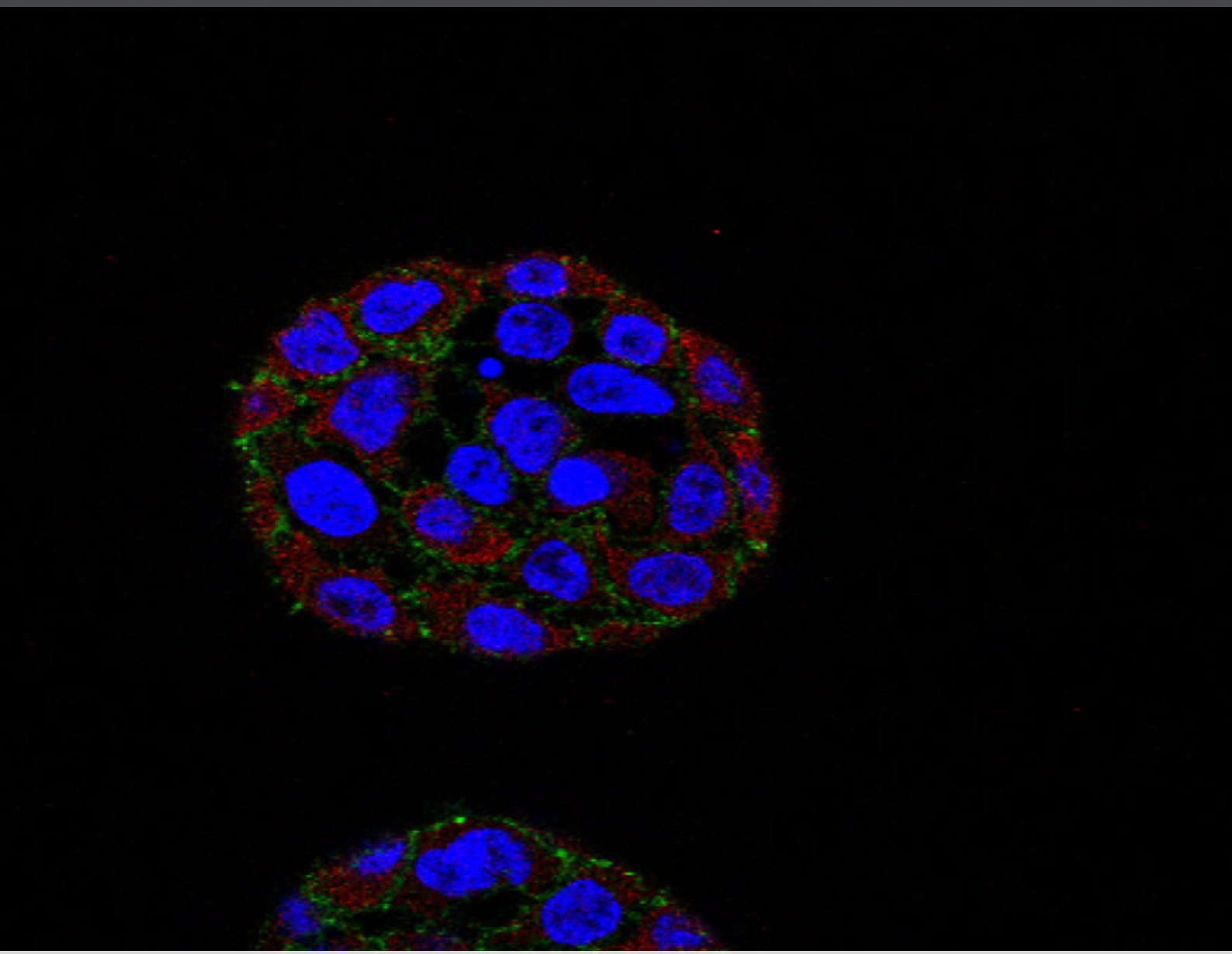
DIACYLGLYCEROL KINASE SIGNALLING

EDITED BY: Isabel Merida, Andrea Graziani and Fumio Sakane
PUBLISHED IN: Frontiers in Cell and Developmental Biology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-335-1
DOI 10.3389/978-2-88945-335-1

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

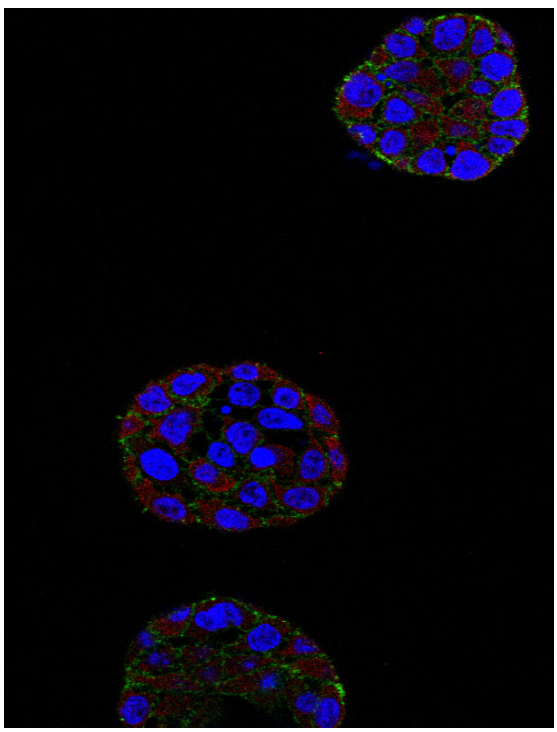
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

DIACYLGLYCEROL KINASE SIGNALLING

Topic Editors:
Isabel Merida, National Center of Biotechnology-CSIC, Spain
Andrea Graziani, University Vita e Salute San Raffaele Milan, Italy
Fumio Sakane, Chiba University, Japan



DGKalpha expression in 3D cultured breast cancer cells. MCF-10A cells seeded in matrigel and cultured for 6 days show the characteristic acinar-like structures. Cells were fixed and stained with DAPI to visualize nuclei (blue), DGKα (red) and actin (green).

Image courtesy of Pedro Torres and Antonia Avila-Flores.

Diacylglycerol kinases (DGKs) phosphorylate diacylglycerol (DG), catalyzing its conversion into phosphatidic acid (PA). This reaction attenuates membrane DG levels, limiting the localization/activation of signaling proteins that bind this lipid. Initially recognized as modulators of classical and novel PKC family members, the function of the DGK has further expanded with the identification of novel DG effectors including Ras Guanyl nucleotide-releasing proteins (RasGRP)

and chimaerin Rac GTPases. The product of the DGK reaction, PA, is also a signaling lipid that mediates activation of multiple proteins including the mammalian target of rapamycin (mTOR). The DGK pathway thus modulates two lipids with important signaling properties that are also key intermediates in lipid metabolism and membrane trafficking. The DGK family in eukaryotes comprises 10 different members grouped into five different subfamilies characterized by the presence of particular regulatory motifs. These regions allow the different DGK isoforms to establish specific complexes and/or to be recruited to specific subcellular compartments. The subtle regulation of DG and PA catalyzed byspecific DGKs is sensed by a restricted set of molecules, providing the means for spatio-temporal regulation of signals in highly specialized cell systems.

In the recent years, multiple studies have unveiled the functions of specific isoforms, their mechanisms of regulation and their participation in different pathways leading to and from DG and PA. Animal models have greatly helped to understand the specialized contribution of DGK mediated signals, particularly in the immune and central nervous systems. Mice deficient for individual DGK isoforms show defects in T and B cell functions, dendritic spine maintenance, osteoclast and mechanical-induced skeletal muscle formation. Studies in flies and worms link DGK mediated DAG metabolism with mTOR- mediated regulation of lifespan and stress responses. In plants DGK mediated PA formation contributes to plant responses to environmental signals.

Aberrant DGK function has been recently associated with pathological states, an expected consequence of the essential role of these enzymes in the regulation of multiple tissue and systemic functions. DGK mutations/deletions have been related to human diseases including diabetes, atypical hemolytic-uremic syndrome, Parkinson disease and bipolar disorders. On the contrary DGK upregulation emerges as a non-oncogenic addition of certain tumors and represents one of the main mechanism by which cancer evades the immune attack. As a result, the DGK field emerges an exciting new area of research with important therapeutic potential.

Citation: Merida, I., Graziani, A., Sakane, F., eds. (2017). Diacylglycerol Kinase Signalling. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-335-1

Table of Contents

frontiers in Cell and Developmental Biology

EDITORIAL
published: 21 September 2017
doi: 10.3389/fcell.2017.00084

Editorial: Diacylglycerol Kinase Signalling

Isabel Merida[1]*, *Andrea Graziani*[2,3] *and Fumio Sakane*[4]

[1] Department of Immunology and Oncology, CNB-CSIC, Madrid, Spain, [2] Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy, [3] Division of Experimental Oncology, School of Medicine, Vita-Salute San Raffaele University, Milan, Italy, [4] Department of Chemistry, Graduate School of Science, Chiba University, Chiba, Japan

Keywords: lipid signaling, immune system, synaptic transmission, immunotherapy of cancer, cytotoxic t cells, synaptic plasticity (LTP/LTD)

Editorial on the Research Topic

Diacylglycerol Kinase Signalling

By gathering 10 reviews from leading scientists in a fast-evolving field, this Research Topic illustrates the contribution of diacylglycerol kinase (DGK) family members to the regulation of cell responses, and how their malfunction contributes to human pathologies.

DGK transform diacylglycerol (DG) into phosphatidic acid (PA). This simple reaction alters the levels of two lipids with central functions as second messengers, as phospholipid precursors, and as membrane modulators. The first DGK was purified and cloned by the Hideo Kanoh group in 1990; since then, the study of DGK regulation and functions has expanded and grows daily. Mammalian DGK comprise 10 isoforms that are particularly abundant in brain and hematopoietic organs. Not surprisingly, DAG effectors such as protein kinase C family members, mammalian Unc13 homologs, the chimaerin family of Rac GTPases, and the Ras GEF RasGRPs have critical functions in the regulation of nervous and immune synapses.

The mammalian DGK isoforms are grouped into five subtypes based on the presence of distinct regulatory domains. At least eight DGK isoforms are detected in the mammalian brain. In their review, Lee et al. summarize studies that link various DGK isoforms with certain features of synaptic plasticity. The generation of knockout mice for specific DGK isoforms has demonstrated their involvement in distinct aspects of brain function. These findings imply precise, isoform-specific control of lipid homeostasis that creates local environments suitable for triggering synaptic plasticity. Genome-wide association studies suggest relationships between specific DGK and human diseases that include bipolar disease and Parkinson's disease. Sakane et al. review these findings and discuss others such as mutations in the *DGKE* gene and its link to epilepsy and Huntington disease, or the more recent discovery of *DGKE* mutations associated with atypical hemolytic-uremic syndrome (aHUS).

The predominance of DGKε malfunction and disease might be related to this isoform's unique properties. DGKε is the only DGK family member that lacks regulatory domains, that is membrane-bound, and has substrate specificity for arachidonate-containing DG. Two articles in this series review DGKε properties and functions in depth, and cast new light on the links between DGKε defects and disease. Epand et al. describe the structural features that lend DGKε its arachidonoyl-DG specificity, its regulation by phosphorylation, and the identification of DGKε-interacting proteins. They discuss at length the unanticipated connection between recessive *DGKE* mutations and aHUS, recently uncovered by whole-exome sequencing. Although dysregulated complement activation is generally associated with all aHUS forms, the vast majority of aHUS patients that harbor *DGKE* mutations do not show this defect. DGKε is found at the endoplasmic reticulum (ER), a specialized site in membrane phospholipid synthesis and transport. In their review, Nakano et al. discuss the DGKε-specific contribution at the ER by generating arachidonoyl PA that

OPEN ACCESS

Edited and reviewed by:
Pedro M. Fernández-Salguero, University of Extremadura, Spain

Correspondence:
Isabel Merida
imerida@cnb.csic.es

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *27 July 2017*
Accepted: *06 September 2017*
Published: *21 September 2017*

Citation:
Merida I, Graziani A and Sakane F (2017) Editorial: Diacylglycerol Kinase Signalling. Front. Cell Dev. Biol. 5:84. doi: 10.3389/fcell.2017.00084

feeds directly into the PI cycle. DGKε malfunction impairs PA generation and facilitates DG lipase-dependent DG degradation to 2-arachidonoyl glycerol, an endocannabinoid with important contributions to retrograde synaptic transmission.

All 10 mammalian DGK isoforms share a conserved catalytic domain and two protein kinase C conserved type I (C1) domains. The exception, with three C1 domains, is DGKθ, the lone member of the type V DGK class and the least-studied. In their detailed review, Tu-Sekine et al. cover DGKθ structure, enzymology and the latest results, which suggest its contribution to regulation of neurotransmission.

In addition to this notable role in brain, the DGK contribution to immune response control is another area of intensive research. T lymphocyte recognition of antigens presented on the antigen-presenting cell surface generates a polarized increase in membrane DG at the contact area. Sustained DG generation at the immune synapse facilitates activation of the Ras/ERK and PKC pathways, which ultimately dictate the fate of differentiated T cell populations. Chen et al. extensively describe the studies with DGKα- and ζ-deficient mice that have defined the contribution of these two isoforms to differentiation of CD8 T effector and memory populations, development of regulatory T cells (Treg), and lineage development of invariant natural killer cells. At difference from DGKα, expressed predominantly in T cells, DGKζ is expressed ubiquitously in hematopoietic cells. Singh and Kambayashi's review explores the immunomodulatory function of DGKζ in several cell types. The dual contribution of DGKζ, which can either potentiate or limit immune responses, adds new interest to its targeting. In addition to promoting defense against infection or cancer, DGKζ inhibition might have benefits by limiting immune responses in pathological conditions such as allergy and septic shock.

Due to their negative regulatory T cell function, DGKα and ζ have recently attracted interest as prospective targets, which might improve T cell-mediated tumor destruction. Two articles in this series examine the findings from their own and other groups that identify DGKα and ζ targeting potential in the fight against cancer. The contribution of DGKα as a cytosolic immune checkpoint is detailed reviewed in Noessner. On their review, Riese et al. consider DGKα and ζ contributions to limiting anti-tumor functions of engineered cytotoxic T cells. In both cases, pharmacological DGKα inhibitors induce recovery of cytotoxic functions by tumor-infiltrating cells, although the precise role of each isoform and the consequences of specific DGK ζ inhibition remain to be addressed. The continued search for new, more effective DGK inhibitors is a critical issue that is addressed by Sakane's group, who review the recent characterization of a new DGKα inhibitor.

DGK phosphorylation of DAG to PA is the principal pathway of DAG signaling termination. Through PA generation, DGK enzymes promote local alteration of membrane composition and electrostatic charge, which leads to recruitment and activation of additional sets of proteins. Baldanzi et al. illustrate how PA generation in specific membrane subdomains affects polarized responses such as immune synapse formation or cell migration.

We are deeply indebted to all the authors who have contributed to this Research Topic and to the dedicated reviewers who helped us achieve the highest quality standards. We gratefully acknowledge the valuable support of the Frontiers team in manuscript processing.

AUTHOR CONTRIBUTIONS

IM wrote the editorial, AG and FS approved it.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 31 August 2016
doi: 10.3389/fcell.2016.00092

Diacylglycerol Kinases in the Coordination of Synaptic Plasticity

Dongwon Lee[1], *Eunjoon Kim*[1,2] *and Keiko Tanaka-Yamamoto*[3]*

[1] Center for Synaptic Brain Dysfunctions, Institute for Basic Science, Daejeon, South Korea, [2] Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, South Korea, [3] Center for Functional Connectomics, Korea Institute of Science and Technology, Seoul, South Korea

Synaptic plasticity is activity-dependent modification of the efficacy of synaptic transmission. Although, detailed mechanisms underlying synaptic plasticity are diverse and vary at different types of synapses, diacylglycerol (DAG)-associated signaling has been considered as an important regulator of many forms of synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD). Recent evidences indicate that DAG kinases (DGKs), which phosphorylate DAG to phosphatidic acid to terminate DAG signaling, are important regulators of LTP and LTD, as supported by the results from mice lacking specific DGK isoforms. This review will summarize these studies and discuss how specific DGK isoforms distinctly regulate different forms of synaptic plasticity at pre- and postsynaptic sites. In addition, we propose a general role of DGKs as coordinators of synaptic plasticity that make local synaptic environments more permissive for synaptic plasticity by regulating DAG concentration and interacting with other synaptic proteins.

Keywords: diacylglycerol kinase, synaptic plasticity, long-term potentiation, long-term depression, protein kinase C

OPEN ACCESS

Edited by:
Isabel Merida,
Spanish National Research Council (CSIC) - Spanish National Centre for Biotechnology (CNB), Spain

Reviewed by:
Piero Crespo,
Consejo Superior de Investigaciones Científicas - Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), Spain
Daniel M. Raben,
The Johns Hopkins University School of Medicine, USA

***Correspondence:**
Keiko Tanaka-Yamamoto
keikoyamat@gmail.com

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *16 June 2016*
Accepted: *17 August 2016*
Published: *31 August 2016*

Citation:
Lee D, Kim E and Tanaka-Yamamoto K (2016) Diacylglycerol Kinases in the Coordination of Synaptic Plasticity. Front. Cell Dev. Biol. 4:92. doi: 10.3389/fcell.2016.00092

INTRODUCTION

Alterations in the efficacy of synaptic transmission are believed to be cellular mechanisms of learning and memory. Two well-studied forms of such synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD), which have been widely observed at different types of synapses in many brain areas (Citri and Malenka, 2008). The signaling mechanisms of synaptic plasticity vary to some extent depending on the types of synapses and stimulation. One of the main differences is the synaptic site of expression: some forms of LTP and LTD rely on changes in presynaptic neurotransmitter release, but others rely on changes in postsynaptic receptor numbers or properties (Malenka and Bear, 2004; Castillo, 2012; Huganir and Nicoll, 2013). Nevertheless, there are some properties shared by several forms of LTP and LTD. A common property of all forms of LTP and LTD is that potentiation and depression are triggered by transient stimulation, whereas the potentiation and depression last for a long time. For the long-term maintenance of altered synaptic strength, translational, and transcriptional regulations are often involved (Citri and Malenka, 2008). The protocol triggering LTP or LTD usually consists of strong or repeated synaptic stimulation, which likely activates synaptic receptors and triggers increases in the levels of signaling molecules, such as calcium and diacylglycerol (DAG). In fact, signaling mechanisms associated with calcium or DAG have been shown to be required for several forms of LTP and LTD (Sossin and Farah, 2009).

