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Diacylglycerol kinases in immune cell function and self-tolerance

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Summary

Both diacylglycerol (DAG) and phosphatidic acid (PA) are important second messengers involved in signal transduction from many immune cell receptors and can be generated and metabolized through multiple mechanisms. Recent studies indicate that diacylglycerol kinases (DGKs), the enzymes that catalyze phosphorylation of DAG to produce PA, play critical roles in regulating the functions of multiple immune cell lineages. In T cells, two DGK isoforms, α and ζ , inhibit DAG-mediated signaling following T cell receptor engagement and prevent T cell hyperactivation. DGK α and ζ synergistically promote T cell anergy and are critical for T cell tolerance. In mast cells, DGK ζ plays differential roles in their activation by promoting degranulation but attenuating cytokine production following engagement of the high affinity receptor for IgE. In dendritic cells and macrophages, DGK ζ positively regulates Toll-like receptor-induced proinflammatory cytokine production through its product PA, and is critical for host defense against *Toxoplasma gondii* infection. These studies demonstrate pivotal roles of DGKs in regulating immune cell function by acting both as signal terminator and initiator.

Keywords

Diacylglycerol kinase; phosphatidic acid; signal transduction; T cell receptor; mast cell; Toll like receptor

Introduction

Immune cells are tightly regulated during their development and activation to ensure proper immune function and self-tolerance. Immune cell development and function are regulated by signaling from various cell surface receptors. Stimulation of many receptors including the T cell receptor (TCR), the B cell receptor, the high affinity receptor for IgE (Fc ϵ RI), chemokine receptors, and Toll-like receptors (TLRs) results in generation of DAG and/or PA. Both DAG and PA are important second messengers for controlling immune cell activation by activating multiple signaling cascades. Given their important roles in receptor signaling and immune cell function, DAG and PA concentrations must be tightly regulated to maintain normal homeostasis of the immune system. In this review, we will discuss recent studies that demonstrate the critical roles of DGKs in immune cell development and function.

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DGKs: an overview

Since the cloning of the first mammalian DGK in 1990, ten DGKs have been identified in mammals (Figure 1)(1-3). All mammalian DGKs contain a kinase domain and at least two cysteine-rich C1 domains that are homologue to the DAG/phorbol ester binding C1 domain of protein kinase Cs (PKCs). Although it has been proposed that the C1 domains of DGKs may serve as a substrate binding motif, most C1 domains of DGKs, with the exception of DGK β and γ , do not show high DAG/phorbol ester binding affinity due to the lack of several key residues (4). In addition, disruption of the C1 domains of DGK α or the naturally occurring absence of C1 domains in plant DGKs does not abolish DGK enzyme activity, indicating that the C1 domain is not essential for substrate binding by DGKs (5-8). Instead of serving as a substrate binding domain, the C1 domains of DGK γ , θ , and ζ have been found to mediate cytoplasm membrane translocation of these enzymes through interaction with proteins such as β -arrestin and lipids (6, 9-13).

In addition to the common structural features described above, DGK isoforms also contain distinct structural motifs that divide mammalian DGKs into five types (1-3). Type I (α , β , γ) DGKs contain an N-terminal recoverin homology (RVH) domain, and two calcium binding EF-hand motifs between the RVH domain and the C1 domains (14-17). The RVH domain is homologous to the N-termini of the recoverin family of neuronal calcium sensors. Deletion of the RVH domain results in loss of Ca^{++} -dependent activation of these enzymes (18). The EF-hands in type I DGKs bind Ca^{++} , resulting in activation of the enzymes. Deletion of the EF hands results in constitutive activation of type I DGKs. Thus, the EF hands auto-inhibit the enzymatic activity of type I DGKs and binding of Ca^{++} to the EF hands relieves the inhibition and induces the activation of their enzymatic activities (18). Type II (δ , η , and κ) DGKs contains a pleckstrin homology (PH)-domain in their N-termini (19-21). PH domains are involved in lipid-protein and protein-protein interactions. Treatment of HEK-293 human embryonic kidney cells with PMA, a DAG functional analogue resistant to DGK activity, induces cytoplasm membrane translocation of DGK δ that requires an intact PH domain (22, 23). In addition to the PH domain, DGK δ and η contain a sterile alpha motif (SAM) domain at their C-termini. SAM domains are found widely in signaling and nuclear proteins and mediate protein-protein interactions and homodimerization/oligomerization. The SAM domain of DGK δ mediates oligomerization of this enzyme (22). In addition, in NIH3T3 fibroblasts, the SAM domain of DGK δ acts as an ER-targeting motif (24). DGK κ does not have a SAM domain but contains a PDZ domain binding motif (PDZ-BM) and a proline/glutamic acid-rich region at its C-terminus and N-terminus, respectively (21). DGK ϵ is the single type III DGK identified to date, the simplest form of DGKs. DGK ϵ contains only two C1 domains and a catalytic domain, with no additional detectable domains or motifs. Type IV (ζ and τ) DGKs contain a myristoylated alanine-rich C-kinase substrate (MARCKS) motif between the C1 domains and the catalytic domain, a PDZ-BM at their C-termini, and several ankyrin-repeats (ANK) between the PDZ-BM and catalytic domain (25-27). The MARCKS domain, which also contains a nuclear localization sequence, can be phosphorylated by PKC α , which attenuates DGK ζ activity and nuclear translocation (28). The PDZ-BM of DGK ζ interacts with γ 1-syntrophin and regulates Rac1 activation and membrane ruffle formation (29-31). DGK θ is the only type V DGK and contains three C1 domains, a PH domain-like region between the C1 domains and the catalytic domain, and a glycine/proline rich region at its N-terminus (32). Mutations in each of these domains inhibit DGK θ enzyme activity (33). Of the ten DGK isoforms, six (β , γ , δ , η , ζ , and τ) have at least two alternatively spliced isoforms, which further increases the structural diversity of the DGK family and may add additional mechanisms to regulate DGK function.

Most DGK isoforms are expressed in multiple tissues, particularly in the hematopoietic system and the neuronal system. Within one tissue, multiple DGK isoforms may be present.

For example, DGK α , δ , and ζ have been detected in T cells (34-36) and five DGK isoforms are expressed in mast cells (our unpublished observations). DAG is the sole substrate for mammalian DGKs and most DGK isoforms do not have obvious preference for specific acyl chains of DAG. Only DGK ϵ manifests a substrate preference for DAG with a 2-arachidonoyl chain (37). The lack of substrate selectivity by DGK isoforms is distinct from other families of enzymes, like the PKC family, in which each isoform may selectively target specific structural motifs. Despite the lack of substrate selectivity for their enzymatic activity, some DGK isoforms exhibit differences in regulatory functions. The distinct structural features residing in individual DGK isoforms may determine their specificity in receptor signaling and in regulating cellular function. DGKs may also function independently of their enzymatic activity. For instance, the presence of multiple motifs/domains involved in protein-protein and protein-lipid interactions in most DGK isoforms suggests that DGKs may function as a scaffold to mediate formation of multi-molecular complexes. Some DGK isoforms have indeed been found in protein complexes involved in signal transduction, vesicle transportation, and cytoskeleton rearrangement. As an example, DGK δ promotes ER to Golgi retrotranslocation through its SAM domain and PH domain in a kinase activity independent manner (24).

