

Diacylglycerol kinase ζ controls diacylglycerol metabolism at the immunological synapse

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ABSTRACT Diacylglycerol (DAG) generation at the T cell immunological synapse (IS) determines the correct activation of antigen-specific immune responses. DAG kinases (DGKs) α and ζ act as negative regulators of DAG-mediated signals by catalyzing DAG conversion to phosphatidic acid (PA). Nonetheless, the specific input of each enzyme and their spatial regulation during IS formation remain uncharacterized. Here we report recruitment of endogenous DGK α and DGK ζ to the T cell receptor (TCR) complex following TCR/CD28 engagement. Specific DGK gene silencing shows that PA production at the activated complex depends mainly on DGK ζ , indicating functional differences between these proteins. DGK ζ kinase activity at the TCR is enhanced by phorbol-12-myristate-13-acetate cotreatment, suggesting DAG-mediated regulation of DGK ζ responsiveness. We used GFP-DGK ζ and -DGK α chimeras to assess translocation dynamics during IS formation. Only GFP-DGK ζ translocated rapidly to the plasma membrane at early stages of IS formation, independent of enzyme activity. Finally, use of a fluorescent DAG sensor confirmed rapid, sustained DAG accumulation at the IS and allowed us to directly correlate membrane translocation of active DGK ζ with DAG consumption at the IS. This study highlights a DGK ζ -specific function for local DAG metabolism at the IS and offers new clues to its mode of regulation.

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INTRODUCTION

T lymphocytes respond to antigen-presenting cell (APC) interactions through T cell receptor (TCR)-induced signals that promote the formation of a surface subdomain at the T cell-APC contact zone, termed the immunological synapse (IS) (Smith-Garvin *et al.*, 2009; Fooksman *et al.*, 2010). At the IS, signaling components and adaptor proteins are recruited and reorganized, orchestrating T cell responses (Lin and Weiss, 2001). TCR triggering leads to phosphatidylinositol-4,5 bisphosphate hydrolysis by phospholipase C γ 1 (PLC γ 1) to generate inositol-1,4,5 trisphosphate and diacylglycerol

(DAG), which control intracellular Ca²⁺ intake and activation of DAG effectors, respectively. TCR-triggered DAG accumulation at the IS results in DAG-mediated recruitment and allosteric activation of proteins with conserved protein kinase C type I (C1) domains. These include the Ras-guanyl nucleotide-releasing protein 1 (RasGRP1), classic and novel protein kinase C (PKC) family members, protein kinase D, and β -chimaerin (Ebinu *et al.*, 2000; Spitaler *et al.*, 2006; Siliceo and Merida, 2009; Quann *et al.*, 2011). The need for a polarized DAG pool to ensure appropriate T cell polarization and activation suggests that generation and consumption of this lipid must be precisely regulated (Carrasco and Merida, 2007; Quann *et al.*, 2009).

Diacylglycerol kinases (DGKs) participate in DAG metabolism, catalyzing its conversion to phosphatidic acid (PA). There are 10 mammalian DGKs, classified in five groups (types I–V) based on specific regulatory domains (Merida *et al.*, 2008). Type I DGK α and type IV DGK ζ are the two main isozymes expressed in lymphoid tissues, and their exact contribution to TCR-triggered, DAG-mediated signals is of great interest. Gain-of-function experiments in the Jurkat T cell line demonstrated a role for both isoforms as negative regulators of Ras activation by impairing the DAG-mediated

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Abbreviations used: APC, antigen-presenting cell; DAG, diacylglycerol; DGK, diacylglycerol kinase; IS, immunological synapse; PA, phosphatidic acid; PKC, protein kinase C; PM, plasma membrane; PMA, phorbol-12-myristate-13-acetate; TCR, T cell receptor.

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membrane localization and activation of RasGRP1 (Sanjuan *et al.*, 2003; Zhong *et al.*, 2003). These early data suggest overlapping functions for the two isoforms in the regulation of the Ras/ERK pathway, although structural–functional analysis shows striking differences probably based on their different mechanisms of regulation. DGK α contains Ca²⁺-responsive regions at its N-terminus, whose deletion improves its negative regulation of RasGRP1 membrane localization (Sanjuan *et al.*, 2003). Removal of the DGK ζ C-terminal region, containing ankyrin repeats and a PDZ-binding motif, enhances its negative regulation of TCR-induced Ras-ERK1/2 activation (Zhong *et al.*, 2002).

The phenotypes of knockout (KO) mouse models for these two isoforms confirmed their major role as brakes on TCR-mediated RasGRP1-Ras-ERK1/2 activation. DGK α - and DGK ζ -deficient mice show enhanced Ras activation and increased ERK1/2 phosphorylation compared with wild-type littermates. As a result, DGK α - and DGK ζ -deficient mice are resistant to anergy, a nonresponsive T cell state caused by TCR triggering in the absence of costimulation and originated by selective generation of Ca²⁺ signals without simultaneous activation of the RasGRP1-Ras-ERK1/2-AP1 pathway (Zhong *et al.*, 2003; Olenchok *et al.*, 2006). Anergy induction helps to maintain peripheral T cell tolerance, and its impairment is predicted to result in self-reactive T cell activation and autoimmunity. Nonetheless, DGK α - and DGK ζ -deficient T cells do not develop autoimmune diseases, suggesting that one isoform may compensate for the loss of the other to maintain T cell tolerance. In agreement with this argument, mice deficient in both isoforms develop chronic autoimmune hepatitis (Guo *et al.*, 2008), confirming DGK α and DGK ζ synergy in the control of T cell tolerance and development.

The analysis of genetically modified mice has greatly helped to confirm the physiological relevance of DGK α and DGK ζ . There are nonetheless important questions that remain to be addressed. One of the most imperative is the nature of the regulatory mechanisms that control DGK α and DGK ζ enzymatic activity and their spatiotemporal subcellular localization in response to TCR stimulation. Studies in T cells that ectopically express the muscarinic type I receptor showed that each isoform responds with different translocation kinetics as the result of their distinct activation mechanisms (Sanjuan *et al.*, 2001; Santos *et al.*, 2002). DGK α relocates to the membrane with rapid and transient kinetics that require tyrosine kinase activation and Ca²⁺ elevation (Sanjuan *et al.*, 2001). DGK ζ on the other hand shows sustained translocation kinetics regulated by PKC-mediated phosphorylation of its myristoylated alanine-rich C kinase substrate (MARCKS) domain (Santos *et al.*, 2002). Enzyme activity is dispensable for DGK ζ membrane relocation, whereas DGK α inactivation promotes sustained membrane localization of the inactive enzyme (Sanjuan *et al.*, 2001; Santos *et al.*, 2002). The relevance of these mechanisms in the regulation of DGK α and DGK ζ localization and activity during IS formation has not been explored. There are no data showing direct DGK recruitment to the TCR–coreceptor complex, although signaling protein recruitment to scaffolds and adaptor proteins in close proximity to the TCR is the immediate consequence of T cell–APC interaction. Finally, whether the DGKs contribute to DAG metabolism outside the IS as a mechanism to promote polarization of DAG-mediated signals or whether they consume the DAG generated at the T cell–APC contact site is still debated.