In many types of cells, including neurons, DAG is generally produced after the activation of G-protein coupled receptors (GPCRs) through the metabolization of phosphatidylinositol

4,5-bisphosphate by phospholipase C (PLC) (Rhee, 2001). At synapses, an increase in DAG level by the activation of GPCRs likely regulates its target molecules required for synaptic plasticity (Brose et al., 2004). A well-known target molecule of DAG is protein kinase C (PKC), which has been shown to be involved in many forms of LTP or LTD. Therefore, the regulation of DAG concentrations at synapses is crucial for the regulation of these forms of LTP or LTD.

The conversion of DAG to phosphatidic acid (PA) by diacylglycerol kinase (DGK) is the major pathway for the termination of DAG signaling (Sakane et al., 2007). Ten mammalian DGK isoforms have been identified so far, and at least 8 of them are readily detected in the mammalian brain, suggesting the important roles of DGKs in the brain (Tu-Sekine and Raben, 2011). Interestingly, the expression pattern of each isoform in the brain is different, and their subcellular localizations are distinct (Tu-Sekine and Raben, 2011; Ishisaka and Hara, 2014), suggesting that different isoforms of DGKs have unique neuronal or synaptic functions. In this review, we summarize the roles of DGKs in the regulation of synaptic plasticity (see **Table 1** for summary), focusing on specific types of synaptic plasticity and individual DGK isoforms involved. In addition, we would like to emphasize the emerging notion that DGKs may be involved in the generation of local environments suitable for synaptic plasticity.

DGKs INVOLVED IN SEVERAL FORMS OF HIPPOCAMPAL SYNAPTIC PLASTICITY

DGKε for LTP at Perforant Path-Dentate Granule Cell Synapses

The involvement of DGKs in synaptic plasticity was first demonstrated for LTP at synapses of hippocampal dentate granular cells receiving inputs from the perforant path of the entorhinal cortex. LTP at these synapses requires the action of postsynaptic NMDA-type glutamate receptors (NMDARs) and increases in calcium concentrations (Colino and Malenka, 1993; Kleschevnikov and Routtenberg, 2001). However, it is controversial whether this LTP is presynaptically or postsynaptically expressed. Several studies demonstrated that LTP at these synapses was expressed by an increase in the probability of presynaptic neurotransmitter release (Christie and Abraham, 1994; Wang et al., 1996; Min et al., 1998), which is controlled by the retrograde lipid messenger, platelet-activating factor (Kato and Zorumski, 1996; Chen et al., 2001). On the other hand, other studies showed that LTP was expressed by an increase in the number of postsynaptic AMPA-type glutamate receptors (Wang et al., 1996; Reid and Clements, 1999; Moga et al., 2006).

Despite the undefined locus of LTP expression, PKC has been demonstrated to be involved in this form of LTP. PKC activation rescues LTP blocked by an NMDAR antagonist

TABLE 1 | Involvement of DGK isoforms in several forms of synaptic plasticity.

DGK isoform	Synapse	Localization	Tested forms of synaptic plasticity	Reported effects in KO mice	Known/expected functions of DGKs
DGKε	Perforant path-dentate granule cell synapses	N.D.	LTP	Reduction	Regulating amounts of DAG and PKC activity required for LTP, and regulating the lipid signaling leading to the production of retrograde messengers required for LTP
DGKζ	Hippocampal SC-CA1 synapses	Postsynaptic density	Postsynaptic LTP	Enhancement	Regulating amounts of DAG produced by mGluR activation, and balancing PKC activity, which is a modulator of LTP and LTD
			Postsynaptic LTD	Reduction	
DGKβ	Hippocampal SC-CA1 synapses	Membranes including synaptic areas	Postsynaptic LTP	Reduction	Regulating basal DAG levels
DGKι	Hippocampal SC-CA1 synapses	Presynaptic areas	Presynaptic LTD	Reduction	Reducing DAG levels and consequently preventing the activation of target molecules that antagonize LTD
DGKκ	Hippocampal SC-CA1 synapses	N.D.	Postsynaptic LTP	Reduction	Regulating basal DAG levels
			Postsynaptic LTD	Enhancement	
DGKζ	Cerebellar parallel fiber-Purkinje cell synapses	Postsynaptic areas	Postsynaptic LTP	Normal	–
			Postsynaptic LTD	Reduction	Targeting PKCα required for LTD at synapses, maintaining optimal PKCα activity levels via reducing basal DAG levels, and receiving inhibition from PKCα

N.D. stands for "not described or undetermined."

(Kleschevnikov and Routtenberg, 2001), suggesting involvement of PKC activation in LTP. In contrast, PKC activation by metabotropic glutamate receptors (mGluRs) prior to LTP induction inhibits subsequent LTP induction (Gisabella et al., 2003), suggesting that maintaining minimum PKC activity at the basal state is required for LTP. These results indicate that the regulation of PKC activity is critical for LTP induction.

In mice lacking DGKε (DGKε$^{-/-}$), which is the only DGK that can act on *sn*-2 arachidonoyl-DAG, LTP at dentate granular cell synapses was impaired (Rodriguez de Turco et al., 2001). A possible function of DGKε is to reduce DAG concentrations and PKC activity at the basal state, considering the dynamics of PKC activity required for LTP induction. Another possibility is that DGKε regulates lipid metabolism, so that the synthesis of platelet-activating factor is suppressed, leading to impairment of LTP in DGKε$^{-/-}$ mice (Rodriguez de Turco et al., 2001).

Role of DGKζ in Postsynaptic LTP and LTD at SC-CA1 Synapses

Synaptic plasticity has been extensively investigated at hippocampal Schaffer-collateral (SC)-CA1 synapses, in which LTP and LTD are postsynaptically expressed (Citri and Malenka, 2008). Many studies have demonstrated that PKC is involved in both LTP and LTD at SC-CA1 synapses (Akers et al., 1986; Malinow et al., 1989; Klann et al., 1993; Thiels et al., 2000), suggesting the importance of DAG regulation in the postsynaptic area. DGKζ is mainly present at postsynaptic sites and directly interacts with PSD-95 family proteins (Kim et al., 2009). In DGKζ$^{-/-}$ mice, LTP at SC-CA1 synapses is enhanced, whereas LTD is reduced (Seo et al., 2012). Importantly, pharmacological inhibition of PLC and PKC restores abnormal LTP and LTD in DGKζ$^{-/-}$ mice, suggesting that enhanced PLC-PKC signaling by DGKζ deficiency may lead to an altered balance of LTP and LTD (Seo et al., 2012). Therefore, DGKζ appears to limit excessive increases in DAG level and PKC activity for proper modulation of bidirectional synaptic plasticity at hippocampal SC-CA1 synapses.

Role of DGKβ in Postsynaptic LTP at SC-CA1 Synapses

In addition to DGKζ, DGKβ has also been reported to regulate postsynaptically expressed LTP at hippocampal SC-CA1 synapses. However, its functions appear to be different from those of DGKζ, because LTP was reduced in DGKβ$^{-/-}$ mice, contrary to the enhanced LTP in DGKζ$^{-/-}$ mice (Shirai et al., 2010). DGKβ is expressed at high levels in the hippocampal pyramidal cell layer (Goto and Kondo, 1999) and shows unique localization patterns at the plasma membrane (Caricasole et al., 2002) and postsynaptic compartments (Hozumi et al., 2008). Thus, both DGKβ and DGKζ are localized around the postsynaptic area, excluding a possibility that this accounts for the differences in their functions. Another possibility is that DGKβ and DGKζ are responsible for metabolizing DAG under different contexts. DGKβ deficiency resulted in a reduction in PA production and an increase in DAG level even without stimulation (Shirai et al., 2010), whereas DGKζ deficiency resulted in a significant reduction in PA production only when stimulation was applied without changes under basal conditions (Kim et al., 2009). These results suggest that whereas DGKζ converts DAG to PA after synaptic stimulation to maintain DAG at appropriate levels that are required for synaptic plasticity, DGKβ is mainly responsible for lowering DAG levels at the basal state.

Role of DGKι in mGluR-Dependent, Presynaptic LTD at SC-CA1 Synapses

DGKι, which shares a similar domain structure with DGKζ, binds to PSD-95 (Yang et al., 2011). However, unlike DGKζ, DGKι is also present in axon terminals in addition to postsynaptic sites, being detected in the presynaptic plasma membrane and synaptic vesicles (Yang et al., 2011). In DGKι$^{-/-}$ mice, postsynaptic LTP and LTD are not altered at hippocampal SC-CA1 synapses, presumably because DGKβ and DGKζ even in the absence of DGKι are functionally sufficient to regulate the postsynaptic DAG metabolism that is required for synaptic plasticity. In contrast, mGluR-dependent LTD at these synapses is suppressed in the hippocampus of neonatal (2-week old) DGKι$^{-/-}$ mice. It has been shown that mGluR-dependent LTD in SC-CA1 synapses of neonatal mice relies mainly on the reduction of presynaptic release probability (Fitzjohn et al., 2001; Zakharenko et al., 2002; Rammes et al., 2003; Nosyreva and Huber, 2005; Yang et al., 2011). Consistently with the idea that presynaptic LTD is suppressed at DGKι$^{-/-}$ SC-CA1 synapses, mGluR stimulation did not cause an activity-dependent reduction in release probability in these mice (Yang et al., 2011). Furthermore, inhibition of the binding of DAG to its target molecules or inhibition of PKC in DGKι$^{-/-}$ mice rescued mGluR-dependent LTD as well as activity-dependent reduction of release probability (Yang et al., 2011). Thus, DGKι may work during normal mGluR-LTD to remove DAG at presynaptic terminals and to suppress the activity of DAG targets, such as PKC and Munc13, as well as enhancement of neurotransmitter release.

Role of DGKκ in Postsynaptic LTP and LTD at SC-CA1 Synapses

A recent study demonstrated that the reduction of DGKκ expression levels using an shRNA resulted in reduced LTP and increased LTD at hippocampal SC-CA1 synapses (Tabet et al., 2016). This result is similar to the abovementioned reduced LTP observed in DGKβ$^{-/-}$ mice, and suggests that DGKκ may also be required for lowering DAG levels under basal conditions and inducing normal LTP. In line with this idea, in knockout mice lacking the fragile X mental retardation protein where DGKκ translation is impaired, DAG levels are increased under basal conditions, but not after mGluR stimulation. It is possible that DGKκ may cooperate with DGKβ to maintain low levels of DAG under basal conditions for normal induction of LTP at SC-CA1 synapses.

DGKζ IS REQUIRED FOR CEREBELLAR LTD

In addition to hippocampal synaptic plasticity, DGKζ regulates cerebellar LTD that is postsynaptically expressed at the synapses of cerebellar Purkinje cells receiving inputs from parallel fibers, as supported by impaired LTD, but not LTP, in DGKζ$^{-/-}$ mice (Lee et al., 2015). Cerebellar LTD has long been studied, and the importance of PKC in LTD has also been well established (Linden and Connor, 1991; De Zeeuw et al., 1998), with the PKC isoform PKCα being critical (Leitges et al., 2004). Our results showed that DGKζ bound to not only PSD-93, a PSD-95 relative abundant in Purkinje cells, but also to PKCα in Purkinje cells, and such binding functions of DGKζ were required for LTD (Lee et al., 2015). In addition, we have shown that LTD induction causes the dissociation of DGKζ and PKCα, and that the catalytic function of DGKζ is also required for LTD. These results collectively suggest the following mechanisms. PSD-93-bound DGKζ interacts with and promotes the synaptic localization of PKCα, but suppresses PKCα activity under basal conditions by reducing DAG concentrations. When LTD is triggered, PKCα dissociates from DGKζ and gets activated to promote the induction of cerebellar LTD.

GENERAL ROLES OF DGKs IN SYNAPTIC PLASTICITY

As summarized above, 5 different isoforms of mammalian DGKs have so far been reported to be involved in some forms of synaptic plasticity (**Table 1**). Generally, the 10 known isoforms of DGKs are categorized into five types based on their distinct functional domain structures, which display differential distribution patterns in the brain (Ishisaka and Hara, 2014). These diversities in the domain structures and distribution patterns of brain DGKs may influence their distinct subcellular localization, spectrum of binding proteins, and regulation of specific aspects of LTP and LTD.

Although, the current results suggest that individual DGK isoforms distinctly regulate several forms of LTP or LTD, they can be considered to play a conceptually common role in synaptic plasticity. In general, molecules involved in synaptic plasticity fall into two categories based on their functions, namely, mediators or modulators. Mediators are directly responsible for triggering synaptic plasticity, whereas modulators are molecules modulating the ability to trigger synaptic plasticity or playing a permissive role (Citri and Malenka, 2008). Given that all isoforms of DGKs described above do not directly mediate the expression of synaptic plasticity, they should be categorized as modulators. Furthermore, DGKs may be specifically considered as coordinators because they function to prepare synapses to undergo synaptic plasticity. As described above, in some cases, DGKs reduce DAG signaling under basal conditions so that DAG signaling can be effectively enhanced after the induction of synaptic plasticity. In other cases, DGKs control DAG signaling "after" the initiation of the induction of synaptic plasticity. Therefore, DGK-dependent modulation of DAG tones before and after the induction of synaptic plasticity may create synaptic environments appropriate for triggering synaptic plasticity. Such a coordinating role would be accomplished by the ability of DGKs to localize itself at synapses, to bind, recruit, and temporally suppress the activity of downstream effector molecules of DAG, such as PKCα, required for synaptic plasticity, and to timely terminate DAG signaling to suppress excessive induction of synaptic plasticity (**Figure 1**).

REMAINING QUESTIONS

Studies using knockout mice of specific isoforms of DGKs have demonstrated the involvement of DGKs in some forms of synaptic plasticity, and based on these studies, the general role of DGKs in synaptic plasticity has been proposed. The next question that naturally arises is whether other DGK isoforms that are abundantly expressed in the brain, such as DGKα, DGKγ, DGKη, and DGKθ, also play roles in some forms of synaptic plasticity. Future studies to address this question should support or even strengthen the idea that DGKs function as coordinators of synaptic plasticity.

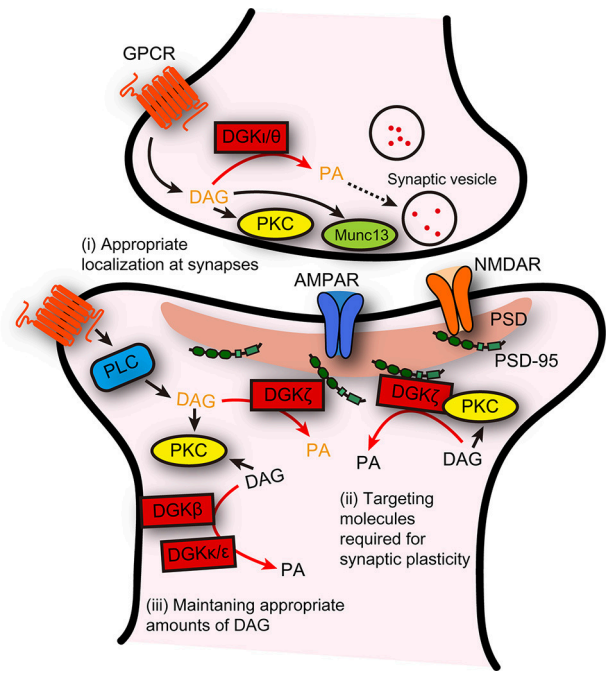


FIGURE 1 | Possible distinct functions of DGK isoforms, and their general role as a coordinator of synaptic plasticity. Distinct functions of individual DGK isoforms in several forms of synaptic plasticity are summarized. In addition, DGKs could be considered to play a general role: DGKs act to create a synaptic environment that is suitable for triggering synaptic plasticity, through their abilities to (i) localize itself appropriately at presynaptic or postsynaptic areas, (ii) interact with and facilitate the synaptic targeting of the molecules required for triggering synaptic plasticity, and (iii) maintain DAG at levels that can adequately contribute to the induction of synaptic plasticity. DAG/PA in black and in dark yellow indicates DAG/PA produced under basal conditions and after stimulation, respectively.

Although, we have described some molecular mechanisms as to how DGK isoforms are involved in synaptic plasticity, there are still several questions regarding the molecular mechanisms, including the two following straightforward ones. The first is why there are three isoforms of DGKs–DGKβ, DGKζ, and DGKκ–that are required for the regulation of synaptic plasticity in hippocampal CA1 synapses. One possibility is that they play distinct roles based on their specific subcellular localizations and catalytic properties: DGKζ localized at synapses via binding with PSD-95 (Kim et al., 2009) may metabolize only high concentrations of DAG produced after synaptic stimulation, while DGKβ and DGKκ may be capable of metabolizing low concentrations of DAG around synapses (**Figure 1**). Although, the subcellular localization of DGKκ in neurons still remains unclear, the reported specific localization of DGKβ at the plasma membrane (Caricasole et al., 2002) may render distinct functions to DGKκ and DGKβ under basal conditions.

The second question is how DGK isoforms are localized to presynaptic terminals to regulate presynaptically expressed synaptic plasticity. DGKι is shown to be involved in presynaptic LTD (Yang et al., 2011). Although, the involvement of DGKθ in synaptic plasticity is not directly tested, DGKθ was shown to regulate synaptic vesicle recycling presumably via the production of PA (Goldschmidt et al., 2016). Except for common structural domains of DGKs (C1 and catalytic domains) DGKι and DGKθ do not share domains that may mediate the interaction with presynaptic molecules. Identification of such mechanisms would further advance our understanding of the DGK-dependent regulation of presynaptically expressed synaptic plasticity.

Considering that DGKs create synaptic environments appropriate for triggering synaptic plasticity, DGKs may be adequate regulators of metaplasticity. Metaplasticity refers to activity-dependent synaptic changes that modulate the ability to induce subsequent synaptic plasticity (Abraham and Bear, 1996). The activities of DGKs likely rely on their localization or posttranslational modifications (Shulga et al., 2011), and DGK protein levels may also alter the overall activity of DAG metabolism. Therefore, it is possible that activity-dependent regulation of DGK protein levels, localization, or modification leads to metaplasticity. Intriguingly, it has recently been shown that the cellular microRNA miR-34a targets DGKζ mRNA, and DGKζ expression was decreased via the stimulation-dependent upregulation of miR-34a in immune cells (Shin et al., 2013). A similar regulation of DGKζ or other DGK isoforms may be achieved at synapses as mechanisms of metaplasticity.

Finally, it has not yet been intensively investigated as to how DGK-dependent regulation of synaptic plasticity contributes to learning and memory, although a study has shown that DGKβ is necessary for hippocampus-dependent spatial reference memory formation (Shirai et al., 2010), for which LTP at CA1 synapses is implicated (Martin et al., 2000). To avoid compensation by other DGKs or other mechanisms in knockout mice, it would be needed to employee additional approaches such as conditional knockout of DGKs, mutations in specific domains, or temporal control of such modifications. Nevertheless, considering the role of DGKs in the coordination of synaptic environments for synaptic plasticity, it would be highly valuable to understand how DGK-dependent regulations of synaptic plasticity affect learning and memory at the behavior level.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Korea Institute of Science and Technology Institutional Program (Project No. 2E26190) and the Institute for Basic Science (IBS-R002-D1).