DAG and PA in receptor signaling and DGK function

During receptor stimulation, several enzymes produce DAG (Figure 2). Phosphatidylinositol (PI) dependent phospholipase Cs (PI-PLCs) produce DAG after hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂). Phosphatidylcholine (PC) dependent PLCs (PC-PLCs) hydrolyze PC to produce DAG and phosphoryl choline (P-Cho). Sphingomyelin synthase (SMS) generates sphingomyelin (SM) and DAG from PC and ceramide (38). Sequential hydrolysis of PC by phospholipase Ds (PLDs) and PA phosphohydrolases (PAPs) leads to generation of DAG. DAG binds to the C1 domain of numerous effector molecules and regulates the activity and/or subcellular localization of these molecules. The classic and novel types of protein kinases (PKCs), Ras guanyl nucleotide releasing proteins (RasGRPs), protein kinase Ds (PKDs), Munc-13s, α and β chimaerins, and the transient receptor potential cation channel (TRPC) are the most well-known DAG effector molecules (39). PKCs and PKDs are both serine/threonine kinases that participate in numerous receptor signaling events. RasGRPs promote formation of the GTP-bound Ras and induce Ras activation. Munc13s are adaptor molecules playing essential roles in synaptic vesicle priming (40, 41). Chimaerins are GTPase-activating proteins for Rac1, a member of the Rho family of GTP-binding proteins (42).

PA can be produced from DAG and PC via the enzymatic activities of DGKs and PLDs, respectively. PA has been implicated as a mediator of the mitogenic action of various growth factors and hormones in several types of mammalian cells by associating with and regulating many effector molecules. Additionally, PA promotes vesicle transportation and granule releases and has many other activities (43). Known PA-binding proteins include tyrosine phosphatase SHP-1 (44), the mammalian target of Rapamycin (mTOR) (45), the protein phosphatase-1 (PP1) (46), phosphatidylinositol-4-phosphate 5-kinase (PIP5K) (47-49), RasGAP (50), Raf-1 kinase (51), p47^{phox}, a key cytosolic subunit required for activation of phagocyte NADPH oxidase (52), and the Ras guanine nucleotide exchange factor Son of sevenless (Sos) (53). Among these effector molecules, SHP-1 negatively regulates signaling from multiple immune cell receptors. Deficiency of SHP-1 causes multi-organ autoimmune disease due to dysregulated activation of T cells, B cells, and myeloid cells (54). mTOR governs cell growth, proliferation, survival, and autophagy by mediating mitogen- and nutrient-induced signaling (55). Inhibition of mTOR by Rapamycin induces death of CD4⁺CD8⁺ DP thymocytes, suggesting a role of the mTOR pathway in T cell development (56). Most studies have found that PLD-derived PA modulates function of

these PA-effector molecules. Several recent reports indicate that PA produced by DGK ζ plays important roles in receptor signaling. DGK ζ -derived PA has been found to promote mTOR signaling, PIP5K activity, and TLR-induced IL-12 production (57-59).

The diverse functions of DAG and PA in receptor signaling and the complexity of the DGK family pose a significant challenge to uncovering the physiological function of each DGK isoform. Nevertheless, significant progress has been made in understanding the roles of DGKs in cellular function in the last few years. DGK α can promote or inhibit cell proliferation, inhibit apoptosis, promote cells migration, regulate secretory vesicular traffic, and regulate T cell activation (60-63). DGK β inhibits respiratory burst during Fc γ receptor-mediated phagocytosis by inhibiting PKC β activity (64). DGK γ interacts with and activates β 2-chimaerin, resulting in inhibition of platelet-derived growth factor-induced Rac1 activation, cytoskeleton reorganization, and lamellipodium formation (65-67). DGK δ positively regulates epidermal growth factor receptor (EGFR) signaling by inhibiting PKC α activity. DGK δ deficient mice die within 24 hours after birth. DGK δ deficiency increases DAG-mediated PKC α activation which, in turn, phosphorylates EGFR at threonine 654 leading to accelerated degradation of EGFR (68). In addition to regulating EGFR degradation, DGK δ has been recently found to regulate clathrin-dependent endocytosis (69). DGK ϵ participates in PI turnover. Studies in DGK ϵ deficient mice have revealed its important role in seizure susceptibility and long-term potentiation by regulating rapid kindling epileptogenesis through arachidonoyl-inositol lipid signaling (70, 71). DGK ζ has been the most studied DGK family member. DGK ζ regulates signaling from the TCR, TLR, and Fc ϵ RI and plays important roles regulating immune cell function. DGK ζ associates with and is activated by protein tyrosine kinase c-Src following gonadotropin-releasing hormone (GnRH) stimulation and may control the transcription of luteinizing hormone β (72). DGK ζ also regulates cytoskeletal rearrangement following association with γ 1-syntrophin and Rac1 (29-31), attenuates pressure overload-induced or angiotensin II and phenylephrine-induced cardiac hypertrophy (73, 74), and promotes mTOR signaling and PIP5K activity (57, 58). Another DGK member, DGK τ , binds to RasGRP3 and inhibits its activation of the GTPase-activating protein Rap1, leading to enhanced Ras activation and tumorigenesis (75).

It is important to note that while some DGKs function similarly, some other DGKs perform distinct functions in receptor signaling. For example, both DGK α and γ promote cell proliferation and survival but DGK ζ inhibits cell cycle progression and plays a negative role in cell survival (60, 76-78). Many DGKs inhibit Ras activation while DGK τ enhances Ras activation (35, 75, 79, 80). How DGK isoforms perform such distinct functions and how their activities are regulated in different cells and/or receptor signaling are important issues to be investigated in the future.