Here we evaluated DGK α and DGK ζ recruitment to the TCR complex using affinity purification of active TCR. We then analyzed DGK activity at the TCR complex and used RNA interference (RNAi)-mediated gene silencing to determine the contribution of each isozyme to PA generation. We used GFP-fused DGK α and DGK ζ

constructs to assess translocation dynamics during IS formation and a fluorescent DAG sensor to evaluate the effect of DGK translocation on DAG metabolism at the cell–cell contact site. Our data showed recruitment of both isoforms to the TCR complex, where DGK ζ is the major DAG regulatory isozyme. Videomicroscopy analysis indicated rapid, sustained DGK ζ mobilization to the T cell plasma membrane (PM) after APC encounter and confirms that this translocation correlates with rapid consumption of DAG at the IS. We propose a positive feedback mechanism by which IS formation promotes membrane recruitment of DGK ζ that is further activated at this site by DAG-regulated mechanisms. Our results suggest that this complex mechanism of DGK ζ regulation in response to TCR triggering contributes to ensure accurate DAG:PA balance during IS formation.

RESULTS

Isolation of activated TCR complexes using anti-CD3/CD28-coated beads

The TCR complex is composed of eight transmembrane chains—the TCR α/β heterodimer and the CD3 $\epsilon\gamma$, $\epsilon\delta$, and $\zeta\zeta$ dimers. Each CD3 cytosolic chain bears an immunoreceptor tyrosine kinase cytosolic motif (ITAM) rich in tyrosine residues, which serve as a platform to recruit protein tyrosine kinases and adaptor molecules. To isolate the protein complex recruited to TCR after T cell activation, we used magnetic beads coated with anti-CD3 and/or anti-CD28 antibodies to act as surrogate APC and partially mimic IS-mediated actin and PM reorganization (Viola *et al.*, 1999). We tested bead recognition by immunofluorescence (Figure 1A). Anti-CD3-coated beads showed phospho-ERK (pERK) induction and accumulation around the bead; we also observed formation of an actin cup that was not seen with anti-CD28 alone, which was subsequently used to discriminate TCR-activated clusters. Cells in complex with antibody-coated beads were lysed in a mild nonionic detergent, Brij 96V (2-[(Z)-octadec-9-enoxy]ethanol), to preserve noncovalent interactions between TCR and signaling components (Beyers *et al.*, 1992). We monitored tyrosine-phosphorylated proteins recruited to beads alone or coupled to anti-CD3, anti-CD28, or anti-CD3/CD28 (Figure 1B). As predicted, full stimulation with anti-CD3/CD28 induced greater tyrosine kinase activation than did anti-CD3, whereas anti-CD28 beads or uncoated beads did not (Figure 1B). These optimum stimulation and affinity purification conditions were used for specific isolation of the active TCR complex.

DGKs are recruited to activated TCR complexes

Using our model for isolation of activated TCR, we tested whether the two main DGKs in T cells were recruited directly to TCR complexes. DGK α and DGK ζ segregated with the TCR immune complex after activation in a manner comparable to phospho-PLC γ , as early as the first minutes of stimulation (Figure 2A). In contrast, ERK1/2 was activated by anti-CD3/CD28 beads but was not enriched at the TCR complex, indicating that beads do not precipitate nonspecific proteins stimulated by, but not associated to, TCR complexes.

DGK are activated downstream of the TCR, and DGK activity is induced by T cell activation (Sanjuan *et al.*, 2003); however, there are no studies of this lipid kinase activity associated to the TCR complex. To determine whether DGK recruitment contributes to DAG consumption at the TCR complex, we measured TCR complex-associated DGK activity using exogenous DAG as substrate and monitored PA production by [³²P]ATP incorporation. After 20 min of stimulation with anti-CD3/CD28 beads, DGK activity increased at the TCR complex, correlating with DGK ζ and DGK α recruitment

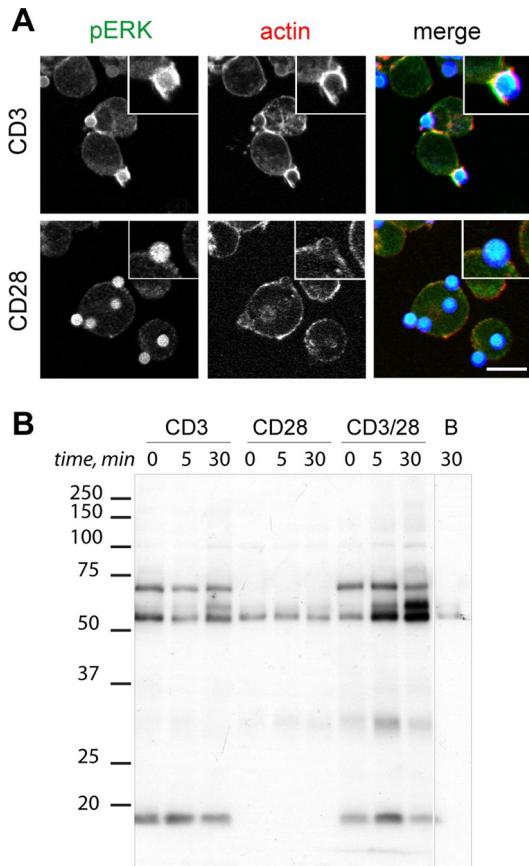


FIGURE 1: Isolation of TCR immune complexes using anti-CD3/CD28-coated beads. (A) Jurkat T cells were stimulated with sheep anti-mouse IgG Dynabeads coated with human anti-CD3 or anti-CD28 (10 min). Immunostaining is shown for activation (pERK) and actin dynamics markers (phalloidin-rhodamine). Insets, zoomed area. Bar, 10 μ m. (B) Jurkat T cells (10^7 cells/ml) serum starved in DMEM, 0.5% BSA (2 h), were incubated with anti-CD3, anti-CD28, or anti-CD3/CD28 on ice, followed by Dynabeads (37°C) for indicated times. Time 0 shows cells incubated with antibody and Dynabeads on ice. Associated protein complexes were resolved by SDS-PAGE and tyrosine-phosphorylated proteins developed in WB. Left, molecular weight markers. Control immunoprecipitation was performed with Dynabeads alone (lane B).

(Figure 2B). To determine the contribution of each DGK isozyme to PA production, we analyzed the effect of DGK α or DGK ζ RNAi on DGK activity. Isozyme-specific depletion showed that DGK α RNAi had little effect on DGK activity, whereas activity was significantly reduced when DGK ζ was silenced (Figure 2B). To exclude any potential disadvantage for DGK α , we performed additional DGK assays in the presence of phosphatidylserine and Ca²⁺, the two cofactors that maximize type I DGK activation. Under these conditions, we observed similar results (Supplemental Figure S1). As reported in KO mice (Zhong *et al.*, 2003; Olenchock *et al.*, 2006), attenuation of DGK α or DGK ζ resulted in sustained ERK phosphorylation kinetics (Figure 2C). We conclude that DGK ζ is the main isozyme controlling DAG metabolism at the TCR immune complex after engagement.

Regulation of DGK ζ at the TCR complex

Several studies proposed a role for PKC α in the regulation of DGK ζ membrane recruitment and activation (Santos *et al.*, 2002; Abramovici *et al.*, 2003; Luo *et al.*, 2003). We monitored DGK activity at the TCR complex after cotreatment with phorbol-12-myristate-13-ace-

tate (PMA), a potent PKC α activator that is not consumed by DGK, and we observed a notable increase in DGK activity in response to PMA treatment (Figure 3, A and B). We performed RNAi gene silencing of both DGK α and DGK ζ in Jurkat T cells (Figure 3A) and observed that this increase in activity was dependent on DGK ζ but not DGK α expression (Figure 3, A and B). We used bisindolylmaleimide II (BIM), a broad PKC inhibitor with a lower IC₅₀ for PKC α , to determine PKC activity input on DGK activity following TCR signaling. PKC inhibition apparently did not alter TCR-recruited DGK activity (Figure 3B).

