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Role of diacylglycerol kinases in T cell development and function

Sruti Krishna and
Department of Pediatrics-Allergy and Immunology and Department of Immunology, Duke University Medical Center

Xiao-Ping Zhong, MD, PhD
Department of Pediatrics-Allergy and Immunology, Rm. 133 MSRB-I, Research Drive, Box 2644, Duke University Medical Center, Durham, NC 27710. Phone: 919-681-9450; Fax: 919-668-3750

Sruti Krishna: ss195@duke.edu; Xiao-Ping Zhong: zhong001@mc.duke.edu

Abstract

Diacylglycerol (DAG), a second messenger generated by phospholipase Cγ1 activity upon T cell receptor (TCR) engagement, triggers several signaling cascades that play important roles in T cell development and function. A family of enzymes called diacylglycerol kinases (DGKs) catalyzes the phosphorylation of DAG to phosphatidic acid, acting as a braking mechanism that terminates DAG-mediated signals. Two DGK isoforms, α and ζ, are predominantly expressed in T cells and synergistically regulate the development of both conventional αβ T cells and invariant NKT cells in the thymus. In mature T cells, the activity of these DGK isoforms aids in the maintenance of self-tolerance by preventing T cell hyper-activation upon TCR stimulation and by promoting T cell anergy. In CD8 cells, reduced DGK activity is associated with enhanced primary responses against viruses and tumors. Recent work has also established an important role for DGK activity at the immune synapse and identified partners that modulate DGK function. In addition, emerging evidence points to previously unappreciated roles for DGK function in directional secretion and T cell adhesion. In this review, we discuss the multitude of roles played by DGKs in T cell development and function, while emphasizing recent advances in the field.

Keywords

Diacylglycerol kinase; phosphatidic acid; signal transduction; T cell receptor

I. INTRODUCTION

Diacylglycerol kinases (DGKs) are a family of enzymes that catalyze the conversion of lipid second messenger diacylglycerol (DAG) to phosphatidic acid (PA). Work from several groups, including ours, has shown that DGKs serve as a braking mechanism in immune cell signaling, dampening DAG levels after receptor stimulation and preventing hyper-activation of immune cells.[1–4] Ten isoforms of DGK have been identified in mammals, many of which are expressed in cells of the immune system.

Correspondence to: Xiao-Ping Zhong, zhong001@mc.duke.edu.

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Notably, both the substrate and product of the DGK-catalyzed reaction, DAG and PA, are bioactive lipids that can act as second messengers.[5–8] DGK activity therefore serves as a switch to simultaneously dampen DAG-mediated signals and boost PA-mediated signals. In T cells, DAG recruits RasGRP1 and PKCθ to the cell membrane, leading to signaling via the RasGRP1/Ras/ERK and PKCθ/IKK/NF-κB pathways.[9,10] Previous studies have shown that PA, on the other hand, can bind to signaling molecules such as mammalian target of rapamycin (mTOR), SHP-1, RasGAP, Sos, PI5Kα, and p47(phox).[8,11–17]

All mammalian DGKs contain a catalytic kinase domain, consisting of a conserved motif and an accessory domain, and at least two cysteine-rich DAG-binding C1 domains. However, DGKs also possess other distinct structural domains, based on which they are classified into five types (Figure 1). Two DGK isoforms, the type-I α isoform and the type-IV ζ isoform, are highly expressed in T cells.[18,19] The type-I DGK isoforms α, β and γ possess an N-terminal recoverin homology domain and two Ca^{2+}-binding EF hand motifs. While the recoverin homology domain is related to the N terminal region of the recoverin family of neuronal calcium sensors, the EF hands are involved in auto-inhibition.[20] Type-IV DGK isoforms, ζ and ι, contain a myristoylated alanine rich C-kinase substrate (MARCKS) motif, four ankyrin repeats, and a C-terminal PDZ binding domain. As its name suggests, the MARCKS domain can be phosphorylated by PKC isoforms. Studies in cell lines have shown that PKCα can phosphorylate the DGKζ MARCKS motif to negatively regulate both DGKζ's catalytic activity and its ability to interact with other proteins.[21,22] In addition, the MARCKS motif contains a nuclear localization sequence.[23] The ankyrin repeats and PDZ binding motif are thought to play a role in protein-protein interactions, with the latter binding to PDZ domains on proteins such as syntrophins.[24,25] Similar to some other DGKp isoforms such as β, δ, and η, DGKζ contains several alternative splicing isoforms.[26,27] The functional differences among these DGKζ isoforms in the immune system remain to be clearly defined.

Members of the DGK family show substantial diversity in the cell types they are expressed in and their localization within those cells. Notably, DGK isoforms are highly expressed in cells of the hematopoietic and nervous systems. Multiple DGK isoforms are often expressed simultaneously in a given cell type. Though DGK α and ζ isoforms predominate in T cells, we can be detected multiple DGK isoforms by reverse transcriptase-PCR in T cells (α, γ, δ, ζ, θ), macrophages (α, β, γ, δ, ζ, ι) and mast cells (α, γ, δ, ε, ζ, ι)(References 28, 29 and our unpublished observations). Due to their distinct structural domains, different types of DGKs tend to localize to specific subcellular compartments and are regulated by unique cues in the intracellular milieu.[30] Six DGK isoforms – α, γ, δ, ζ, ι, and θ - have been observed to reside in or move to the nucleus upon stimulation in different cell types.[31] For instance, stimulation via the TCR leads to the nuclear translocation of DGKα and its binding to the nuclear matrix in primary rat T cells.[32] Immunohistochemical analyses have also revealed that DGKζ localizes to the nucleus in neurons in various parts of the rat brain, and that this subcellular distribution is specifically disrupted in hippocampal pyramidal neurons in a model of forebrain ischemia.[33] Nuclear localization of DGKζ has also been demonstrated in other cell types.[23,24] Nuclear translocation of DGKs could control nuclear DAG and PA concentrations and/or prevent DGKs from terminating DAG in the cytoplasm membrane. Whether DGKζ localizes to the nucleus and the functional importance of nuclear localization of DGK isoforms in T cells remain to be defined.

From an organismal standpoint, it is interesting to note that DGKα protein expression appears to be restricted to certain cell types such as T-lineage cells and oligodendrocytes,[34,35] while DGKζ is expressed more ubiquitously in the brain, lungs, heart, hematopoietic system and skeletal muscles.[27,36,37] However, as stated previously, our unpublished data suggests that DGKα, at least at the mRNA level, may also be expressed in

other hematopoietic cells including mast cells and macrophages. In this review, we discuss the varied roles played by DGKs in T cell development and function, while emphasizing recent advances that have helped move the field forward.

