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Protein Kinase C-θ Clustering at Immunological Synapses Amplifies Effector Responses in NK Cells

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In lymphocytes, stimulation of cell surface activating receptors induces the formation of protein microclusters at the plasma membrane that contain the receptor itself, along with other signaling molecules. Although these microclusters are generally thought to be crucial for promoting downstream cellular responses, evidence that specifically links clustering potential to signaling output is lacking. We found that protein kinase C-θ (PKCθ), a key signaling molecule in multiple lymphocyte subsets, formed microclusters in activated NK cells. These microclusters coalesced within the immunological synapse between the NK cell and its target cell. Clustering was mediated by the regulatory region of PKCθ and specifically required a putative phosphotyrosine-binding site within its N-terminal C2 domain. Whereas expression of wild-type PKCθ rescued the cytokine production defect displayed by PKCθ-deficient NK cells, expression of a PKCθ point-mutant incapable of forming microclusters had little to no effect. Hence, PKCθ clustering was necessary for optimal effector function. Notably, only receptors containing ITAMs induced PKCθ microclusters on their own, explaining previous observations that ITAM-coupled receptors promote stronger activating signals and effector responses than do receptors lacking these motifs. Taken together, our results provide a cell biological basis for the role of PKCθ clustering during NK cell activation, and highlight the importance of subcellular compartmentalization for lymphocyte signal transduction. The Journal of Immunology, 2012, 189: 000–000.
PKCα microclusters amplify NK cell effector responses

(14, 15). A number of distinct lymphocyte activating receptors are known to form microclusters in response to ligand binding (16–19). It is generally thought that this clustering facilitates signal transduction by providing activating receptors and receptor proximal enzymes privileged access to their substrates and binding partners while at the same time excluding negative regulators. It has been difficult, however, to assess the specific effects of microcluster formation on signaling output.

The subcellular distribution of PKCα in the NK cell IS has not been characterized. In this study, we demonstrated that PKCα forms transient microclusters at the NK cell IS in response to activating receptor stimulation. These microclusters, which were specific for PKCα and not other PKC isozymes, were enriched for phosphotyrosine (pTyr) and phosphorylated signaling molecules, indicative of a role in activating signaling. Intriguingly, we found that only ITAM-coupled receptors, but not non–ITAM-coupled receptors, could drive PKCα clustering on their own. Because these data correlated with previous studies indicating that ITAM-coupled receptors induce stronger downstream responses than their non–ITAM-coupled counterparts (20–23), we investigated whether PKCα clustering might be important for promoting effector function. Indeed, using a point mutation in PKCα that disrupted cluster formation without altering other PKCα functionality, we demonstrated that PKCα clustering is required for optimal effector responses. These results provide new insight into the importance of subcellular compartmentalization for effective signal transduction in lymphocytes.

Materials and Methods

DNA constructs and recombinant proteins
cDNAs encoding full-length KIR2DS2, mouse Bc110, and human DAP12 were subcloned into a murine stem cell virus retrotranslational plasmid upstream of either GFP or mCherry. Retroviral expression vectors for full-length mouse PKC constructs fused to GFP or Tag-RFP-T have been described (8). Constructs encoding the following fragments were obtained by PCR of the full-length PKCα gene: C2 (amino acids 1–151), tandem C1 (160–281), C2–C1 (1–282). Chimeric PKC proteins were constructed using a three-step PCR approach in which different domains from PKCε and PKCα were amplified from either PKCε or PKCα cdNA and stitched together in two consecutive PCR steps. The chimeric PCR products were then cloned into the murine stem cell virus-GFP vector. In all cases, PKC proteins or fragments were positioned upstream (i.e., N-terminal) of the fluorescent protein.

Chimeric constructs encoding full-length PKCα or PKCα-H63D linked by an internal ribosome entry site to the extracellular domain of human CD2 were prepared by subcloning into the next coding sequence for PKCα into the pMigR2 plasmid. Biotinylated HLA-Cw3, ULBP3, and ICAM-1 were prepared as described [16]. HLA-Cw3 used for these studies contained a mutation that blocks binding of the inhibitory receptor ILT2 (16). The extracellular region of CD2 was prepared by cloning the coding sequence for CD2 into the pMigR2 plasmid. Biotinylated HLA-Cw3, ULBP3, and ICAM-1 were prepared as described (16). HLA-Cw3 used for these studies contained a mutation that blocks binding of the inhibitory receptor ILT2 (16). The extracellular region of CD2 was prepared by cloning the coding sequence for CD2 into the pMigR2 plasmid. Biotinylated HLA-Cw3, ULBP3, and ICAM-1 were prepared as described (16). HLA-Cw3 used for these studies contained a mutation that blocks binding of the inhibitory receptor ILT2 (16). The extracellular region of CD2 was prepared by cloning the coding sequence for CD2 into the pMigR2 plasmid. Biotinylated HLA-Cw3, ULBP3, and ICAM-1 were prepared as described (16).

Primary NK cells and NKL cells
Primary mouse NK cells were purified from splenocytes by negative selection using Abs against erythroid cells (TER-119), CD4 (GK1.5), CD8α (YTS169.4), CD5 (53-7.3.4), CD19 (1D3), and GR-1 (RB6-8C5; all Abs from the University of California, San Francisco, Monoclonal Antibody Core). Enriched NK cells were cultured in RPMI 1640 supplemented with 10% FCS and 1000 IU/ml of human IL-2. NKL cells were maintained in RPMI 1640 with 10% FCS and 200 IU/ml IL-2. Retroviral production and transduction of NKL cells was performed as described (16). Expression of transduced proteins was quantified 48 h after transduction by flow cytometry (BD LSR II), using either the transduced fluorescent protein label or an Ab against KIR2 (DX27; BD Biosciences) for detection. NKL cells expressing the transduced protein (typically representing 2–10% of the total population) were isolated by FACS 1–2 wk after transduction and maintained as stable cell lines.