Regulation of TCR signaling, T cell activation, and T cell tolerance by DGK α and ζ

DAG in TCR signaling and T cell activation

T cell-mediated adaptive immune responses are critical for host defense against microbial infection and tumor immune surveillance but can also contribute to many autoimmune diseases if not properly controlled. T cell activation is initiated by engagement of the TCR with antigenic peptide presented by MHC complexes expressed on dendritic cells (DCs) and other antigen presenting cells (APCs). Following TCR engagement, proximal tyrosine kinases Lck and Zap70 are activated and phosphorylate many substrates including adaptor molecules and enzymes (Figure 3)(81-83). The formation of multi-molecular signal complexes orchestrated by adaptor molecules and the coordinated action of these signaling molecules results in activation of PLC γ 1 (84-88). Activated PLC γ 1 hydrolyzes PIP $_2$ to

inositol 1,4,5 trisphosphate (IP₃) and DAG (89). IP₃ triggers Ca⁺⁺ influx, leading to the activation of calcineurin which, in turn, dephosphorylates NFAT to induce NFAT nuclear translocation (90, 91). DAG associates with and allosterically activates RasGRP1, PKD, and PKCθ through association with the C1 domains of these molecules (92-94). RasGRP1, together with Sos, promotes GTP binding to Ras and activates Ras, leading to the activation of the Raf1-Mek1/2-Erk1/2-AP1 pathway (92, 95-97). AP1 dimerizes with NFAT, and cooperative binding of the AP1/NFAT dimer to the composite AP1/NFAT site on the IL-2 and other cytokine promoters is critical for T cell activation and cytokine production (90). Activated PKCθ phosphorylates RasGRP1 to promote its activity (98). In addition, activated PKCθ phosphorylates and recruits Carma1 and Bcl10, leading to the activation of NFκB (99-105). Following activation by PKC-mediated serine phosphorylation, PKD1 phosphorylates histone deacetylase 7 to regulate intrathymic T cell development (106-108). Via RasGRP1, PKCθ, and PKD, DAG plays a critical role in T cell development and activation. Deficiency of DAG generation due to deficiency of PLCγ1 inhibits T cell activation (109). In contrast, phorbol esters cause maximal T cell activation. Given the importance of DAG and its effector molecules in T cells, DAG concentration must be tightly regulated to maintain normal T cell homeostasis.

Regulated expression of DGK isoforms in T cells

DGK-mediated phosphorylation represents one mechanism to inactivate DAG. Several DGK isoforms, α, ζ, and δ, are detected in T cells. DGKζ has at least two splicing isoforms, ζ1 and ζ2, with molecular weights of 115 and 130 kDa, respectively. DGKζ1 and ζ2 differ only at their N-termini due to alternative splicing of the first and second exons to the third exon (110). In addition to T cells, most hematopoietic lineages such as B cells, mast cells, dendritic cells, macrophages, neutrophils, and platelets express DGKζ. DGKζ1 is the predominant form expressed in most of these immune cells. However, during T cell development, DGK α2 is expressed at the highest level in CD4⁺CD8⁻ double negative (DN) thymocytes and its expression decreases as T cells mature. In contrast, DGKζ1 is expressed at a low level in DN thymocytes and its expression increases with T cell maturation. In mature T cells, DGKζ1 is the predominant DGKζ isoform present (35). No specific motifs or domains encoded by alternatively spliced exons 1 and 2 have been identified at the N-termini of ζ1 or ζ2 and it is unclear if there are any functional differences between DGKζ1 and ζ2 in T cells.

DGK α is also expressed in thymocytes and peripheral T cells (34, 80) but its expression levels in different stages of T cell development have not been reported. Expression of both DGKα and ζ is influenced by T cell activation status. Both DGKα and ζ are expressed at high levels in naive T cells and downregulated after T cell activation. Anergic T cells express elevated DGKα, but similar levels of DGKζ, as compared to naive T cells (36, 80, 111). As will be discussed later, elevated DGK activity appears to be critical for T cell anergy. The mechanisms that control DGKα and ζ expression during T cell development, activation, and anergy remain to be determined.

Inhibition of TCR signaling and T cell activation by DGKα and ζ in T cell line models

To investigate a potential role of DGK activity in TCR signaling and T cell activation, Koretzky, Zhong, and colleagues first used a gain-of-function approach by transiently overexpressing DGKζ in Jurkat T cell lines. Enhanced DGKζ activity in this system potently inhibits TCR-induced Ras-Erk1/2-AP1 activation and upregulation of the T cell activation marker CD69. Such inhibition is dependent on DGKζ kinase activity and can be overcome by PMA, suggesting that the inhibition is mediated by terminating DAG signaling (35). In addition, the N-terminus of DGKζ, where the C1 domains are located, is critical for DGKζ-mediated inhibition of TCR-induced AP1 activation. In contrast, the C-terminus of

DGK ζ , which includes the ANK repeats and the PDZ-BM motif, is not essential for DGK ζ -mediated inhibition of TCR-induced AP1 activation (35). Topham and Prescott have found that DGK ζ interacts with RasGRP1, suggesting that DGK ζ may be recruited to attenuate TCR signaling by association with RasGRP1 (112). However, it is unclear if DGK ζ -RasGRP1 association is critical for DGK ζ -mediated inhibition of TCR signaling.

DGK α also inhibits TCR-induced activation of the RasGRP1-Ras-Erk1/2-AP1 pathway in cell line models. Merida's and Gajewsky's groups have shown that DGK α inhibits translocation of RasGRP1 to the cytoplasm membrane in a Jurkat variant and to the immune synapse in a Th1 cell line, respectively (80, 113). Overexpression of a dominant negative form of DGK α enhances TCR-induced Erk1/2 activation while overexpression of a constitutively active form of DGK α has the opposite effect (114). We have also shown that overexpression of DGK α inhibits TCR-induced AP1 activation (36). Collectively, data from *in vitro* cell line models demonstrate that both DGK α and ζ can negatively regulate TCR signaling and T cell activation, suggesting that these two isoforms may perform overlapping function in T cells. Functional differences between these two isoforms during TCR signaling have also been revealed. While DGK α readily translocates to cytoplasm membrane in Jurkat cells following TCR stimulation, TCR stimulation can only induce DGK ζ translocation to the cytoplasm membrane after its C-terminus, including the ANK repeats and PDZ-BM motif, is truncated (34).

Enhanced T cell activation in the absence of DGK α and ζ

To further establish the physiological importance of DGK function in T cells, Koretzky, Zhong, and colleagues generated and analyzed DGK ζ knockout mice (79). DGK ζ deficient mice are born at the expected Mendelian ratio and do not manifest obvious developmental defects. T cell numbers in these mice are not altered compared to wild type (WT) mice. However, DGK ζ deficiency results in decreased but not completely abolished DAG phosphorylation following TCR stimulation, indicating the involvement of DGK ζ in inactivating DAG. Signaling events downstream of DAG, such as Ras activation and phosphorylation of Mek1/2 and Erk1/2, which correlate with activation of these enzymes, are elevated in DGK ζ deficient T cells compared to WT controls. In contrast, signaling events independent of DAG, such as Jnk1/2 activation, PLC γ 1 phosphorylation, and Ca⁺⁺ influx, are not elevated in DGK ζ deficient T cells following TCR stimulation.