To evaluate whether the increased DGK activity correlated with enhanced enzyme recruitment, we analyzed TCR-associated proteins after PMA or BIM cotreatment (Figure 3C). We observed no major changes in DGK ζ recruitment to the TCR after either condition, although BIM treatment resulted in greater TCR recruitment of a faster-migrating DGK ζ band. Our data thus suggest that the increased DGK activity observed after PMA treatment does not correspond to greater enzyme recruitment but could reflect posttranslational regulation. In fact, we also detected PKC α recruited to the activated TCR complex, with greater mobilization after PMA treatment (Figure 3C). PKC function is related to the control of actin dynamics in T cells (Sims *et al.*, 2007; Beal *et al.*, 2008), so we assessed Ras and actin recruitment under the different conditions as a measure of TCR clustering and a marker of cytoskeletal reorganization. Actin recruitment was markedly blocked with PMA cotreatment and enhanced following PKC α inhibition, further confirming that this isoform contributes to the regulation of TCR signals.

DGK ζ migrated as a doublet, indicative of putative posttranslational regulation, and showed a molecular weight shift following stimulation with anti-CD3/CD28 beads in total cell lysates (Figure 3D). This shift was greater after PMA cotreatment and was not observed in the presence of the src kinase inhibitor PP2. Taken together, these data suggest that endogenous DGK ζ is recruited to the TCR complex, where it might be activated in response to DAG-mediated signals in a feedback loop in which DAG-regulated signals contribute to its consumption.

DGK ζ translocation dynamics during immune synapse formation

We used the green fluorescent protein (GFP)-DGK ζ fusion protein to examine DGK ζ translocation dynamics to TCR complexes in live T cells. Our anti-CD3/CD28 complex isolation strategy showed that the chimera was recruited to the TCR complex without altering recruitment of endogenous DGK ζ (Supplemental Figure S2A). Microscopy analysis showed that GFP-DGK ζ was recruited to the bead-cell contact area as soon as the bead contacted the cell (Supplemental Figure S2B). We stimulated Jurkat T cells using Raji B cells loaded with the staphylococcal enterotoxin E (SEE) superantigen as APC (Fraser *et al.*, 2000; Friedl and Storim, 2004). Time-lapse fluorescence microscopy showed that, as in the case of antibody-coated beads, GFP-DGK ζ redistributed to the PM within minutes of cell-cell contact (Figure 4A, Supplemental Figure S2C, and Supplemental Movie 1), and this localization was sustained for up to 30 min after T/B cell encounter. This suggests that DAG level control is important from early signal transduction stages and precedes IS maturation. Quantification of GFP-DGK ζ fluorescence density showed significant DGK ζ accumulation at the IS compared with cytosol (Figure 4A), correlating with biochemical estimates. To visualize simultaneous changes in T cell morphology and DGK ζ translocation after antigen presentation, we coexpressed GFP-DGK ζ with actin-Cherry, followed by double fluorescence time-lapse microscopy. DGK ζ shuttled to the PM in the initial T lymphocyte contact

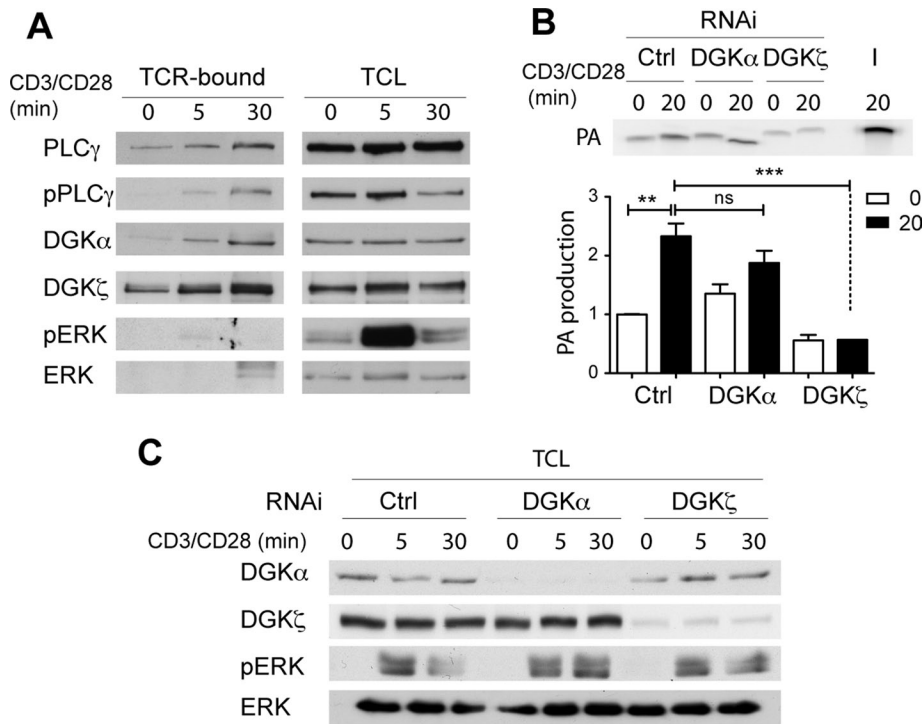


FIGURE 2: DGK recruitment to active TCR complex; DGK ζ is necessary for PA production. (A) Jurkat T cells were presented to anti-CD3/CD28-coated Dynabeads for various times. Cell treatment and lysate precipitation were as in Figure 1B. TCR immune complexes were eluted (TCR bound), resolved by SDS-PAGE, and probed in WB with DGK- and activation marker-specific antibodies. Unprecipitated samples were used as activation controls (total cell lysate [TCL]). (B, C) DGK ζ is the main PA-generating enzyme recruited to the TCR complex. RNAi was used to silence DGK α and DGK ζ ; at 72 h posttreatment, cells (10^7 cells/ml) were starved as in Figure 1B and stimulated with anti-CD3/CD28 beads for various times. TCR-bound samples or TCL (input, I) were used as an enzyme source for the DAG kinase assay (type I; see *Materials and Methods*); a representative experiment is shown (B). PA production is shown as the mean \pm SEM ($n = 4$). A fraction of the samples used in B was analyzed by WB to confirm RNAi efficiency and activation (C).

with the APC, and accumulated in an F-actin-enriched fraction (Figure 4B and Supplemental Movie 2).

PKC-dependent phosphorylation of DGK ζ at the MARCKS region (Supplemental Figure S3A) is necessary for enzyme localization to the PM in response to G protein-coupled receptor stimulation in both lymphoid and nonlymphoid cell lines (Santos *et al.*, 2002; Abramovici *et al.*, 2003). We examined translocation of DGK ζ mutated in the MARCKS domain (Ser-to-Asp [SD] or Ser-to-Ala [SA], which mimicked or impaired phosphorylation, respectively) in response to T cell-APC contact. The SA mutant was unresponsive to antigen presentation and remained diffuse in cytosol, whereas the SD mutant behaved like the wild-type form after stimulation (Figure 4C). MARCKS-domain phosphorylation is believed to release the negative restriction imposed by the DGK ζ C-terminal domain, which bears ankyrin repeat sequences and a PDZ-binding motif (Santos *et al.*, 2002). Accordingly, the SA mutant recovered PM localization in response to T cell activation following truncation of its C-terminal domain (SA- Δ Ank; Supplemental Figure S3B). As shown for endogenous DGK ζ , BIM treatment did not affect GFP-DGK ζ translocation to the PM, although some intracellular vesicular staining was detected. GFP-DGK ζ dynamics was also unaffected by rottlerin, an inhibitor of novel PKC (Supplemental Figure S3C). These findings further demonstrate that PM relocation of DGK ζ is independent of PKC activity and suggest that phosphorylation of the DGK ζ MARCKS region could take place prior to T cell activation, possibly as a priming step.