Functional assays

Plastic surfaces for stimulation of primary murine NK cells were prepared using 48-well plates coated first with DOTAP (Sigma-Aldrich) solution and then with Abs (5 μg/ml) against mouse B24 (mB24; BioLegend), Ly49H (3D10; eBioscience), NK1.1 (PK136; eBioscience), or NK2D2 (A10; eBioscience). After coating, 1 × 10^5 NK cells were stimulated in each well for 8 h at 37°C. Cells were then fixed and stained for intracellular IFN-γ (XMG1.2; eBioscience), CD49b (DX-5; BioLegend), and CD3 (17A2; eBioscience). Flow cytometric analysis was performed on the CD49b^CD3^ population. To assess degranulation, fluorescently conjugated Abs against CD107a (1D4B; BD Biosciences) were added at the start of the experiment, and the staining was evaluated after fixation and labeling of the other Abs. In certain experiments, cells were stimulated in the presence of 10 μM PP2 (Sigma-Aldrich) or 0.5 μM BAY61-3606 (Calbiochem) to inhibit Src- or Syk-family kinases, respectively. NKL cells were stimulated in plastic wells coated with streptavidin followed by biotinylated ICAM-1 (1 μg/ml), HLA-Cw3 (1 μg/ml), CD48 (1 μg/ml), ULBP3 (1 μg/ml), or Abs against NK2D2 (1 μg/ml). For comparative studies in which one or more constituents were left out, a nonstimulatory biotinylated mouse MHC molecule (either I-E^b^ or H2-D^D^) was added to keep the total protein concentration for that experiment constant. To assess degranulation, fluorescently conjugated Abs against CD107a were included at the start of the experiment. After washing, CD107a staining was quantified by flow cytometry. All flow cytometric data were analyzed using FlowJo software.

Killing assay

IL-2–cultured NK cells were incubated with 51Cr-labeled target cells for 4 h at 37°C, after which supernatants were analyzed for 51Cr release. Maximum (MAX) lysis was determined by repeated freeze–thaw lysis of labeled target cells. Spontaneous (MIN) lysis was determined from wells containing target cells in the absence of effectors. Individual samples were run in triplicate. Percent specific lysis = (Lysissample – LysisMIN)/LysisMAX – LysisMIN × 100%.

Supported lipid bilayers

Bilayers were generated as described from small unilamellar vesicles containing a 10:1 mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine and biotinyl cap phosphoethanolamine (Avanti Polar Lipids) (16). After formation, bilayers were incubated with streptavidin, washed with PBS, and then incubated with biotinylated NK receptor ligands or biotinylated Abs. ULBP3, HLA-Cw3, and CD48 were used at 0–2 μg/ml and ICAM was used at 2 μg/ml. Biotinylated Abs against NK1.1 (PK136; eBioscience), Ly49H (3D10; eBioscience), NK2D2 (A10; eBioscience), and B24 (mB24; BioLegend) were used at 0–10 μg/ml. Stimulatory bilayers for SC.C7 T cells were prepared using biotinylated I-E^b^ containing the stimulatory mothe cytochrome c peptide, B7-1, and ICAM-1 (all proteins at 0.5 μg/ml). For comparative studies in which one or more constituents were left out of the bilayer, a nonstimulatory biotinylated mouse MHC molecule (either I-E^b^ containing the null Hb peptide or H2-D^D^) was added to keep the total protein concentration for that experiment constant. After protein loading, bilayers were stored at room temperature for up to 4 h prior to use.

Immunocytochemistry

NK cells were incubated on stimulatory bilayers for 15 min at 37°C and then fixed with 2% paraformaldehyde in PBS. After extensive washing in PBS, cells were permeabilized with Triton X-100, blocked with BSA, and then incubated with primary Abs against pSrc (Cell Signaling), pZap70 (Cell Signaling), pTyr (4G10; Millipore), PKCθ (C-18; Santa Cruz Bio technology), or GFP (Invitrogen), followed by staining with Alexa 594–conjugated Abs and Alexa Fluor 488 or 594 (Invitrogen) for 1 h. After a final PBS wash, cells were imaged by total internal reflection fluorescence (TIRF) microscopy. In certain experiments, stimulation was performed in the presence of 10 μM PP2 or 0.5 μM BAY61-3606 to inhibit Src- or Syk-family kinases, respectively.

Kinase assay

Phoenix E cells were transfected with plasmids containing GFP-labeled PKCθ-H63D, PKCθ-Y90F, PKCθ, or GFP only. Cells were collected after 2 d and lysed in ice-cold 10 mM Tris/Ci, pH 7.5; 150 mM NaCl, 0.5 mM EDTA, 0.5 mM Na3EDTA, with 1× protease inhibitor mixture (Roche) freshly added. Lysates were incubated with GFP-Trap beads (ChromoTek) for 2 h at 4°C, washed 4 times with ice-cold wash buffer (10 mM Tris/Ci, pH 7.5; 150 mM NaCl; 0.5 mM N3EDTA, with 1× protease...
inhibitor mixture freshly added), and resuspended in wash buffer. To 39.5 μl lysis buffer was added 2 μg purified Marsck1l (MLP, a PKC substrate) or mutant Marsck1l lacking the Ser103 and Ser104 consensus PKC phosphorylation sites (MLP*), together with 5 μl l kinase buffer (final concentration, 25 mM Tris/Cl, pH 7.5; 10 mM MgCl2; 1 mM NaF). The reaction was started by adding 0.5 μl ATP (final concentration, 1 mM) and incubated at 37°C for 30 min. Then 6X SDS loading buffer was added to stop the reaction, and samples were analyzed by immunoblot using α-phosphoserine PKC substrate Ab (Cell Signaling). Total PKC levels were assessed using α-GFP Ab (Invitrogen).

