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*J Immunol* 2007; 179:5649-5652; doi: 10.4049/jimmunol.179.9.5649

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Cutting Edge: Atypical PKCs Regulate T Lymphocyte Polarity and Scanning Behavior

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Leukocyte locomotion is a polarized process with diverse regulatory assemblies segregating along an anterior-posterior axis that defines two regions within the cell, the leading edge and the uropod. However, the mechanisms that generate T cell asymmetry downstream of chemokine receptors are ill defined. In this study we show that the atypical protein kinases C (aPKCs), PKCβ and PKCε, are required for an early symmetry breaking step. Once the polarity is established, aPKCs also drive uropod formation. These effects depend on the interaction between Par6 and aPKCs. Finally, failure to transduce aPKC-dependent signals reduces T cell motility and their ability to scan dendritic cells. Altogether, our findings suggest that lymphocyte motor activity is regulated by a signaling cascade that relays chemokinetic input to aPKCs. The Journal of Immunology, 2007, 179: 5649–5652.

The adaptive immune system is composed of dispersed cellular elements that continuously survey peripheral tissues for potential pathogenic agents and convey information to one another to effectively respond to infections. Naïve T lymphocytes traffic through an extensive network of secondary lymphoid organs, dwelling for limited periods in specific compartments and then returning to the blood circulation if no Ag is detected.

The homeostatic chemokines CCL21 and CCL19 are the main high endothelial venule (HEV) signatures in peripheral lymph nodes (1). All naïve T cells express the matching receptor CCR7 and are able to arrest at HEVs. It has been recently shown that dendritic cells (DCs) bear CCL21 at their surfaces (2) and can secrete CCL19 (3). Therefore, it is likely that DCs can also be a source of chemokines in vivo.

Cell migration depends on the functional specialization of discrete regions and is therefore intrinsically related to cell polarity (4). The protrusive forces for locomotion are generated at the lamellipodium, owing to an intense actin-polymerizing activity concentrated at this site. The middle of the cell or lamella integrates adhesion to traction generation (5). The uropod, at the back of the cell, provides the contractile forces that allow the cell body to translocate (6).

The Par6-PKCε complex (where PKC is protein kinase C) has been described in many cell types as a broad regulator of polarity (7). The function of this complex resides in the enzymatic activity of the atypical PKCs (aPKCs), PKCβ or PKCε/ PKCA, which are controlled by Par6. Interestingly, previous results have shown that a minute amount of PKCε is activated in leukocytes exposed to chemoattractants (8, 9), raising the possibility that the Par6-PKCε complex might be an effector of polarity in these cells. We have addressed this hypothesis and provide evidence that aPKCs specify the topography of the two poles of the cell.

Materials and Methods

Plasmid constructs

The FLAG-PKCε constructs were purchased from Addgene. The myc-Par6 and GST-PKCβ constructs were described (10, 11). The pEGFPmax (Amaxa) or pEGFP-C3 (Clontech Laboratories) vectors were used in cotransfection experiments.

RT-PCR

Total RNA from human T cells was extracted using TRIzol reagent (Invitrogen Life Technologies). cDNA was prepared using the Advantage RT-for-PCR kit (BD Biosciences). PCR amplification of cDNA samples was conducted using specific primers and PCR products were resolved on a 1% agarose gel.

Stimulatory and staving reagents

CCL19 was provided by PeproTech and used for cell stimulation at 100 ng/ml in solution under stirring conditions at 37°C. F-actin was stained with phalloidin conjugated to TRITC or Texas Red (Molecular Probes). The anti-FLAG Ab conjugated to FITC was purchased from Sigma-Aldrich. The anti-PKCar was from BD PharMingen. The anti-Myc and anti-PKCε (clone C-20) Abs were from Santa Cruz Biotechnology. Anti-GST was from Amersham Biosciences. Fluorescent anti-rabbit or anti-mouse IgG were all from Jackson ImmunoResearch.

Cells

Human peripheral blood T lymphocytes (PBTs) were isolated by Ficoll density gradient centrifugation from healthy blood donors. PBMCs were depleted of non-T cells with a human T lymphocyte enrichment set (BD Biosciences). DCs...
were provided by Immuno-Designed Molecules (IDM) (12). All cells were cultured in complete RPMI 1640 medium containing 10% AB serum (Cambrex) and were transfected as described (13).