II. ROLE AND REGULATION OF DAG IN TCR SIGNALING

Engagement of the TCR by a cognate peptide-MHC complex triggers a multitude of signaling pathways that, in the presence of additional signals, cooperate to turn on a transcriptional program of T cell activation (Figure 2). Following TCR engagement, Lck, a Src family tyrosine kinase that associates with the cytoplasmic tails of CD4 and CD8, is activated by CD45-mediated dephosphorylation.[38, 39] Active Lck, in turn, phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) on CD3, leading to the recruitment of the kinase ZAP-70.[40, 41] Lck phosphorylates and activates ZAP-70, which then phosphorylates the adaptor protein LAT.[42, 43] Phosphorylated LAT recruits a number of signaling molecules, including adaptor SLP-76 and phospholipase PLCγ1, to the cell membrane.[44,45]

Active PLCγ1 hydrolyzes membrane phospholipid PIP_2 to produce two second messengers - DAG and inositol triphosphate (IP_3). IP_3 binds to its receptors on the endoplasmic reticulum (ER) to trigger the release of intracellular ER calcium stores. Depletion of calcium in the ER lumen causes a conformational change in the ER-associated calcium sensors STIM1 and STIM2.[46] This conformational change activates the calcium-release activated calcium (CRAC) channel Orai1 on the cell membrane, leading to an influx of calcium from the extracellular milieu.[47] Increasing calcium levels in the cytosol activate the phosphatase calcineurin, which dephosphorylates the transcription factor NFAT to trigger its nuclear translocation.[48] Signaling via the NFAT pathway is important for T cell development, activation, anergy, and in the function of T_{reg}, T_{FH}, T_H17, and CD8 T cells.[49,50]

Membrane-associated DAG, on the other hand, recruits PKCθ, PKD1, and Ras family guanine nucleotide exchange factor RasGRP1 to the cell membrane through their C1 domains.[10,51–53] Activated RasGRP1, along with Sos, helps convert the small GTPase Ras from its GDP-bound inactive form to a GTP-bound active form, and active Ras then activates the kinase Raf.[54, 55] In turn, Raf activates MEK1/2, which subsequently activate MAP kinases ERK1/2. ERK activity increases the expression of transcription factor c-Fos and also phosphorylates c-Fos, leading to its dimerization with c-Jun to form the transcription factor AP-1.[56, 57] NFAT and AP-1 interact with each other and bind cooperatively to composite binding elements on the promoters of several genes, including IL-2.[58–60] The RasGRP1-Ras-ERK1/2 pathway has been shown to play a critical role in positive selection during intrathymic T cell development and in the activation of peripheral T cells.[61–64]

Active PKCθ phosphorylates the adaptor CARD11, leading to the formation of a signalosome with Bcl10 and MALT1.[65] This signalosome activates the three-subunit IKK complex, which phosphorylates IκB. Phosphorylation of IκB eventually leads to its degradation, allowing active NF-κB dimers to translocate to the nucleus.[66, 67] The PKCθ-IKK-NFκB pathway is not essential for conventional T cell maturation, but is critical for *i*NKT cell and regulatory T cell development and for peripheral T cell activation,[68–71] particularly for effective T_H2 immune responses.[72–74] It is important to note here that DAG-independent mechanisms may also play important roles in PKCθ activation. For instance, CD28 can directly recruits PKCθ to the immunological synapse to promote its activation.[75,76]

DAG-activated PKD1 phosphorylates the transcriptional repressor HDAC7, leading to its export from the nucleus and the de-repression of its target genes, such as Nur77.[77,78] Membrane-localized and cytosolic forms of PKD1 may have distinct functions during thymocyte development.[79] The individual DAG-mediated pathways outlined above have also been shown to cooperate with each other. For example, PKCθ phosphorylates RasGRP1 and PKD1 to enhance their activation.[80] Thus, by recruiting PKCθ, RasGRP1, and PKD1, DAG controls signaling via a number of interconnected pathways in response to TCR engagement.

DAG-mediated activation of the Ras-ERK1/2 and NFκB pathways also indirectly activates signaling via the PI3K-mTOR pathway. The mTOR pathway and its tight regulation play an important role in T cell development, homeostasis, activation, and differentiation,[81–88] while PI3K isoforms function redundantly to promote T cell development, activation, survival, and self-tolerance.[89, 90] Studies in cell lines have shown that active Ras can bind to and activate PI3K,[91, 92] which catalyzes the conversion of PIP_2 to PIP_3. While it was known that PI3K activity can recruit the kinases PDK1 and Akt to activate the Akt-mTOR complex 1 pathway,[93] recent studies using primary mouse thymocytes and T cells have provided direct evidence that DAG-mediated activation of the Ras-MEK1/2-ERK1/2 pathway can also initiate signaling through mTOR complex 1 and mTOR complex 2.[94,95]

Given the nature and number of signaling pathways activated by DAG, it stands to reason that DAG levels must be tightly regulated to prevent T cell hyper-activation. This hypothesis is supported by findings that dysregulation of individual DAG-mediated signaling pathways can have profound effects on T cell function and iNKT cell development.[71,96, 97] DGKs play a critical role in regulating intracellular DAG levels, removing DAG through phosphorylation to produce PA. DGKα and DGKζ are the predominant isoforms expressed in T cells, as mentioned previously. Two splice variants of DGKζ, DGKζ1 (130 kDa) and DGKζ2 (115 kDa), are expressed in thymocytes and matureT cells.[19] Splicing of the first coding exon directly to the third generates the smaller splice variant, while transcription from an alternative promoter at the second exon and subsequent splicing to the third generates the longer variant.[26,98] Interestingly, the splicing isoforms are expressed in a complementary fashion, with higher expression of DGKζ1 in $CD4^-$ $CD8^-$ and CD4+ CD8+ thymocytes, and higher expression of DGKζ2 in mature (CD4SP and CD8SP) thymocytes and peripheral T cells. The mechanisms that control the differential expression of DGKζ1/ζ2 during T cell development are currently unclear, as are the functional differences between these two isoforms. Experiments with deletion mutants in Jurkat cells showed that the N-terminal end of DGKζ, but not the C-terminal end, is essential for optimal inhibition of TCR signaling.[19] Other studies have shown that DGKα is expressed in thymocytes and peripheral T cells,[34, 99,100] but its expression levels at different stages of T cell development remain to be examined. As discussed below, synergistic regulation of DAG signaling by DGKα and DGKζ is essential for normal T cell development and function.