DGK ζ regulates DAG at the immunological synapse

Videomicroscopy analysis showed that T cell-APC contact results in rapid GFP-DGK ζ recruitment to the entire PM and not only to the IS. To examine the DGK ζ contribution to the regulation of DAG generated at the IS, we used the C1ab tandem domain of PKC θ previously characterized as a DAG sensor in living T cells (Carrasco and Merida, 2004). Nonstimulated cells expressing the PKC θ C1ab domain fused to the red fluorescent protein Cherry (Cherry-C1ab) showed fluorescence accumulation mainly in internal membranes, as reported for this domain when fused to GFP (Carrasco and Merida, 2004; Supplemental Figure S4). Following APC contact, the DAG sensor shuttled rapidly to the cell-cell contact zone and also labeled the Golgi-positive pool that polarized to the IS (Figure 5A and Supplemental Movie 3).

Coexpression of GFP-DGK ζ did not alter the subcellular distribution of Cherry-C1ab in resting cells (Supplemental Figure S4), but during APC contact the sensor failed to accumulate as efficiently at the IS, although internal staining was observed (Figure 5B and Supplemental Movie 4). Translocation of Cherry-C1ab was quantified as the ratio of signal intensity at the IS compared with cytosol (Figure 5G). To confirm that membrane localization of DGK ζ is necessary for DAG consumption, we performed a similar experiment with the DGK ζ SA mutant. Enzymatic activity of this mutant is similar to that of the wild-type enzyme (Santos *et al.*, 2002),

but the mutant did not translocate to the PM in response to antigen presentation (Figure 4C). GFP-DGK ζ SA and Cherry-C1ab cotransfection did not alter DAG generation at the IS, as determined by Cherry-C1ab construct accumulation (Figure 5, C and G). To evaluate the requirement for enzymatic activity, we next used a GFP-DGK ζ construct with a mutation in the conserved GGDG motif that impairs catalytic activity (Santos *et al.*, 2002). Cotransfection of this GFP-DGK ζ KD and Cherry-C1ab did not affect the rapid accumulation of the DAG sensor at the IS (Figure 5, D and G). Image analysis showed partial colocalization of the DAG sensor with the GFP-DGK ζ KD at the IS, whereas the sensor was excluded from regions enriched in WT and SA forms (Figure 5D). Taken together, our data confirm the key role of DGK ζ as a modulator of DAG levels at the IS and link DGK ζ subcellular dynamics with the control of DAG-dependent signals.

We also evaluated translocation dynamics of a GFP-DGK α construct, as well as its effect on DAG sensor localization. Tests of GFP-DGK α expression on Cherry-C1ab accumulation at the IS showed neither PM accumulation of this isoform nor significant changes on C1ab synapse accumulation (Figure 5, E and G). A kinase-inactive GFP-DGK α KD mutant showed enhanced membrane localization, in accordance with previous findings (Sanjuan *et al.*, 2001), but it did not alter DAG generation at the IS (Figure 5, F and G).

To evaluate the functional impact of GFP-DGK ζ on DAG consumption during IS formation, we monitored interleukin-2 (IL-2)

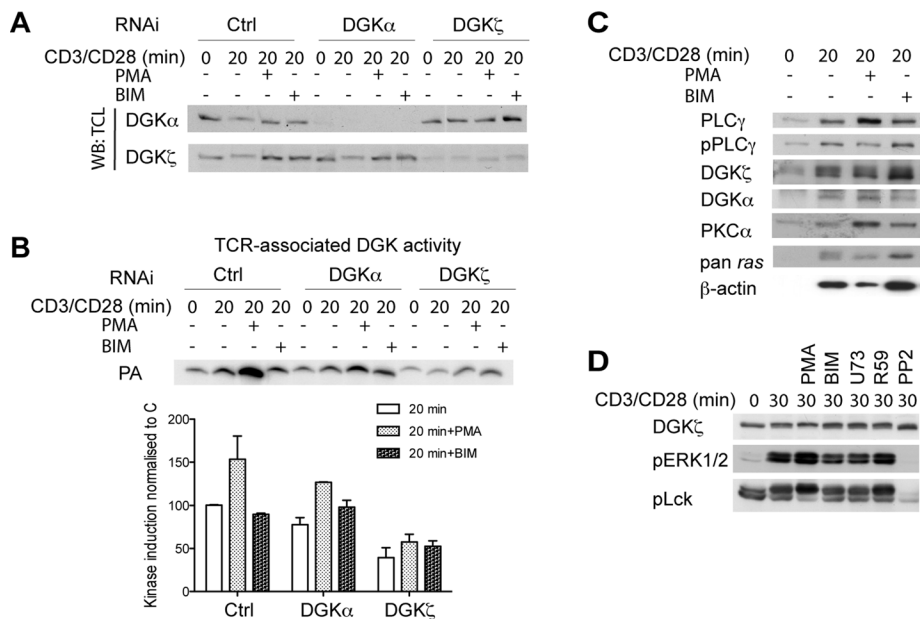


FIGURE 3: PMA promotes DGK ζ activity at the TCR complex. (A, B) DGK α and DGK ζ were silenced and DAG kinase activity measured at the TCR complex. Where indicated, Jurkat T cells were pretreated with 200 ng/ml PMA or 50 nM BIM (15 min). (A) DGK α and DGK ζ expression levels from total cell lysates (TCL) for each condition. (B) DGK assay measured at the TCR complex from cells treated (as in A). A representative kinase assay (PA) is shown (top), percentage PA production after TCR induction, normalized to controls, is represented based on three individual experiments (bottom). Data show mean \pm SD; $n = 3$. (C) Jurkat T cells, untreated or pretreated with PMA or BIM (as in A), were stimulated with anti-CD3/CD28 beads and TCR immune complexes collected at times 0 and 20 min poststimulation. Eluted complexes were resolved by SDS-PAGE and processed in WB with antibodies to endogenous proteins. (D) Jurkat T cells were stimulated with anti-CD3/CD28 beads, untreated or pretreated as in (A) with 200 ng/ml PMA, 50 nM BIM, 1 μ M U73122, 30 μ M R59949, or 30 μ M PP2, and whole-cell extract analyzed by WB using specific antibodies. (C, D) Representative WB images from at least three individual experiments are shown.

promoter induction in reporter luciferase assay using cells expressing the distinct DGK ζ constructs (Figure 5H). Expression of either DGK ζ -WT or the SD mutant reduced IL-2 promoter induction by 50% compared with controls, whereas the SA mutant had no effect. These findings were not due to altered kinase activity, which is similar in both mutants (Santos *et al.*, 2002; unpublished results). These results further demonstrate that membrane localization of DGK ζ is required for its negative function in T cell signaling.

DISCUSSION

DGK enzymes regulate the balance between DAG and PA, and impairment of their function is linked to the onset of various diseases, including diabetes and bipolar disorders (Yakubchik *et al.*, 2005; Chibalin *et al.*, 2008; Kakefuda *et al.*, 2010). Recent evidence shows the importance of DGK α and DGK ζ in T cell activation and tolerance and places them at the front line as important brakes to immune functions (Zhong *et al.*, 2008). Nonetheless, their regulation downstream of TCR signaling is not well characterized and, although their relocation to the TCR activation site might appear conceptually trivial, no direct proof of this dynamic regulation has been reported.

Here we developed a biochemical tool using CD3- and CD28-coated beads with which to study DGK in activated TCR complexes. In addition, we used mild nonionic detergent to partially solubilize membrane structures while preserving proximal TCR complexes. Beads are used successfully for biochemical studies that analyze TCR supramolecular complexes (Viola *et al.*, 1999; Harder and Kuhn,

2001; de Wet *et al.*, 2011). In our studies, both DGK α and DGK ζ were recruited to the TCR complex, which correlated with an increase in associated DGK activity. DGK ζ was the main isozyme controlling PA generation at this site. This, however, does not rule out DGK α as a player in TCR signaling but points out that its activity at the TCR complex can possibly be compensated by DGK ζ . In addition, DGK α could contribute on a different time scale, which warrants further study, and probably correlates with the milder phenotype of DGK α compared with that of DGK ζ -KO mice (Zhong *et al.*, 2003; Olenchock *et al.*, 2006).