Results
PKC0 forms membrane microclusters at the NK cell IS
To explore the cellular basis for PKC0 function in NK cells, we analyzed the subcellular localization of PKC0 at the NK cell IS. IL-2–cultured mouse NK cells were stimulated on supported lipid bilayers containing the adhesion molecule ICAM-1 and cross-linking Abs against the activating receptors NK1.1, Ly49H, and 2B4. After contact formation, cells were fixed and stained for PKC0 and F-actin, and then imaged using TIRF microscopy, which enables high-resolution analysis of the plasma membrane attached to the bilayer. Activated NK cells formed radially symmetric synapses under all conditions, often characterized by a ring of dense F-actin in the periphery surrounding a region of hypo-dense F-actin at the center, consistent with previous observations (17, 25). In cells stimulated through NK1.1 and Ly49H, both of which are ITAM-coupled receptors, PKC0 formed distinct microclusters in the plasma membrane that localized inside the peripheral actin ring (Fig. 1A). In contrast, PKC0 clusters were not observed in cells stimulated through 2B4, which does not contain ITAMs (Fig. 1A). The extent of PKC0 clustering was quantified by calculating the fraction of synapse area taken up by high-intensity PKC0 staining. This analysis demonstrated that the apparent differences in clustering observed between 2B4-activated cells and cells stimulated through ITAM-coupled receptors were highly significant (Fig. 1B). Importantly, all receptor crosslinking Abs used for these experiments induced robust Ca2+ flux in addition to Src kinase phosphorylation at the NK cell IS (Fig. 1C, 1D). These early signaling responses were much stronger than those observed in cells plated on ICAM-1 alone, indicating that all of the crosslinking Abs were effective at stimulating their cognate NK receptors.

Consistent with previous reports (20–23), we found that stimulation of NK1.1 and Ly49H induced stronger degranulation and cytokine production than did stimulation of 2B4 in both resting and IL-2–cultured NK cells (Fig. 2). Hence, the ability of each activating receptor to induce PKC0 microclusters correlated with both the presence of ITAMs and the potential to generate robust effector responses.

Clustering is specific for PKC0 but does not require ITAM signaling
To characterize the dynamics and molecular composition of PKC0 microclusters, we developed a system for routine live imaging of cluster formation in the human NK cell line NKL. As model non–ITAM-coupled receptors, we used 2B4 and NKG2D, both of which are expressed in NKL cells. The receptor 2B4 recognizes the Ig domain protein CD48, whereas NKG2D binds to a set of proteins (the MIC and ULBP families in humans) that are up-regulated in response to infection and cellular transformation (26). As a model ITAM-coupled receptor, we used KIR2DS2, which associates with the ITAM-containing signaling adaptor DAP12 (26). The affinity of KIR2DS2 for its ligand, the class I MHC protein HLA-Cw3, is too low to mediate effective signaling. Therefore, we introduced a point mutation (Y45F) known to enhance ligand binding (Supplemental Fig. 1A, 1B) (27). NKL cells expressing KIR2DS2-Y45F (called simply KIR2DS2 hereafter) degranulated in response to immobilized HLA-Cw3, CD48, and ULBP3 (Supplemental Fig. 1B, 1C), validating KIR2DS2, 2B4, and NKG2D, respectively, as activating receptors in this system.

To examine the recruitment of PKC0 under various stimulus regimens, we prepared NKL cells expressing KIR2DS2, together with GFP-labeled PKC0, and then imaged them using TIRF microscopy on bilayers presenting ICAM-1 and either HLA-Cw3,
Only PKC\(\text{a}\) formed KIR2DS2-induced microclusters (Fig. 3B), indicating that clustering is a specific property of this isofrom.

Previous studies have shown that NKG2D and 2B4, when stimulated together, induce stronger activating signals and effector responses than when either receptor is triggered alone (21, 28). To assess whether synergy between NKG2D and 2B4 could promote PKC\(\text{a}\) clustering, we imaged NKL cells expressing GFP-labeled PKC\(\text{a}\) on bilayers containing CD48 and ULBP3. Remarkably, PKC\(\text{a}\) formed microclusters under these conditions (Fig. 3A). These data imply that PKC\(\text{a}\) clustering does not result from ITAM signaling per se, but rather that it is correlated with the overall strength of activating signals and their potential to induce robust effector responses.

**PKC\(\text{a}\) colocalizes with activating receptors and signaling molecules**

In B cells, T cells, and NK cells, activating receptors form plasma membrane microclusters at the IS in response to ligand binding (16, 18, 19). To explore the relationship between these activating receptor microclusters and PKC\(\text{a}\), we labeled DAP12 and transduced it into NKL cells expressing KIR2DS2, together with GFP-labeled PKC\(\text{a}\). Using TIRF microscopy, we observed that stimulatory bilayers containing HLA-Cw3 specifically induced the formation of DAP12 microclusters in these cells. Microclusters formed initially during cell spreading and began to traffic centripetally toward the center of the synapse once the size of the contact had stabilized (Fig. 4A, Supplemental Video 2). This behavior is quite similar to what has been observed for Ag receptors in T cells and B cells (18, 19). Clusters of DAP12 eventually coalesced into a stable aggregate at the center of the synapse that persisted for the duration of our experiments (typically 20–40 min). Because this central accumulation behaved analogously to the central supra-molecular activation cluster (cSMAC) that has been observed in T cell synapses (19), we refer to it as a cSMAC.