DGK ζ deficient T cells are hyper-responsive to TCR stimulation. Following TCR stimulation, they express higher levels of the T cell activation markers CD69 and CD25 than WT T cells. They proliferate more vigorously than WT T cells following *ex vivo* stimulation through the TCR or after adoptive transfer to lymphopenic hosts. Furthermore, DGK ζ deficient mice mount stronger antiviral immune responses than WT mice following lymphocytic choriomeningitis virus (LCMV) infection, manifested by enhanced expansion and activation of CD8 T cells, increased virus-specific T cells that produce IFN γ , and accelerated clearance of the pathogen from the hosts (79). Thus, both *in vitro* and *in vivo* data support the importance of DGK ζ in regulating DAG levels in T cells following TCR stimulation and indicate that DGK ζ is an important physiological negative regulator for T cell function. Since DAG phosphorylation is only partially inhibited in DGK ζ deficient T cells following TCR engagement, it suggests that other DGK isoforms may also participate in DAG metabolism in T cells.

To investigate whether DGK α may function redundantly with DGK ζ and compensate for DGK ζ deficiency, we further generated DGK α knockout mice. The immune phenotype of DGK α deficient mice is similar to that of DGK ζ deficient animals. DGK α deficient T cells are also hyper-responsive to TCR stimulation, correlating with decreased DAG phosphorylation and enhanced Ras and Erk1/2 activation (36). An more striking phenotype

is observed in mice deficient in both DGK α and ζ (DGK $\alpha\zeta$ DKO). Deficiency of both DGK α and ζ causes an incomplete blockade of T cell maturation at the CD4⁺CD8⁺ double positive (DP) to CD4⁺CD8⁻ and CD4⁻CD8⁺ single positive (SP) step (our unpublished observations). In peripheral lymphoid organs, both CD4 and CD8 T cells are present in DGK $\alpha\zeta$ DKO mice but their ratios to other immune cell lineages are significantly decreased compared to WT controls. DGK $\alpha\zeta$ DKO CD4 and CD8 T cells exhibited upregulated CD69 surface expression and an effector/memory T cell phenotype, CD44^{hi}CD62L^{low} and they readily produce IFN γ following four hour *ex vivo* stimulation with PMA and ionomycin. Both DGK $\alpha\zeta$ DKO CD4 and CD8 T cells proliferate when cultured *ex vivo* in the absence of anti-TCR stimulation (our unpublished observations). Collectively, these observations demonstrate that DGK α and ζ synergistically control T cell maturation and T cell homeostasis.

Contribution of DGK α and ζ to peripheral T cell tolerance

T cell anergy and active suppression by regulatory T cells (Tregs) represent two important mechanisms for peripheral T cell tolerance. Activation of naïve T cells requires both TCR engagement and co-stimulation (115, 116). T cells become anergic after stimulation through their TCR without a co-stimulatory signal (117-120). Treatment of T cells with the Ca⁺⁺ ionophore ionomycin alone can also induce anergy (111, 117, 121, 122). Anergic T cells do not proliferate or make IL-2 following antigen restimulation in the presence of appropriate co-stimulation (123-125). T cell anergy represents a mechanism that prevents self-reactive T cells from full activation that could lead to self-destruction. Several studies into the signaling abnormalities of anergic T cells have led to the discovery that Ras activation is decreased and that AP1 nuclear localization is impaired in anergic cells following TCR stimulation, while NFAT activity is normal (126-128). A subsequent study by Gajewski and colleague has found that RasGRP1 translocation to the immune synapse is impaired in anergic T cells. Furthermore, ectopic expression of constitutively active Ras in anergic T cells restores Erk1/2 activation and reverses the anergic state (80). Together, these studies indicate that selective Ca⁺⁺ signaling and NFAT activation without simultaneous activation of the RasGRP1-Ras-Erk1/2-AP1 pathway causes T cell anergy. Since DAG and IP₃ are produced by PLC γ 1 at an equal molar ratio, an important question to understand is how selective Ca⁺⁺ signaling is achieved during anergy induction via TCR stimulation.

In an effort to understand the mechanisms controlling T cell anergy, Rao's group and Gajewski's group performed gene chip analyses to identify genes that are differentially expressed in ionomycin-induced anergic T cells that revealed elevated DGK α expression in anergic T cells (80, 111). Overexpression of DGK α in a Th1 cell line and in primary T cells inhibited translocation of RasGRP1 to immune synapse and rendered these cells anergic (80). Complementary to these results from gain of function studies, we have found that DGK α or DGK ζ deficient T cells are resistant to anergy induction (36). When stimulated through the TCR for 48 hours in the presence of CTLA4-Ig, which competes with CD28 for ligands and blocks co-stimulation, T cells deficient in either DGK α or ζ undergo several rounds of cell division. Furthermore, these cells produce significant amounts of IL-2 upon subsequent restimulation through the TCR and CD28. The amount of IL-2 produced is even greater when DGK ζ deficient T cells are treated with a DGK α inhibitor, suggesting that these two enzymes may function synergistically to promote T cell anergy. In contrast, WT T cells do not proliferate following 48 hour TCR and CTLA-4Ig stimulation and do not produce IL-2 following TCR and CD28 restimulation. These *ex vivo* experiments indicate that both DGK α and ζ contribute to induction of T cell anergy.

Studies utilizing the *in vivo* staphylococcal enterotoxin B (SEB) superantigen-induced anergy model further support that DGK activity contributes to T cell anergy. In this model, SEB injected into WT mice selectively stimulates T cells expressing the V β 8 TCR (129,

130). WT V β 8⁺ but not other T cells are then either deleted or become unresponsive following *ex vivo* restimulation with SEB. Injection of SEB into DGK α deficient mice does not affect SEB-mediated deletion of V β 8⁺ T cells in these animals. However, DGK α deficient V β 8⁺ T cells produce more IL-2 and proliferate more vigorously than WT T cells during *ex vivo* restimulation with SEB (36).

Impairment of T cell anergy is expected to result in activation of self-reactive T cells and autoimmunity. Although T cells lacking either DGK α or ζ are hyper-responsive to TCR stimulation, there is no obvious autoimmune disease in DGK α or ζ deficient mice, suggesting that DGK α or ζ may compensate for the loss of the other isoform to maintain T cell tolerance. In contrast to these single DGK knockout mice, DGK $\alpha\zeta$ DKO mice have enlarged livers and develop chronic autoimmune hepatitis (AIH) that ultimately leading to fibrosis and liver failure (our unpublished observations). DGK $\alpha\zeta$ DKO T cells can transfer AIH to T cell deficient mice, indicating a critical role for T cells in the pathogenesis of the disease. Furthermore, Tregs are functionally impaired in DGK $\alpha\zeta$ DKO mice, and transfer of WT Tregs into these animals inhibits the development AIH, indicating that impairment of Treg function contributes to AIH in DGK $\alpha\zeta$ DKO mice.

Taken together, the studies described above demonstrate that both DGK α and ζ are physiological inhibitors of T cell activation. They synergistically promote T cell maturation, T cell anergy, and Treg function, and are critical for T cell tolerance to the liver.