III. ROLE OF DGKα AND DGKζ IN T CELL DEVELOPMENT

Lymphoid progenitor cells generated in the bone marrow migrate to the thymus, where they travel through the cortex and medulla, developing into mature T cells.[101, 102] Successive developmental stages of a thymocyte can be distinguished by the combination of CD4 and CD8 co-receptors expressed on its surface. Early committed T cells do not express TCR, CD4, or CD8 on the cell surface and are called $CD4^-$ $CD8^-$ double-negative (DN) cells. DN cells rearrange V, D, and J gene segments at the TCRβ locus, leading to the expression of a pre-TCR. Cells that express a functional pre-TCR pass through the so-called “β-selection” checkpoint, while others undergo apoptosis. The β-selected DN cells undergo several rounds of proliferation, maturing into $CD4^+$ $CD8^+$ double-positive (DP) cells that constitute about

90 percent of all thymocytes. DP cells rearrange V and J gene segments at the TCRa locus, leading to the expression of a unique TCR on the cell surface.

Following TCR expression, DP thymocytes are subjected to processes called positive and negative selection,[103,104] that ensure the generation of a functional, non self-reactive T cell repertoire. In order to be positively selected, DP cells must express a TCR that is able to recognize self-peptide-MHC complexes expressed by thymic epithelial cells or bone-marrow-derived dendritic cells in the thymus. In general, DP cells with TCRs that fail to recognize self-peptide-MHC complexes are eliminated at this stage, as they fail to receive survival signals. On the other hand, DP cells with TCRs that recognize self-peptide-MHC with high affinity also undergo apoptosis, a process referred to as negative selection. Thus, only DP cells with TCRs that recognize self-peptide-MHC molecules with low affinity survive positive and negative selection, developing further into mature $CD4^+$ $CD8^-$ single positive (CD4SP) or $CD4^-$ $CD8^+$ single positive (CD8SP) cells.

Several signaling pathways, including MAP kinase, NF-κB, and NFAT pathways, are known to play critical roles in thymocyte selection. PLCγ1 deficiency in thymocytes impairs both positive and negative selection processes, suggesting a potential role for DAG-mediated signals in T cell development.[105] Numerous studies have shown that defects in DAG-effector pathways profoundly impact thymocyte development, lending further credence to this notion. For instance, thymocytes deficient in RasGRP1 display severely impaired positive selection, with a marked paucity of mature single positive cells[106] Expression of a dominant negative form of Ras or MEK1 inhibits positive but not negative selection,[107, 108] leading to a block in thymocyte development at the DP stage. While thymocytes lacking ERK1 experience a partial developmental block at the DP stage with a concomitant reduction of mature thymocytes,[109] combined deficiency of ERK1 and ERK2 has been shown to impair positive but not negative selection.[110,111] The Ras-MEK1/2-ERK1/2 pathway is thus thought to play a critical role in the positive selection of thymocytes. On the other hand, signaling via the p38 and JNK MAP kinase pathways is thought to play an essential role in negative selection.[112] Though deficiency of PKCθ or IKKβ does not appear to affect conventional αβ T cell maturation,[70, 113] a recent study has revealed a differential role for NF-κB in the selection and survival of CD4 and CD8 thymocytes.[114] Moreover, PKCθ-mediated signaling is pivotal for natural regulatory T cell and iNKT cell development.[115] The importance of DAG-triggered Ras-ERK and PKCθ-NF-κB pathways in thymocyte selection processes thus suggests that tight regulation of DAG levels by DGKs may be critical for normal T cell development.

Previous studies have shown that signaling via the pre-TCR increases DGKα expression in thymocytes.[116] Although pharmacological inhibition of DGKα activity (with the inhibitor R59949) suggested that DGKα could promote DP thymocyte survival via a Bcl-xL mediated pathway,[116] other studies have revealed that genetic deficiency of either DGKα or ζ does not obviously alter thymocyte populations.[117,118] Additional studies should determine whether this type-I DGKα inhibitor may possess off-target activities that are yet to be identified or if other type-I DGKs are expressed in developing thymocytes that may compensate for DGKα deficiency. More recent work from our group has provided genetic evidence that DGKα and DGKζ synergistically regulate T cell development.[119] Combined deficiency of the DGKα and ζ isoforms led to a severe block in murine thymocyte development at the DP stage, with a dramatic reduction in the number of mature CD4SP and CD8SP cells. Crossing with HY TCR transgenic mice revealed that combined DGKαζ deficiency was associated with impaired positive selection, but not negative selection. Reduced DGK activity in the DGKαζ double knockout (DKO) thymocytes was associated with increased levels of intracellular DAG after TCR stimulation, and enhanced signaling via the Ras-ERK pathway. However, the developmental blockade was partially overcome by

PA treatment, suggesting that DGKs play a critical role in thymocyte development not only by terminating DAG-mediated signaling but also by initiating PA-mediated signals. How PA promotes T cell maturation is a critical question that remains to be addressed.

A novel role for DGKs in regulating mTOR activity in thymocytes has also emerged recently.[94] Upon TCR stimulation, DGKαζ DKO thymocytes showed elevated levels of S6K1 and 4E-BP1 phosphorylation, suggestive of increased mTOR complex 1 activity. This was correlated with increased ERK1/2 and Rsk1 activation. Phosphorylation of Akt at S473 was also increased in DKO thymocytes, indicating enhanced mTOR complex 2 activity. Inhibition of MEK1/2 dramatically reduced TCR-induced mTOR activation in both wild-type (WT) and DGKαζ DKO thymocytes, indicating that DGK activity inhibits TCR-induced mTOR activation by attenuating signaling via the RasGRP1-Ras-ERK1/2 pathway. In non-T cell lines, ERK1/2 phosphorylate and inactivate TSC2, a negative regulator of mTOR complex 1 activation, to promote signaling via mTOR complex 1.[120] Whether the RasGRP1-Ras-ERK1/2 pathway activates mTOR complex 1 signaling via similar mechanisms in T cells needs to be confirmed. In addition, the mechanisms by which DGK (α and ζ) activity inhibits and RasGRP1-Ras-ERK1/2 signaling promotes mTORC2 signaling remain to be explored.