Analysis of cells expressing labeled DAP12 and PKC\(\text{a}\) revealed extensive overlap between the two proteins. This overlap, however, was incomplete, and it changed as the NK cell IS matured (Fig. 4A, Supplemental Video 2). PKC\(\text{a}\) was not recruited to activating receptor microclusters as they formed in the periphery (e.g., yellow arrowheads in Fig. 4A), but rather accumulated in these microclusters when they approached the center of the contact. Subsequently, as the clusters coalesced into a mature cSMAC, PKC\(\text{a}\) accumulation faded. Hence, PKC\(\text{a}\) association with activating microclusters is transient, beginning as the clusters approach the center of the synapse and diminishing as a stable cSMAC is formed.

It is generally thought that activating signals propagate from receptor microclusters at the periphery of IS and that these signals are downregulated as microclusters accumulate in the cSMAC (29–32). Consistent with this idea, we observed robust staining for pTyr in peripheral DAP12 microclusters, but proportionately less staining in the cSMAC that formed at later time points (Fig. 4B). It remained unclear, however, whether microclusters containing PKC\(\text{a}\), which are centrally located, still engage in activating signaling. To address this issue, we stimulated NKL cells expressing GFP-labeled PKC\(\text{a}\) on bilayers containing HLA-Cw3 and then stained them with Abs against pTyr and the phosphorylated form of the Syk-family kinase Zap70 (pZap70). Unambiguous colocalization was observed between these markers and PKC\(\text{a}\) (Fig. 4C). Indeed, both pTyr and pZap70 were enriched 4- to 5-fold in PKC\(\text{a}\) clusters relative to the rest of the IS (Fig. 4D). This finding suggests that activating receptor microclusters are still signaling while PKC\(\text{a}\) is associated. Taken together, our data indicate that PKC\(\text{a}\) is transiently recruited to microclusters containing activating receptors.
where it participates in activating signaling before dissociating as the microclusters are downregulated in the cSMAC. In T cells, it has been shown that PKC\(\mu\) induces activation of the transcription factor NF-\(\kappa\)B by phosphorylating a scaffolding complex containing the adaptor molecules CARMA1, Bcl10, and Malt1 (CBM complex) (33). A recent imaging study suggested that PKC\(\mu\) associates with the CBM complex in microclusters at the T cell IS, consistent with the importance of PKC\(\mu\) for NF-\(\kappa\)B signaling (15). To investigate whether analogous coclustering occurs in NK cells, we imaged GFP-labeled Bcl10, together with RFP-labeled PKC\(\mu\). Although Bcl10 did form microclusters upon stimulation of KIR2DS2, we observed little to no overlap between these clusters and clusters of PKC\(\mu\) (Fig. 4D, 4E), suggesting that PKC\(\mu\) does not associate with the CBM complex at the NK cell IS. This result is consistent with previous observations that NF-\(\kappa\)B signaling is unaffected in NK cells lacking PKC\(\mu\) (5).

PKC\(\mu\) clustering requires Src-family, but not Syk-family, kinases

To address the intracellular signaling requirements for PKC\(\mu\) microcluster formation, we monitored PKC\(\mu\) recruitment dynamics in the presence of PP2 and BAY61-3606, which are small-molecule inhibitors of Src- and Syk-family kinases, respectively. Treatment with PP2 completely disrupted microcluster formation in NKL cells, whereas BAY61-3606 had no effect on the process (Fig. 5A). Both compounds completely blocked IFN-\(\gamma\) secretion from murine NK cells after stimulation of NK1.1, confirming their efficacy as inhibitors (Fig. 5B). Together, these results suggest that PKC\(\mu\) clustering operates downstream of Src kinases but that it is independent of Syk kinase activity. This idea is consistent with our observation that simultaneous stimulation of NKG2D and 2B4, which are not known to signal through Syk kinases, nevertheless induced PKC\(\mu\) microcluster formation (Fig. 3A).

PKC\(\mu\) clustering requires the putative pTyr binding site in its C2 domain

Subcellular localization of PKC\(\mu\) is mediated by its N-terminal regulatory region, which contains both tandem C1 domains and a C2 domain (9, 34). To explore the requirements for PKC\(\mu\) clustering, we assessed the recruitment behavior of various GFP-labeled PKC\(\mu\) fragments in NKL cells. We found that the C2 domain and the tandem C1 domains were insufficient on their own

FIGURE 2. ITAM-coupled receptors induce stronger NK cell effector responses than do non–ITAM-coupled receptors. Purified murine NK cells were stimulated using plate-bound Abs against the indicated activating receptors. IFN-\(\gamma\) production (A) and degranulation (B) were quantified by intracellular cytokine staining and CD107a staining, respectively, of CD49b\(^+\)CD3\(^+\) cells. In each panel, resting NK cells are shown on the top and IL-2 cultured NK cells on the bottom. The percentage of responding cells is indicated in blue in each graph. Results are representative of at least two independent experiments.
FIGURE 3. PKCε forms synaptic microclusters in NKL cells in response to ITAM receptor stimulation. (A) NKL cells expressing GFP-labeled PKCε and KIR2DS2 were stained with phalloidin and imaged using TIRF microscopy on bilayers containing ICAM-1 and the indicated activating receptor ligands. **Top left panel**, Representative images showing GFP-labeled PKCε (blue) and phalloidin (red). **Bottom left panel**, Line scans showing fluorescence intensity for PKCε (blue) and F-actin (red). Line scans were calculated using the magenta lines shown in each image above. **Right panel**, Mean area of clustered fluorescence per cell for NKL cells stimulated through the indicated receptors. Each bar represents the average value calculated from ≥34 cells (DS2, KIR2DS2; 2D, NKG2D). (B) NKL cells expressing KIR2DS2 and the indicated GFP-labeled PKC isoform were stained with phalloidin and imaged using TIRF microscopy on bilayers containing ICAM-1 and HLA-Cw3. **Left panel**, shows representative images of the indicated GFP-labeled PKCε (blue) and phalloidin (red). **Right panel** shows mean area of clustered fluorescence per cell for NKL cells expressing the indicated labeled PKC isoform. Each bar represents the average value calculated from ≥30 cells. Scale bars, 10 µm. All data are representative of at least three independent experiments. ***p < 0.001, ns = p > 0.05.

to form synaptic clusters (Fig. 6A). However, a fragment containing both the C2 and the tandem C1 domains completely reconstituted clustering behavior, indicating that the entire N-terminal regulatory region participates in the process.