A model of antigen presentation with SEE-pulsed APC demonstrated rapid DGK ζ translocation to the PM, concomitant with initial actin recruitment. This concurs with reports in other cell systems, in which DGK ζ colocalized with filamentous actin to modulate lipid signals for correct actin organization and cell migration (Abramovici *et al.*, 2003; Yakubchik *et al.*, 2005). The regulatory mechanism observed in our study closely resembles that described for the carbachol-triggered muscarinic type I receptor (Santos *et al.*, 2002) or in C2C12 myoblasts (Abramovici *et al.*, 2003); in both cases, phosphorylation of the DGK ζ MARCKS region was critical for localization to PM. Whereas the DGK ζ SD mutant (which mimics phosphorylation) showed PM translocation when T cells were in contact with SEE-loaded APC, the SA mutant (which impaired MARCKS-domain phosphorylation) failed to do so.

Although eukaryotic DGK ζ has not yet been characterized structurally, mutational analysis suggests that its C-terminal region has a negative regulatory function that maintains the protein in a closed conformation. MARCKS-domain phosphorylation appears to facilitate an open conformation, exposing the C1 domain and the catalytic region (Santos *et al.*, 2002; Nelson *et al.*, 2007); this would facilitate membrane-protein interactions to further control DGK ζ subcellular localization, as well as regulation of restricted DAG pools. In accordance with this hypothesis, the SA mutant lacking the C-terminal region translocated to the PM during antigen presentation.

Several studies identified PKC α as the kinase responsible for DGK ζ phosphorylation at the MARCKS domain (Luo *et al.*, 2003). Our data showed direct recruitment of these two enzymes to the TCR complex. There were nonetheless striking differences in DGK ζ dynamics during IS formation compared with other models in which PKC α strictly controls GFP-DGK ζ translocation. Indeed, pharmacological inhibition of PKC activity under the conditions tested blocked neither GFP-DGK ζ membrane localization nor recruitment of the endogenous enzyme to the TCR complex. This could suggest that kinases other than PKC can activate DGK ζ , possibly by releasing the negative restriction imposed by the C-terminal region.

PMA cotreatment does not promote further recruitment to the TCR but activates recruited DGK ζ , suggesting further DAG-mediated regulation at this site. Changes in DGK activity did not correlate with enhanced DGK ζ recruitment, indicating that this activation was due to posttranslational modifications and/or protein-protein

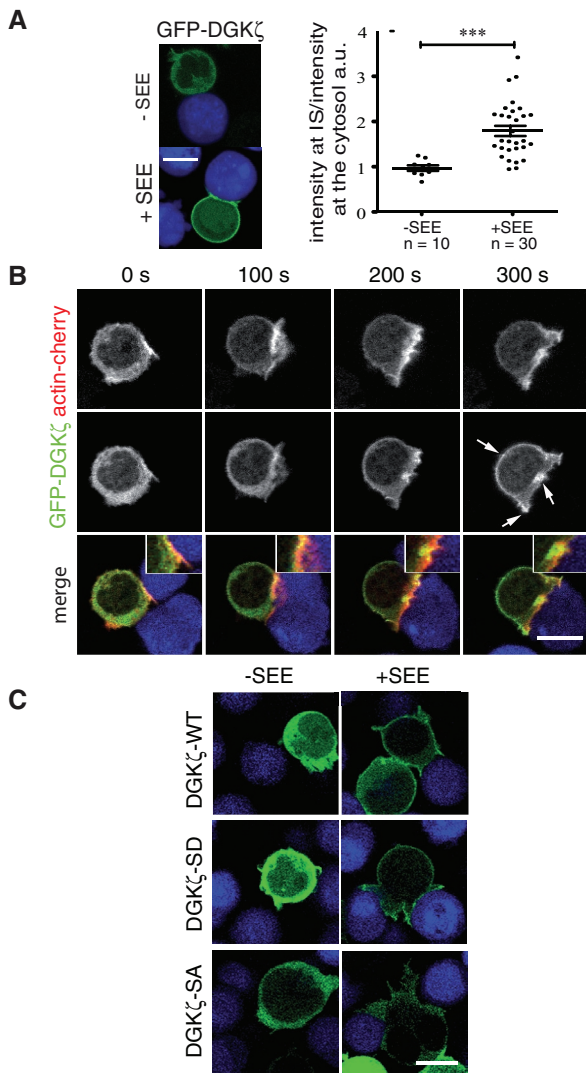


FIGURE 4: GFP-DGK ζ translocates to the PM in response to APC-driven antigen presentation. (A, B) Jurkat T cells transfected with GFP-DGK ζ alone (A) or cotransfected with actin-Cherry (B) were collected after 24 h in HBSS, 2% FBS in poly-L-lysine-coated chambers, and placed on a warmed microscope stage (37°C) for image acquisition; Raji B cells, alone or pulsed with 1 μ g/ml SEE, were prestained with the fluorescent marker CMAC (blue) and added in droplets. (A) Representative images (left). Quantitative image analysis of GFP-DGK ζ accumulated at the IS (right; see *Materials and Methods*). Each dot shows the synapse:cytosol intensity ratio for accumulated GFP-DGK ζ constructs in a T cell after APC encounter. Bars, mean \pm SEM (***p* < 0.001; Kolmogorov-Smirnov test). (B) Dynamic translocation of GFP-DGK ζ (green) with actin-Cherry (red) in the presence of SEE-pulsed APC (blue). Arrows, DGK ζ accumulation; time 0 represents first cell-cell contact. Bar, 10 μ m. (C) Translocation of DGK ζ wild type (WT) vs. MARCKS-domain mutants (SD, SA) after antigen presentation. At 48 h posttransfection, Jurkat T cells were collected and incubated with APC; representative confocal images of live cells are shown.

interactions at the IS. This regulatory mechanism would enhance DGK ζ activation at the synapse as a direct result of local DAG changes and warrants further analysis. PMA activates critical DAG-dependent targets, including PKC and RasGRP1 (Geiger *et al.*, 2003). PKC θ is the main isoform recruited to the T cell synapse (Isakov and Altman, 2002), although our data show that endogenous PKC α is also recruited to the TCR complex. While BIM treat-

ment alters the DGK ζ migration pattern, suggesting an altered phosphorylation status, it does not block activity. Other DAG-regulated kinases, such as ERK or novel PKC, should also be considered, since DGK ζ has consensus sites for ERK and PKC phosphorylation that could contribute to the changes seen in enzyme activity (Abramovici *et al.*, 2003). An alternative possibility to be considered, based on studies of non-T cells, is that recruited PKC α associates with DGK ζ and alters its activation status (Luo *et al.*, 2003). PMA would release this association, maintaining PKC α and DGK ζ in active states, whereas BIM would promote association of inactive PKC α with DGK ζ . All of these are positive feedback mechanisms, which would guarantee adequate DGK ζ activation at the site of DAG generation.