IV. ROLE OF DGKα AND DGKζ IN *i*NKT CELL DEVELOPMENT

Natural killer T (NKT) cells are a rare subset of lymphocytes that express both NK family receptors (such as NK1.1 in mice) and a semi-invariant TCR.[121–123] Unlike conventional αβ T cells, the TCR on NKT cells recognizes glycolipids presented on MHC-like CD1d molecules. Capable of producing an array of cytokines within minutes to hours of stimulation, NKT cells have been shown to modulate several important immune phenomena including responses to infection and cancer, allergy, and autoimmunity. A majority of NKT cells in humans and mice are characterized by their unique usage of TCR Vα24 (human) or Vα14 (murine) and Jα18 segments and a limited TCR Vβ repertoire. Such NKT cells are called type I or invariant NKT (*i*NKT) cells. Developing *i*NKT cells are classified into successive developmental stages from 0 to 3, based on the surface expression of CD24, NK1.1, and CD44[124] Invariant NKT cell development and function remain actively investigated.

In the thymus, DP cells that express appropriate TCRs to enter the NKT lineage are thought to undergo selection processes analogous to those of conventional αβ T cells, which require signaling from the *i*Vα14TCR.[125, 126] However, unlike conventional αβ thymocytes that are selected on thymic epithelial cells, NKT thymocytes are selected on fellow CD1d-expressing DP thymocytes.[127–129] Results from previous studies have also suggested that developing *i*NKT cells may differ from αβ thymocytes in certain signaling requirements for proper development. For example, a SLAM/SAP/Fyn/PKCθsignaling pathway is critical for *i*NKT cell ontogeny but exerts minimal impact on conventional αβT cell development.[130–134] Several recent reports have demonstrated that DAG-mediated signaling and its proper regulation are pivotal for normal *i*NKT cell development and homeostasis. Absence of RasGRP1 or expression of a dominant negative Ras impairs *i*NKT cell development at the earliest stage.[97,135] In contrast, hyper-activation of this pathway by the expression of constitutively active K-Ras in thymocytes causes defective *i*NKT cell terminal maturation, correlating with decreased T-bet expression.[71] Similarly, absence of PKCθ impairs *i*NKT cell development and overactive IKKβ severely reduces *i*NKT cell numbers.[69, 71] Recent studies have also provided genetic evidence that individual deficiency of DGKα or DGKζ does not significantly affect *i*NKT development. However, simultaneous deficiency of both isoforms led to a severe *i*NKT cell-intrinsic developmental blockade/homeostasis defect and a concomitant paucity of *i*NKT cells in the thymus, spleen

and liver. In DGKαζDKO thymocytes, both Ras-ERK1/2 and PKCθ-IKK signaling are elevated. These observations have not only revealed the importance of DAG-effector signaling pathways in *i*NKT cell development but also elucidated the requirement of DGKαζ activity for normal *i*NKT cell ontogeny via tight control of these pathways. It remains unclear whether DGKα and ζ promote *i*NKT cell development solely by terminating DAG signaling or also by initiating PA-mediated signaling. Further studies are required to determine how dysregulation of DAG-mediated signaling might affect *i*NKT cell function. The generation of mice that allow for conditional deletion of DGKα or ζ isoforms is likely to prove instrumental in defining the role of DAG-mediated signaling in mature *i*NKT cell homeostasis and function.

V. ROLE OF DGKα AND DGKζ IN T CELL FUNCTION

A. DGK activity in T cell activation and anergy

Mice deficient in DGKζ have slightly fewer T cells in the periphery than WT counterparts.[118] DGK $\zeta^{-/-}$ T cells show selective perturbations in DAG-mediated signaling including enhanced Ras-ERK activation and reduced PA production upon TCR stimulation. However, DAG-independent events including TCR-induced calcium mobilization remain unaffected. Upon TCR cross-linking with anti-CD3 antibodies, a greater proportion of DGK $\zeta^{-/-}$ T cells upregulate surface markers of activation, such as CD69 and CD25, as compared to DGKζ-sufficient counterparts. In addition, T cells deficient in DGKζ proliferate more readily and rapidly than WT T cells upon *ex vivo* stimulation with anti-CD3 or transfer to lymphopenic hosts. Thus, deficiency of DGKζ enhances T cell activation and proliferation.

T cell numbers in the spleens and lymph nodes of DGK $\alpha^{-/-}$ mice are comparable to those of WT littermates.[117] DGK $\alpha^{-/-}$ T cells resemble DGK $\zeta^{-/-}$ counterparts in showing enhanced activation of the Ras-ERK pathway and increased proliferation in response to TCR stimulation. However, unlike DGK $\zeta^{-/-}$ T cells, DGK $\alpha^{-/-}$ T cells show normal PA production upon TCR stimulation, suggesting that these isoforms may somehow differ in activity or substrate specificity. Taken together, studies with DGK $\alpha^{-/-}$ and DGK $\zeta^{-/-}$ mice establish important and non-redundant roles for these isoforms in regulating T cell activation and proliferation in response to TCR stimulation.

Proper immune function is critically dependent on the ability of the immune system to distinguish between self and non-self antigens. While mounting effective immune responses to foreign pathogens is important for host defense, retaining tolerance to self-antigens is necessary to prevent autoimmunity. Rendering auto-reactive T cells functionally inactive (a state termed anergy) is an important means of generating peripheral tolerance.[136, 137] Anergized T cells are refractory to subsequent stimulation and fail to proliferate or produce IL-2, even in the presence of co-stimulation. E3 ubiquitin ligases such as Cbl-b, Itch and GRAIL are upregulated in response to anergizing stimuli, and act as anergy effectors by mechanisms that include preventing PI3K recruitment by CD28 and promoting lysosomal trafficking of endocytosed signaling molecules.[138–142]

In keeping with the two-signal model,[143] binding of TCR to cognate peptide-MHC must be accompanied by co-stimulation (for instance via the CD28 receptor) to fully trigger all TCR-coupled signaling pathways and result in T cell activation. In the absence of co-stimulation, TCR engagement selectively activates the Ca^{2+}/calcineurin/NFAT pathway (downstream of IP_3) to trigger the transcription of anergy-inducing genes.[144, 145] Treatment of T cells with the Ca^{2+} ionophore ionomycin is sufficient to induce anergy. Given these observations and the equimolar production of DAG and IP_3 following TCR engagement, it stands to reason

that DGKs may play a role in anergy induction by selectively dampening DAG-mediated signals in the absence of co-stimulation.