To confirm and extend these results, we prepared a set of chimeric constructs in which portions of the PKCε regulatory region were replaced by the corresponding part of PKCe, a related isoform that does not form microclusters at the NK cell IS (Fig. 3B). We also generated reciprocal chimeras in which domains of PKCe were introduced into PKCε. The clustering behavior of each protein was then analyzed by TIRF in NKL cells (Fig. 6B). Only constructs containing the PKCε C2 domain formed activation-induced microclusters, indicating that this specific domain is crucial for the response. Conversely, the tandem C1 domains of both PKCe and PKCε were permissive for clustering, providing the PKCε C2 domain was present.

Tetramer together with the results obtained from isolated PKCε fragments, these data indicate that both the C2 and the tandem C1 domains contribute to clustering behavior, but that the C1 domains merely serve as a generic DAG binder, possibly to localize the protein to the DAG-rich synaptic membrane (see Discussion). By contrast, the C2 domain of PKCε plays a much more specialized and isoform-specific role in this process. To further explore this role, we analyzed two different PKCε mutants bearing single amino acid substitutions in the C2 domain. It has been shown that the Src kinase Lck phosphorylates PKCε at Tyr 90 within the C2 domain, and that this residue is required for optimal transcriptional and proliferative responses to TCR stimulation (35). To assess the importance of Tyr 90 for PKCε clustering in NK cells, we examined the recruitment behavior of a PKCε mutant in which Tyr90 was changed to Phe (PKCε-Y90F). In NKL cells, this mutant formed clusters that were indistinguishable from those of wild-type PKCε (Supplemental Fig. 2A; compare with Fig. 4C), indicating that Lck-mediated phosphorylation of the C2 domain is not involved in synaptic microcluster formation.

It has also been shown that the C2 domain of PKCε, the PKC isoform most closely related to PKCθ, can specifically recognize pTyr-containing peptides (10). pTyr recognition requires a histidine residue in the PKCε C2 domain that is conserved in PKCθ (His63 in PKCε). Accordingly, we mutated His63 to Asp and examined the recruitment behavior of the resulting protein at the NK cell IS. Experiments using NKL cells expressing either wild-type or mutant PKCε labeled with GFP indicated that the H63D mutation completely disrupted microcluster formation (Fig. 6C).

Both proteins exhibited robust accumulation at the NK cell–target cell interface (Supplemental Fig. 2B), indicating that the distinct clustering behavior we observed did not result from differences in IS recruitment. To guard against artifacts related to the comparison of independently derived stable cell lines, we expressed both mCherry-labeled wild-type PKCε and GFP-labeled PKCε-H63D together in the same NKL cells. Strikingly, stimulation of KIR2DS2 induced no observable clustering of PKCε-H63D, despite the fact that wild-type PKCε clustered robustly in the same synapses (Fig. 6D, Supplemental Video 3). Hence, the putative pTyr binding site within the C2 domain is required for PKCε microcluster formation in NK cells.

The C2 domain pTyr binding site is not required for PKCε recruitment in T cells

To further explore the role of the putative pTyr binding site within the C2 domain, we examined the effects of the H63D mutation on PKCε dynamics during T cell activation. TCR signaling induces
Figure 4. PKC ε is transiently recruited to microclusters containing activating receptors and other signaling proteins. (A) NKL cells expressing GFP-labeled PKC ε, mCherry-labeled DAP12, and KIR2DS2 were imaged using TIRF microscopy on bilayers containing ICAM-1 and HLA-Cw3. A representative time-lapse montage is shown. Several peripheral DAP12 microclusters lacking visible PKC εu in the second frame. (B) NKL cells expressing GFP-labeled DAP12 and KIR2DS2 were stimulated on bilayers containing ICAM-1 and HLA-Cw3 and then fixed and stained with Abs against pTyr. Representative TIRF images from two time points are shown. A cSMAC is readily apparent in the cell from the 30-min time point. (C) NKL cells expressing GFP-labeled PKC ε and KIR2DS2 were stimulated on bilayers containing ICAM-1 and HLA-Cw3 and then fixed and stained with Abs against pTyr (top) or pZap70 (bottom). Representative TIRF images are shown. (D) Enrichment of pTyr (pY), pZap70 (pZap), and Bcl110 (Bcl) within PKC ε microclusters (see Materials and Methods). Each bar represents the average value calculated from 20 cells. (E) NKL cells expressing RFP-labeled PKC ε, GFP-labeled Bcl110, and KIR2DS2 were stimulated on bilayers containing ICAM-1 and HLA-Cw3. Representative TIRF images are shown. Scale bars, 10 μm. All data are representative of at least two independent experiments. ***p < 0.001.