REFERENCES

Abraham, W. C., and Bear, M. F. (1996). Metaplasticity: the plasticity of synaptic plasticity. *Trends Neurosci.* 19, 126–130. doi: 10.1016/S0166-2236(96)80018-X

Akers, R. F., Lovinger, D. M., Colley, P. A., Linden, D. J., and Routtenberg, A. (1986). Translocation of protein kinase C activity may mediate hippocampal long-term potentiation. *Science* 231, 587–589.

Brose, N., Betz, A., and Wegmeyer, H. (2004). Divergent and convergent signaling by the diacylglycerol second messenger pathway in mammals. *Curr. Opin. Neurobiol.* 14, 328–340. doi: 10.1016/j.conb.2004.05.006

Caricasole, A., Bettini, E., Sala, C., Roncarati, R., Kobayashi, N., Caldara, F., et al. (2002). Molecular cloning and characterization of the human diacylglycerol kinaseβ (DGKβ) gene: alternative splicing generates DGKβ isotypes with different properties. *J. Biol. Chem.* 277, 4790–4796. doi: 10.1074/jbc.M110249200

Castillo, P. E. (2012). Presynaptic LTP and LTD of excitatory and inhibitory synapses. *Cold Spring Harb. Perspect. Biol.* 4:a005728. doi: 10.1101/cshperspect.a005728

Chen, C., Magee, J. C., Marcheselli, V., Hardy, M., and Bazan, N. G. (2001). Attenuated LTP in hippocampal dentate gyrus neurons of mice deficient in the PAF receptor. *J. Neurophysiol.* 85, 384–390.

Christie, B. R., and Abraham, W. C. (1994). Differential regulation of paired-pulse plasticity following LTP in the dentate gyrus. *Neuroreport* 5, 385–388. doi: 10.1097/00001756-199401120-00003

Citri, A., and Malenka, R. C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33, 18–41. doi: 10.1038/sj.npp.1301559

Colino, A., and Malenka, R. C. (1993). Mechanisms underlying induction of long-term potentiation in rat medial and lateral perforant paths *in vitro*. *J. Neurophysiol.* 69, 1150–1159.

De Zeeuw, C. I., Hansel, C., Bian, F., Koekkoek, S. K., van Alphen, A. M., Linden, D. J., et al. (1998). Expression of a protein kinase C inhibitor in Purkinje cells blocks cerebellar LTD and adaptation of the vestibulo-ocular reflex. *Neuron* 20, 495–508. doi: 10.1016/S0896-6273(00)80990-3

Fitzjohn, S. M., Palmer, M. J., May, J. E., Neeson, A., Morris, S. A., and Collingridge, G. L. (2001). A characterisation of long-term depression induced by metabotropic glutamate receptor activation in the rat hippocampus *in vitro*. *J. Physiol.* 537(Pt 2), 421–430. doi: 10.1111/j.1469-7793.2001.00421.x

Gisabella, B., Rowan, M. J., and Anwyl, R. (2003). Mechanisms underlying the inhibition of long-term potentiation by preconditioning stimulation in the hippocampus *in vitro*. *Neuroscience* 121, 297–305. doi: 10.1016/S0306-4522(03)00440-8

Goldschmidt, H. L., Tu-Sekine, B., Volk, L., Anggono, V., Huganir, R. L., and Raben, D. M. (2016). DGKθ catalytic activity is required for efficient recycling of presynaptic vesicles at excitatory synapses. *Cell Rep.* 14, 200–207. doi: 10.1016/j.celrep.2015.12.022

Goto, K., and Kondo, H. (1999). Diacylglycerol kinase in the central nervous system–molecular heterogeneity and gene expression. *Chem. Phys. Lipids* 98, 109–117. doi: 10.1016/S0009-3084(99)00023-7

Hozumi, Y., Fukaya, M., Adachi, N., Saito, N., Otani, K., Kondo, H., et al. (2008). Diacylglycerol kinase β accumulates on the perisynaptic site of medium spiny neurons in the striatum. *Eur. J. Neurosci.* 28, 2409–2422. doi: 10.1111/j.1460-9568.2008.06547.x

Huganir, R. L., and Nicoll, R. A. (2013). AMPARs and synaptic plasticity: the last 25 years. *Neuron* 80, 704–717. doi: 10.1016/j.neuron.2013.10.025

Ishisaka, M., and Hara, H. (2014). The roles of diacylglycerol kinases in the central nervous system: review of genetic studies in mice. *J. Pharmacol. Sci.* 124, 336–343. doi: 10.1254/jphs.13R07CR

Kato, K., and Zorumski, C. F. (1996). Platelet-activating factor as a potential retrograde messenger. *J. Lipid Mediat. Cell Signal.* 14, 341–348. doi: 10.1016/0929-7855(96)00543-3

Kim, K., Yang, J., Zhong, X. P., Kim, M. H., Kim, Y. S., Lee, H. W., et al. (2009). Synaptic removal of diacylglycerol by DGKζ and PSD-95 regulates dendritic spine maintenance. *EMBO J.* 28, 1170–1179. doi: 10.1038/emboj.2009.44

Klann, E., Chen, S. J., and Sweatt, J. D. (1993). Mechanism of protein kinase C activation during the induction and maintenance of long-term potentiation probed using a selective peptide substrate. *Proc. Natl. Acad. Sci. U.S.A.* 90, 8337–8341. doi: 10.1073/pnas.90.18.8337

Kleschevnikov, A. M., and Routtenberg, A. (2001). PKC activation rescues LTP from NMDA receptor blockade. *Hippocampus* 11, 168–175. doi: 10.1002/hipo.1034

Lee, D., Yamamoto, Y., Kim, E., and Tanaka-Yamamoto, K. (2015). Functional and physical interaction of diacylglycerol kinaseζ with protein kinase cα is required for cerebellar long-term depression. *J. Neurosci.* 35, 15453–15465. doi: 10.1523/JNEUROSCI.1991-15.2015

Leitges, M., Kovac, J., Plomann, M., and Linden, D. J. (2004). A unique PDZ ligand in PKCα confers induction of cerebellar long-term synaptic depression. *Neuron* 44, 585–594. doi: 10.1016/j.neuron.2004.10.024

Linden, D. J., and Connor, J. A. (1991). Participation of postsynaptic PKC in cerebellar long-term depression in culture. *Science* 254, 1656–1659.

Malenka, R. C., and Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* 44, 5–21. doi: 10.1016/j.neuron.2004.09.012

Malinow, R., Schulman, H., and Tsien, R. W. (1989). Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. *Science* 245, 862–866.

Martin, S. J., Grimwood, P. D., and Morris, R. G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annu. Rev. Neurosci.* 23, 649–711. doi: 10.1146/annurev.neuro.23.1.649

Min, M. Y., Asztely, F., Kokaia, M., and Kullmann, D. M. (1998). Long-term potentiation and dual-component quantal signaling in the dentate gyrus. *Proc. Natl. Acad. Sci. U.S.A.* 95, 4702–4707. doi: 10.1073/pnas.95.8.4702

Moga, D. E., Shapiro, M. L., and Morrison, J. H. (2006). Bidirectional redistribution of AMPA but not NMDA receptors after perforant path simulation in the adult rat hippocampus *in vivo*. *Hippocampus* 16, 990–1003. doi: 10.1002/hipo.20227

Nosyreva, E. D., and Huber, K. M. (2005). Developmental switch in synaptic mechanisms of hippocampal metabotropic glutamate receptor-dependent long-term depression. *J. Neurosci.* 25, 2992–3001. doi: 10.1523/JNEUROSCI.3652-04.2005

Rammes, G., Palmer, M., Eder, M., Dodt, H.-U., Zieglgänsberger, W., and Collingridge, G. L. (2003). Activation of mGlu receptors induces LTD without affecting postsynaptic sensitivity of CA1 neurons in rat hippocampal slices. *J. Physiol.* 546, 455–460. doi: 10.1113/jphysiol.2002.033514

Reid, C. A., and Clements, J. D. (1999). Postsynaptic expression of long-term potentiation in the rat dentate gyrus demonstrated by variance-mean analysis. *J Physiol.* 518(Pt 1), 121–130. doi: 10.1111/j.1469-7793.1999.0121r.x

Rhee, S. G. (2001). Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* 70, 281–312. doi: 10.1146/annurev.biochem.70.1.281

Rodriguez de Turco, E. B., Tang, W., Topham, M. K., Sakane, F., Marcheselli, V. L., Chen, C., et al. (2001). Diacylglycerol kinase epsilon regulates seizure susceptibility and long-term potentiation through arachidonoyl- inositol lipid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4740–4745. doi: 10.1073/pnas.081536298

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007). Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* 1771, 793–806. doi: 10.1016/j.bbalip.2007.04.006

Seo, J., Kim, K., Jang, S., Han, S., Choi, S. Y., and Kim, E. (2012). Regulation of hippocampal long-term potentiation and long-term depression by diacylglycerol kinase ζ. *Hippocampus* 22, 1018–1026. doi: 10.1002/hipo.20889

Shin, J., Xie, D., and Zhong, X. P. (2013). MicroRNA-34a enhances T cell activation by targeting diacylglycerol kinase ζ. *PLoS ONE* 8:e77983. doi: 10.1371/journal.pone.0077983

Shirai, Y., Kouzuki, T., Kakefuda, K., Moriguchi, S., Oyagi, A., Horie, K., et al. (2010). Essential role of neuron-enriched diacylglycerol kinase (DGK), DGKβ in neurite spine formation, contributing to cognitive function. *PLoS ONE* 5:e11602. doi: 10.1371/journal.pone.0011602

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011). Regulation and functions of diacylglycerol kinases. *Chem. Rev.* 111, 6186–6208. doi: 10.1021/cr1004106

Sossin, W. S., and Farah, C. A. (2009). "Synaptic plasticity: diacylglycerol signalling role A2," in *Encyclopedia of Neuroscience,* ed L. R Squire (Oxford: Academic Press), 747–755.

Tabet, R., Moutin, E., Becker, J. A., Heintz, D., Fouillen, L., Flatter, E., et al. (2016). Fragile X Mental Retardation Protein (FMRP) controls diacylglycerol kinase activity in neurons. *Proc. Natl. Acad. Sci. U.S.A.* 113, E3619–E3628. doi: 10.1073/pnas.1522631113

Thiels, E., Kanterewicz, B. I., Knapp, L. T., Barrionuevo, G., and Klann, E. (2000). Protein phosphatase-mediated regulation of protein kinase C during long-term depression in the adult hippocampus *in vivo*. *J. Neurosci.* 20, 7199–7207.

Tu-Sekine, B., and Raben, D. M. (2011). Regulation and roles of neuronal diacylglycerol kinases: a lipid perspective. *Crit. Rev. Biochem. Mol. Biol.* 46, 353–364. doi: 10.3109/10409238.2011.577761

Wang, S., Wojtowicz, J. M., and Atwood, H. L. (1996). Synaptic recruitment during long-term potentiation at synapses of the medial perforant pathway in the dentate gyrus of the rat brain. *Synapse* 22, 78–86.

Yang, J., Seo, J., Nair, R., Han, S., Jang, S., Kim, K., et al. (2011). DGKι regulates presynaptic release during mGluR-dependent LTD. *EMBO J.* 30, 165–180. doi: 10.1038/emboj.2010.286

Zakharenko, S. S., Zablow, L., and Siegelbaum, S. A. (2002). Altered presynaptic vesicle release and cycling during mGluR-dependent LTD. *Neuron* 35, 1099–1110. doi: 10.1016/S0896-6273(02)00898-X

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 17 August 2016
doi: 10.3389/fcell.2016.00082

Diacylglycerol Kinases as Emerging Potential Drug Targets for a Variety of Diseases: An Update

*Fumio Sakane *, Satoru Mizuno and Suguru Komenoi*

Department of Chemistry, Graduate School of Science, Chiba University, Chiba, Japan

Ten mammalian diacylglycerol kinase (DGK) isozymes (α–κ) have been identified to date. Our previous review noted that several DGK isozymes can serve as potential drug targets for cancer, epilepsy, autoimmunity, cardiac hypertrophy, hypertension and type II diabetes (Sakane et al., 2008). Since then, recent genome-wide association studies have implied several new possible relationships between DGK isozymes and diseases. For example, DGKθ and DGKκ have been suggested to be associated with susceptibility to Parkinson's disease and hypospadias, respectively. In addition, the DGKη gene has been repeatedly identified as a bipolar disorder (BPD) susceptibility gene. Intriguingly, we found that DGKη-knockout mice showed lithium (BPD remedy)-sensitive mania-like behaviors, suggesting that DGKη is one of key enzymes of the etiology of BPD. Because DGKs are potential drug targets for a wide variety of diseases, the development of DGK isozyme-specific inhibitors/activators has been eagerly awaited. Recently, we have identified DGKα-selective inhibitors. Because DGKα has both pro-tumoral and anti-immunogenic properties, the DGKα-selective inhibitors would simultaneously have anti-tumoral and pro-immunogenic (anti-tumor immunogenic) effects. Although the ten DGK isozymes are highly similar to each other, our current results have encouraged us to identify and develop specific inhibitors/activators against every DGK isozyme that can be effective regulators and drugs against a wide variety of physiological events and diseases.

Keywords: diacylglycerol kinase, bipolar disorder, hypospadias, Parkinson's disease, inhibitor, cancer, anti-tumor immunity

OPEN ACCESS

Edited by:
Bin Ren,
Medical College of Wisconsin, USA

Reviewed by:
Francisco Iñesta-Vaquera,
University of Dundee, UK
Tomas Obsil,
Charles University, Czech Republic

****Correspondence:***
Fumio Sakane
sakane@faculty.chiba-u.jp

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 July 2016*
Accepted: *29 July 2016*
Published: *17 August 2016*

Citation:
Sakane F, Mizuno S and Komenoi S (2016) Diacylglycerol Kinases as Emerging Potential Drug Targets for a Variety of Diseases: An Update. Front. Cell Dev. Biol. 4:82. doi: 10.3389/fcell.2016.00082

INTRODUCTION

Mammalian diacylglycerol kinase (DGK) represents a large enzyme family (Goto et al., 2006; Sakane et al., 2007; Mérida et al., 2008; Topham and Epand, 2009). To date, ten mammalian DGK isozymes, α (Sakane et al., 1990; Schaap et al., 1990), β (Goto and Kondo, 1993), γ (Goto et al., 1994; Kai et al., 1994), δ (Sakane et al., 1996), ε (Tang et al., 1996), ζ (Bunting et al., 1996; Goto and Kondo, 1996), η (Klauck et al., 1996), θ (Houssa et al., 1997), ι (Ding et al., 1998), and κ (Imai et al., 2005), have been identified. Moreover, several alternative splicing products—such as δ1 and δ2 (Sakane et al., 2002); η1–η3 (Murakami et al., 2003; Shionoya et al., 2015); ζ1 and ζ2 (Ding et al., 1997), and ι1–ι3 (Ito et al., 2004)—have also been found. These isozymes contain two or three characteristic protein kinase C (PKC)-like C1 domains (cysteine-rich, zinc finger structures) and the catalytic region in common and are subdivided into five groups, type I (α, β and γ), II (δ, η and κ), III (ε), IV (ζ and ι), and

V (θ), according to their structural features (Goto et al., 2006; Sakane et al., 2007; Mérida et al., 2008; Topham and Epand, 2009). Each group is characterized by the subtype-specific functional domains, such as EF-hand motifs (type I), pleckstrin homology and sterile α motif domains (type II), ankyrin repeats (type IV), and ras-associating and pleckstrin homology domains (type V).

Our previous review (Sakane et al., 2008) showed that many interesting studies on DGK have brought DGK to the center stage of diverse biological events such as growth factor/cytokine-dependent cell proliferation and motility, seizure activity, immune responses, cardiovascular responses, and glucose metabolism. Therefore, from a medical point of view, DGK isoforms are implicated in the pathogenesis of a wide variety of diseases, for example, cancer, epilepsy, autoimmunity, cardiac hypertrophy, hypertension, and type II diabetes. Thus, DGKs have emerged as potential and attractive drug targets for curing these diseases.

Recent advances in genotyping technology have allowed for rapid genome-wide screening of common variants in large populations, launching a new era in the investigation of the genetic basis of complex diseases. DGK is no exception. Since our review was published (Sakane et al., 2008), additional interesting reports using genome-wide association studies (GWASs) have successively implied several new possible relationships between DGK isozymes and diseases. For example, DGKη (Baum et al., 2008; Ollila et al., 2009; Squassina et al., 2009; Weber et al., 2011; Zeng et al., 2011), DGKκ (van der Zanden et al., 2011; Carmichael et al., 2013), and DGKθ (Pankratz et al., 2009; Simón-Sánchez et al., 2011) have been suggested to be associated with susceptibility to bipolar disorder (BPD), hypospadias, and Parkinson's disease, respectively.

Among these isozymes, based on the results obtained for the GWASs of *DGKH* (DGKη gene), we recently investigated the relationship between DGKη and BPD. For this purpose, we generated DGKη-knockout (KO) mice and used these mice to perform behavioral and pharmacological tests. Intriguingly, we found that DGKη-knockout mice showed lithium (BPD remedy)-sensitive mania-like behaviors, suggesting that DGKη is one of key enzymes of the pathogenesis of BPD (Isozaki et al., 2016).

As mentioned in our previous review (Sakane et al., 2008), the development of DGK isozyme-specific inhibitors/activators is important both for fundamental research and for developing therapeutic strategies to treat a wide variety of pathological disorders. However, there was no available DGK isozyme-specific inhibitor/activator until recently. We have recently identified DGKα-selective inhibitors using a newly established high-throughput screening method (Sato et al., 2013). Because DGKα has both pro-tumoral and anti-immunogenic properties, the DGKα-selective inhibitors would simultaneously have anti-tumoral and pro-immunogenic (anti-tumor immunogenic) effects.

This mini review will focus primarily on the two abovementioned topics, recent GWASs and the development of DGK isozyme-specific inhibitors.