Flow cytometry

T cells cotransfected with GFP together with indicated constructs were stimulated or not stimulated, fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and blocked. They were stained with relevant Abs and analyzed on a FACScan (BD Biosciences).

Microscopy

For immunocytochemistry stainings, PBTs were processed as described in the previous paragraph. Slides were then mounted on coverslips and samples were visualized on an Eclipse TE2000-E inverted microscope (Nikon) equipped with a cooled charge-coupled device camera (CoolsNAPHQ; Photometrics). Image capture and quantitative analysis were done with MetaVue imaging software (Universal Imaging). To monitor the live migration dynamics of T cells, GFP+ cells cotransfected with the appropriate construct were compared. Cells were washed and added to the DC layer. Image acquisition was performed with the MetaFluor imaging software (Universal Imaging). Quantification of the trajectories of individual T cells was performed with the Metamorph (Universal Imaging) or Imaris (Bitplane) tracking software. A linear fit to the trace corresponding to the mean distance traveled vs the square root of time was overlaid. This slope represents the motility coefficient, $M$, given by $M = x^2/4t$ where $x =$ mean distance from origin at time $t$.

Results and Discussion

Chemoattractant-driven T cell polarization is a multistep process

We have analyzed cellular morphologies and F-actin distribution during T cell polarization induced by CCL19. Upon stimulation, F-actin accumulated within seconds in multiple pseudopods around the periphery of cells (Fig. 1). One minute after the initial stimulus, F-actin had polarized to one side in most cells and an axis of polarity was already clearly distinguishable. During the minute that followed, an important fraction of the cells developed a uropod that was even reinforced at later times. Thus, T cell polarization occurs through a series of sequential steps.

aPKCs drive T lymphocyte polarization

Studies in diverse model systems have converged into a model whereby Cdc42 and PI3K act in concert to control polarization with respect to external gradients (14). We initially hypothesized that PI3K could mediate these changes in T cells. However, an in-depth analysis performed in our laboratory has shown that PI3K does not play a major role in T cell polarization (15).
Therefore, in an attempt to screen for molecular components involved in T cell polarization, we used kinase activity mutants of PKCζ and PKCs. Both isoforms are expressed in PBTs (data not shown) and activated by CCR7 signaling (9).

We overexpressed the wild-type (WT) enzyme and the T410A and K281W kinase-dead (KD) mutants and determined their effect on cell morphology and actin distribution. Following CCL19 stimulation, most control and WT PKCζ-transfected cells showed a strong asymmetric F-actin staining that was accompanied by the formation of a distinctive uropod (Fig. 2, a and b). In contrast, lymphocytes expressing the activation loop T410A PKCζ mutant, whose activity cannot be upregulated but who keeps its ability to bind its partners, were not able to develop these rear structures (45 vs 5%; \( p < 0.005 \)) and an important fraction accumulated F-actin randomly around the perimeter of the cell (20 vs 3%; \( p < 0.005 \)). We observed the same phenotype when K281W PKCζ was overexpressed, although the effects were less pronounced (24% had uropods and 6% showed random F-actin), possibly due to the lower levels of expression of this mutant. The same tendency was observed in T cells transfected with a KD mutant of PKCs. The proportion of fully polarized PBTs dropped from 48% in control conditions to 26% in cells expressing KD PKCζ (\( p = 0.008 \)) (Fig. 2c). Thus, these results suggest that aPKCs have a role in T cell polarization.

Notably, not all signaling events were affected by expression of the mutant forms of aPKCs. For example, the increase in F-actin levels was found to be not significantly different between control, T410A PKCζ-transfected (Fig. 2d), and KD PKCζ-transfected cells (Fig. 2e), which confirmed that the observed defects in cell polarization were not related to the process of F-actin assembly itself. In addition, the increase in phosphorylated Akt was normal (data not shown), excluding a nonspecific effect on PDK1-dependent signaling.