The DAG-dependent accumulation of PKC ε in regions of the plasma membrane containing activated receptors (8). The kinetics and strength of this accumulation can be quantified using a photochemical approach in which TCRs in a defined region of the plasma membrane are activated with a pulse of UV light (36). Using this assay, we compared the recruitment dynamics of wild-type PKC ε and PKC ε-H63D, and found no difference between the two proteins (Supplemental Fig. 2C). These results are consistent with our previous data suggesting that the DAG-binding C1 domains primarily mediate plasma membrane association of PKC ε in this context (8).

As mentioned above, stimulation of CD28 together with the TCR induces the formation of PKC ε microclusters that coalesce into a ring around the cSMAC (14). This localization pattern can be visualized readily by TIRF imaging of T cells on lipid bilayers containing agonist peptide-MHC along with the CD28 ligand B7-1. Under these conditions both wild-type PKC ε and PKC ε-H63D formed microclusters (Supplemental Fig. 2D), suggesting that determinants other than the putative pTyr binding site in the C2 domain are required for CD28-dependent clustering in T cells.

The absence of an observable recruitment defect in T cells implies that the H63D mutation does not substantially alter the structure of full-length PKC ε. Consistent with this idea, the kinase activity of PKC ε-H63D is comparable to that of both wild-type PKC ε and PKC ε-Y90F (Supplemental Fig. 2E). We conclude that the H63D mutation selectively disrupts PKC ε microcluster formation in NK cells without substantially altering other properties of the protein.

PKC ε clustering is required for robust NK cell effector responses

It has been shown previously that PKC ε is required for optimal NK cell cytokine secretion (5). Consistent with these results, we found that IFN-γ production in response to ITAM receptor stimulation was suboptimal in PKC ε-deficient NK cells (Supplemental Fig. 3A). We also observed decreased levels of degranulation in the absence of PKC ε, implying that a defect in cytotoxicity might be present (Supplemental Fig. 3A). To further explore this possibility, we performed in vitro killing assays using three different types of target cell. We observed no differences between wild-type and PKC ε-deficient NK cells in these assays (Supplemental Fig. 3B), in agreement with previous work (5). This finding indicates that the degranulation defect resulting from loss of PKC ε does not lead to reduced cytotoxicity.

The ability of the H63D mutation to disrupt the synaptic clustering of PKC ε in NK cells without altering its other recruitment properties enabled us to assess whether microcluster formation is required for PKC ε to promote effector responses. If clustering is required, one would predict that expression of wild-type PKC ε in PKC ε-deficient NK cells would restore normal cytokine production and degranulation, and that expression of PKC ε-H63D would not. Accordingly, we transduced hematopoietic stem cells (HSCs) from PKC ε knockout mice, with retroviruses expressing either...
derived from the PKC\(\mu\) served to identify transduced cells (Supplemental Fig. 4). The three distinct populations of cells: 1) a CD45.1+CD45.2 expressing the congenic marker CD45.1. After 8 wk, the splenic cells hereafter); 2) a CD45.1 population derived from the host (Gate 1 in Fig. 7A, called “host” population derived from PKC\(\mu\)-H63D together with human CD2, which thereby served as an internal positive control for fully functional NK cells. This finding presumably reflected the fact that untransduced cells were PKC\(\mu\)–, whereas host cells were PKC\(\mu\)+. By contrast, IFN-\(\gamma\) production and degranulation in transduced cells expressing wild-type PKC\(\mu\) were comparable to that of host cells, consistent with the importance of PKC\(\mu\) for effector function. Remarkably, little to no rescue was observed in transduced cells expressing PKC\(\mu\)-H63D. These results strongly suggest that PKC\(\mu\) microcluster formation is required for optimal effector responses in NK cells.

**Discussion**

Although it is generally thought that microcluster formation at the IS plays a crucial role in lymphocyte activation, little evidence to date directly links the clustering potential of a specific signaling molecule with the strength of downstream responses. In this article, we showed that strong activating stimulation, through either ITAM-coupled receptors or a synergistic combination of non–ITAM-coupled receptors, induces the recruitment of PKC\(\mu\) into activating receptor microclusters at the NK cell IS. Using a mutation that selectively disrupts clustering behavior, we also showed that PKC\(\mu\) microcluster formation is required for optimal effector responses. Indeed, the similarity in phenotype displayed by PKC\(\mu\)-deficient NK cells and NK cells expressing PKC\(\mu\)-H63D implies that clustering is necessary for a substantial fraction of PKC\(\mu\)-dependent IFN-\(\gamma\) production and degranulation in this cell type. These results lay the groundwork for future analysis of the functional relevance of signaling microclusters during NK cell activation.

The PKC\(\mu\) microclusters we have characterized bear a superficial similarity to the clusters of PKC\(\theta\) that form in response to CD28 stimulation in T cells (14). Close examination of the requirements and dynamic properties of both types of cluster, however, suggests that they are fundamentally distinct structures. PKC\(\theta\) microclusters in NK cells fade as the cSMAC matures, whereas the T cell clusters appear to persist for the lifetime of the IS. In T cells, PKC\(\theta\) clustering requires the YINM receptor CD28, whereas in NK cells the ITAM-coupled receptors more effectively promote microcluster formation. Indeed, YINM signaling from NKG2D is insufficient, on its own, to induce PKC\(\theta\) microclusters in NK cells and must be supplemented by coactivation through B24. Finally, a recent study indicated that the V3 linker between the tandem C1 domains and the kinase domain is necessary and sufficient for recruitment to the cSMAC in T cells (37). In contrast, we have shown that clustering in NK cells requires the putative pTyr binding site in the C2 domain.