GWAS—NEW POSSIBLE RELATIONSHIPS BETWEEN DGK ISOZYMES AND DISEASES

DGKη

BPD is a highly heritable neuropsychiatric illness characterized by recurrent episodes of depression and mania or hypomania and affects up to 4% of the adult population worldwide (Bauer and Pfennig, 2005; Merikangas et al., 2007). Approximately 20% of the patients die of suicide (Kilbane et al., 2009). Recent GWASs of BPD have proposed novel genetic candidates, including *DGKH*, which encodes DGKη. Baum et al. for the first time, reported a strong association between BPD and three SNPs (rs9315885, rs1012053, and rs1170191) located in the first intron of *DGKH* by a GWAS in two independent samples of European origin (Baum et al., 2008; **Table 1**). Next, SNP rs9315885 was demonstrated to be associated with BPD in a Finnish family cohort (Ollila et al., 2009). In addition, six SNPs in *DGKH* including rs1170191 were associated with BPD in a German sample as well (Weber et al., 2011). Moreover, an association of *DGKH* with BPD has also been found in Sardinian (Squassina et al., 2009) and Chinese (Zeng et al., 2011) samples at the haplotype level. In addition, another study showed that BPD samples displayed significantly increased *DGKH* gene expression levels (25% higher than in controls; Moya et al., 2010). These data imply that mutations of the *DGKH* gene are involved in BPD. However, other studies have not confirmed this association (Sklar et al., 2008; Tesli et al., 2009; Yosifova et al., 2009). Moreover, GWAS itself does not directly indicate a relationship between SNPs and diseases. Therefore, it has been difficult to definitively conclude whether *DGKH* is related to BPD.

All of the SNPs in *DGKH* that are implicated in the etiology of BPD by GWASs are located in introns and 3′-flank region (**Table 1**). For example, the SNPs rs9315885 and rs1170191, which are identified in multiple independent reports (Baum et al., 2008; Ollila et al., 2009; Weber et al., 2011), are located in the first intron of *DGKH*. Therefore, it is likely that the SNPs lead to dysregulation of the expression and generation of splice variants of DGKη, which probably cause BPD.

DGKη is known to be most abundantly expressed in the brain (Klauck et al., 1996; Usuki et al., 2015). Interestingly, the expression of DGKη increased between 1 and 4 weeks after birth, which coincides with synapse formation in the brain (Usuki et al., 2015). Moreover, a substantial amount of DGKη was detected in layers II–VI of the cerebral cortex; in the CA1, CA2, and dentate gyrus regions of the hippocampus; in the mitral cell and glomerular layer of the olfactory bulb; and in the Purkinje cells in the cerebellum of one—to 32-week-old mice (Usuki et al., 2015).

To test the association between DGKη and BPD, DGKη-KO mice are required. However, the generation of DGKη-KO mice has not been accomplished until recently. In our recent study, we succeeded in generating DGKη-KO mice, and performed a comprehensive behavioral analysis of the mice (Isozaki et al., 2016) to investigate the role of DGKη in higher brain functions and the relationship between this isozyme and BPD. DGKη-KO mice exhibited increased open field activity (the frequency of behavioral switching hyperactivity), increased open field center time/frequency (antianxiety), increased open arm

TABLE 1 | Summary of disease-associated SNPs of *DGKη*, *κ*, *θ*, *γ*, *δ*, and *ι*.

SNP name	Allele	Location	Gene	Disease/medical condition	References
rs9315885	T	13q14.11	DGKη (Intron 1)	BPD	Baum et al., 2008; Ollila et al., 2009
rs1012053	A	13q14.11	DGKη (Intron 1)	BPD	Baum et al., 2008
rs1170191	C/A	13q14.11	DGKη (Intron 1)	BPD	Baum et al., 2008
				UPD	Weber et al., 2011
rs1170169	G	13q14.11	DGKη (Intron 1)	BPD	Weber et al., 2011
				UPD	Weber et al., 2011
				ADHD	Weber et al., 2011
rs2148004	G	13q14.11	DGKη (Intron 1)	UPD	Weber et al., 2011
rs994856	G	13q14.11	DGKη (Intron 3)	BPD	Weber et al., 2011
				UPD	Weber et al., 2011
				ADHD	Weber et al., 2011
rs9525580	A	13q14.11	DGKη (Intron 3)	BPD	Weber et al., 2011
				UPD	Weber et al., 2011
				ADHD	Weber et al., 2011
rs9525584	C	13q14.11	DGKη (Intron 7)	BPD	Weber et al., 2011
				UPD	Weber et al., 2011
rs1170101	G	13q14.11	DGKη (Intron 20)	BPD	Weber et al., 2011
				UPD	Weber et al., 2011
rs347405	C	13q14.11	DGKη (Intron 26)	ADHD	Weber et al., 2011
rs2122246	G	13q14.11	DGKη (Intron 14)	BPD	Zeng et al., 2011
rs1170099	A	13q14.11	DGKη (Intron 20)	SCZ	Zeng et al., 2011
rs1934179	A/G	Xp11.22	DGKκ (Intron 1)	Hypospadias	van der Zanden et al., 2011; Carmichael et al., 2013
rs7063116	A	Xp11.22	DGKκ (5′ upstream)	Hypospadias	van der Zanden et al., 2011; Carmichael et al., 2013
rs5961179	G	Xp11.22	DGKκ (Exon 15, synonymous codon)	Hypospadias	Carmichael et al., 2013
rs7882950	T	Xp11.22	DGKκ (Intron 14)	Hypospadias	Carmichael et al., 2013
rs12556919	T	Xp11.22	DGKκ (Intron 13)	Hypospadias	Carmichael et al., 2013
rs17003341	T	Xp11.22	DGKκ (Intron 10)	Hypospadias	Carmichael et al., 2013
rs1934190	G	Xp11.22	DGKκ (Intron 8)	Hypospadias	Carmichael et al., 2013
rs4143304	T	Xp11.22	DGKκ (Exon 6, synonymous codon)	Hypospadias	Carmichael et al., 2013
rs1934188	T	Xp11.22	DGKκ (Intron 4)	Hypospadias	Carmichael et al., 2013
rs17328236	G	Xp11.22	DGKκ (Intron 1)	Hypospadias	Carmichael et al., 2013
rs9969978	C	Xp11.22	DGKκ (Intron 1)	Hypospadias	Carmichael et al., 2013
rs1934183	T	Xp11.22	DGKκ (Intron 1)	Hypospadias	Carmichael et al., 2013
rs6614511	T	Xp11.22	DGKκ (Intron 1)	Hypospadias	Carmichael et al., 2013
rs5961183	C	Xp11.22	DGKκ (Intron 1)	Hypospadias	Carmichael et al., 2013
rs7876567	T	Xp11.22	DGKκ (Intron 1)	Hypospadias	Carmichael et al., 2013
rs1564282	T/A	4p16.3	DGKθ (3′ downstream)	Parkinson's disease	Pankratz et al., 2009
rs11248060	T/A	4p16.3	DGKθ (Intron 2)	Parkinson's disease	Pankratz et al., 2009
rs7647305	C	3q27.2	DGKγ (3′ downstream)	BMI	Melén et al., 2010
rs6798931	G/C	3q27.2	DGKγ (Intron 19)	BMI	Melén et al., 2010
rs11706414	T/A	3q27.2	DGKγ (3′ downstream)	Asthma	Melén et al., 2010
rs888383	C/G	3q27.2	DGKγ (Intron 19)	Asthma	Melén et al., 2010
rs1550532	C	2q37.1	DGKδ (Intron 1)	Bone density	O'Seaghdha et al., 2013
rs161339	G	7q32.3	DGKι (3′ downstream)	Obesity/BMI	Laramie et al., 2009

BPD, bipolar disorder; UPD, unipolar depression; ADHD, attention deficit hyperactivity disorder; SCZ, schizophrenia; BMI, body mass index.

time/frequency in elevated plus maze (antianxiety), and increased antidepressant-like behavior (Isozaki et al., 2016). Moreover, these phenotypes were sensitive to a BPD remedy, lithium. The behavioral profile (hyperactivity, lower anxiety, lower depressive states, and cognitive impairment) of DGKη-KO mice is similar in behavioral dimensions to BPD patients in the manic state

(Martinowich et al., 2009), including the disappearance of the phenotypes upon lithium treatment. These lithium-sensitive phenotypes have been commonly observed in representative BPD model mice, such as neurocan-KO (Miró et al., 2012), clock-KO (Roybal et al., 2007), glutamate receptor 6-KO (Shaltiel et al., 2008), DGKβ-KO (Kakefuda et al., 2010; Shirai et al., 2010), and glycogen synthase kinase 3β-transgenic (Spittaels et al., 2000; Prickaerts et al., 2006) mice. Therefore, these findings strongly suggest that DGKη is one of the key enzymes related to BPD pathogenesis and support the GWAS results. The lack of availability of suitable animal models of mania has been one of the greatest impediments in the field. Our results indicate that the DGKη-KO mice would represent a bona fide model of human BPD with mania. Therefore, it is likely that these mice are particularly useful for studying the pathophysiology of mania. Moreover, DGKη-specific inhibitors can be good remedies for BPD patients in the depressive state.

DGKη has also been found to be associated with attention deficit hyperactivity disorder (ADHD) by GWAS (Weber et al., 2011). Moreover, mania-like behaviors are similar to ADHD symptoms. Therefore, DGKη-KO mice could also represent a model for ADHD, and there may be a possible link between DGKη and ADHD in addition to BPD (**Table 1**). GWASs have also implied that DGKη is associated with unipolar depression (Weber et al., 2011), and schizophrenia (Zeng et al., 2011). It is also interesting to investigate the relationship between DGKη and unipolar depression/schizophrenia. DGKη may commonly play pivotal roles in the pathology of these four psychoses.

DGKη-KO mice showed impairment in glycogen synthase kinase 3β signaling (Isozaki et al., 2016), which is closely related to BPD (Spittaels et al., 2000; Prickaerts et al., 2006). However, it is still unclear how DGKη is involved in the etiology of BPD. Phosphatidylinositol turnover has been hypothesized to play an important role in the mechanism of action of lithium (Martinowich et al., 2009). DGK is one of the components of phosphatidylinositol turnover (Goto et al., 2006; Sakane et al., 2007; Mérida et al., 2008; Topham and Epand, 2009). Moreover, we recently found that the pleckstrin homology domain of DGKη is selectively and strongly bound to phosphatidylinositol 4,5-bisphosphate, a product of phosphatidylinositol turnover (Kume et al., 2016). We also revealed that DGKη is a unique enzyme with high affinity for DG (Komenoi et al., 2015). In addition, DGKη is a positive regulator of the epidermal growth factor receptor/Raf/MEK/ERK pathway (Yasuda et al., 2009), which drives phosphatidylinositol turnover and is related to BPD (Sklar et al., 2008). It will be interesting to determine what role DGKη plays in the phosphatidylinositol turnover-related, lithium-sensitive molecular mechanisms of BPD pathogenesis.

DGKκ

Hypospadias is a common congenital hypoplasia of the penis, affecting ∼1 in 750 births in Europe. It is believed that hypospadias is caused by sex hormonal disturbances. In fact, genetic polymorphisms in endocrine-related genes such as estrogen receptors have been associated with hypospadias (Ban et al., 2008). To further identify the genetic variants in hypospadias, van der Zanden et al. performed the first GWAS using European samples of anterior or middle hypospadias patients and found that two SNPs, rs1934179 and rs7063116, in *DGKκ*, which mapped to Xp11.22 and encodes DGKκ, exhibited a significant association (van der Zanden et al., 2011; **Table 1**). The authors also found SNPs in *DGKκ* in additional Dutch and Swedish cohorts of anterior or middle hypospadias cases. Carmichael et al. confirmed that *DGKκ* variants are associated with hypospadias in a more racially/ethnically diverse study population of California births (Carmichael et al., 2013). In addition to rs1934179 and rs7063116, several other SNPs in *DGKκ* are associated with the disease. DGKκ mRNA is most abundant in the testis and placenta (Imai et al., 2005), and the study of van der Zanden et al. showed that expression of *DGKκ* was lower in preputial tissues in carriers of the risk allele rs1934179 (van der Zanden et al., 2011). These results indicate that *DGKκ* is a major risk gene for hypospadias.

DGKθ

Parkinson's disease (PD) is a second most common chronic neurodegenerative disease with a cumulative prevalence of greater than one per thousand people (Kuopio et al., 1999). Mutations in five genes have been identified to influence PD risk in fewer than 5% of those with PD (Pankratz and Foroud, 2007). Three genes, *PARK2* (*parkin*), *PARK7* (*DJ1*), and *PINK1*, are typically transmitted with autosomal recessive inheritance and two, *SNCA* and *LRRK2*, are inherited in an autosomal dominant fashion. Mutations in all but *LRRK2* are typically found in early onset PD.

In addition to those five genes, two SNPs, rs1564282 and rs11248060, in the *GAK* (cyclin G associated kinase, a cell cycle regulator)/*DGKQ* (DGKθ) region were repeatedly reported to be associated with PD by Pankratz et al. (2009), and Simón-Sánchez et al. (2011) (**Table 1**). DGKθ is abundantly expressed in the brain (Houssa et al., 1997). Thus, these data suggest the identification of new susceptibility alleles for PD in the *GAK/DGKQ* region.

Other DGK Isozymes

genome-wide association studies have suggested that several other DGK isozymes are associated with diseases and medical conditions as follows: DGKγ: asthma (rs11706414, s888383) and obesity (rs7647305, rs6798931) in children (Melén et al., 2010); DGKδ (rs1550532): bone mineral density (O'Seaghdha et al., 2013); and DGKι (rs161339): obesity/body mass index (Laramie et al., 2009; **Table 1**).

SPECIFIC INHIBITORS FOR DGK ISOZYMES

DGKα (Sakane et al., 1990; Schaap et al., 1990) is highly expressed in hepatocellular carcinoma and melanoma cells (Yanagisawa et al., 2007; Takeishi et al., 2012). DGKα expression is involved in hepatocellular carcinoma progression and is a positive regulator of the proliferative activity of hepatocellular carcinoma through the Ras/Raf/MEK/ERK pathway (Takeishi et al., 2012). In melanoma cells, DGKα positively regulates

tumor necrosis factor-α-dependent nuclear factor-κB (p65) activation via the PKC ζ-mediated Ser311 phosphorylation of p65 (Kai et al., 2009). The growth of colon and breast cancer cell lines was significantly inhibited by DGKα-siRNA and R59949 (Torres-Ayuso et al., 2014). The DGKα/atypical PKC/β1 integrin signaling pathway is essential for matrix invasion of breast carcinoma cells (Rainero et al., 2014). Therefore, the suppression of DGKα activity is expected to inhibit the progression of these cancers. On the other hand, DGKα is abundantly expressed in T lymphocytes, where it facilitates the non-responsive state known as anergy (Olenchock et al., 2006; Zha et al., 2006). Anergy induction in T cells represents the main mechanism by which advanced tumors avoid immune action. Therefore, if a DGKα-selective inhibitor is identified and developed, it would reversely attenuate cancer cell proliferation and simultaneously activate T cell function and can be a dual effective compound.

We started the "Dual effective DGKα-selective inhibitor project" in 2009. To develop highly effective and DGKα-selective inhibitors, a system for high-throughput screening is required; however, the conventional DGK assay is quite laborious and requires technical skill. For example, the conventional assay requires the use of a radioisotope ([γ-^{32}P]ATP) and the manipulation of thin-layer chromatography with multiple extraction steps. We recently established a simple DGK assay (Sato et al., 2013) that is useful for constructing a high-throughput screening system for detecting DGK inhibitors from chemical compound libraries.

We screened a library containing core 9600 compounds (Drug Discovery Initiative, The University of Tokyo) using a high-throughput chemiluminescence-based assay. We obtained several compounds that inhibited the α-isozyme of DGK. Among the compounds, CU-3, 5-[(2E)-3-(2-furyl)prop-2-enylidene]-3-[(phenylsulfonyl)amino]-2-thioxo-1,3-thiazolidin-4-one was identified as a potent and selective inhibitor against the DGKα (Liu et al., 2016). Compared with commercially available DGK inhibitors, such as R59022 and R59949 (Sato et al., 2013), CU-3 exhibited higher efficiency and selectivity against DGKα. The IC_{50} value of CU-3 (0.6 μM) was markedly lower than the values of R59022 and R59949 (~25 and 18 μM, respectively; Sato et al., 2013). R59022 and R59949 only semi-selectively inhibited type I, III and V DGKs α, ε, and θ, and type I and II DGKs α, γ, δ, and κ, respectively (Sato et al., 2013). However, the IC_{50} value of CU-3 for DGKα was at least ~12 times lower than the values for other DGK isozymes. Therefore, this study is the first report of a highly α-isozyme selective inhibitor. The target of CU-3 is the catalytic domain of DGKα, and CU-3 competitively reduced the affinity of DGKα for ATP but not diacylglycerol or phosphatidylserine, strongly suggesting that CU-3 competes with ATP binding.

CU-3 induced apoptosis in HepG2 hepatocellular carcinoma and HeLa cervical cancer cells (Liu et al., 2016). Supporting our results, Torres-Ayuso et al. (Torres-Ayuso et al., 2014) also demonstrated that the growth of colon and breast cancer cell lines was significantly inhibited by DGKα-siRNA and R59949. In addition, Dominguez et al. reported that DGKα-siRNA and R59022 negatively affected the proliferation of glioblastoma, melanoma, breast cancer, and cervical cancer cells (Dominguez et al., 2013). The authors also observed that in marked contrast to cancer cells, R59022 did not weaken the growth of non-cancerous astrocytes and fibroblasts (Dominguez et al., 2013). CU-3 also failed to increase the caspase 3/7 activity of the non-cancer-derived COS-7 cells. These findings suggest that CU-3 selectively induces apoptosis.

In addition to the induction of cancer cell apoptosis, we found that CU-3 promoted IL-2 production, which is one of the indicators of T cell activation. Because inactivation (anergy induction) of T cells is the main mechanism by which advanced tumors to avoid immune action, it is expected that CU-3 is able to activate cancer immunity.

General anti-cancer drugs inhibit the proliferation and function of both cancer and bone marrow cells (Chabner and Roberts, 2005; Pérez-Herrero and Fernández-Medarde, 2015). Therefore, they induce not only the attenuation of cancer cell proliferation but also bone marrow suppression/myelosuppression, which is one of the most commonly observed side-effects of anti-cancer drugs. However, there is no drug that has both pro-tumoral and anti-immunogenic effects. The DGKα-selective inhibitor would simultaneously have anti-tumoral and pro-immunogenic effects (**Figure 1**). Therefore, in addition to the direct effects on apoptosis induction in cancer cells, CU-3 can indirectly induce the death of cancer cells through activation of the immune system. Moreover, CU-3 can be an effective tool for biological science concerning cancer and immunity.