Regulating Fc ϵ RI signaling and mast cell function by DGK ζ

Mast cells play important roles in normal innate and adaptive immune responses, as well as in the immune pathogenesis of asthma and other allergic disorders. Fc ϵ RI is one of several cell surface receptors critical for mast cell function (131-133). Engagement of IgE-loaded Fc ϵ RI by allergens induces activation of the Src family protein tyrosine kinases Lyn and Fyn and the tyrosine kinase Syk (Figure 4). These kinases then phosphorylate and activate many substrates, such as PI3Ks, PLC γ , PKCs, RasGRP1, MAPKs, PLDs, and several adaptor molecules (134-143). Similar to TCR signaling, both DAG and PA are produced following Fc ϵ RI stimulation and play important roles in mast cell function. In cell line models, DAG and its functional analogues induce mast cells to degranulate and release active mediators in the presence of Ca⁺⁺ ionophore (144, 145). A defect in generating DAG due to PLC γ 2-deficiency results in impaired Fc ϵ RI-induced degranulation and cytokine secretion and resistance to IgE-mediated passive cutaneous anaphylaxis (PCA), an established *in vivo* measurement of mast cell function (137, 146). Studies of mice deficient in several DAG effector molecules have revealed differential roles for these molecules in Fc ϵ RI-induced mast cell activation. While RasGRP1 and PKC β promote mast cell activation (139), PKC δ -deficient mast cells respond more vigorously to suboptimal Fc ϵ RI stimulation with a more sustained calcium mobilization and a significantly higher level of degranulation than that of WT mast cells (140). Both DGKs and PLDs produce PA in mast cells following Fc ϵ RI crosslinking. Diversion of PLD-derived PA production to phosphatidyl butanol by 1-butanol treatment of mast cells or knockdown of PLD1 and PLD2 expression suppresses the translocation of both classic and novel types of PKCs to the cytoplasm membrane and reduces degranulation following Fc ϵ RI engagement (143). These observations suggest that PLD-derived PA is critical for mast cell activation. Mice deficient of PLDs have not been reported. It would be interesting to examine if DGKs and PLDs function synergistically to regulate mast cell activation.

Regulation of Fc ϵ RI signaling after antigen-cross linking

The ability of DGKs to regulate both DAG and PA concentrations leads to the hypothesis that DGKs may be important regulators of mast cell activation. Using RT-PCR, we have

detected five DGK transcripts (δ , ϵ , τ , ζ , and θ) in WT bone marrow derived mast cells (BMMCs, our unpublished observations). In addition, overexpression of DGK ζ in the mast cell line RBL-2H3 inhibits Fc ϵ RI-induced degranulation (our unpublished observations). Inhibition of mast cell degranulation by overexpression of DGK ζ is consistent with the importance of RasGRP1 and PKC β for mast cell degranulation, since activation of both RasGRP1 and PKC β requires DAG. It remains to be determined whether DAG levels and activation of these DAG effector molecules are indeed decreased following Fc ϵ RI stimulation in DGK ζ overexpressed mast cells.

Using DGK ζ deficient BMMCs, Olenchok et al investigated how deficiency of this specific DGK isoform may affect mast cell activation (77). Similar numbers of mast cells have been detected in multiple tissues in DGK ζ deficient mice as compared to WT mice. DGK ζ deficient BMMCs express similar levels of cell surface Fc ϵ RI and c-Kit and contain similar numbers of granules and β -hexosaminidase as WT BMMCs, suggesting that DGK ζ deficiency does not globally affect mast cell development.

When stimulating IgE-loaded DGK ζ deficient BMMCs through the Fc ϵ RI, PA production is decreased by approximately 50% compared with that observed in WT BMMCs, indicating that DGK ζ is involved in the conversion of DAG to PA in mast cells following crosslinking of Fc ϵ RI. Ras, Mek1/2, and Erk1/2 activation are enhanced in DGK ζ deficient BMMCs following Fc ϵ RI crosslinking (77). Since RasGRP1 is important for PI3K activation but dispensable for Erk1/2 activation (141), this suggests that DAG may promote Erk1/2 activation through other effector molecules.

The impact of DGK ζ deficiency on Fc ϵ RI signaling is not limited to DAG and its downstream signaling pathways. Fc ϵ RI-induced tyrosine phosphorylation of both PLC γ 1 and γ 2 is decreased in DGK ζ deficient BMMCs as compared to WT BMMCs. The decreases of PLC γ phosphorylation is correlated with decreases of PLC γ enzyme activity as both IP $_3$ production and Ca $^{++}$ responses are decreased in DGK ζ deficient BMMCs following Fc ϵ RI engagement. Pretreatment of IgE-sensitized WT BMMCs with PMA or 1,2-dioctanoyl-*sn*-glycerol can also greatly inhibit Ca $^{++}$ flux following subsequent Fc ϵ RI crosslinking (77, 147). Thus, elevated DAG concentration, due either to the addition of DAG analogues or to decreased DAG inactivation in the absence of DGK ζ , can inhibit Fc ϵ RI-induced Ca $^{++}$ flux in mast cells. Together, these observations suggest that DAG may trigger a negative feedback mechanism to inhibit upstream PLC γ activation and that DGK ζ prevents such feedback inhibition by inactivating DAG.

Many potential mechanisms could participate in DAG-mediated feedback inhibition. For example, PKC β can induce serine phosphorylation of Bruton's tyrosine kinase (Btk) at an inhibitory site, leading to inhibition of Btk activation in mast cells (148). Since Btk phosphorylates and activates PLC γ in mast cells following Fc ϵ RI stimulation, elevated PKC β activity due to increased DAG in mast cells could indirectly inhibit PLC γ activation through Btk. Additionally, Erk1/2 can phosphorylate linker for activation of T cells (LAT) at a threonine residue, resulting in the decrease of LAT-PLC γ association and PLC γ activation in T cells (149). Initial analyses have revealed that Fc ϵ RI-induced membrane translocation of PKC β II is decreased and LAT-PLC γ 2 association is not affected in DGK ζ deficient BMMCs (77). However, LAT phosphorylation, LAT-PLC γ 1 association, and Btk phosphorylation at the inhibitory serine residue have not been examined. Additional studies are needed to determine how DGK ζ deficiency causes inhibition of PLC γ activation.