The idea that PKC\(\theta\) microclusters in NK cells are not directly analogous to CD28-dependent clusters in T cells is consistent with results from our laboratory and others suggesting that PKC\(\theta\) induces distinct downstream signaling responses in NK cells relative to T cells. Phosphorylation of the CBM complex by PKC\(\theta\) in T cells is crucial for NF-\(\kappa\)B activation, and components of the CBM complex appear to colocalize with CD28 and PKC\(\theta\) at the T cell IS (15, 33). In contrast, NK cells lacking PKC\(\theta\) do not display any defects in NF-\(\kappa\)B signaling (5), and we did not observe co-clustering between PKC\(\theta\) and Bcl10 at the NK cell IS. Given the apparent functional differences between PKC\(\theta\) clusters...
in T cells and NK cells, it will be interesting in future studies to explore the biological consequences of disrupting each type of clustering behavior. Access to the H63D and V3 mutations should facilitate this line of investigation.

The C2 domains of PKC\(\delta\) and PKC\(\upsilon\) are highly conserved. Indeed, all of the amino acid residues directly involved in the recognition of pTyr-containing peptides by PKC\(\delta\) are identical in PKC\(\upsilon\) (10). Hence, it is surprising that PKC\(\delta\) does not form detectable microclusters in our hands. A likely explanation for this discrepancy is that compartmentalization within the NK cell synapse is a combinatorial process requiring multiple regulatory domains. We have found that both the tandem C1 domains and the C2 domain are required for PKC\(\upsilon\) microcluster formation. However, the role of the C1 region is likely to be quite generic, as the corresponding portion of PKC\(\varepsilon\) is permissive for clustering. In T cells, the C1 domains of PKC\(\upsilon\) and PKC\(\varepsilon\) mediate DAG-dependent recruitment of the protein to the IS (8, 38). In contrast, PKC\(\delta\) does not accumulate synaptically, but instead associates with lysosomal compartments (39). Hence, it is tempting to speculate that PKC\(\upsilon\) localization at the NK cell IS cells is a two-step process involving initial, C1-dependent recruitment to the synaptic membrane, followed by C2-dependent recruitment into activating receptor microclusters.

FIGURE 6. PKC0 clustering requires the putative phosphotyrosine binding site in its C2 domain. (A and B) NKL cells expressing KIR2DS2 and the indicated GFP-labeled fragments of PKC0 (A) or the indicated GFP-labeled PKC0-PKCe chimeras (B) were imaged by TIRF microscopy on bilayers containing HLA-Cw3 and ICAM-1. Center, Representative images are shown. Left, Mean area of clustered fluorescence per cell for NKL cells expressing the indicated construct. ***p < 0.001. (C) NKL cells expressing KIR2DS2 and the indicated GFP-labeled PKC0 proteins were stained with phalloidin and imaged by TIRF microscopy on bilayers containing HLA-Cw3 and ICAM-1. Top and center, Representative TIRF images are shown. Bottom, Line scans showing fluorescence intensity for wild-type PKC0 (blue, left) and PKC0-H63D (blue, right), together with F-actin (red). (D) NKL cells expressing KIR2DS2, mCherry-labeled wild-type PKC0, and GFP-labeled PKC0-H63D were imaged on bilayers containing HLA-Cw3 and ICAM-1. Top, Representative TIRF images are shown. Bottom left, A line scan showing fluorescence intensity for mCherry-labeled wild-type PKC0 (red) and GFP-labeled PKC0-H63D (blue). Bottom right, a “before–after” graph showing mean area of clustered fluorescence for both wild-type PKC0 and PKC0-H63D calculated from 11 cells expressing mCherry-labeled wild-type PKC0 and GFP-labeled PKC0-H63D. Paired values are connected by lines. Line scans in (C) and (D) were calculated using the magenta lines shown in the corresponding images above. Scale bars, 10 \(\mu\)m [in (A) and (C)]; 5 \(\mu\)m [in (D)]. All data are representative of at least two independent experiments.
FIGURE 7. PKCε clustering is required for optimal NK cell effector responses. Splenic NK cells were isolated from PKCε-reconstituted chimeric mice, cultured for 1 wk in IL-2, and then stimulated using immobilized Abs against NK1.1. (A) Representative contour plot showing the three populations of NK cells (identified as CD49b⁺CD3⁻ cells) typically present in chimeric mice. (B and C) Representative intracellular IFN-γ (B) and degranulation (C) responses for each of the three NK cell populations, derived from chimeric mice expressing either wild-type PKCε (top) or PKCε-H63D (bottom). The percentage of responding cells is indicated in blue in each graph. (D) Mean rescue ratios (see Materials and Methods) for both untransduced (un) and transduced (tr) populations of transferred NK cells, calculated using three wild-type PKCε chimeras (WT) and four PKCε-H63D chimeras (Mut). Rescue ratios for IFN-γ production are shown to the left, and for degranulation to the right. *p < 0.05, **p < 0.01, ***p < 0.001, or ns.
former relative to the latter would presumably be useful for enabling target cell killing in cases in which concomitant inflammatory responses might be unnecessary or even harmful.

Taken together, our results suggest that PKC0 clustering is an early and necessary step toward optimal NK cell activation. It is presumably has access to substrates and other interacting proteins that would not otherwise be available. The identification and characterization of these signaling partners will be crucial for determining the mechanism of PKC0-dependent signal amplification and remains an area for future investigation.

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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure Legends

**Figure S1. Validation of activating receptors in NKL cells.** (A) Surface representation of the hydrophobic pocket in HLA-Cw3 recognized by Tyr45 in KIR2DS2 and the corresponding Phe45 in KIR2DL2 (PDB IDs 1M4K and 1EFX respectively). Phe fits more snugly into the pocket, explaining the difference in affinity for HLA-Cw3 observed between KIR2DL2 and KIR2DS2. (B-C) NKL cells expressing either wild type KIR2DS2 (B, top) or KIR2DS2-Y45F (B, bottom, and C) were stimulated on plastic surfaces bearing the indicated immobilized ligands. Degranulation was quantified by surface expression of CD107a. Data are representative of three independent experiments.