CU-3 still does not have sufficient isozyme selectivity and efficiency as an excellent inhibitor. Moreover, comprehensive studies where other kinase groups are tested have not been performed. Further refinement of CU-3 and/or identification/development of new candidates using larger chemical compound libraries are required. Finally, our current results encourage us to identify and develop specific

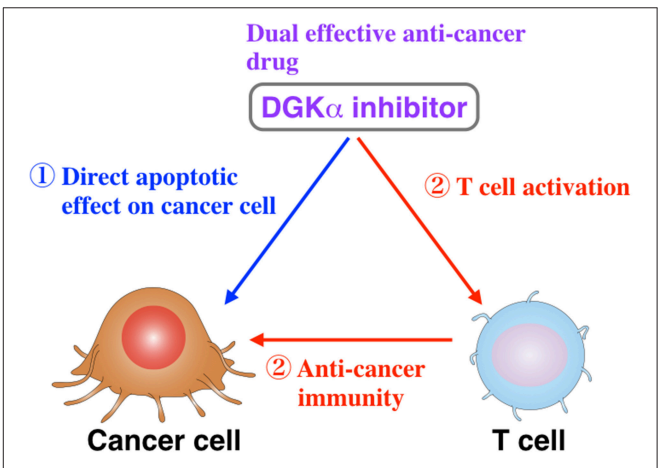


FIGURE 1 | A DGKα-selective inhibitor would directly attenuate cancer cell proliferation and simultaneously activate T cell function, which includes anti-tumor immunogenic activity (Liu et al., 2016).

inhibitors/activators against every DGK isozyme that can be effective regulators and drugs against a wide variety of physiological events and diseases, although the ten DGK isozymes are highly similar to each other.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Ban, S., Sata, F., Kurahashi, N., Kasai, S., Moriya, K., Kakizaki, H., et al. (2008). Genetic polymorphisms of ESR1 and ESR2 that may influence estrogen activity and the risk of hypospadias. *Hum. Reprod.* 23, 1466–1471. doi: 10.1093/humrep/den098

Bauer, M., and Pfennig, A. (2005). Epidemiology of bipolar disorders. *Epilepsia* 46(Suppl. 4), 8–13. doi: 10.1111/j.1528-1167.2005.463003.x

Baum, A. E., Akula, N., Cabanero, M., Cardona, I., Corona, W., Klemens, B., et al. (2008). A genome-wide association study implicates diacylglycerol kinase eta (DGKH) and several other genes in the etiology of bipolar disorder. *Mol. Psychiatry* 13, 197–207. doi: 10.1038/sj.mp.4002012

Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Molecular cloning and characterization of a novel human diacylglycerol kinase zeta. *J. Biol. Chem.* 271, 10230–10236. doi: 10.1074/jbc.271.17.10237

Carmichael, S. L., Mohammed, N., Ma, C., Iovannisci, D., Choudhry, S., Baskin, L. S., et al. (2013). Diacylglycerol kinase K variants impact hypospadias in a California study population. *J. Urol.* 189, 305–311. doi: 10.1016/j.juro.2012.09.002

Chabner, B. A., and Roberts, T. G. Jr. (2005). Timeline: chemotherapy and the war on cancer. *Nat. Rev. Cancer* 5, 65–72. doi: 10.1038/nrc1529

Ding, L., Traer, E., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1998). The cloning and characterization of a novel human diacylglycerol kinase, DGKiota. *J. Biol. Chem.* 273, 32746–32752. doi: 10.1074/jbc.273.49.32746

Ding, L., Bunting, M., Topham, M. K., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1997). Alternative splicing of the human diacylglycerol kinase ζ gene in muscle. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5519–5524. doi: 10.1073/pnas.94.11.5519

Dominguez, C. L., Floyd, D. H., Xiao, A., Mullins, G. R., Kefas, B. A., Xin, W., et al. (2013). Diacylglycerol kinase α is a critical signaling node and novel therapeutic target in glioblastoma and other cancers. *Cancer Discov.* 3, 782–797. doi: 10.1158/2159-8290.CD-12-0215

Goto, K., and Kondo, H. (1993). Molecular cloning and expression of a 90-kDa diacylglycerol kinase that predominantly localizes in neurons. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7598–7602. doi: 10.1073/pnas.90.16.7598

Goto, K., and Kondo, H. (1996). A 104-kDa diacylglycerol kinase containing ankylin-like repeats localizes in the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11196–11201. doi: 10.1073/pnas.93.20.11196

Goto, K., Funayama, M., and Kondo, H. (1994). Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* 91, 13042–13046. doi: 10.1073/pnas.91.26.13042

Goto, K., Hozumi, Y., and Kondo, H. (2006). Diacylglycerol, phosphatidic acid, and the converting enzyme, diacylglycerol kinase, in the nucleus. *Biochim. Biophys. Acta* 1761, 535–541. doi: 10.1016/j.bbalip.2006.04.001

Houssa, B., Schaap, D., van der Val, J., Goto, K., Kondo, H., Yamakawa, A., et al. (1997). Cloning of a Novel Human Diacylglycerol Kinase (DGKθ) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping ras-associating domain. *J. Biol. Chem.* 272, 10422–10428. doi: 10.1074/jbc.272.16.10422

Imai, S., Kai, M., Yasuda, S., Kanoh, H., and Sakane, F. (2005). Identification and characterization of a novel human type II diacylglycerol kinase, DGKκ. *J. Biol. Chem.* 280, 39870–39881. doi: 10.1074/jbc.M500669200

Isozaki, T., Komenoi, S., Lu, Q., Usuki, T., Tomokata, S., Matsutomo, D., et al. (2016). Deficiency of diacylglycerol kinase eta induces lithium-sensitive mania-like behavior. *J. Neurochem.* 138, 448–456. doi: 10.1111/jnc.13661

Ito, T., Hozumi, Y., Sakane, F., Saino-Saito, S., Kanoh, H., Aoyagi, M., et al. (2004). Cloning and characterization of diacylglycerol kinase ι splice variants in rat brain. *J. Biol. Chem.* 279, 23317–23326. doi: 10.1074/jbc.M312976200

Kai, M., Sakane, F., Imai, S., Wada, I., and Kanoh, H. (1994). Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells. *J. Biol. Chem.* 269, 18492–18498.

Kai, M., Yasuda, S., Imai, S., Toyota, M., Kanoh, H., and Sakane, F. (2009). Diacylglycerol kinase α enhances protein kinase Cζ-dependent phosphorylation at Ser311 of p65/RelA subunit of nuclear factor-κB. *FEBS Lett.* 583, 3265–3268. doi: 10.1016/j.febslet.2009.09.017

Kakefuda, K., Oyagi, A., Ishisaka, M., Tsuruma, K., Shimazawa, M., Yokota, K., et al. (2010). Diacylglycerol kinase β knockout mice exhibit lithium-sensitive behavioral abnormalities. *PLoS ONE* 5:e13447. doi: 10.1371/journal.pone.0013447

Kilbane, E. J., Gokbayrak, N. S., Galynker, I., Cohen, L., and Tross, S. (2009). A review of panic and suicide in bipolar disorder: does comorbidity increase risk? *J. Affect. Disord.* 115, 1–10. doi: 10.1016/j.jad.2008.09.014

Klauck, T. M., Xu, X., Mousseau, B., and Jaken, S. (1996). Cloning and characterization of a glucocorticoid-induced diacylglycerol kinase. *J. Biol. Chem.* 271, 19781–19788. doi: 10.1074/jbc.271.33.19781

Komenoi, S., Takemura, F., Sakai, H., and Sakane, F. (2015). Diacylglycerol kinase η1 is a high affinity isozyme for diacylglycerol. *FEBS Lett.* 589, 1272–1277. doi: 10.1016/j.febslet.2015.03.032

Kume, A., Kawase, K., Komenoi, S., Usuki, T., Takeshita, E., Sakai, H., et al. (2016). The pleckstrin homology domain of diacylglycerol kinase eta strongly and selectively binds to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* 291, 8150–8161. doi: 10.1074/jbc.M115.648717

Kuopio, A. M., Marttila, R. J., Helenius, H., and Rinne, U. K. (1999). Changing epidemiology of Parkinson's disease in southwestern Finland. *Neurology* 52, 302–308. doi: 10.1212/WNL.52.2.302

Laramie, J. M., Wilk, J. B., Williamson, S. L., Nagle, M. W., Latourelle, J. C., Tobin, J. E., et al. (2009). Multiple genes influence BMI on chromosome 7q31-34: the NHLBI Family Heart Study. *Obesity* 17, 2182–2189. doi: 10.1038/oby.2009.141

Liu, K., Kunii, N., Sakuma, M., Yamaki, A., Mizuno, S., Sato, M., et al. (2016). A novel diacylglycerol kinase α-selective inhibitor, CU-3, induces cancer cell apoptosis and enhances immune response. *J. Lipid Res.* 57, 368–379. doi: 10.1194/jlr.M062794

Martinowich, K., Schloesser, R. J., and Manji, H. K. (2009). Bipolar disorder: from genes to behavior pathways. *J. Clin. Invest.* 119, 726–736. doi: 10.1172/JCI37703

Melén, E., Himes, B. E., Brehm, J. M., Boutaoui, N., Klanderman, B. J., Sylvia, J. S., et al. (2010). Analyses of shared genetic factors between asthma and obesity in children. *J. Allergy Clin. Immunol.* 126, 631–637.e631–e638. doi: 10.1016/j.jaci.2010.06.030

Mérida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* 409, 1–18. doi: 10.1042/BJ20071040

Merikangas, K. R., Akiskal, H. S., Angst, J., Greenberg, P. E., Hirschfeld, R. M., Petukhova, M., et al. (2007). Lifetime and 12-month prevalence of bipolar spectrum disorder in the National Comorbidity Survey replication. *Arch. Gen. Psychiatry* 64, 543–552. doi: 10.1001/archpsyc.64.5.543

Miró, X., Meier, S., Dreisow, M. L., Frank, J., Strohmaier, J., Breuer, R., et al. (2012). Studies in humans and mice implicate neurocan in the etiology of mania. *Am. J. Psychiatry* 169, 982–990. doi: 10.1176/appi.ajp.2012.11101585

Moya, P. R., Murphy, D. L., McMahon, F. J., and Wendland, J. R. (2010). Increased gene expression of diacylglycerol kinase eta in bipolar disorder.

Int. J. Neuropsychopharmacol. 13, 1127–1128. doi: 10.1017/S1461145710000593

Murakami, T., Sakane, F., Imai, S., Houkin, K., and Kanoh, H. (2003). Identification and characterization of two splice variants of human diacylglycerol kinase η. *J. Biol. Chem.* 278, 34364–34372. doi: 10.1074/jbc.M301542200

O'Seaghdha, C. M., Wu, H., Yang, Q., Kapur, K., Guessous, I., Zuber, A. M., et al. (2013). Meta-analysis of genome-wide association studies identifies six new Loci for serum calcium concentrations. *PLoS Genet.* 9:e1003796. doi: 10.1371/journal.pgen.1003796

Olenchock, B. A., Guo, R., Carpenter, J. H., Jordan, M., Topham, M. K., Koretzky, G. A., et al. (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* 7, 1174–1181. doi: 10.1038/ni1400

Ollila, H. M., Soronen, P., Silander, K., Palo, O. M., Kieseppä, T., Kaunisto, M. A., et al. (2009). Findings from bipolar disorder genome-wide association studies replicate in a Finnish bipolar family-cohort. *Mol. Psychiatry* 14, 351–353. doi: 10.1038/mp.2008.122

Pankratz, N., and Foroud, T. (2007). Genetics of Parkinson disease. *Genet. Med.* 9, 801–811. doi: 10.1097/GIM.0b013e31815bf97c

Pankratz, N., Wilk, J. B., Latourelle, J. C., DeStefano, A. L., Halter, C., Pugh, E. W., et al. (2009). Genomewide association study for susceptibility genes contributing to familial Parkinson disease. *Hum. Genet.* 124, 593–605. doi: 10.1007/s00439-008-0582-9

Pérez-Herrero, E., and Fernández-Medarde, A. (2015). Advanced targeted therapies in cancer: drug nanocarriers, the future of chemotherapy. *Eur. J. Pharm. Biopharm.* 93, 52–79. doi: 10.1016/j.ejpb.2015.03.018

Prickaerts, J., Moechars, D., Cryns, K., Lenaerts, I., van Craenendonck, H., Goris, I., et al. (2006). Transgenic mice overexpressing glycogen synthase kinase 3beta: a putative model of hyperactivity and mania. *J. Neurosci.* 26, 9022–9029. doi: 10.1523/JNEUROSCI.5216-05.2006

Rainero, E., Cianflone, C., Porporato, P. E., Chianale, F., Malacarne, V., Bettio, V., et al. (2014). The diacylglycerol kinase alpha/atypical PKC/beta1 integrin pathway in SDF-1alpha mammary carcinoma invasiveness. *PLoS ONE* 9:e97144. doi: 10.1371/journal.pone.0097144

Roybal, K., Theobold, D., Graham, A., DiNieri, J. A., Russo, S. J., Krishnan, V., et al. (2007). Mania-like behavior induced by disruption of CLOCK. *Proc. Natl. Acad. Sci. U.S.A.* 104, 6406–6411. doi: 10.1073/pnas.0609625104

Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C., and Tanabe, T. (1990). Porcine diacylglycerol kinase sequence has zinc finger and E-F hand motifs. *Nature* 344, 345–348. doi: 10.1038/344345a0

Sakane, F., Imai, S., Kai, M., Wada, I., and Kanoh, H. (1996). Molecular cloning of a novel diacylglycerol kinase isozyme with a pleckstrin homology domain and a C-terminal tail similar to those of the EPH family of protein tyrosine kinase. *J. Biol. Chem.* 271, 8394–8401. doi: 10.1074/jbc.271.14.8394

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007). Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* 1771, 793–806. doi: 10.1016/j.bbalip.2007.04.006

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2008). Diacylglycerol kinases as emerging potential drug targets for a variety of diseases. *Curr. Drug Targets* 9, 626–640. doi: 10.2174/138945008785132394

Sakane, F., Imai, S., Yamada, K., Murakami, T., Tsushima, S., and Kanoh, H. (2002). Alternative splicing of the human diacylglycerol kinase δ gene generates two isoforms differing in their expression patterns and in regulatory functions. *J. Biol. Chem.* 277, 43519–43526. doi: 10.1074/jbc.M206895200

Sato, M., Liu, K., Sasaki, S., Kunii, N., Sakai, H., Mizuno, H., et al. (2013). Evaluations of the selectivities of the diacylglycerol kinase inhibitors R59022 and R59949 among diacylglycerol kinase isozymes using a new non-radioactive assay method. *Pharmacology* 92, 99–107. doi: 10.1159/000351849

Schaap, D., de Widt, J., van der Wal, J., Vandekerckhove, J., van Damme, J., Gussow, D., et al. (1990). Purification, cDNA-cloning and expression of human diacylglycerol kinase. *FEBS Lett.* 275, 151–158. doi: 10.1016/0014-5793(90)81461-V

Shaltiel, G., Maeng, S., Malkesman, O., Pearson, B., Schloesser, R. J., Tragon, T., et al. (2008). Evidence for the involvement of the kainate receptor subunit GluR6 (GRIK2) in mediating behavioral displays related to behavioral symptoms of mania. *Mol. Psychiatry* 13, 858–872. doi: 10.1038/mp.2008.20

Shionoya, T., Usuki, T., Komenoi, S., Isozaki, T., Sakai, H., and Sakane, F. (2015). Distinct expression and localization of the Type II diacylglycerol kinase isozymes δ, η and κ in the mouse reproductive organs. *BMC Dev. Biol.* 15:6. doi: 10.1186/s12861-015-0055-z

Shirai, Y., Kouzuki, T., Kakefuda, K., Moriguchi, S., Oyagi, A., Horie, K., et al. (2010). Essential role of neuron-enriched diacylglycerol kinase (DGK), DGKβ in neurite spine formation, contributing to cognitive function. *PLoS ONE* 5:e11602. doi: 10.1371/journal.pone.0011602

Simón-Sánchez, J., van Hilten, J. J., van de Warrenburg, B., Post, B., Berendse, H. W., Arepalli, S., et al. (2011). Genome-wide association study confirms extant PD risk loci among the Dutch. *Eur. J. Hum. Genet.* 19, 655–661. doi: 10.1038/ejhg.2010.254

Sklar, P., Smoller, J. W., Fan, J., Ferreira, M. A., Perlis, R. H., Chambert, K., et al. (2008). Whole-genome association study of bipolar disorder. *Mol. Psychiatry* 13, 558–569. doi: 10.1038/sj.mp.4002151

Spittaels, K., Van den Haute, C., Van Dorpe, J., Geerts, H., Mercken, M., Bruynseels, K., et al. (2000). Glycogen synthase kinase-3beta phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. *J. Biol. Chem.* 275, 41340–41349. doi: 10.1074/jbc.M006219200

Squassina, A., Manchia, M., Congiu, D., Severino, G., Chillotti, C., Ardau, R., et al. (2009). The diacylglycerol kinase eta gene and bipolar disorder: a replication study in a Sardinian sample. *Mol. Psychiatry* 14, 350–351. doi: 10.1038/mp.2008.93

Takeishi, K., Taketomi, A., Shirabe, K., Toshima, T., Motomura, T., Ikegami, T., et al. (2012). Diacylglycerol kinase alpha enhances hepatocellular carcinoma progression by activation of Ras-Raf-MEK-ERK pathway. *J. Hepatol.* 57, 77–83. doi: 10.1016/j.jhep.2012.02.026

Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. *J. Biol. Chem.* 271, 10237–10241. doi: 10.1074/jbc.271.17.10230

Tesli, M., Kähler, A. K., Andreassen, B. K., Werge, T., Mors, O., Mellerup, E., et al. (2009). No association between DGKH and bipolar disorder in a Scandinavian case-control sample. *Psychiatr. Genet.* 19, 269–272. doi: 10.1097/YPG.0b013e32832d302f

Topham, M. K., and Epand, R. M. (2009). Mammalian diacylglycerol kinases: molecular interactions and biological functions of selected isoforms. *Biochim. Biophys. Acta* 1790, 416–424. doi: 10.1016/j.bbagen.2009.01.010

Torres-Ayuso, P., Daza-Martín, M., Martín-Pérez, J., Ávila-Flores, A., and Mérida, I. (2014). Diacylglycerol kinase α promotes 3D cancer cell growth and limits drug sensitivity through functional interaction with Src. *Oncotarget* 5, 9710–9726. doi: 10.18632/oncotarget.2344

Usuki, T., Sakai, H., Shionoya, T., Sato, N., and Sakane, F. (2015). Expression and localization of type II diacylglycerol kinase isozymes delta and eta in the developing mouse brain. *J. Histochem. Cytochem.* 63, 57–68. doi: 10.1369/0022155414559130

van der Zanden, L. F., van Rooij, I. A., Feitz, W. F., Knight, J., Donders, A. R., Renkema, K. Y., et al. (2011). Common variants in DGKκ are strongly associated with risk of hypospadias. *Nat. Genet.* 43, 48–50. doi: 10.1038/ng.721

Weber, H., Kittel-Schneider, S., Gessner, A., Domschke, K., Neuner, M., Jacob, C. P., et al. (2011). Cross-disorder analysis of bipolar risk genes: further evidence of DGKH as a risk gene for bipolar disorder, but also unipolar depression and adult ADHD. *Neuropsychopharmacology* 36, 2076–2085. doi: 10.1038/npp.2011.98