Regulation of mast cell activation by DGK ζ

Production of cytokines and degranulation to release active mediators are two of the most important parameters for measurement of mast cell activation. DGK ζ deficient BMMCs

produce 2 to 3 fold more IL-6 than WT mast cells following FcεRI crosslinking, which is consistent with elevated Ras-Erk1/2 pathway activation in these cells. Although the total content of β-hexosaminidase, an enzyme residing in mast cell granules, is comparable between WT and DGKζ deficient BMMCs, FcεRI-induced release of β-hexosaminidase by DGKζ deficient BMMCs is decreased compared with that of WT BMMCs. However, PMA plus ionomycin stimulation induces similar level of β-hexosaminidase release in WT and DGKζ deficient BMMCs, suggesting that the defect causing impairment of mast cell degranulation in DGKζ deficient BMMCs is proximal to DAG and Ca⁺⁺. The impairment of mast cell degranulation has been confirmed by measurement PCA responses, which are much weaker in DGKζ deficient mice than in WT mice (77).

The mechanism that leads to impaired degranulation of DGKζ deficient mast cells is unclear. Ca⁺⁺ is critical for mast cell degranulation. A decreased Ca⁺⁺ response in DGKζ deficient mast cells could contribute to impairment of degranulation. PKCβII positively regulates mast cell degranulation while PKCδ performs an opposite role. FcεRI-induced PKCβII, but not PKCδ, translocation to cell membrane is decreased in DGKζ deficient mast cells, which may also contribute to inhibition of degranulation. At present, the mechanism by which DGKζ deficiency causes decreased PKCβII translocation is unknown. Additionally, DGKζ-derived PA could function to promote mast cell degranulation similar to PA produced by PLDs. DGK and PLD derived PA may differ in the composition of their acyl chains. Since deprivation of PLD-derived PA causes inhibition of mast cell degranulation (143, 150), DGK-derived PA may not be able to compensate fully for the loss of PLD-derived PA. It is possible that DGK-derived PA may play a partial overlapping function with PLD-derived PA or may regulate mast cell function in different subcellular compartments.

Regulating mast cell survival and monomeric IgE-induced FcεRI signaling by DGKζ

In addition to being activated after multimeric crosslinking, FcεRI can trigger signaling events after binding to monomeric IgE (mIgE) (151-153). To investigate whether DGKζ is involved in regulating mIgE-induced FcεRI signaling, Olenchok et al first asked whether DAG is induced in mast cells by measuring DAG phosphorylation to produce PA. In WT BMMCs, mIgE induces an approximately ten-fold increase of DAG phosphorylation, indicating that DAG is indeed induced and DGK activity converts DAG to PA during mIgE stimulation of mast cells. In addition to inducing DAG production, Ras and Erk1/2 are both activated in WT mast cells following mIgE stimulation, suggesting that mIgE-induced signaling shares common pathways to those induced by crosslinking of IgE loaded FcεRI with multivalent antigens. Compared to WT BMMCs, mIgE-induced DAG phosphorylation is decreased in DGKζ deficient BMMCs. In contrast, Ras and Erk1/2 activation are increased and prolonged (77). These observations indicate that DGKζ is involved in inactivating DAG in BMMCs during mIgE-induced FcεRI signaling and also negatively inhibits Ras and Erk1/2 activation. The identity of RasGRPs and other DAG effector molecules that mediate mIgE-induced Ras activation have not been identified.

Survival and expansion of BMMCs *ex vivo* requires IL-3 (154, 155). IL-3 receptor (IL-3R)-mediated signal transduction includes STAT phosphorylation and activation of MAPKs and the PI3K-Akt pathway (156). In the presence of IL-3, WT and DGKζ deficient BMMCs survive and expand similarly. However, an obvious difference in survival between WT and DGKζ deficient mast cells can be detected after IL-3 withdrawal. Whereas WT BMMCs die rapidly, most DGKζ deficient BMMCs remain viable during the five day culture period. The survival advantage of DGKζ deficient BMMCs is correlated with elevated Akt phosphorylation, suggesting that DGKζ promotes Akt activation through the receptor for IL-3 or other growth factors (77).

Collectively, these data demonstrate that DGK ζ plays differential roles in mast cells. It regulates Fc ϵ RI signaling after crosslinking of the receptor and after mIgE association. DGK ζ promotes mast cell survival and Fc ϵ RI-induced degranulation but attenuates cytokine production.

Regulation of TLR-mediated innate immunity by DGK ζ

Susceptibility of DGK ζ deficient mice to Toxoplasmosis

Toxoplasma (T.) gondii is an intracellular opportunistic protozoan pathogen that causes widespread infection in humans and animals. *T. gondii* causes serious and sometimes fatal diseases in immunocompromised patients (157). Host defense against *T. gondii* is initiated by DCs, macrophages (M ϕ), and other APCs after recognition of pathogen-associated molecular patterns through the TLRs. TLR-induced activation of these APCs leads to production of IL-12 and subsequent induction of IFN γ and Th1 immune responses that are critical to control the infection (158, 159). TLR2 and TLR11 have been demonstrated to participate in *T. gondii* recognition and deficiency of these TLRs increases host susceptibility to *T. gondii* infection (160-162). Signals from these TLRs are mediated by the myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which is utilized by most TLRs except TLR3. This pathway is initiated after association of MyD88 with the TLRs during microbial recognition (Figure 5). MyD88 in turn recruits IL-1R-associated kinase 1 and 4 (IRAK1, IRAK4), TNF receptor associated factor 6 (TRAF6) and other signaling molecules to the cytoplasm membrane, leading to the activation of I κ B kinase (IKK) $\alpha/\beta/\gamma$ complex. IKK $\alpha/\beta/\gamma$ phosphorylates I κ B, causing I κ B degradation and nuclear translocation of NF κ B to induce expression of its target genes such as IL-12 and DC maturation (163). Mice deficient of MyD88, IL-12, or IFN γ are highly susceptible to *T. gondii* infection (161, 164).

Although T cell activation is enhanced in DGK ζ deficient mice and DGK ζ deficient mice mount enhanced anti-LCMV immune responses, a collaborative work between our laboratory and Aliberti's laboratory has surprisingly revealed that DGK ζ deficient mice are susceptible to *T. gondii* infection. When challenged with 20 to 40 *T. gondii* cysts through the peritoneal cavity, WT C57BL/6 mice can control and survive the infection. By contrast, DGK ζ deficient C57BL/6 mice are unable to control *T. gondii* infection and died between 40 to 50 days after challenged with the parasite. In the brain of DGK ζ deficient mice, *T. gondii* cysts are increased in both numbers and size with an accompanying diffusive mononuclear cell infiltration (59).