**Figure S2. Characterization of the Y90F and H63D PKCθ mutants.** (A) NKL cells expressing GFP-labeled PKC0-Y90F and KIR2DS2 were stimulated on bilayers containing ICAM-1 and HLA-Cw3 and then fixed and stained with antibodies against pZap70. Above, a representative TIRF image. Below, linescans showing fluorescence intensity for PKC0-Y90F (blue) and pZap70 (red). Linescans were calculated using the magenta line shown in the image above and to the right. (B) NKL cells expressing GFP-labeled wild type PKC0 or PKC0-H63D were mixed with .221 target cells, fixed, and imaged by epifluorescence microscopy. GFP fluorescence from representative NKL-target cell conjugates is shown in pseudocolor (warmer colors indicate higher fluorescence intensity) on a background brightfield image. (C-D) T cell blasts expressing the 5C.C7 TCR were transduced with GFP-labeled wild type PKC0 or PKC0-H63D. (C) T cells were attached to glass surfaces containing immobilized, photoactivatable peptide-MHC specific for the 5C.C7 TCR. Localized UV irradiation was then used to activate TCRs in a small region of membrane, followed by TIRF imaging of PKC0 recruitment responses. Above, representative timelapse montages (1 min intervals) are shown, with the time and place of
UV irradiation indicated by red circles. Below, PKCθ accumulation, expressed as the normalized mean fluorescence intensity (ΔF/F) in the irradiated region as a function of time. UV irradiation is indicated by a purple line. Curves were derived from at least seven cells each, and bars along curves = s.e.m. (D) Transduced T cells were imaged by TIRF microscopy on bilayers containing agonist peptide-MHC (pMHC) and B7-1 (B7) as indicated. Representative images of PKCθ or PKCθ-H63D are shown. (E) Immunoprecipitates containing the indicated PKCθ-GFP fusion proteins were used in kinase reactions with either wild type Marcksl1 (MLP) or Marcksl1 lacking its consensus PKC phosphorylation sites (MLP*) as substrates. Phosphorylation was evaluated by immunoblot using an antibody against the phosphorylated PKC consensus sequence. The lane to the far right contains a control kinase reaction performed using GFP immunoprecipitates. Scale bars in (A) and (C) = 5 µm. Scale bars in (B) and (D) = 10 µm. All data are representative of at least two independent experiments.

Figure S3. Effector responses in PKCθ−/− NK cells. (A) Purified, IL-2 cultured NK cells from wild type or PKCθ deficient mice were stimulated using plate-bound antibodies against NK1.1 as indicated. IFNγ production (left) and degranulation (right) were quantified by IFNγ and CD107a staining, respectively, of CD49b+CD3− cells. Percentage of IFNγ+ and CD107a+ cells are indicated in blue in each graph. (B) Purified IL-2 cultured NK cells from wild type (blue) or PKCθ deficient (red) mice were mixed with the YAC-1, BaF3-Rae1, or BaF3-m157 target cells at the indicated ratios, and specific lysis was assessed by chromium release assay after 4 h. Error bars = s.e.m. All results are representative of at least two independent experiments.

Figure S4. Strategy for preparing bone marrow chimeras. PKCθ−/− bone marrow was transduced with PKCθ constructs and then injected into congenically marked hosts.
Supplemental Video Legends

Video S1. PKCθ forms synaptic microclusters in response to ITAM-receptor stimulation. Related to Fig. 2. NKL cells expressing GFP-labeled PKC0 and KIR2DS2 were imaged using TIRF microscopy (Olympus IX-81) on bilayers containing HLA-Cw3 and ICAM-1. Frames were taken every 30 seconds. A representative 150X timelapse video is shown.

Video S2. PKCθ is transiently recruited to activating receptor microclusters. Related to Fig. 3. NKL cells expressing GFP-labeled PKC0, mCherry-labeled DAP12, and KIR2DS2 were imaged using TIRF microscopy (Olympus IX-81) on bilayers containing HLA-Cw3 and ICAM-1. Frames were taken every 10 seconds. A representative 50X timelapse video is shown, with PKC0 images to the left, DAP12 images in the center, and the overlay to the right.

Video S3. The H63D mutation disrupts PKCθ microcluster formation. Related to Fig. 5. NKL cells expressing GFP-labeled PKC0-H63D, mCherry-labeled PKC0, and KIR2DS2 were imaged using TIRF microscopy (Olympus IX-81) on bilayers containing HLA-Cw3 and ICAM-1. Frames were taken every 30 seconds. A representative 150X timelapse video is shown, with PKC0-H63D images to the left, PKC0 images in the center, and the overlay to the right.
A
KIR2DL2(F45)  KIR2DS2(Y45)

B
- Unstim.
- ICAM
- \(\alpha\)-NKG2D
- HLA-Cw3 + ICAM

C
- Unstim.
- CD48 + ICAM
- ICAM
- ULBP3 + ICAM

Merino et al., Figure S1
Merino et al., Figure S2
A

Unstim.  NK1.1

Unstim.  NK1.1

Wild type

PKC0−/−

B

Specific lysis (%)

YAC-1

BaF3-Rae1

BaF3-m157

Effector:target

Effector:target

Effector:target

Merino et al., Figure S3
PKCθ⁻/⁻ BM

Retroviral transduction

CD2 IRES PKCθ wt
CD2 IRES PKCθ H63D

PKC⁻/⁻ CD45.2

B6 CD45.1

8 weeks

Reconstituted mice

NK cell isolation

NK cell activation
Degranulation and IFNγ

Merino et al., Figure S4