Cell fractionation experiments in leukocytes provided evidence for a partial redistribution of the PKCζ pool from a particulate fraction to the plasma membrane, but PKCζ translocation has not been directly visualized (8, 9). In another study, an immunocytochemistry analysis concluded that PKCζ was mostly cytosolic (16). To clarify this issue, we have determined the intracellular distribution of aPKCs by immunofluorescence. We have used the cytosolic staining of PKCζ as an internal control to correct for nonspecific accumulation of cytoplasm. The aPKCs PKCα and PKCζ colocalized with PKCα in resting and fully polarized cells (Fig. 2f). We have found the same pattern of colocalization at the early time points (data not shown). Importantly, we did not observe any obvious translocation of aPKCs to the plasma membrane. Thus, T lymphocyte polarization requires aPKCs but does not involve distinct polarization of the enzyme.

Par6 also contributes to T cell polarization

Par6 is a scaffold protein that constitutively associates with aPKCs in numerous models (17). The most common form of Par6, Par6C, and a low level of Par6B could be detected in T lymphocytes by RT-PCR (Fig. 3a), raising the possibility that the components of this complex might have been conserved in lymphocyte polarization.

Because all Par6 isoforms bind to aPKCs via an N-terminal (NT) PB1 domain (17), we have overexpressed this region alone (NT-Par6) to interfere with the regulation of aPKCs.

Contrary to chemokine-stimulated control cells that accumulated F-actin asymmetrically and were capable of protruding uropods (Fig. 3b), NT-Par6-expressing cells formed fewer uropods (25 vs 45%) and a comparatively higher fraction failed to respond to the stimulus (25 vs 11%). Although these defects were only partial, Par6, like aPKCs, seems to contribute to a fully polarized phenotype.

T cell scanning of DCs depends on PKCζ activity

Because aPKCs are necessary to assemble the cellular domains that support these specialized activities, we hypothesized that lymphocyte migration would be equally dependent on aPKCs. To test this, we have visualized the scanning behavior of control, WT, and T410A PKCζ-transfected T cells interacting with DCs. Fig. 4a depicts the position of representative WT and T410A PKCζ-expressing cells at different time points. As control cells, WT-PKCζ-expressing cells were motile and interacted sequentially with several DCs (movie 1). In contrast, T410A PKCζ cells were less motile (movie 2), had lower top speeds, and only made contacts with DCs in their immediate vicinity (50 vs 22% migrated more than three times the cell size). In addition, although these cells were not inert they failed to polarize. The mean velocities and motility coefficients (8.33 vs 4.61) were also consistently decreased (Fig. 4, b–d), showing that T410A PKCζ cells moved comparatively more slowly and for shorter distances than control ones. Thus, PKCζ-dependent polarization favors effective lymphocyte migration.

In this study, we provide evidence that the key effectors of epithelial and neuronal polarity are similarly implicated in T cell polarization downstream of chemokine receptors. Our results are consistent with a model whereby Par6 and aPKCs control a series of steps leading to the assembly of spatially confined subdomains within the lymphocyte. Cellular locomotion is an
intrinsically polarized process such that the disruption of cell polarity should have a negative impact on the locomotory function. Our results confirm this view. Indeed, αPKCs were shown to exert direct control over the establishment of axial polarity upon chemokine stimulation. Interestingly, a recent paper has shown no effect of PKCθ on chemokinesis but a role for this isoform in breaking the symmetry of the immunological synapse to initiate migration (18). It is thus possible that αPKCs and PKCθ share sequential roles in the control of T cell motility in vivo.

Nevertheless, our results suggest that T cells with impaired αPKC signaling not only failed to segregate the lamellipodium along a single axis but were also inefficient in forward movement. This in turn was shown to compromise the Ag-independent scanning activity of these cells, which could not engage into multiple sequential interactions with DCs and often meandered around the same spot. Thus, our data suggest that αPKC activity may be essential for T cell function in adaptive immunity, because the detection of rare foreign Ags relies on the ability of lymphocytes to scan a large number of DCs in an adequate time frame.

Acknowledgments
We thank A. Toker, M.W. Wooten and S.G. Martin for sharing reagents, Immuno-Designed Molecules (IDM) for providing DCs, and D. Fruman, A. Trautmann, G. Bismuth, and C. Randriamampita for critical reading of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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