Studies have revealed a critical role for DGK isoforms, particularly DGKα, in the induction and enforcement of T cell anergy. In primary T cells, both DGKα and ζ are expressed at higher levels in the anergic state than in the activated state.[117] Similarly, anergic CD4 (T_H1 clone) cells express five-fold to ten-fold more DGKα and two-fold more DGKζ than control CD4 cells [100] Overexpression of DGKα in T_H1 cells resulted in an anergy-like state, characterized by suppressed Ras-ERK activation and reduced IL-2 transcription in response to stimulation with anti-CD3 and anti-CD28. DGKα overexpression also produced an anergy-like state in 2C TCR transgenic CD8 cells, as seen by impaired recruitment of RasGRP1 to the plasma membrane. Pharmacological inhibition of DGK activity led to a dose-dependent recovery of IL-2 production by anergic T_H1 cells *ex vivo*, and anergic 2C cells *in vivo*. In an *in vivo* model of anergy induction with staphylococcal enterotoxin B (SEB), T cells from DGKα$^{-/-}$ mice (in contrast to WT counterparts) were resistant to the induction of anergy and retained the ability to produce IL-2 and proliferate when re-stimulated with SEB *ex vivo*, providing direct genetic evidence of the role of DGKα in enforcing T cell anergy.[117] When CD8-depleted splenocytes were stimulated under anergy-inducing conditions (anti-CD3 and CTLA4-Ig) *ex vivo*, very few surviving WT cells divided in 48 hours. In contrast, DGKα$^{-/-}$ and DGKζ$^{-/-}$ T cells were relatively resistant to anergy induction and underwent two to three rounds of cell division. When DGKζ$^{-/-}$ cells were stimulated in a similar fashion, but in the presence of a DGKα inhibitor, they showed growth and division comparable to WT cells receiving anti-CD3 and anti-CD28 stimulation. Taken together, results from these studies reveal a key role for DGKs in regulating whether a T cell gets activated or anergized in response to signals via the TCR. They also lend credence to a model of T cell anergy in which DGKα and DGKζ (both of which are expressed at high levels in naïve T cells and down-regulated upon productive activation) selectively dampen DAG-mediated signals in the absence of co-stimulation to promote the induction and enforcement of anergy.

B. DGK localization and regulation at the immune synapse

The immunological synapse is an interface formed between a T cell and an antigen-presenting cell by membrane apposition when a TCR on the former recognizes a cognate peptide-MHC complex on the latter.[146] Previous studies using Jurkat and other cell lines have demonstrated the accumulation of DAG at the immunological synapse,[147] and the translocation of DGKα and DGKζ to the cell membrane upon TCR crosslinking.[18,148] A recent study has revealed a critical role for DGKζ in regulating DAG metabolism at the immune synapse.[149] In this study, examination of TCR complexes isolated from Jurkat cells directly demonstrated the recruitment of endogenous DGKα and DGKζ isoforms to TCR engagement. RNA interference experiments revealed complexes upon TCR and CD28 a critical role for DGKζ, but not DGKα, in PA production in these complexes. The use of GFP fusion proteins also showed rapid translocation of DGKζ, but not DGKα, to the cell membrane at early stages of immunological synapse formation. Future studies are required to dissect the relative contributions of DAG-binding and protein-protein interactions towards the recruitment of DGKs to the immune synapse. The functional consequences of DGK recruitment to the synapse also remain to be determined.

Experiments with HeLa cell lines have shown that DGKη may act as an adaptor protein during EGF-mediated ERK1/2 activation,[150] raising the possibility that DGK isoforms may serve in a similar capacity in T cells. A proteomics-based approach revealed that sorting nexin 27 (SNX27), a PDZ-domain containing protein that participates in vesicular and protein trafficking, could interact with DGKζ in a PDZ-dependent manner.[151] While more recent studies have suggested that SNX27 localizes to the immune synapse after TCR

engagement, results from co-localization experiments with tagged SNX27 and DGKζ overexpression argue against a role for DGKζ in recruiting SNX27 to the immune synapse.[152] Further studies are needed to thoroughly examine a possible role for DGKα andζ isoforms as scaffolding proteins at the T cell synapse.

Emerging evidence points to the existence of multiple positive and negative regulators of DGK activity. Lck-dependent phosphorylation at Y335 was recently shown to be critical for membrane association and enzymatic function of DGKα, in studies with Jurkat cell lines.[153] The Y335 residue is located at a hinge region between the catalytic domain and C1 domains of DGKα. Results from cell fractionation experiments indicated that Y335-phosphorylated DGKα localized specifically to membranes. Unlike its WT counterpart, the Y335F mutant form failed to translocate to the cell membrane in response to TCR stimulation, when transfected into Jurkat cells. In addition, while expression of WT DGKα in HEK293 cells reduced ERK phosphorylation in response to PMA stimulation, expression of the Y335F mutant did not. Together, these findings suggest an important role for Lck-mediated phosphorylation of DGKα at Y335 in membrane translocation and function of the enzyme.

In addition to the Y335 residue, Y218 on DGKα can be phosphorylated by tyrosine kinase c-Abl in NIH 3T3 cells following serum stimulation. Y218 phosphorylation contributes to the spatio-temporal regulation of DGKα in NIH 3T3 cell lines.[154] Results from this study showed that GFP-tagged DGKα moves from the cytoplasm to the nucleus in response to serum starvation, and in the opposite direction in response to serum restoration. Knockdown of c-Abl impaired DGKα export from the nucleus after serum restoration, and Y218 on DGKα was identified as the site of c-Abl mediated phosphorylation. At present, it is unclear whether Y218 is similarly phosphorylated in T cells and what the functional significance of such phosphorylation in T cells might be. Similar to DGKα, the distribution of DGKζ between the nucleus and cytoplasm is regulated by phosphorylation events. Studies with COS-7 cells have shown that PKCα or PKCγ can phosphorylate DGKζ, and that this phosphorylation promotes the nuclear export of DGKζ.[23]

Other work has demonstrated a positive effect of DAG itself (and its analog PMA) on DGKζ activity in Jurkat cells,[149] suggesting the existence of a feedback loop by which DAG can activate DGKζ to promote its own consumption. On the other hand, recent studies have implicated the adaptor SLAM-associated protein (SAP) as a negative regulator of DGKα activity during T cell activation.[155] SAP is essential for SLAM-mediated signaling, and mutations in SAP are associated with X-linked lympho-proliferative disease (XLP) in humans.[156] Experiments using primary blood lymphocytes and Jurkat cell lines demonstrated a loss of DGKα activity (without changes in its protein levels), following stimulation via the TCR and CD28/SLAM. Inhibition of DGKα activity was dependent on SAP expression, and overexpression of SAP was sufficient to impair DGKα activity. SAP-dependent blunting of DGK activity was isoform-specific, and not seen with DGKζ. In addition, pharmacological inhibition or siRNA knockdown of DGKα activity was able to rescue TCR-mediated signaling in SAP-deficient Jurkat cells and T cells from XLP patients.