The increased susceptibility of DGK ζ deficient mice to *T. gondii* infection is correlated with inhibition of Th1 immune responses *in vivo* (59). Serum IFN γ level, which is upregulated in WT mice following *T. gondii* infection, is significantly lower in DGK ζ deficient mice than in WT mice 7 days after *T. gondii* infection. T cells from *T. gondii* infected DGK ζ deficient mice show impaired Th1 recall response to *T. gondii*-soluble tachyzoite antigens (STAg) 15 and 30 days after *T. gondii* infection. The impairment of *T. gondii* specific Th1 response is unlikely due to the impairment of T cell activation in DGK ζ deficient mice, as more activated CD4 and CD8 T cells can be detected in these mice than in WT mice after *T. gondii* infection. Furthermore, purified naive CD4 T cells from DGK ζ deficient mice produce similar levels of IFN γ as WT CD4 T cells under Th1 polarizing conditions, indicating that there is no intrinsic defect of DGK ζ deficient T cells to differentiate from the Th1 lineage (our unpublished observations). These observations prompt us to hypothesize that DGK γ may participate in regulating innate immune response against *T. gondii* infection. In support of this hypothesis, several studies have demonstrated that both DAG and PA are produced in M ϕ following lipopolysaccharide (LPS, a TLR4 ligand) and lipopeptide (a TLR2 ligand) stimulation (11-13), suggesting that enzymes involved in DAG/

PA metabolism are indeed activated during TLR stimulation. Furthermore, inhibition of DAG and PA generation by chemical inhibitors of PC-PLCs or by diversion of PLD-derived PA to phosphatidyl butanol decreases LPS- and lipopeptide-induced TNF α production (11-13). These observations, while suggesting a positive role of DAG and/or PA in TLR-induced responses, cannot rule out off-target effects of these chemical inhibitors.

Inhibition of TLR-induced IL-12 and TNF α production in DGK ζ deficient DCs and M ϕ

To investigate a role of DGK ζ in innate immunity, Liu et al first determined expression of this protein in DCs and M ϕ (59). Both DGK ζ 1 and DGK ζ 2 are expressed in DCs and M ϕ with the ζ 1 isoform predominantly expressed. In addition, DGK ζ expression is upregulated following 24-hours of LPS stimulation. Thus, DGK ζ is not only expressed in DCs and M ϕ but is also regulated by TLR4 signaling. Injection of TLR ligands into mice induces rapid production of proinflammatory cytokines by DCs, M ϕ , and neutrophils and thus provides an easy experimental approach to detect TLR-induced response *in vivo*. Six hours after injection of LPS and STAg, which activates at least TLR2 and TLR11 (160, 162), into WT and DGK ζ deficient mice serum TNF α and IL-12 levels are much lower in DGK ζ deficient mice than in WT mice. The decreased serum IL-12 and TNF α correlate with decreased mRNA levels of these cytokines in DGK ζ deficient spleens. Since DGK ζ deficiency does not cause reduction of DCs, monocytes, and neutrophils in mice, these observations suggest that deficiency of DGK ζ results in decreased transcription or decreased mRNA stability of these cytokines in response to LPS and STAg stimulation *in vivo* (59).

Additional *in vitro* experiments provide clear evidence supporting that DGK ζ promotes TLR-mediated proinflammatory cytokine production (59). DGK ζ deficient bone marrow derived M ϕ (BMM ϕ) and purified splenic DCs produced much less IL-12 and TNF α after being stimulated with LPS, STAg, Poly(I:C) (a TLR3 ligand), or Pam3CSK4 (a TLR2 ligand) than WT controls. The effects of DGK ζ deficiency appears selective to cytokine production, since LPS-induced nitric oxide production, DC migration, and DC maturation are not inhibited by DGK ζ deficiency (our unpublished observations). Together, these data indicate that DGK ζ positively contributes to multiple TLR-induced proinflammatory cytokine production.

Negative regulation of the PI3K-Akt pathway by DGK ζ in TLR signaling

With the role of DGK ζ in TLR-induced proinflammatory responses determined, additional studies were undertaken to determine how DGK ζ controls TLR signaling. I κ B α degradation, a central event downstream of the MyD88 pathway, is slightly increased in DGK ζ deficient BMM ϕ following LPS stimulation as compared to WT BMM ϕ . MAPK p38 activation is not obviously altered in DGK ζ deficient BMM ϕ following LPS stimulation. I κ B α degradation and p38 activation are both critical for TLR-induced proinflammatory responses (59). These results suggest I κ B α and p38 signaling events do not account for decreased proinflammatory cytokine production by DGK α deficient M α . The MAPK Erk1/2 have been reported to negatively regulate IL-12 production induced by several TLR ligands (165, 166). However, Erk1/2 phosphorylation is not obviously enhanced in DGK α deficient M α following LPS stimulation. As mentioned earlier, Erk1/2 activation is elevated in DGK α deficient T cells. Thus, different mechanisms might be involved in regulating Erk1/2 activation during TCR and TLR stimulation.

Elevated PIP $_3$ levels and enhanced, prolonged Akt phosphorylation have been noted in DGK ζ deficient BMM ϕ , compared with WT BMM ϕ , following LPS stimulation. Importantly, treatment of DGK ζ deficient BMM ϕ with a PI3K inhibitor, Ly294002, can restore LPS-induced IL-12 production to a level similar to WT BMM ϕ (59). Thus, elevated activation of the PI3K-Akt pathway contributes to the impairment of TLR-induced

proinflammatory cytokine production by DGK ζ deficient BMM ϕ . These data are consistent with several studies demonstrating that the PI3K-Akt pathway can negatively regulate TLR-induced responses (167-169). DCs deficient of the p85 α regulatory subunit of the class I α PI3Ks express high levels of IL-12 following stimulation with multiple TLR ligands. p85 α -deficient BALB/c mice become resistant to *Leishmania major* infection due to enhanced Th1 responses (167). Our data identify DGK ζ as an upstream negative regulator of PIP $_3$ level. It remains to be determined whether DGK ζ inhibits PI3K or promotes PTEN activity.

DGK ζ deficiency should affect both DAG and PA concentrations. While the effects of DGK ζ deficiency on DAG/PA levels following TLR stimulation remain to be evaluated, data indicate that DGK ζ -derived PA is critical for TLR-induced IL-12 production. For example, DGK ζ deficient BMM ϕ produce less IL-12 than WT BMM ϕ following LPS stimulation, but high levels of IL-12 were induced in both cell types treated with PA plus LPS (59). Thus, DGK ζ may function as a signal initiator in TLR signaling by producing PA. Whether PA treatment may restore normal activation of PI3K-Akt pathway in DGK ζ deficient DCs and M ϕ s is an important question to be studied. It is important to note that PA produced through other mechanisms such as PLDs should be intact in DGK ζ deficient M ϕ . Thus, inhibition of either DGK-derived PA or PLD-derived PA decreases TLR-induced cytokine production, indicating that PA from these two different sources cannot fully compensate for the loss of the other. It would be interesting to further determine whether DGK and PLD synergistically regulate TLR-induced proinflammatory responses and whether these two enzymes may function in different subcellular locations during TLR stimulation.

Collectively, these studies indicate that DGK ζ plays a critical role in host defense against *T. gondii*. DGK ζ promotes TLR-induced IL-12 production by negatively regulating the PI3K-Akt pathway. The positive role of DGK ζ in TLR-induced proinflammatory responses is likely mediated by its product PA.