Localized DAG production not only acts as a signaling platform to recruit DAG effectors, but it also assures polarization of the microtubule-organizing center (MTOC; Quann *et al.*, 2009). Pharmacological inhibition of DGK in primary T cells affects DAG gradient formation and MTOC reorientation, suggesting that DGK activity helps to prevent DAG diffusion across the PM (Quann *et al.*, 2009). DGK ζ distribution to the entire PM following T cell-APC contact hinted at a role for this isoform in the control of DAG production outside the IS. Use of a DAG sensor enabled direct evaluation of the functional effect of DGK ζ on DAG levels during TCR activation. Our results demonstrate that although DGK ζ relocates to the entire PM, DAG is consumed at IS, which correlates with recruitment of active DGK ζ to the TCR complex. Indeed, the kinase-inactive DGK ζ did not disrupt DAG pool formation at the IS, thus confirming that enzyme activity is needed to control DAG levels at the IS. In addition, membrane localization was required both for DAG consumption and for DGK ζ to exert its negative control over T cell signal as detected by TCR-dependent IL-2 induction.

In summary, our results indicate that DGK ζ recruitment to the membrane is important for maintaining negative control of DAG accumulation at the IS. On the basis of our data, we propose a model in which mature DGK ζ in cytosol relocates to the PM in response to T cell-APC encounter; DAG production at the IS catalyzes its enzymatic activity to restrict local DAG levels. These findings correlate with the proposed role of DGK ζ in mouse models and suggest that isoform-specific inhibitors would be beneficial in modulating the amplitude of the immune response.

MATERIALS AND METHODS

Reagents and antibodies

Leupeptin and aprotinin were purchased from Roche (Indianapolis, IN). Orthovanadate, phenylmethylsulfonyl fluoride, poly-D-lysine, paraformaldehyde, Igopal CA-630 (NP40), PMA, and BIM were from Sigma-Aldrich (St. Louis, MO). U73122, R59949, and PP2 were all from Calbiochem (La Jolla, CA). Purified mouse anti-human CD28 (CD28.2) and mouse anti-human CD3 (HIT3a) were from BD PharMingen (San Diego, CA). Anti-mouse immunoglobulin G (IgG)-coated Dynabeads and anti-GFP antibody were from Invitrogen (Carlsbad, CA). Antibody to endogenous DGK ζ and DGK α were kind gifts of Matthew Topham (Huntsman Cancer Institute, Salt Lake City, UT) and Wim J. Van Blitterswijk (Netherlands Cancer Institute, Amsterdam, Netherlands), respectively; anti-PKC α was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-pERK1/2, anti-pZAP70 (Y319), and anti-psrc (Y416) were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-mouse and rat IgG were from Dako (Glostrup, Denmark). Lipids for DGK assays were from Avanti Polar Lipids (Alabaster, AL).

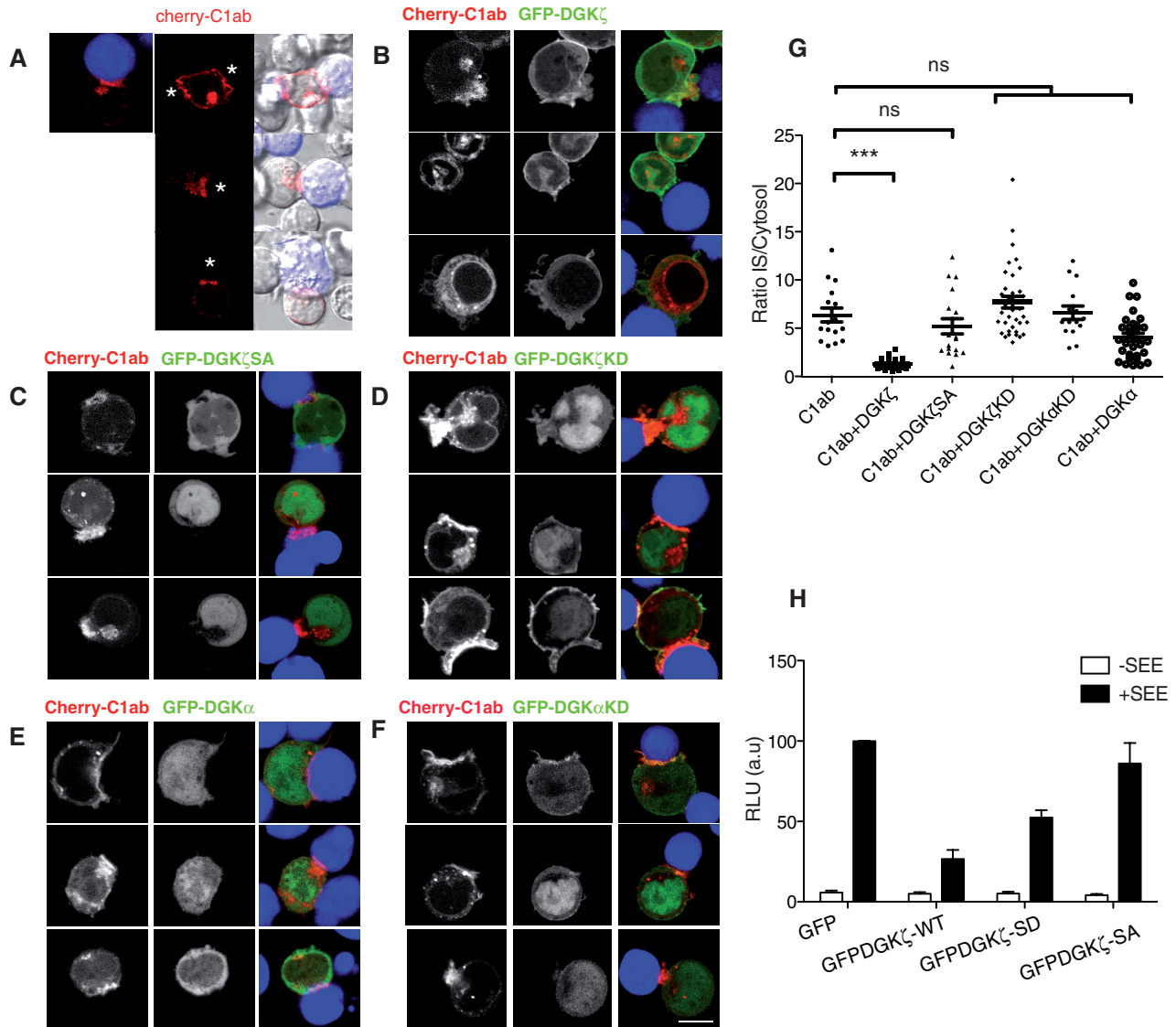


FIGURE 5: DGK localization modulates local DAG pools. (A–F) Jurkat T cells were transfected with the tandem C1 domain of PKC θ fused to Cherry (Cherry-C1ab) alone (A) or cotransfected with GFP-tagged constructs of DGK α or ζ wild type (WT) (B and E, respectively) or mutated DGK ζ SA, DGK ζ KD, or DGK α KD (C, D, and F, respectively), to visualize DAG generation by videomicroscopy during antigen presentation. Raji B cells were treated as in Figure 4; asterisks, Raji B cell position. Representative images from different fields during in vivo imaging are shown (A–F). Bar, 5 μ m. (G) Quantitative analysis of Cherry-C1ab accumulation at the IS in cells transfected with Cherry-C1ab alone or with GFP-DGK constructs taken from three individual experiments. Each dot represents the synapse:cytosol intensity ratio of Cherry C1ab after APC encounter. Bars, mean \pm SEM, $n \geq 15$ (***) $p < 0.001$; Kolmogorov–Smirnov test). (H) Reporter assay for the IL-2 promoter. Jurkat T cells were cotransfected with 15 μ g of IL-2 luciferase reporter/10 ng *Renilla* and with GFP alone or with GFP-DGK ζ WT or mutant (SD or SA). At 48 h posttransfection, cells were stimulated at a 1:1 ratio with APC alone or SEE loaded (24 h). Data show mean \pm SD; $n = 3$ from individual assays.