C. DGK activity in CD8 cell function

When CD8 cells recognize a cognate antigen in an appropriate milieu of co-stimulatory molecules and cytokines, the ensuing immune response consists of three distinct phases. First, the CD8 cells undergo exponential clonal expansion, reaching peak numbers at around seven days after infection. Once the infection is cleared, a majority of the CD8 cells undergo apoptosis in the contraction phase, leaving behind a small pool of memory cells in the maintenance phase.[157,158] In an early study, DGKζ$^{-/-}$ mice showed a greater increase in CD8^{+} splenocyte numbers upon infection with lymphocytic choriomeningitis virus (LCMV), as compared to WT counterparts.[118] In addition, a higher percentage of CD8 cells

in DGKζ$^{-/-}$ mice showed an activated phenotype, as evidenced by up-regulation of CD44 and down-regulation of CD62L markers on the cell surface. A recent study investigated in further detail the effect of DGK deficiency on CD8 T cell responses to LCMV.[159] DGKα$^{-/-}$ and DGKζ$^{-/-}$ mice showed increased CD8 T cell expansion upon infection with LCMV, and more DGK-deficient CD8 cells produced IFNγ than WT counterparts. These changes were determined to be CD8 cell intrinsic in DGKζ$^{-/-}$, but not DGKα$^{-/-}$ mice, by adoptive transfer experiments. Fewer memory cells were generated/maintained in the absence of either DGK isoform. When equal numbers of WT or DGK-deficient LCMV-specific CD8 memory cells were transferred into WT recipients and re-challenged with LCMV, DGK-deficient memory cells showed impaired expansion but normal cytokine production. Of note, impaired recall response of DGK-deficient memory T cells is correlated with increased S6 phosphorylation, an event that is usually dependent on mTOR activity. Since mTOR signaling promotes primary but inhibits memory CD8 T cell responses, it would be interesting to determine if DGK activity controls CD8 T cell responses in part via modulating mTOR signaling. Taken together, studies with the LCMV model have revealed that DGK activity may differentially regulate primary and memory CD8 immune responses.

Apart from their role in responding to pathogens, CD8 cells play a critical role in defending against tumors.[160] Recent experiments have shown that DGKζ$^{-/-}$ mice develop smaller tumors than WT mice upon implantation with EL4 lymphoma cells expressing ovalbumin.[161] An increased proportion of CD44^{hi} CD62L^{lo} "effector memory" type CD8 cells was found in the spleens of DGKζ$^{-/-}$ mice, and a greater proportion of tumor-infiltrating CD8 cells was proliferating (as shown by Ki-67 staining) in DGKζ$^{-/-}$ mice than WT counterparts. Adoptive transfer of congenically marked WT OT1 or DGKζ$^{-/-}$ OT1 cells into WT mice that were subsequently injected with EL4-Ova cells produced similar results, arguing for a CD8 cell-intrinsic role of DGKζ deficiency in enhancing anti-tumor responses. While WT and DGKζ$^{-/-}$ CD8 cells lysed target cells comparably *ex vivo*, DGKζ$^{-/-}$ cells showed enhanced IL-2 production and proliferation.

A higher expression of DGKα was found in tumor-infiltrating CD8 cells from renal cell carcinoma patients, as compared to non-tumor kidney-infiltrating cells, in another recently published study.[162] While the tumor-infiltrating cells showed normal TCR proximal signaling, distal events such as phosphorylation of ERK, JNK, Akt, and IκB were impaired. No such defects were observed in CD8 cells residing outside tumors. The signaling defects in tumor-infiltrating cells also correlated with functional impairment in lytic activity and cytokine production. Treatment of tumor-infiltrating CD8 cells with a DGK inhibitor or with low-dose IL-2 was found to enhance ERK phosphorylation and lytic granule exocytosis, suggesting that enhancement of DGK expression/activity may be a possible mechanism by which infiltrating T cells are rendered less potent by the tumor micro-environment. Interestingly, a recent study has found that FoxO1 and FoxO3 can bind to the DGKα promoter to activate its transcription.[163] It is known that FoxO proteins are sequestered in the cytosol after Akt-mediated phosphorylation,[164] and that ERK1/2 can promote PI3K/Akt signaling in T cells.[94] Decreased ERK1/2 and Akt activity in the tumor-infiltrating CD8 T cells may therefore cause enhanced FoxO function and DGKα transcription.

Taken together, the findings from these studies argue that restraining DGK activity in T cells may prove valuable in generating more vigorous immune responses against pathogens and tumors. However, decreased DGK activity was found to promote thymic lymphomagenesis in mice bearing the HY transgenic TCR, suggesting that the development of therapeutic solutions involving DGK inhibition may not be entirely straightforward.[119] Perhaps the increased incidence of thymic lymphomas in these mice should not be surprising, considering that the highly oncogenic Ras-ERK1/2 and PI3K pathways are hyper-activated by TCR stimulation in the presence of reduced DGK activity. Future work should attempt to

delineate strategies that manipulate DGK activity to enhance CD8 cell function while minimizing the risk of triggering oncogenesis.

D. DGK activity in directional secretion and T cell adhesion

Single-cell photo-activation experiments have recently revealed that polarization of the T cell microtubule-organizing center (MTOC) toward the immune synapse (with an antigen-presenting cell or target cell) is driven by localized DAG accumulation in the cell membrane.[165] Polarization of the MTOC is thought to play an important role in the directional secretion of cytokines, cytolytic molecules, and other soluble factors by T cells.[166] While previous studies have shown that the MTOC aligns itself with the immune synapse within minutes of TCR stimulation, the exact mechanisms linking TCR stimulation to MTOC re-alignment were previously unknown. Recent work has demonstrated that DAG-mediated recruitment of three distinct PKC isoforms (θ, ε, and η) to the immune synapse promotes MTOC reorientation.[167] MTOC polarization was blocked by PLCγ inhibition (but not by Ca^{2+} blockade), suggesting that DAG may play a critical role in this process. Photo-activation in the presence of a DGK inhibitor was associated with failure to establish a stable DAG gradient (as reported by C1 domain-GFP fusion proteins) and defective MTOC polarization. MTOC recruitment toward the synapse was spatially correlated with and temporally preceded by DAG accumulation. Experiments with a photo-activated form of DAG also showed that localized DAG signaling was sufficient to drive transient MTOC polarization. Treatment with agents such as PMA and DGK inhibitors that perturb MTOC polarization impaired the ability of cytotoxic T cells to kill target cells. Taken together, these observations suggest the hypothesis that DGK isoforms may play a critical role in T cell function by regulating MTOC-directing DAG gradients.

