U/ml) for 4 h were used in all experiments. Detailed procedures of cell cultures are provided in Supplementary materials and methods.

Adhesion and migration assays
The slides with TNF-α-treated ECs were mounted in a parallel-plate flow chamber and connected to the perfusion loop system, as previously described (Chiu et al., 2003). The chamber was placed on the stage of an inverted microscope (Axiovert 200M; Zeiss), to which a CCD video camera (CCD-72; Dage-MTI) was attached. The video image was transmitted to a video monitor (HR-1000; Dage-MTI) and recorder (SR9090U; JVC), enabling the recording of results in the video fields. $3 \times 10^6$–$6 \times 10^6$ PBTLs were perfused over the EC monolayers in the chamber and settled for 3 min to allow adherence. After incubation, non-adherent PBTLs were removed by gentle washing with culture medium for 1 min. Arrested (or adherent) PBTLs were defined as cells that did not detach or roll during the 1-min sampling time. The arrested cells on ECs were then subjected to a laminar flow with a shear stress at $7 \text{ dynes/cm}^2$ for 10 min, as previously described (Chen et al., 2006). Images were analyzed by using the image analysis software AxioVision Tracking Module (Zeiss) and a MacIntosh computer (Macintosh). The net migration distance was obtained by comparing the initial and final positions over 10 min, and the total distance traversed was obtained for the same 10-min experiment by summing the individual distances traversed during successive 10-sec periods. The ratio of total distance to net distance provides an index of randomness of migration. DI was determined using the formula described in the following section. In some experiments, images of migratory PBTLs were also taken every 10 sec with Metamorph image software to acquire time-lapse photography and assembled into a QuickTime movie using Photoshop CS (Adobe Systems).

Deformation index
To evaluate the degree of morphological changes of migratory PBTLs under flow, cells were fixed in 4% (w/v) paraformaldehyde and stained for CD45. DI was determined as described (Fanning et al., 2005). Briefly, 30 cells from six randomly selected fields of view were analyzed by using the Metamorph image software with the following formula. DI = elongation index/circularity index, where elongation index = major ellipse diameter/ minor ellipse diameter, and circularity index = $4 \pi \times \text{area/ perimeter}^2$. DI larger than 10 is considered high degree of cell deformation (Fanning et al., 2005).

Rac1 and RhoA activity assay
Control and stimulated PBTLs were extracted with a lysis buffer (300 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% NP40, 10 mM MgCl$_2$, 1 mM DTT, and protease inhibitor cocktail) and centrifuged with 14000 rpm at 4°C for 10 min. Six hundred micrograms of each sample were used to analyze Rac1 and RhoA activities. The Rac/Cdc42 binding domain of PAK and the Rho-GTP binding domain (RBD) of the human Rhotekin are tagged with GST (PAK-GST and Rhotekin-RBD-GST, respectively) and bounded to colored glutathione sepharose beads (Cytoskeleton) to pull-out the active forms of Rac1 and RhoA, respectively. Detailed procedures of assessing Rac1 and RhoA activities, respectively, are provided in Supplementary materials and methods.

Statistical analysis
Results are expressed as mean ± SEM from 3–5 independent experiments. Statistical analysis was performed by using an independent Student t-test for two groups of data and analysis of variance (ANOVA) followed by Schefee’s test for multiple comparisons with the statistical package Statistica software (StatSoft). A P-value < 0.05 is considered significant.

Detailed procedures of immunofluorescence staining, flow cytometric analysis, western blot analysis, native polyacrylamide gel electrophoresis (PAGE), compartmental protein isolation and immunoprecipitation, reverse transcription-polymerase chain reaction (RT–PCR), and small interfering RNA (siRNA) transfection assay are provided in Supplementary materials and methods.

Supplementary material
Supplementary material is available at Journal of Molecular Cell Biology online.

Funding
This work was supported by ‘Ministry of Science and Technology grants MOST-103-2321-B-400-001 (to J.-J.C.) and MOST-103-2325-B-016-003 (to M.-C.T. and J.-J.C.).

Conflicts of interest: none declared.

References