Yanagisawa, K., Yasuda, S., Kai, M., Imai, S., Yamada, K., Yamashita, T., et al. (2007). Diacylglycerol kinase α suppresses tumor necrosis factor-α-induced apoptosis of human melanoma cells through NF-κB activation. *Biochim. Biophys. Acta* 1771, 462–474. doi: 10.1016/j.bbalip.2006.12.008

Yasuda, S., Kai, M., Imai, S., Takeishi, K., Taketomi, A., Toyota, M., et al. (2009). Diacylglycerol kinase η augments C-Raf activity and B-Raf/C-Raf heterodimerization. *J. Biol. Chem.* 284, 29559–29570. doi: 10.1074/jbc.M109.043604

Yosifova, A., Mushiroda, T., Stoianov, D., Vazharova, R., Dimova, I., Karachanak, S., et al. (2009). Case-control association study of 65 candidate genes revealed a possible association of a SNP of HTR5A to be a factor susceptible to

bipolar disease in Bulgarian population. *J. Affect. Disord.* 117, 87–97. doi: 10.1016/j.jad.2008.12.021

Zeng, Z., Wang, T., Li, T., Li, Y., Chen, P., Zhao, Q., et al. (2011). Common SNPs and haplotypes in DGKH are associated with bipolar disorder and schizophrenia in the Chinese Han population. *Mol. Psychiatry* 16, 473–475. doi: 10.1038/mp.2010.86

Zha, Y., Marks, R., Ho, A. W., Peterson, A. C., Janardhan, S., Brown, I., et al. (2006). T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α. *Nat. Immunol.* 7, 1166–1173. doi: 10.1038/ni1394

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 18 October 2016
doi: 10.3389/fcell.2016.00112

Diacylglycerol Kinase-ε: Properties and Biological Roles

Richard M. Epand[1], Vincent So[1,2], William Jennings[1], Bijendra Khadka[1], Radhey S. Gupta[1] and Mathieu Lemaire[2,3,4]**

[1] Department of Biochemistry and Biomedical Sciences, McMaster University Health Sciences Centre, Hamilton, ON, Canada, [2] Nephrology Division and Cell Biology Program, Hospital for Sick Children, Toronto, ON, Canada, [3] Department of Biochemistry, University of Toronto, Toronto, ON, Canada, [4] Institute of Medicine, University of Toronto, Toronto, ON, Canada

In mammals there are at least 10 isoforms of diacylglycerol kinases (DGK). All catalyze the phosphorylation of diacylglycerol (DAG) to phosphatidic acid (PA). Among DGK isoforms, DGKε has several unique features. It is the only DGK isoform with specificity for a particular species of DAG, i.e., 1-stearoyl-2-arachidonoyl glycerol. The smallest of all known DGK isoforms, DGKε, is also the only DGK devoid of a regulatory domain. DGKε is the only DGK isoform that has a hydrophobic segment that is predicted to form a transmembrane helix. As the only membrane-bound, constitutively active DGK isoform with exquisite specificity for particular molecular species of DAG, the functional overlap between DGKε and other DGKs is predicted to be minimal. DGKε exhibits specificity for DAG containing the same acyl chains as those found in the lipid intermediates of the phosphatidylinositol-cycle. It has also been shown that DGKε affects the acyl chain composition of phosphatidylinositol in whole cells. It is thus likely that DGKε is responsible for catalyzing one step in the phosphatidylinositol-cycle. Steps of this cycle take place in both the plasma membrane and the endoplasmic reticulum membrane. DGKε is likely present in both of these membranes. DGKε is the only DGK isoform that is associated with a human disease. Indeed, recessive loss-of-function mutations in DGKε cause atypical hemolytic-uremic syndrome (aHUS). This condition is characterized by thrombosis in the small vessels of the kidney. It causes acute renal insufficiency in infancy and most patients develop end-stage renal failure before adulthood. Disease pathophysiology is poorly understood and there is no therapy. There are also data suggesting that DGKε may play a role in epilepsy and Huntington disease. Thus, DGKε has many unique molecular and biochemical properties when compared to all other DGK isoforms. DGKε homologs also contain a number of conserved sequence features that are distinctive characteristics of either the rodents or specific groups of primate homologs. How cells, tissues and organisms harness DGKε's catalytic prowess remains unclear. The discovery of DGKε's role in causing aHUS will hopefully boost efforts to unravel the mechanisms by which DGKε dysfunction causes disease.

Keywords: diacylglycerol kinase-ε, phosphatidylinositol cycle, lipid acyl chains, atypical hemolytic-uremic syndrome, re-entrant helix, arachidonic acid, rodents- and primates-specific signatures

OPEN ACCESS

Edited by:
Isabel Merida, Centro Nacional de Biotecnología (CSIC), Spain

Reviewed by:
Pedro A. Lazo, Instituto de Biología Molecular y Celular del Cancer (CSIC), Spain
Fumio Sakane, Chiba University, Japan

****Correspondence:***
Richard M. Epand
epand@mcmaster.ca
Mathieu Lemaire
mathieu.lemaire@sickkids.ca

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *19 July 2016*
Accepted: *27 September 2016*
Published: *18 October 2016*

Citation:
Epand RM, So V, Jennings W, Khadka B, Gupta RS and Lemaire M (2016) Diacylglycerol Kinase-ε: Properties and Biological Roles. Front. Cell Dev. Biol. 4:112. doi: 10.3389/fcell.2016.00112

INTRODUCTION

Among the many isoforms and gene-splicing variants of mammalian DGK, the DGKε isoform is one of the most unique in its properties. DGKε is the smallest known isoform, it is the only one with no domain for binding a specific ligand, it is the only form that has a predicted transmembrane segment, and it is unique in having specificity for the acyl chain composition of the substrate. Mammalian DGKs can be divided into 5 types. DGKε is the only Type 3 isoform (**Figure 1**).

DGKε may also have a unique functional role in catalyzing one of the steps in the phosphatidylinositol-cycle (PI-cycle). The importance of the biological role of DGKε is suggested by the fact that it is the only DGK isoform that is associated with a human disease, namely atypical hemolytic uremic syndrome (aHUS).

INTERACTION WITH DIACYLGLYCEROL

Atypical C1 Domains

DGK catalyzes the reaction between DAG and ATP to produce PA and ADP. The C1 domains of protein kinase C bind DAG and phorbol esters directly via interactions mediated by select residues (Colón-González and Kazanietz, 2006). Amino acid sequence analysis revealed that all mammalian DGKs harbor at least two segments homologous to the prototypical C1 domain (Hurley et al., 1997). Multiple teams independently demonstrated that for most DGKs, these domains do not bind DAG or phorbol esters (Ahmed et al., 1991; Sakane et al., 1996); [DGKγ (Shindo et al., 2001, 2003) and DGKβ (Shindo et al., 2003) are notable exceptions]. Interestingly, some truncated DGKs devoid of all C1 domains have preserved catalytic activity (Sakane et al., 1996). Data suggest that DGKε may be unique among these DGKs: truncation resulted in complete abrogation of 1-stearoyl-2-arachidonoyl glycerol (SAG) phosphorylation (Tang et al., 1996). The biological function of these "atypical C1 domains" (Hurley et al., 1997) remains elusive to this day.

Specificity for DAG with Certain Acyl Chains

Except for DGKε, all other isoforms of DGK phosphorylate DAG at rates that are largely independent of the nature of the acyl chains of DAG (Topham and Prescott, 1999). In contrast, the reaction catalyzed by DGKε is very sensitive to the acyl chains displayed by DAG: its peak activity is when the DAG substrate is SAG (Tang et al., 1996; Pettitt and Wakelam, 1999). The DAG molecule harboring these specific acyl chains is a critical lipid intermediate of the PI-cycle (**Figure 2**). In addition to DGKε, another enzyme that has specificity for lipid substrates with 1-stearoyl-2-arachidonoyl is CDP-diacylglycerol synthase 2 (CDS2) (D'Souza et al., 2014). The possible role of these two enzymes in enriching the lipid intermediates of the PI-cycle is discussed below. In addition, acyl chain remodeling of phosphatidylinositol (PI) through the Land's cycle, also contributes to acyl chain enrichment (Gijón et al., 2008).

In vitro Detergent-Based Assays

Data supporting DGKε's exquisite substrate specificity remain incomplete. *In vitro* assays used to test its activity all rely on simultaneous co-solubilization with detergent of DGKε from cells together with DAG (Epand and Topham, 2007). A recent report suggests that the detergent used in these assays can exert a strong influence on the degree of substrate specificity (Natalini et al., 2013). The use of membrane bilayers in the form of liposomes would avoid possible artifacts caused by the presence of detergent and would more closely simulate a biological membrane. Until now, we have not been able to develop a liposome-based assay using extracts from cells overexpressing DGKε. However, we have recently succeeded in purifying human DGKε, thereby facilitating its incorporation into liposomes: this will be the first detergent-free enzyme activity assay for DGKε. We anticipate that the liposome-based assays will confirm the specificity of DGKε for SAG since this substrate specificity has also been demonstrated *in vivo* (see below) (Rodriguez de Turco et al., 2001; Milne et al., 2008).

sn-2 Arachidonoyl Specificity

There have been more recent studies showing that the preference for an arachidonoyl chain in the *sn-2* position of DAG is very specific (Shulga et al., 2011a). The ability of several species of DAG having a stearoyl chain at the *sn-1* position and a polyunsaturated acyl chain at the *sn-2* position to act as a substrate for DGKε was determined. The activity of DGKε when presented with 18:0/20:4-DAG was ∼5-fold higher than 18:0/18:2-DAG, and DGKε is unable to phosphorylate 18:0/22:6-DAG (Shulga et al., 2011a). On the basis of these results and others, it is clear that maximal activity requires that the DAG substrate have an arachidonoyl chain (20:4) at the *sn-2* position. We propose that the arachidonoyl group at the *sn-2* position fits into a specific binding site in DGKε.

sn-1 Stearoyl Specificity

While an arachidonoyl group at the *sn-2* position is critical to DGKε activity by itself, it is not sufficient to make a good lipid substrate for DGKε. The monoglyceride 2-arachidonoyl-glycerol is a poor substrate for DGKε, and can even act as an inhibitor at higher concentrations (Gantayet et al., 2011). DGKε has essentially no activity in phosphorylating 1-monoacylglycerol substrates, but it does have 8% of the activity of SAG in phosphorylating 2-monoacylglycerol. However, this activity against monoglycerides, is not very different for DGKε compared with several other mammalian DGK isoforms (Sato et al., 2016). In addition, changing only the nature of the bond

Abbreviations: aHUS, atypical hemolytic-uremic syndrome; arrb, arrestinβ; CDS, CDP-DAG synthase (Phosphatidatecytidylyltransferase); DGK, diacylglycerol kinase; DAG, diacylglycerol; ECS, electroconvulsive shock; ER, endoplasmic reticulum; GK, glycerol kinase; GPCR, G-protein coupled receptor; HD, Huntington's disease; Htt, Huntington protein; MEFs, mouse embryo fibroblasts; NeoR, neomycin resistant; PA, phosphatidic acid; PI, phosphatidylinositol; PI-cycle, phosphatidylinositol-cycle; PIP2, phosphatidylinositol-(4,5)-bisphosphate; PIP_n, phosphorylated forms of PI; SAG, 1-stearoyl-2-arachidonoyl glycerol; TH, tyrosine hydroxylase.

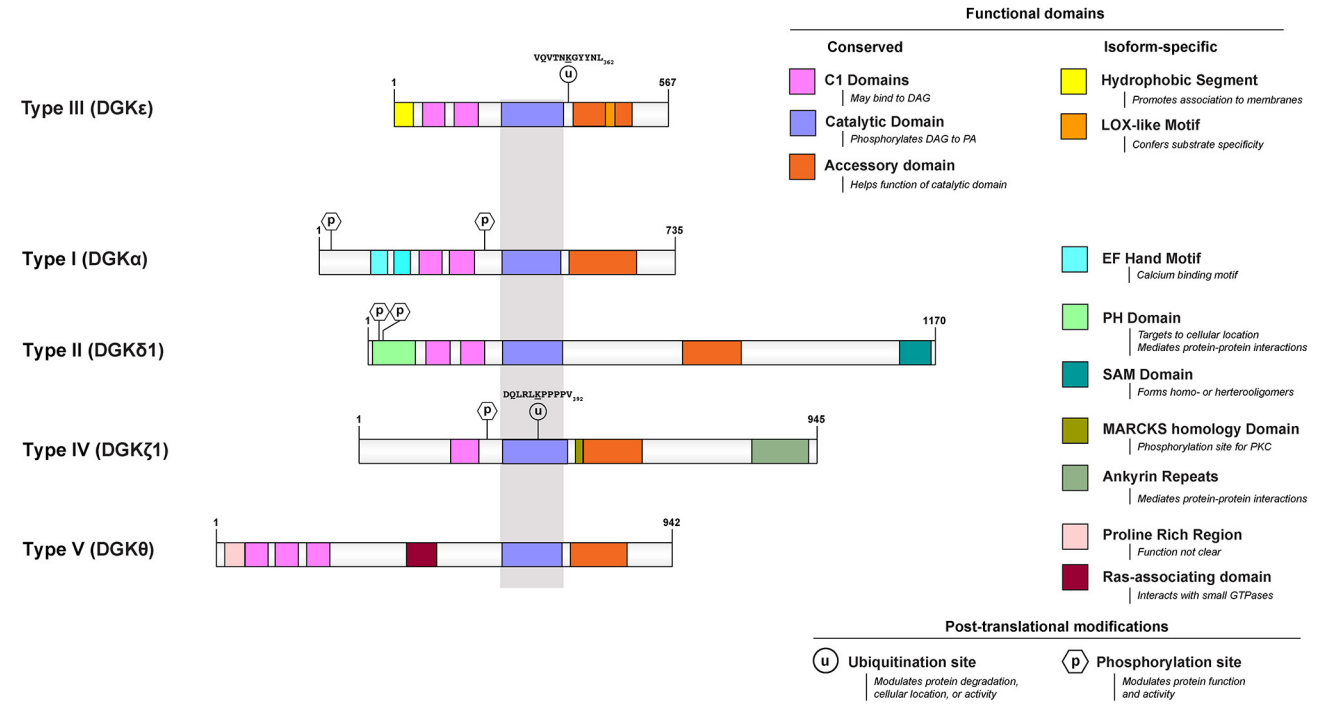


FIGURE 1 | Functional domains of DGKε are contrasted to that of prototypical DGKs from the other 4 subtypes. All protein models were drawn to scale using the Illustrator for Biological Sequences (http://ibs.biocuckoo.org/) (Liu et al., 2015). The proteins are centered around the shared catalytic site (grayed area) because it is the most conserved feature across subtypes. Features that are unique to DGKε and other DGKs are listed on the right side. Known and/or possible phosphorylation (Shirai et al., 2012; Mertins et al., 2013; Park et al., 2015) and ubiquitination (Wagner et al., 2011; Komander and Rape, 2012; Mertins et al., 2013) sites are also displayed (see text and **Table 1** for details). If there are more than one documented isoform, the characteristics of isoform 1 were used for each protein. The accession (and GI) numbers for the proteins illustrated are: DGKε, NP_003638.1 (4503313); DGKα, NP_958853.1 (41872494); DGK δ1, NP_003639.2 (25777596); DGKθ, NP_001338.2 (40806175); DGKζ1, NP_963290.1 (41872522).

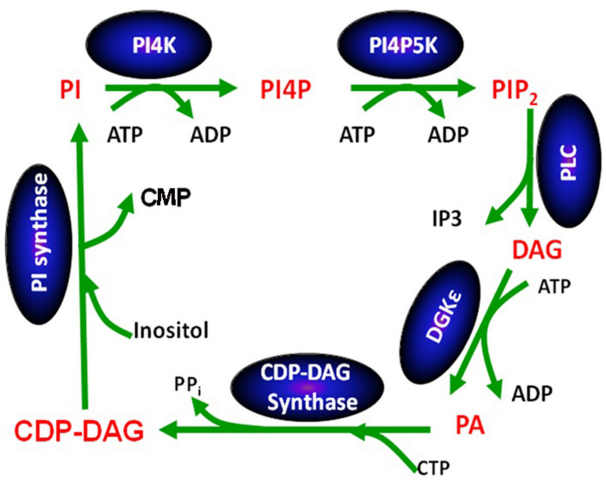


FIGURE 2 | The phosphatidylinositol cycle. Enzymes involved in the catalysis of each step in the cycle are written in a blue oval above the arrow for the reaction using abbreviations. Each lipid intermediate in the cycle is written in red. Figure reproduced from Epand (2016).

at the *sn-1* position of 1-SAG from an ester to an ether linkage (1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycerol) also yielded a poor DGKε substrate (Epand et al., 2004).

The length of the fatty acyl chain at *sn-1* position also influences substrate quality: elongation from 18:0 to 20:0 led to reduced DGKε activity (70% from baseline). On the other hand, DGKε tolerates slightly shorter acyl chains at the *sn-1* position, as DGKε activity was 90% when substituting 18:0 for 16:0 (Lung et al., 2009). Introduction of unsaturation to the acyl chain at the *sn-1* position surprisingly also had only a small effect on the rate of substrate phosphorylation by DGKε. Thus, 20:4/20:4-DAG has about 80% the activity of SAG (Shulga et al., 2011a). Even more striking are the following observations: when compared to SAG, 18:0/18:2-DAG and 18:2/18:2-DAG generate respectively∼20% and ∼60% of the DGKε activity against SAG. Indeed, 18:0 is the optimal saturated acyl chain for the *sn-1* position when the *sn-2* position is occupied by 20:4, but not when the *sn-2* position is 18:2 (Shulga et al., 2011a). Thus, changing the linkage, length, or degree of saturation of the acyl chain at the *sn-1* position of DAG has substantial effects on substrate kinetics but many different acyl chains at the *sn-1* position result in substrates with substantial activity. We propose that the acyl chain at the *sn-1* position has an important, albeit less specific, interaction with DGKε that may indirectly affect the activity of the enzyme. For example, the fatty acyl chain at *sn-1* may modulate a physical property of the membrane surrounding the enzyme or influence the depth of insertion of DAG into the membrane. As a result,

there appears to be a much less stringent requirement for the acyl chain at the *sn-1*, compared with the *sn-2* position of DAG.

Catalytic Accessory Domain: Homology to Lipoxygenase Sequence

We have shown that the most hydrophobic segment of DGKε, which is predicted to bind lipids, can be deleted without loss of enzymatic activity or specificity (Dicu et al., 2007; Lung et al., 2009). When searching for an alternative lipid binding site, we decided to explore a segment of DGKε's accessory domain that is homologous to the arachidonic acid binding site of lipoxygenase (Neau et al., 2009). The finding of such a homologous substrate binding site between lipoxygenase and DGKε was unexpected because the reactions catalyzed by these two enzymes are different, so is the chemical nature of the arachidonoyl groups of the substrates. Remarkably, no other mammalian DGK isoform harbors this novel motif, which is referred to as the "lipoxygenase (LOX)-like motif" (Shulga et al., 2011b).