In addition to secreting cytokines and other soluble effectors, studies have shown that T cells can induce apoptosis of target cells by secreting exosomes that bear membrane-bound FasL.[168] A possible role for DGK activity in negatively regulating the secretion of these exosomes was revealed when inhibition of DGKα activity in human primary T cell blasts was shown to increase the secretion of FasL-bearing exosomes and subsequent activation induced cell death.[169] However, the mechanisms by which DGKα inhibits exosome secretion have remained unclear. Results from a recent study suggest that DGKα may inhibit the formation of FasL-bearing exosomes and multi-vesicular bodies, but aid in their polarization towards the immune synapse, in T cells.[170] Multi-vesicular bodies (MVBs) are late endosomes that contain smaller vesicles inside their lumen. In this study, the activation of T cell lines was found to increase the formation of FasL-containing MVBs, and pharmacological inhibition of DGKα activity increased the number of mature MVBs. In addition, siRNA mediated inhibition of DGKα expression hindered the polarization of MVBs towards the immune synapse. Taken together, these results suggest that DGKα may play a role in regulating both the formation and polarization of FasL-bearing exosomes and vesicles in T cells.

Interaction of T cells with vascular endothelial cells followed by T cell arrest in the microvasculature is an essential step in the process of lymphocyte extravasation. A recent study has identified DGKζ as a critical negative regulator of CXCR4-stimulated T cell firm arrest on surfaces presenting ICAM1 under conditions of shear flow.[171] Binding of CXCR4 to its ligand CXCL12 on the microvasculature converts integrin LFA1 on the vasculature to its active form, in a process called inside-out signaling. The active form of LFA1 can then be bound by ICAM1 on the T cell surface, resulting in firm arrest even in the presence of shear forces. Since DAG-mediated signals play an important role in inside-out activation of LFA1, the authors hypothesized that deficiency of DGKζ would lead to enhanced activation of LFA1 and increased cell arrest under shear flow. Results from flow chamber experiments

showed that DGKζ deficiency in T cells indeed increased firm arrest to ICAM1-coated surfaces and shortened the time to stop without affecting the rolling velocity.

VI. SUMMARY

Recent studies have revealed a host of new functions for DGK isoforms in T cell development and function (Figure 3). Apart from their role in the development of conventional αβ T cells, newer work has unveiled a previously unappreciated requirement for synergistic DGKα and ζ activity during invariant NKT cell development. Several modulators of DGK activity, including Lck, SAP, and c-Abl, have been identified. In addition, the importance of DGK activity in promoting MTOC polarization and directional secretion, and in restraining CD8 T cell responses against LCMV infection and tumors, has come to the fore. However, a number of fundamental questions about DGKs remain unanswered. Transcriptional control and regulation of DGK activity via post-translational modifications and protein-protein interactions during T cell development and immune responses are poorly understood. The spatial and temporal regulation of DAG by individual DGK isoforms in T cells remains to be defined. The importance of DGK-generated PA and the downstream effector pathways controlled by PA in T cells need to be explored. A better understanding of the role and regulation of DGK activity and DAG signaling in T cells can enable us to modulate immune responses, producing better outcomes during vaccination, tumor responses, and autoimmunity.

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LIST OF ABBREVIATIONS

CD4SP	CD4 single positive
CD8SP	CD8 single positive
DAG	diacylglycerol
DKO	double knock out
DGK	diacylglycerol kinase
DN	CD4 CD8 double negative
DP	CD4 CD8 double positive
***i*NKT**	invariant NKT cell
LCMV	lymphocytic choriomeningitis virus
MTOC	microtubule organizing center
MVB	multi-vesicular body
PA	phosphatidic acid
TCR	T cell receptor

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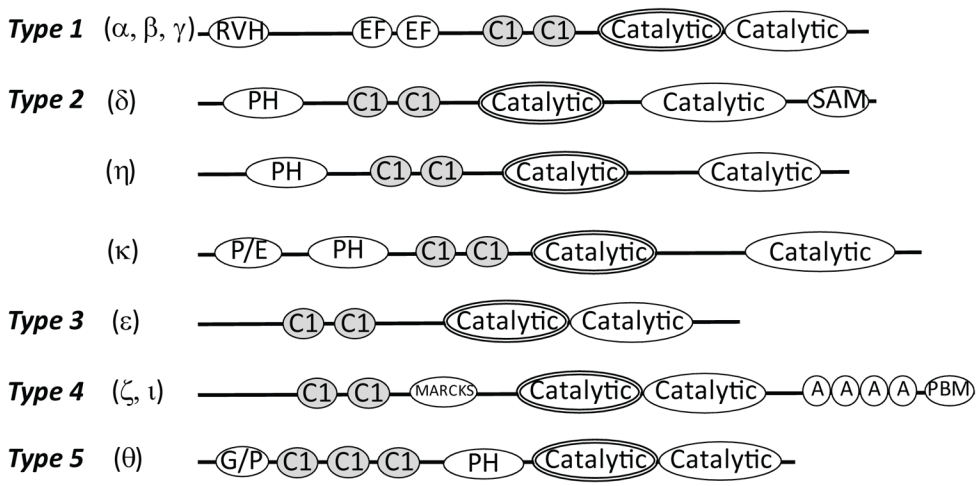

Figure 1. Structure-based classification of mammalian DGK isoforms

Based on the presence of certain structural features, mammalian DGK isoforms are classified into five types. The DGK catalytic domain consists of a conserved motif (shown with double lines) and an accessory domain (shown with a single line). RVH – recoverin homology domain, EF – EF hand, C1- cysteine-rich DAG-binding domain, PH – plextrin homology domain, SAM – sterile alpha motif, P/E- proline/glutamate-rich region, MARCKS – myristoylated alanine-rich C kinase substrate domain, A – ankyrin repeat motif, PBM – PDZ binding motif, G/P – glycine/proline-rich region.