Isolation of TCR immune complexes

Jurkat T cells in exponential growth were collected at $10\text{--}20 \times 10^6$ cells/ml and serum-starved in DMEM with 0.1% bovine serum albumin (BSA; 2 h). Anti-CD3 and anti-CD28 (5 μ g/ml) were added and incubated (10 min, on ice). Anti-mouse IgG-coated Dynabeads (40 μ l) were mixed and incubated (10 min, on ice). Bead–cell complexes were allowed to activate (37°C, 0–30 min or as indicated) and reactions terminated by washing in ice-cold phosphate-buffered saline (PBS). Where stated, cells were preincubated with specific inhibitor (37°C, 30 min). Cell–bead complexes were lysed in

Brij lysis buffer on ice (0.33% Brij 96V, 20 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1 mM MgCl $_2$, 1 mM NaF, containing inhibitor cocktail [10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mM sodium orthovanadate, and 10 mM β -glycerophosphate]) and washed three times in Brij lysis buffer and once in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4. For in vitro kinase assays, TCR-associated complexes were used as an enzyme source; for Western blot, TCR-bound proteins were eluted in Laemmli sample buffer with boiling. Protein complexes were analyzed by Western blot using protein-specific antibodies.

DGK activity assay

Lipid kinase activity was measured at the TCR complex using two conditions. The first one was unbiased and permits the measurement of broad DGK activity, as previously described, with minor modifications (Flores *et al.*, 1996). Briefly, TCR immune complexes were incubated with DAG micelles (0.2 mg/ml 1,2-dioctanoyl-sn-glycerol [C8:0]) in 50 mM HEPES, pH 7.4, and sonicated for 15 min. In addition, we further determined DGK α contribution by measuring DGK activity as in Sakane *et al.* (1991). DAG micelles were formed in octylglucoside (1.2 mg/ml 1,2-dioleoyl-sn-glycerol [C18:1]) and 1.6 mg/ml L- α -phosphatidylserine and dried; lipids were then resuspended in 0.16 M octylglucoside (50 μ M final concentration in the assay) and incubated (15 min, 37°C). Kinase reactions (50 μ l final volume) were initiated by addition of the kinase reaction mixture (assay I was carried out with 20 μ M ATP, 10 mM MgCl₂, 10 μ Ci [γ ³²P]ATP, 50 mM HEPES, pH 7.4; assay II was performed with 1 mM ATP, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithiothreitol, 10 μ Ci of [γ ³²P]ATP, 50 mM HEPES, pH 7.4, and 30 μ M CaCl₂). Reactions continued for 15 min at room temperature and were terminated with 50 μ l of 1 M HCl and 100 μ l of MeOH. Lipids were extracted in CHCl₃ phase and washed once in 1:1 (vol/vol) HCl/MeOH to remove free [γ ³²P]ATP. Lipids were dried in a Speed-Vac, resuspended in 1:1 (vol/vol) MeOH/CHCl₃, and resolved by TLC using silica gel plates in 9:7:2 CHCl₃/MeOH/4 M NH₄OH solvent. PA generation was detected in a phosphorimager (Bio-Rad, Hercules, CA) and quantified using the Quantity One program. Statistical analyses show measurements from at least three independent experiments, normalized to time point 0, corresponding to control cells.

Cell transfection and short hairpin RNA

Jurkat T cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (both from Sigma-Aldrich) at 37°C, 5% CO₂. Cells were collected in exponential growth (1.2 \times 10⁷ cells in 400 μ l) and transfected by electroporation with 20 μ g (for protein expression) or 30 μ g (for short hairpin RNA) of plasmid DNA using a Gene Pulser (975 μ F, 270 mV; Bio-Rad), as described (Rincon *et al.*, 2007). Plasmids used for overexpression studies, encoding wild-type DGK α and DGK ζ fused to GFP, or bearing specific mutations, were reported elsewhere (Sanjuan *et al.*, 2001; Santos *et al.*, 2002). The PKC θ C1ab region was excised from the PKC θ C1ab-GFP construct described elsewhere (Carrasco and Merida, 2004); actin fused to Cherry was a kind gift of Ann Wheeler (Queen Mary University, London, United Kingdom). At 24 h post-transfection, cells were washed and used, or allowed to expand for 24 h. For knockdown analysis of DGK α or DGK ζ , we used specific RNAi to human forms and a negative control that targets murine DGK α (Rincon *et al.*, 2007; A. Avila-Flores and I. Merida, unpublished data), which were cloned into a p-Super Retro plasmid. For maximal down-modulation of target proteins, cells were assayed at 72 or 96 h posttransfection.

Immunofluorescence

Jurkat T cells were preincubated with antibody-coated beads on poly-D-lysine-coated coverslips, fixed in 4% paraformaldehyde (20 min), and permeabilized in 0.2% Triton X100/PBS (5 min, on ice). After washing, fixed cells were blocked in 2% BSA (15 min), incubated with primary antibody (1:50 in 3% FBS/PBS, 30 min, 37°C in a humidified chamber), washed in PBS, and incubated with Alexa-conjugated secondary antibody (1:500, 30 min, 37°C). F-actin was detected with phalloidin-rhodamine (Molecular Probes, Invitrogen (15 min, 37°C) and washed in PBS prior to mounting. Images were captured using a Zeiss (Thornwood, NY) confocal microscope.

Luciferase reporter assay

Jurkat T cells were transiently cotransfected with 15 μ g of GFP-tagged constructs (GFP, GFP-DGK ζ -WT, GFP-DGK ζ -SD, GFP-DGK ζ -SA) and 15 μ g of IL-2-luciferase reporter construct/10 ng *Renilla* luciferase as internal standard. At 48 h posttransfection, cells were incubated with APC nonpulsed or pulsed with 1 μ g/ml SEE at a 1:1 ratio (total 2 \times 10⁶ cells) for 24 h. Each reading was normalized internally to *Renilla* and the percentage induction represented; GFP cells incubated with SEE-loaded APC were considered 100%.

Time-lapse fluorescence microscopy

Chambers were precoated with poly-D-lysine and Jurkat T cells prepared at 0.5 \times 10⁶ cells/ml in HBSS buffer (25 mM HEPES-KOH, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 132 mM NaCl, 0.1% BSA) containing 1% FBS (15 min), then placed on a microscope stage at 37°C. Raji B cells stained with 7-amino-4-chloromethylcoumarin (CMAC) were preincubated with 1 μ g/ml SEE superantigen or left untreated and were added at a 1:1 ratio to adhered Jurkat T cells. Videos were captured on an FV1000 confocal laser-scanning microscope (Olympus, Center Valley, PA) and images processed using ImageJ, version 1.43u (National Institutes of Health, Bethesda, MD), and Adobe Photoshop (San Jose, CA). Still images were captured after 15 min of incubation of Jurkat T cells with pretreated APC.

Analysis of protein accumulation at the T cell-APC contact area

To quantify the amount of GFP-DGK ζ or C1-GFP accumulated at the IS compared with cytosol, we used an ImageJ plugin (Rincon *et al.*, 2011) that measures average intensity value of the image in a small circular area; we monitored background (Bg), cytosol of the T cell (T), and the synapse (S) when the fluorescent protein was expressed by only one of the two cells. Average pixel value was computed for each measurement (Bg, T, S). From these observed values, we separated the contribution of each component (background, T cell, and T cell at the synapse). Finally, we computed the ratio between fluorescence at the synapse and in cytosol. Ratio values were represented as dot plots, with each dot representing an individual cell.

Statistical analysis

To analyze the fluorescence intensity of GFP-DGK ζ or of Cherry-C1ab at the IS, we used a two-sample Kolmogorov-Smirnov test to compare pairs of distribution ratios (IS/T) from different cells. The Bonferroni correction was applied to confidence thresholds to correct for multiple comparisons. Differences were considered nonsignificant (ns) when $p > 0.05$, significant (*) when $p < 0.05$, very significant (**) when $p < 0.01$, and extremely significant (***) when $p < 0.001$.