This motif is characterized by a string of residues: L-$X_{(3-4)}$-R-$X_{(2)}$-L-$X_{(4)}$-G, in which $X_{(n)}$ is n residues of any amino acid. The critical residues are invariant through vertebrate evolution for both DGKε and lipoxygenase (Shulga et al., 2011b). They were identified on the basis that site-specific mutation results in the loss of enzymatic activity or arachidonoyl specificity. The most important reduction of DGKε activity was observed when leucine residues were substituted with more polar and/or sterically smaller amino acids; the impact of substitutions with less polar and/or sterically larger residues was not as dramatic. Most interestingly, substitution of a single amino acid converted DGKα to a LOX-motif-containing DGK that exhibited more specificity for arachidonoyl-containing DAG than unmodified DGKα (Shulga et al., 2011b).

Most mutations of key residues within DGKε's LOX motif resulted in a marked loss in catalytic activity. As a result, accurate assessment of the role of the LOX motif in directing the specific activity of DGKε against arachidonoyl-containing DAG species was challenging. We therefore proceeded to test the impact of mutating residues adjacent to the LOX motif on DGKε substrate specificity (D'Souza and Epand, 2012). This region, adjacent to the LOX motif is a hydrophobic segment contained within the "accessory domain" of DGKε. Unexpectedly, mutagenesis of several residues in this region of DGKε, which is also highly conserved in evolution, had a higher activity toward SAG when compared to wild-type. SAG was the best substrate for all mutants tested, followed by 1,2-diarachidonoyl glycerol. However, each mutant exhibited differences in the relative activity for different DAG substrates. For example, for the wild type enzyme the ratio of activity against 1-stearoyl-2-linoleoyl glycerol vs. SAG is 0.11. However, this ratio ranges from 0.03 to 0.22 for the 5 mutants tested. We conclude that these mutations perturb the lipid binding site, resulting in either enhanced or reduced substrate specificity (D'Souza and Epand, 2012).

DGKε PURIFICATION AND STABILITY

Structural Information Is Lacking for DGKε

There is currently no crystal structure available for any mammalian DGK isoform. Co-crystallization of DGKε with its substrate would be particularly informative. Altogether, these data would be invaluable to drive the discovery of isozyme-specific inhibitors, of which there is only one, for a different DGK isozyme (Liu et al., 2016). However, to do so requires a robust method for expressing and purifying large quantities of DGKε. In our hands, determining the optimal expression system has proven challenging. While bacteria express high levels of recombinant human DGKε, the enzyme recovered is not useful since it is inactive. Human DGKα was successfully expressed in yeast (Abe et al., 2003), but our attempts to express DGKε in the yeast *Pichia* were not successful.

We recently showed that insect (Sf21) cells are excellent bioreactors to produce high amounts of active recombinant DGKε (Prodeus et al., 2013). We recently succeeded to purify full-length and truncated (Δ40) human DGKε to near homogeneity using this cell system coupled to Nickel-affinity chromatography (Jennings, 2016). *In vitro* testing confirmed that both forms retained DGKε's substrate acyl chain specificity.

As mentioned in the Section Integral vs. Peripheral Membrane Protein, DGKε contains a putative membrane-spanning alpha helix at its N-terminus (Decaffmeyer et al., 2008). DGKε proteins generated using our protocol were instrumental in allowing us to test various hypotheses about the role of DGKε's amino terminus in binding to membrane lipids and its relevance to the overall activity and stability of the enzyme (Jennings, 2016).

Glycerol Stabilizes Purified DGKε and DGKεΔ40 Structure

Circular dichroism analysis of purified DGKε and DGKεΔ40 in solution shows that truncating the N-terminal α-helix does not impact the secondary structure of DGKε. Both forms of recombinant DGKε were noted to be highly unstable, losing enzymatic activity and secondary structure in a period of hours after purification. Experiments aimed at monitoring temperature-dependent loss of secondary structure indicates that both constructs undergo a biphasic transition from folded to unfolded states, with transitions occurring at ∼56°C and ∼77°C in a buffer containing 20% glycerol. We demonstrated that adding a high percentage of glycerol to the recombinant DGKε solutions had dramatic stabilizing effects (Jennings, 2016). We also showed that glycerol concentrations higher than 20% were necessary to facilitate the partial refolding of DGKε and DGKεΔ40 after thermal denaturation.

Purified DGKε and DGKεΔ40 Are Active

Activity measurements of purified DGKε and DGKεΔ40 reveal that both constructs retain their acyl chain specificity for SAG (Jennings, 2016). These studies of activity reveal dramatic losses in activity following purification at room temperature, 4°C storage, −80°C storage, and particularly during cycles of freezing/thawing. The incorporation of glycerol into the purification of DGKε as well as during storage dramatically

reduces but does not eliminate the observed losses in activity (Jennings, 2016). The absence of the N-terminal hydrophobic segment does not compromise specific activity in a detergent-phospholipid mixed micelle system and suggests that there are additional regions of DGKε that play critical roles in associating the protein to membranes/micelles. The advancements made in the purification and stabilization of DGKε and DGKεΔ40 will facilitate novel studies utilizing more biologically relevant liposome systems.

In contrast to measuring activity in detergent-phospholipid mixed micelle systems, liposome systems provide insight into how bilayer properties and specific lipid species affect enzyme function. A Ca^{2+}-independent, water soluble DGK has been studied using liposomes (Thomas and Glomset, 1999a,b). However, DGKα and DGKζ are the only specific mammalian isoforms to be studied in a liposome-based system (Fanani et al., 2004). These enzymes were not purified; instead, they were recovered by salt extraction of cell pellets from mammalian cells overexpressing the particular DGK isoform (Fanani et al., 2004). Regardless, the cruder preparation still provided valuable information regarding the critical role of lipids in altering the activity and specificity of DGKα (Fanani et al., 2004). The successful purification of DGKε is facilitating similar studies in liposomes and will lead to novel findings regarding the activation/inhibition of this enzyme. Furthermore, the purification of DGKε is leading the way to more detailed studies of structure and protein interactions. It is also aiding the screening process for the discovery of a DGKε-specific inhibitor.

DGKε HAS A ROLE IN THE PI-CYCLE

The PI-cycle (**Figure 2**) has important roles in signal transduction and in lipid synthesis. Metabolic cycles have particular properties that are intrinsic to their cyclical nature. The concentrations of the PI-cycle intermediates quickly reach a steady-state, which then lasts over prolonged periods of time. The intermediates of the cycle are synthesized at the same rate that they are utilized and they are continually regenerated because they are intermediates within a cycle. The only members of the cycle that are likely to change with time are those that also are either substrates or products of reactions outside of the cycle. In general, the intermediates of the cycle, in addition to being substrates and products of reactions in the cycle, also function as catalysts for the cycle, since the functioning of the cycle neither creates nor destroys these intermediates. They increase the rate of interconversion among intermediates of the cycle, i.e., the rate at which the cycle "turns." The PI-cycle has features that make it different from other metabolic cycles. First, it requires steps that are in two different membrane compartments, namely the plasma and endoplasmic reticulum (ER) membranes (Epand, 2016). DGKε is found in both the plasma membrane (Decaffmeyer et al., 2008) as well as in the ER (Kobayashi et al., 2007), targeted by the amino-terminal segment of DGKε (Matsui et al., 2014). Second, the lipid intermediates of the cycle are normally highly enriched with very specific lipid species that harbor 1-stearoyl-2-arachidonoyl fatty acyl chains.

It is interesting to note that DAG produced by phospholipase C-catalyzed hydrolysis of phosphatidylinositol-(4,5)-bisphosphate (PIP2) is highly enriched with SAG (Pettitt and Wakelam, 1999). This is peculiar because cell membranes contain many other types of DAG. SAG phosphorylation to PA catalyzed by DGKε is one of the key steps of the PI-cycle. It is followed by a series of conversions that ultimately lead to the synthesis of PI in the ER. This pathway includes another 1-stearoyl-2-arachidonoyl-specific enzyme, CDS2 (D'Souza and Epand, 2014). Since the other enzymatic reactions of the PI-cycle have no effect on the fatty acid chains, all intermediates share the 1-stearoyl-2-arachidonoyl backbone.

PI from DGKε-null cells contain less arachidonic acid (Milne et al., 2008) and stearic acid (Lung et al., 2009) than control cells. These data suggest a critical role for DGKε in determining the acyl chain composition of cellular phosphoinositides. Of interest, PI is much more affected than PA when taking into consideration changes in the acyl chain composition (Milne et al., 2008). This is a remarkable finding since PA is the direct product of the reaction catalyzed by DGKε. In contrast to normal cells, many cancer cells do not exhibit enrichment with 1-stearoyl-2-arachidonoyl-containing PIs, but rather are enriched with somewhat shorter and less unsaturated acyl chains (Naguib et al., 2015; Kimura et al., 2016). Differences between the PI-cycles of normal and cancer cells have yet to be thoroughly investigated: we anticipate that the activity of other DGKs and/or CDS enzymes must supplant that of DGKε/CDS2 during oncogeny (Epand, 2016). The fact that patients with complete DGKε deficiency do not appear to have increase cancer risks suggests that this mechanism is unlikely to be a primary driver (See Section Relationship to Disease).

DGKε DEFICIENCY INCREASES INCORPORATION OF GLYCEROL INTO LIPID

Recent studies suggest that $DgkE^{-/-}$ mouse embryonic fibroblasts (MEFs) incubated with ^{3}H-glycerol exhibit increased glycerol incorporation into various glycerolipids (Shulga et al., 2013). Preliminary studies from our laboratory suggest that this finding is likely due to increased glycerol kinase (GK) expression in $Dgk\varepsilon^{-/-}$ MEFs (So et al., 2016). We also found that these cells also consistently exhibit higher than normal p53 levels (So et al., 2016). We investigated this tumor suppressor in more detail because it is known to regulate GK expression (Goldstein et al., 2013). We thus propose that normally, DGKε is a negative regulator of glycerol incorporation through GK, via modulation of p53. In addition, p53 has been shown to exhibit various interactions with lipids. Links to p53 are likely to be complex since it also exhibits strong electrostatic interactions with lipids such as cardiolipin, phosphatidylglycerol, and PA *in vitro* (Li et al., 2010; Goldstein et al., 2013). In addition, its translocation from the nucleus to the mitochondria is modulated by CDS2 (the PI-cycle enzyme mentioned earlier) (Li et al., 2010). Finally,

mutations in p53 have been linked to alterations in the acyl chain composition of PI species in a number of cell cultures of human and mouse origin (Naguib et al., 2015). Since p53 has many well-studied anti-tumorigenic roles in cells, DGKε-specific inhibitors may be useful as potential anti-cancer treatments. Such therapy would be expected to be most efficacious to treat p53-dependent brain cancers since DGKε is highly expressed in brain tissue (Shulga et al., 2011c). However, further studies are required to fully understand how DGKε interacts with p53, and how such an interaction might contribute to or prevent the progression of cancer.

INTEGRAL VS. PERIPHERAL MEMBRANE PROTEIN

Membrane proteins are classified as peripheral or integral, based on their ease of extraction from a membrane. This empirical definition, which is based on experimental observations, is somewhat arbitrary since there is no fundamental difference between these two classes of membrane proteins. In reality, data suggest a continuum of small changes between peripheral and integral membrane-associated proteins. In many respects DGKε is an example of a protein that displays properties that are intermediary between these two protein types. For example, solubilization of overexpressed DGKε did not occur at neutral pH but partial solubilization occurred at alkaline pH (Dicu et al., 2007). It is well-known that varying the pH from neutral to alkaline should have no effect on the extraction of integral proteins from membranes (the structure of the membrane is unaffected by this change). Depending on the context, DGKε proteins may thus exhibit properties that are expected of integral or peripheral membrane proteins.

A number of predictive algorithms suggest that DGKε residues 20–40 can form a transmembrane helix (Glukhov et al., 2007; Jennings et al., 2015). DGKε is the only mammalian DGK isoform with such a putative transmembrane segment. DGKε is also predicted to be the only DGK isoform that is permanently associated with the membrane (Glukhov et al., 2007). Several experimental lines of evidence support this hypothesis. First, a model peptide derived from this DGKε segment, flanked with strings of positively charged lysine residues, was shown to interact with anionic membranes (Glukhov et al., 2007). Third, during synthesis of the protein, glycosylation sites at the amino terminus of DGKε are exposed to the lumen of the ER (Nørholm et al., 2011). These data strongly support the notion that DGKε's hydrophobic segment can form a *bona fide* transmembrane helix in cells.

However, *in silico* calculations suggest that this hydrophobic segment has two possible stable conformations when associated with a membrane: a classic transmembrane helix or a U-shaped, re-entrant helix that enters and leaves the membrane on the same side of the bilayer (Decaffmeyer et al., 2008). If the hydrophobic segment is transmembrane, parts of the amino terminus of DGKε would be expected to be exposed to the extra-cellular environment (**Figure 3**). We tested for this possibility by using a DGKε construct with a FLAG-tag added to the amino terminus. We reasoned that immunofluorescence detection of FLAG-DGKε in non-permeabilized cells should only be possible if the amino terminus crosses the plasma membrane. We showed that permeabilization was required for visualization of FLAG-DGKε, strongly suggesting that DGKε forms mostly re-entrant helices (Decaffmeyer et al., 2008). Interestingly, FLAG-DGKε was detected in non-permeabilized cells after Pro33Ala mutagenesis; this proline residue is predicted to be key to form the U-shaped re-entrant helix [note that in earlier papers, such as reference (Decaffmeyer et al., 2008), we refer to this proline residue as 32, corresponding to its numbering after cleavage of the N-terminal methionine to form the mature protein. In the present article residue numbering includes the N-terminal methionine as residue 1] (Decaffmeyer et al., 2008). The result also suggests that some DGKε locates to the plasma membrane. The presence of DGKε at the plasma membrane was demonstrated by Western blotting of an affinity-purified plasma membrane fraction from 3T3 cells that had been transfected with FLAG-DGKε (Decaffmeyer et al., 2008). Note that this proline residue is invariant in evolution (See Supplementary Materials), suggesting that it plays an important functional role, perhaps by allowing facile interconversion between transmembrane and re-entrant helical conformations.

There is thus conflicting evidence as to whether the amino terminal segment of DGKε forms a transmembrane or a re-entrant helix in cell membranes. Mathematical modeling reveals that the energy difference between these two distinct conformations is small (Decaffmeyer et al., 2008). It is thus possible that one conformation may predominate depending on the discrete properties of the surrounding lipid bilayer (e.g., membrane thickness, charge, the intrinsic curvature of the monolayers, or the presence of specific lipids). Interestingly, many of these parameters differ significantly when the plasma and ER membranes are compared. Our results may not be contradictory, but rather reflect normal comformational changes dictated by environmental conditions or local topogenic signals.

DGKε INTERACTOME

No systematic investigation has focused on identifying the array of proteins that interact with DGKε. The only data available in that regard are from two studies done in HEK293T cells. In the first study, the investigators use immunoprecipitation followed by mass spectrometry (LC-ESI-MS/MS) to delineate the interactomes of 338 Flag-tagged bait proteins, including DGKε. They reported that DGKε pulled down several proteins with no clear links to known DGK biology (see **Table 1** for details): CDCA1, NUDC, NUF2, PAICS, PDHA1, SET (Ewing et al., 2007). The other study used a similar mass spectrometry-based approach to identify the interactomes of arrestinβ1 (ARRB1) and arrestinβ2 (ARRB2), which are important negative regulators of G protein-coupled receptors (GPCR) (Premont and Gainetdinov, 2007). It revealed that DGKε was associated with both ARRB1 and ARRB2, and that these

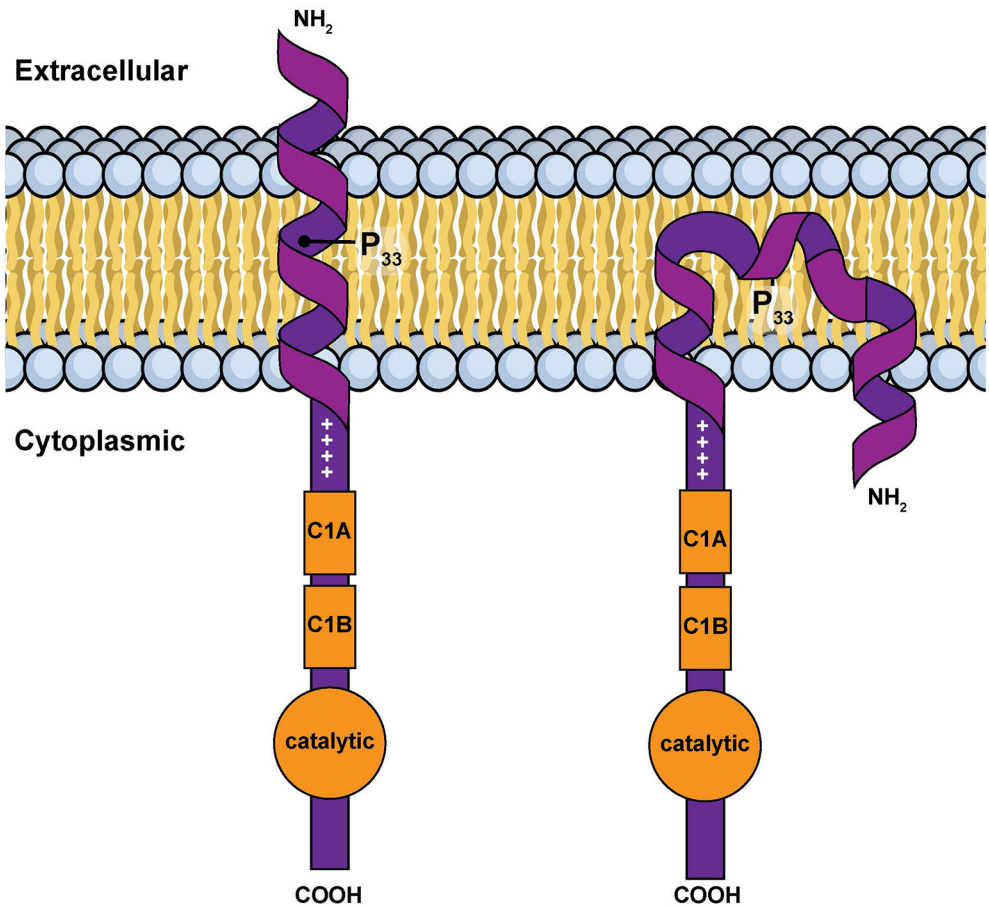


FIGURE 3 | Simple models for the amino terminal segment of DGKε inserted into a membrane either as a bitopic transmembrane helix or as a monotopic re-entrant helix (Nørholm et al., 2011). This illustration is a modified version of **Figure 2** from an article on intracellular protein topogenesis (Blobel, 1980). The approximate position of Pro^{33}, a residue important for the interconversion between these two conformations, is shown. The topological relationships of the intra and extracellular sides of the membrane bilayer, the location of the C1 domains and of the catalytic site are illustrated.

interactions were not modulated by GPCR activation (Xiao et al., 2007). In yet another proteome-wide interactome study performed on liver cell extracts, DGKε was found to interact with MRPL44, a protein involved in mitochondrial ribosomes (**Table 1**).