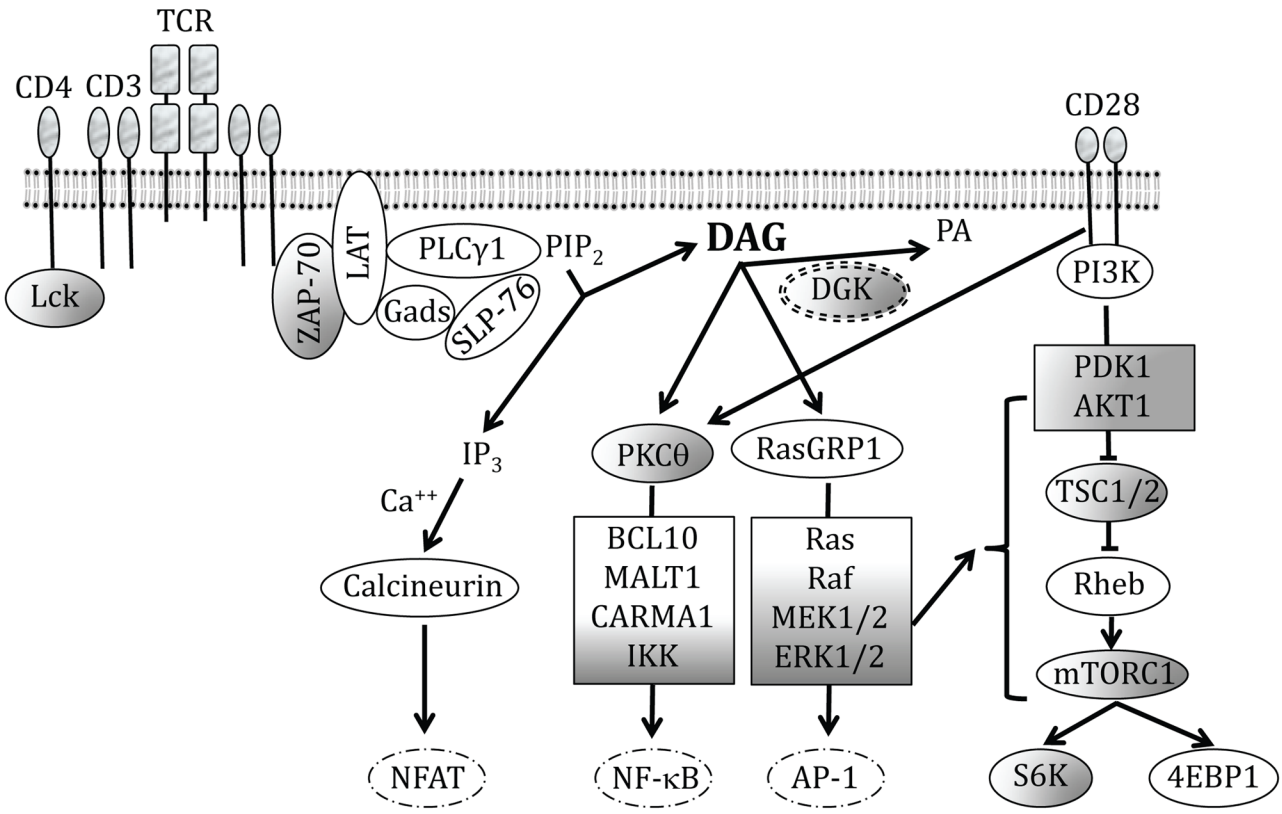

Figure 2. DAG-mediated pathways in T cell receptor signaling

Schematic representation of various signaling pathways activated upon engagement of the T cell receptor and the CD28 co-stimulatory receptor, with an emphasis on DAG-mediated pathways. Please see the text for further details.

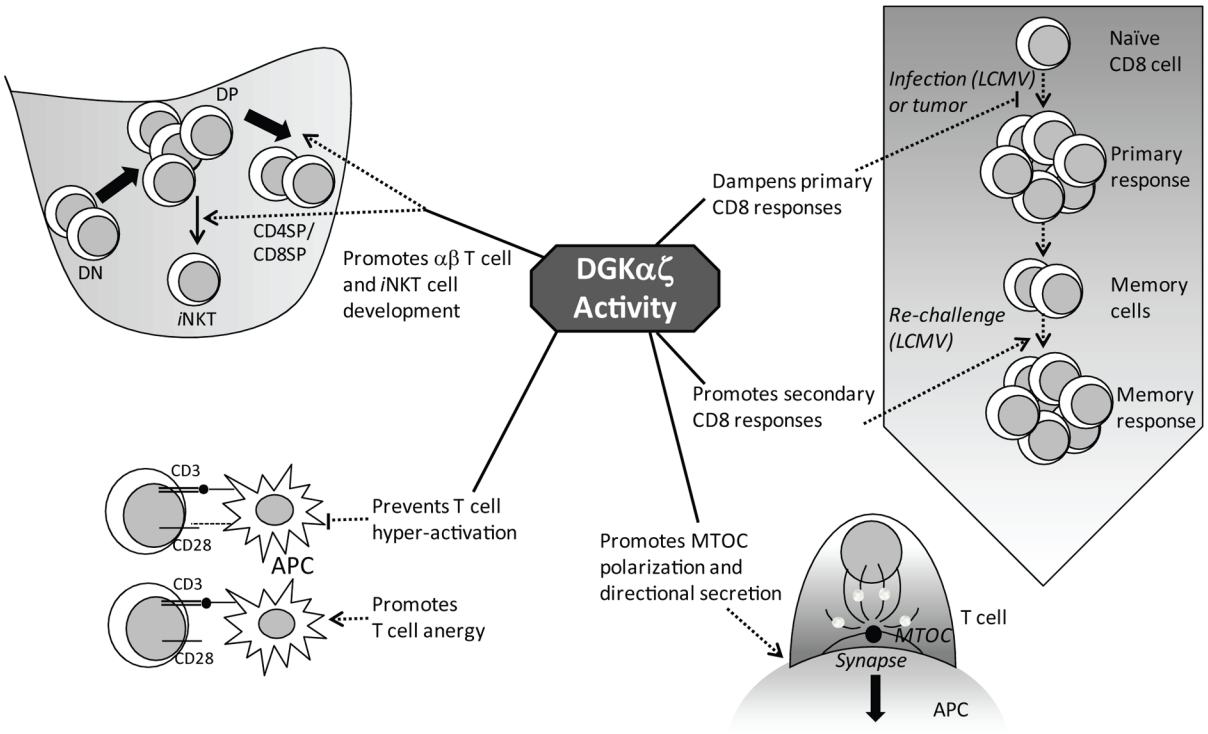

Figure 3. DGKs in T cell development and function

Schematic summary of the numerous roles played by DGK α and ζ in T cell development and function. In the thymus, these two DGK isoforms synergistically promote the development of conventional αβT cells and invariant NKT cells. DGK activity in mature peripheral T cells prevents their hyper-activation upon TCR engagement in the presence of co-stimulatory signals. On the other hand, DGK isoforms are highly expressed in anergic T cells and studies have revealed a critical role for DGK isoforms, particularly DGKα, in promoting T cell anergy. In CD8 cells, DGK α and ζ serve to dampen primary responses against tumor antigens and viral infection (LCMV), while promoting memory responses in the LCMV model. DGKs also play a role in establishing a stable DAG gradient that enables T cells to directionally secrete cytolytic granules and other soluble factors across the immunological synapse.