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REFERENCES

- Abramovici H, Hogan A, Obagi C, Topham M, Gee S (2003). Diacylglycerol kinase-zeta localization in skeletal muscle is regulated by phosphorylation and interaction with syntrophins. *Mol Biol Cell* 14, 4499–4511.
- Beal AM, Anikeeva N, Varma R, Cameron TO, Norris PJ, Dustin ML, Sykulev Y (2008). Protein kinase C theta regulates stability of the peripheral adhesion ring junction and contributes to the sensitivity of target cell lysis by CTL. *J Immunol* 181, 4815–4824.
- Beyers AD, Spruyt LL, Williams AF (1992). Molecular associations between the T-lymphocyte antigen receptor complex and the surface antigens CD2, CD4, or CD8 and CD5. *Proc Natl Acad Sci USA* 89, 2945–2949.
- Carrasco S, Merida I (2004). Diacylglycerol-dependent binding recruits PKCtheta and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes. *Mol Biol Cell* 15, 2932–2942.
- Carrasco S, Merida I (2007). Diacylglycerol, when simplicity becomes complex. *Trends Biochem Sci* 32, 27–36.
- Chibalin AV *et al.* (2008). Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. *Cell* 132, 375–386.
- de Wet B, Zech T, Salek M, Acuto O, Harder T (2011). Proteomic characterization of plasma membrane-proximal T cell activation responses. *J Biol Chem* 286, 4072–4080.
- Ebinu JO, Stang SL, Teixeira C, Bottorff DA, Hooton J, Blumberg PM, Barry M, Bleakley RC, Ostergaard HL, Stone JC (2000). RasGRP links T-cell receptor signaling to Ras. *Blood* 95, 3199–3203.
- Flores I, Casaseca T, Martinez AC, Kanoh H, Merida I (1996). Phosphatidic acid generation through interleukin 2 (IL-2)-induced alpha-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. *J Biol Chem* 271, 10334–10340.
- Fooksman DR, Vardhana S, Vasiliver-Shamis G, Liese J, Blair DA, Waite J, Sacristan C, Victora GD, Zanin-Zhorov A, Dustin ML (2010). Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol* 28, 79–105.
- Fraser J, Arcus V, Kong P, Baker E, Proft T (2000). Superantigens—powerful modifiers of the immune system. *Mol Med Today* 6, 125–132.
- Friedl P, Störöm J (2004). Diversity in immune-cell interactions: states and functions of the immunological synapse. *Trends Cell Biol* 14, 557–567.
- Geiger M, Wrulich OA, Jenny M, Schwaiger W, Grunicke HH, Uberall F (2003). Defining the human targets of phorbol ester and diacylglycerol. *Curr Opin Mol Ther* 5, 631–641.
- Guo R, Wan CK, Carpenter JH, Mousallem T, Boustany RM, Kuan CT, Burks AW, Zhong XP (2008). Synergistic control of T cell development and tumor suppression by diacylglycerol kinase alpha and zeta. *Proc Natl Acad Sci USA* 105, 11909–11914.
- Harder T, Kuhn M (2001). Immunoinhibition of TCR signaling complexes from Jurkat T leukemic cells. *Sci STKE* 2001, pl1.
- Isakov N, Altman A (2002). Protein kinase C(theta) in T cell activation. *Annu Rev Immunol* 20, 761–794.
- Kakefuda K *et al.* (2010). Diacylglycerol kinase beta knockout mice exhibit lithium-sensitive behavioral abnormalities. *PLoS One* 5, e13447.
- Lin J, Weiss A (2001). T cell receptor signalling. *J Cell Sci* 114, 243–244.
- Luo B, Prescott S, Topham M (2003). Protein kinase C alpha phosphorylates and negatively regulates diacylglycerol kinase zeta. *J Biol Chem* 278, 39542–39547.
- Merida I, Avila-Flores A, Merino E (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem J* 409, 1–18.
- Nelson CD, Perry SJ, Regier DS, Prescott SM, Topham MK, Lefkowitz RJ (2007). Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. *Science* 315, 663–666.
- Olenchock BA, Guo R, Carpenter JH, Jordan M, Topham MK, Koretzky GA, Zhong XP (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat Immunol* 7, 1174–1181.
- Quann EJ, Liu X, Altan-Bonnet G, Huse M (2011). A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat Immunol* 12, 647–654.
- Quann EJ, Merino E, Furuta T, Huse M (2009). Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat Immunol* 10, 627–635.
- Rincon E, Saez de Guinoa J, Gharbi SI, Sorzano CO, Carrasco YR, Merida I (2011). Translocation dynamics of sorting nexin 27 in activated T cells. *J Cell Sci* 124, 776–788.
- Rincon E, Santos T, Avila-Flores A, Albar JP, Lalioti V, Lei C, Hong W, Merida I (2007). Proteomics identification of sorting nexin 27 as a diacylglycerol kinase zeta-associated protein: new diacylglycerol kinase roles in endocytic recycling. *Mol Cell Proteomics* 6, 1073–1087.
- Sakane F, Yamada K, Imai S, Kanoh H (1991). Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation. *J Biol Chem* 266, 7096–7100.
- Sanjuan MA, Jones DR, Izquierdo M, Merida I (2001). Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. *J Cell Biol* 153, 207–220.
- Sanjuan MA, Pradet-Balade B, Jones DR, Martinez AC, Stone JC, Garcia-Sanz JA, Merida I (2003). T cell activation in vivo targets diacylglycerol kinase alpha to the membrane: a novel mechanism for Ras attenuation. *J Immunol* 170, 2877–2883.
- Santos T, Carrasco S, Jones DR, Merida I, Eguinoa A (2002). Dynamics of diacylglycerol kinase zeta translocation in living T-cells. Study of the structural domain requirements for translocation and activity. *J Biol Chem* 277, 30300–30309.
- Siliceo M, Merida I (2009). T cell receptor-dependent tyrosine phosphorylation of beta2-chimaerin modulates its Rac-GAP function in T cells. *J Biol Chem* 284, 11354–11363.
- Sims TN *et al.* (2007). Opposing effects of PKCtheta and WASp on symmetry breaking and relocation of the immunological synapse. *Cell* 129, 773–785.
- Smith-Garvin JE, Koretzky GA, Jordan MS (2009). T cell activation. *Annu Rev Immunol* 27, 591–619.
- Spitaler M, Emslie E, Wood CD, Cantrell D (2006). Diacylglycerol and protein kinase D localization during T lymphocyte activation. *Immunity* 24, 535–546.
- Viola A, Schroeder S, Sakakibara Y, Lanzavecchia A (1999). T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283, 680–682.
- Yakubchik Y, Abramovici H, Maillat JC, Daher E, Obagi C, Parks RJ, Topham MK, Gee SH (2005). Regulation of neurite outgrowth in N1E-115 cells through PDZ-mediated recruitment of diacylglycerol kinase zeta. *Mol Cell Biol* 25, 7289–7302.
- Zhong XP, Guo R, Zhou H, Liu C, Wan CK (2008). Diacylglycerol kinases in immune cell function and self-tolerance. *Immunol Rev* 224, 249–264.
- Zhong XP, Hailey EA, Olenchock BA, Jordan MS, Maltzman JS, Nichols KE, Shen H, Koretzky GA (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat Immunol* 4, 882–890.
- Zhong XP, Hailey EA, Olenchock BA, Zhao H, Topham MK, Koretzky GA (2002). Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase zeta. *J Biol Chem* 277, 31089–31098.