Summary and perspective

Significant progress has been made in understanding the importance of DGK isoforms in receptor signaling and cellular function. DGK activity plays critical roles in regulating both innate and adaptive immune responses. DGKs can function as a signal terminator by inhibiting DAG-mediated signaling and as a signal initiator by triggering PA-mediated responses in immune cells. The ability of DGK α and ζ to synergistically regulate T cell development, T cell tolerance, and autoimmunity suggests an evolutionary advantage in that mutations of either DGK α or ζ would not be expected to be deleterious.

Although progress has been made, many questions remain to be addressed. First, how does DGK α and ζ deficiency impact various pathways downstream of DAG and PA? Other than RasGRP1, the ways in which DGKs regulate other DAG effector molecules and PA effector molecules during immune receptor signaling are still not fully understood. DGK α and ζ deficient mice and cell lines may provide invaluable tools to dissect receptor signaling and uncover new pathways important for immune cell development and function. Second, how do DGK α and ζ regulate T cell effector differentiation and memory T cell responses? The spontaneous activation phenotype of DGK $\alpha\zeta$ DKO T cells makes DGK $\alpha\zeta$ DKO mice an imperfect model to address this question. Conditional DGK $\alpha\zeta$ DKO mice would be most useful for further dissecting the role of DGK activity in T cell function. Third, what are the roles of DGKs in other immune cell lineages besides T cells, mast cells, DCs, and M ϕ ? Many receptors in immune cells induce DAG and PA production. It is likely that some DGK isoforms may play critical roles in these immune cells. Fourth, how is expression of each DGK isoform regulated? While DGK α and ζ control immune receptor signaling, their

activities are also regulated by signals from these receptors. Both DGK α and ζ transcripts are differentially regulated by T cell receptor signaling in the presence or absence of co-stimulation. DGK ζ expression is upregulated by LPS stimulation. The mechanisms modulating DGK expression and importance of regulated DGK expression during immune response are not fully understood. At present, *cis*-regulatory elements that control the expression of DGK isoforms have not been identified. Fifth, what mechanism controls DGK enzymatic activity? Src-mediated tyrosine phosphorylation of DGK α at Y355 is important to induce DGK α activity and PKC α phosphorylates and inhibits DGK ζ activity and nuclear translocation in cell line models. The importance of these and other potential modifications and the enzymes that mediate these modifications in immune cells have not been revealed. Sixth, how do DGK isoforms synergistically regulate immune cell function and how are DGKs recruited to regulate specific receptor signaling? It is unclear whether DGK α and ζ target DAG in the same or different subcellular compartments, and whether these occur at the same or different times following engagement of immune receptors. Many proteins have been found to interact with DGK isoforms. DGK α interacts with c-Src (61). DGK ζ has been found to interact with numerous proteins such as RasGRP1 (112), γ -syntrophin (31), β -arrestin (13), PKC α (170), nexin 27 (171), the retinoblastoma protein (pRB) (172), PIP5K (57), and the long form leptin receptor (173). Whether some of these interactions or other yet to be identified proteins are involved in recruiting DGKs to immune cell receptors remains to be demonstrated. Addressing these questions will improve our understanding of the regulation of the immune system and DGK function, and will also provide strategies to modulate immune responses for treatment of autoimmune diseases and for cancer immunotherapy.

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Figure 1. DGK isoforms in mammals

Mammalian DGKs are divided into five subtypes based on distinct structural features. EF, EF-hand; C1, cysteine-rich domain; RVH, recoverin homology domain; PH, Pleckstrin homologous domain; SAM, sterile alpha motif domain; P/E, proline/glutamic acid-rich region; M, MARCKS (myristoylated alanine-rich protein kinase C substrate) motif; A, Ankyrin-repeat; P, PDZ domain binding motif; G/P, glycine/proline rich region.

Figure 2. Major pathways of DAG and PA metabolism during receptor signaling

DAG can be produced by PI-PLC-mediated hydrolysis of PIP₂, by PC-PLC-mediated hydrolysis of PC, and by PAP-mediated hydrolysis of PA, and by SMS. PA can be produced by PLD-mediated hydrolysis of PC and by DGK-mediated phosphorylation of DAG. DAG and PA effector molecules are also listed.

Figure 3. Schematic illustration of TCR signaling

Following TCR engagement, a critical event is the activation of PLC γ following the orchestrated action of proximal tyrosine kinases and adaptor molecules. Activated PLC γ 1 generates IP $_3$ and DAG, leading to the activation of the Ca $^{++}$ -calcineurin-NFAT pathway, the RasGRP1-Ras-Erk1/2-AP1 pathway, the PKC θ -Carma1/Bcl10-NF κ B pathway, and PKD. NFAT alone can induce T cell anergy by increasing expression of anergy promoting molecules including DGK α . This pathway, in concert with DAG-mediated pathways, leads to T cell activation. CD28 strengthens TCR signaling to promote PI3K activation and downregulates DGK α and ζ expression. DGKs convert DAG to PA and may inhibit DAG-mediated signaling.

Figure 4. Schematic illustration of FcεRI signaling

Engagement of IgE-loaded FcεRI induces activation of the Src family protein tyrosine kinases Lyn and Fyn and the tyrosine kinase Syk. These kinases then phosphorylate multiple enzymes such as PI3K, PLCγ, and Btk and adaptor molecules such as Gab2, LAT, and SLP76. Fyn, Gab2 and RasGRP1 promote PI3K activation and mast cell degranulation. PI3K activates Akt to promote mast cell survival and transcription activation of cytokine genes. Activated PLCγs produce DAG and IP₃ to induce PKCβ and RasGRP1 activation and Ca⁺⁺ influx. PKCβ, RasGRP1, and Ca⁺⁺ are required for FcεRI-induced degranulation.

Figure 5. Schematic illustration of TLR signaling

TLR signaling is mediated by the MyD88-dependent and TRIF-dependent pathway. MyD88-dependent pathway is utilized by all TLRs except TLR3 and is initiated by association of MyD88 with the TLRs, followed by subsequent recruitment and activation of IRAKs, TRAF6 and other signaling molecules to the cytoplasm membrane. The pathway leads to activation of IKK and nuclear translocation of NFκB to induce expression of its target genes such as IL-12 and TNFα. The TRIF-dependent pathway is utilized by TLR3 and TLR4 and activates IRF3 (IFN-regulatory factor 3) and transcription of IFNα and co-stimulatory molecules. GSK3α is critical for proinflammatory cytokine production induced by TLRs by promoting NFκB-CBP association. The PI3K-Akt pathway can inhibit TLR-induced proinflammatory cytokine production by phosphorylating and deactivating GSK3α. TAK1, transforming growth factor-β-activated kinase 1; TBK1, TRAF family member associated NFκB activator-binding kinase 1.