These data should be considered as weak evidence of interactions since none of the studies offered independent experimental confirmation. It would have been reassuring to see at least one of the ARRBs in the list of DGKε interactors from the first study given that both studies used the same cells (HEK293T). No subsequent studies have sought to explore the physiologic relevance of these potential DGKε partners. Given DGKε's presumed regulatory role in the signaling pathway of PLC-activated by GPCR, the interactions with the ARRBs are by far the most promising. It is clear that important clues regarding the functions of DGKε in various mammalian cells could be gleaned from studies focused on the DGKε interactome. While the same mass spectrometry-based methodology could be used, other approaches such as BioID (Roux et al., 2013) should also be considered.

POST-TRANSLATIONAL MODIFICATIONS OF DGKε PROTEINS

The extent to which post-translational modifications modulate DGKε function is yet another area of DGKε biology that is understudied. While phosphorylation of several DGKs has been demonstrated experimentally (Shirai et al., 2012), there is no evidence that it plays a major role in modulating DGKε function. Two studies that presented comprehensive phosphorylation data from human samples confirmed that DGKε phosphorylation levels, if present, must be low (Mertins et al., 2013; Park et al., 2015).

TABLE 1 | Data on the possible DGKE interactome abstracted from three proteomics studies.

Bait (OMIM No.)	Prey (OMIM No.)	Full gene name	Other names	Known functions
DGKε[a] (601440)	PAICS (172439)	Phosphoribosylaminoimidazole carboxylase	AIRC	Purine biosynthesis
	NUDC (610325)	Nuclear distribution protein C homolog	–	Nuclear movement protein that associates with dynein
	CDCA1 (611772)	Cell division cycle-associated protein 1	NUF2	Chromosome segregation and the spindle checkpoint
	PDHA1 (300502)	Pyruvate dehydrogenase, alpha-1	PDHCE1A, PDHE1A, PDHA	Conversion of pyruvate into acetyl-CoA
	SET (600960)	Suppressor of variegation, enhancer of zeste, and Trithorax	IGAAD	Inhibitor of protein phosphatase 2A
DGKε[b] (601440)	MRPL44 (611849)	Mitochondrial ribosomal protein l44	–	Mitochondrial ribosome
ARRB1[c] (107940)	DGKε (601440)	Arrestin, beta, 1	ARRB1, BARR1	G protein-coupled receptor desensitization
ARRB2[c] (107941)	DGKε (601440)	Arrestin, beta, 2	ARRB2, BARR2	G protein-coupled receptor desensitization

[a] *HEK 293 cells were transfected with flag-tagged bait (DGKε), followed by immunoprecipitation with anti-Flag antibody. The protein complexes associated with DGKε were analyzed by Mass spectrometry. DGKε was one of 338 baits (Ewing et al., 2007).*

[b] *Liver cells (Wang et al., 2011).*

[c] *HEK 293 cells were transfected with flag-tagged bait, followed by immunoprecipitation with anti-Flag anti-body. The protein complexes associated with the bait were analyzed by Mass spectrometry (Xiao et al., 2007).*

Several proteome-wide screens focused on other post-translational modifications have been published in recent years. The most striking finding is that two distinct studies uncovered the exact same ubiquitination site at lysine 357 in human DGKε (Wagner et al., 2011; Mertins et al., 2013). The cells used in the studies included HEK293T (human embryonic kidney) and MV44-11 (acute monocytic leukemia) in one study, and Jurkat cells (immortalized human T cells) in the other. The same modification was also found at the homologous murine locus, at lysine 354 (Wagner et al., 2012). In the mouse, Dgkε ubiquitination was only observed in proteins extracted from the brain (other tissues tested that did not express ubiquitinated Dgkε included heart, liver, kidney and muscles). This remarkable tissue specificity may be unique to mice since three non-brain cell types were used for the human studies. The so-called "ubiquitin code" is known to control a wide array of biological functions, including enhanced degradation, targeting to specific cellular location, modulation of function, and regulation of protein-protein interactions (Komander and Rape, 2012). It will be important to determine the relevance of ubiquitination at this site and to determine if tissue- and species-specific patterns exist.

NOVEL SEQUENCE FEATURES OF DGKε

Homologs exhibiting a high degree of sequence similarity to DGKε are present in most eukaryotic organisms, except plants, fungi, and some unicellular organisms. Comparison of DGKε sequences from mammalian species reveals a number of interesting differences that are specific for particular groups of animals. For example, the DGKε homologs from the *Muroidea* family of rodent species (e.g., rats, mice, hamsters, gerbils; Catzeflis et al., 1992) contain a conserved 2 amino acid deletion near the N-terminus, which is not found in the homologs from other mammals (**Figure 4**). Conserved inserts and deletions in protein sequences play important roles in mediating novel protein-protein or protein-ligand interactions (Akiva et al., 2008; Singh and Gupta, 2009). Thus, it is likely that this rodent-specific genetic alteration may also affect the biological function of the rodent DGKε in some subtle manner. The functional significance of this alteration may become clearer as the role of the amino terminus is better understood.

A number of other specific changes seen in the DGKε homologs are specific for the Catarrhini subdivision of primates, which includes humans, great apes, gibbons, and old world monkeys (**Figure 5**). At positions 147 and 440 in the human DGKε, homologs from the Catarrhini subdivision contain cysteine and serine residues, respectively, whereas all other mammalian species have serine/threonine or aspartate/asparagine at these positions. Another specific change in the DGKε homologs present at position 48 is a distinctive characteristic of the old world monkeys (*Cercopithecoidea)*. All DGKε homologs from the *Cercopithecoidea* family contain a leucine in this position instead of the glutamine found in all other vertebrates. The high degree of specificity of the noted genetic changes within the indicated groups suggests that these changes are under strong selection pressure and may thus confer some as yet unknown biological advantages to DGKε functions in these primates.

It is of much interest that one of the above noted genetic changes at position 147 is located within one of the C1 domains (C1B) of DGKε. These are highly conserved and cysteine-rich domains that are typically involved in binding of DAG or phorbol esters (Hurley et al., 1997; van Blitterswijk and Houssa, 2000; Sakane et al., 2007; Jennings et al., 2015) (however,

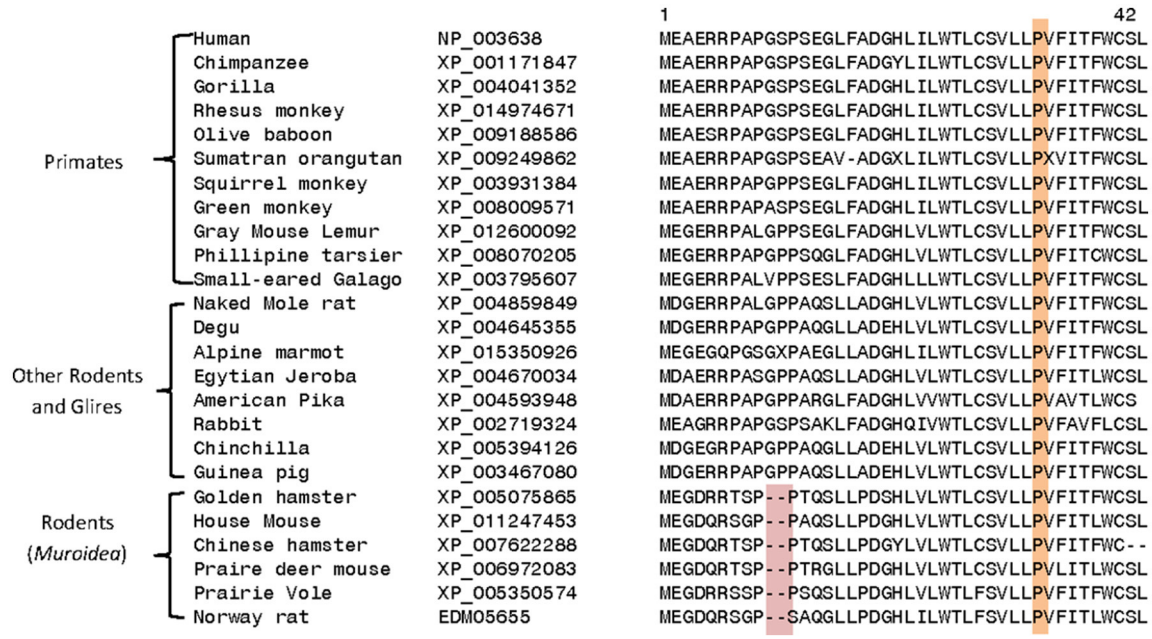


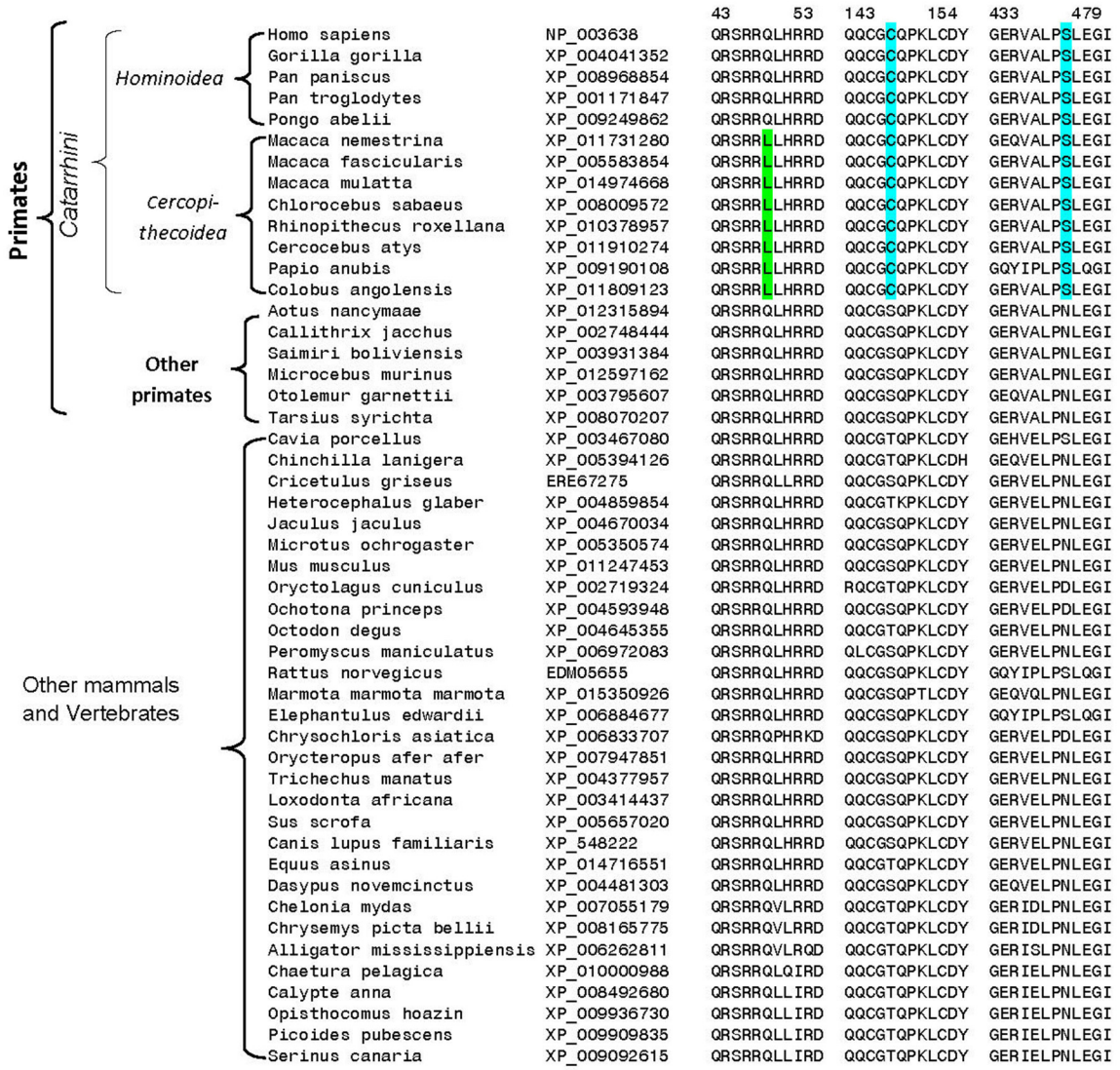
FIGURE 4 | Excerpts from a multiple sequence alignment of DGKε sequences showing two conserved characteristics that are of interest. Protein sequences of DGKε homologs were obtainedfrom the NCBI database (http://www.ncbi.nlm.nih.gov/) and the conserved characteristics noted here were identified in their sequence alignment as previously described (Gupta, 2016). The first characteristic (highlighted in pink) consists of a 2 aa deletion in a conserved region of DGKε that is a unique property of the Muroidea family of rodents, which includes rats, mice and hamsters, but not found in the primates or other mammalian homologs. The sequence information is shown for all rodents and primate homologs and a limited number of other species. The sequence alignment also shows that the proline residue present at position 33 in human DGKε, which is implicated in the re-entrant property of the N-terminal helical region, is a conserved property of all vertebrate DGKε sequences (see also Supplementary Table 1).

see also Section Atypical C1 Domains). As such, they may play a central role in directing the function of DGKs in cells. Alignment of the C1B domains for human DGKε and DGKδ reveals very high degree of sequence homology (**Figure 6A**). Based on the structural information for the most predominant form of DGKδ (Miyamoto et al., 2004), we have created and optimized a homology model of the C1B domain for human DGKε (**Figure 6B**) (Sali and Blundell, 1993; Shen and Sali, 2006; Xu and Zhang, 2011). The superposition of the modeled structure with the structure of DGKδ shows very high degree of structural homology between the two isoforms in the C1B domain and it also shows the location where the Cys substitution specific for the Catarrhini subdivision has occurred. It is possible that this additional cysteine primes the C1 domains to be more avid for DAG-like substrates. This hypothesis can only be confirmed via mutagenesis experiments and by solving the crystal structure of DGKε with its DAG substrate.

Another remarkable finding relates to the near-complete conservation of the hydrophobic segment (positions 21–42) in vertebrate species, including the proline residue at position 33. As discussed in the Section Integral vs. Peripheral Membrane Protein, this segment is hypothesized to act as a membrane-associated domain (Jennings et al., 2015). This proline residue is contained within the very hydrophobic segment near the amino-terminus of DGKε (see hydropathy plot of Figure 1 in Jennings et al., 2015). In this scenario, the proline residue at position 33 plays an important role in the formation of a re-entrant helix (Decaffmeyer et al., 2008). *In vitro* experiments have shown that mutagenesis of this position to alanine results in higher affinity for membranes via conversion of the re-entrant helix to a transmembrane helix (26). This suggests that there might be substantial functional advantage for DGKε to form a re-entrant helix so as to restrict its interactions with lipid bilayers to the inner leaflet. The sequence alignment of DGKε from various vertebrate species (mammals, amphibians, reptiles, birds, and fishes) and also some invertebrates shows that the proline at position 33 is completely conserved and invariant in all examined species (see Supplemental Figure 1). This in turn suggests that the re-entrant potential of this hydrophobic segment is an evolutionarily conserved property of the DGKε from different species.

IN VIVO EVIDENCE FROM DGKε KNOCK-OUT MOUSE

A Dgkε-null mouse was reported in 2001 by investigators that were studying the role of DGKs in the brain (Rodriguez de Turco et al., 2001). No major anomalies were noted despite extensive phenotyping that mostly focused on the neurological system (Rodriguez de Turco et al., 2001). Quantitative phospholipid studies on brain tissue from the Dgkε-null mice revealed deficits



		43 53	143 154	433 479
Homo sapiens	NP_003638	QRSRRQLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Gorilla gorilla	XP_004041352	QRSRRQLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Pan paniscus	XP_008968854	QRSRRQLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Pan troglodytes	XP_001171847	QRSRRQLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Pongo abelii	XP_009249862	QRSRRQLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Macaca nemestrina	XP_011731280	QRSRRLLHRRD	QQCGCQPKLCDY	GEQVALPSLEGI
Macaca fascicularis	XP_005583854	QRSRRLLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Macaca mulatta	XP_014974668	QRSRRLLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Chlorocebus sabaeus	XP_008009572	QRSRRLLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Rhinopithecus roxellana	XP_010378957	QRSRRLLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Cercocebus atys	XP_011910274	QRSRRLLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Papio anubis	XP_009190108	QRSRRLLHRRD	QQCGCQPKLCDY	GQYIPLPSLQGI
Colobus angolensis	XP_011809123	QRSRRLLHRRD	QQCGCQPKLCDY	GERVALPSLEGI
Aotus nancymaae	XP_012315894	QRSRRQLHRRD	QQCGSQPKLCDY	GERVALPNLEGI
Callithrix jacchus	XP_002748444	QRSRRQLHRRD	QQCGSQPKLCDY	GERVALPNLEGI
Saimiri boliviensis	XP_003931384	QRSRRQLHRRD	QQCGSQPKLCDY	GERVALPNLEGI
Microcebus murinus	XP_012597162	QRSRRQLHRRD	QQCGSQPKLCDY	GERVALPNLEGI
Otolemur garnettii	XP_003795607	QRSRRQLHRRD	QQCGSQPKLCDY	GEQVALPNLEGI
Tarsius syrichta	XP_008070207	QRSRRQLHRRD	QQCGSQPKLCDY	GERVALPNLEGI
Cavia porcellus	XP_003467080	QRSRRQLHRRD	QQCGTQPKLCDY	GEHVELPSLEGI
Chinchilla lanigera	XP_005394126	QRSRRQLHRRD	QQCGTQPKLCDH	GEQVELPNLEGI
Cricetulus griseus	ERE67275	QRSRRQLLRRD	QQCGSQPKLCDY	GERVELPNLEGI
Heterocephalus glaber	XP_004859854	QRSRRQLHRRD	QQCGTKPKLCDY	GERVELPNLEGI
Jaculus jaculus	XP_004670034	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Microtus ochrogaster	XP_005350574	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Mus musculus	XP_011247453	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Oryctolagus cuniculus	XP_002719324	QRSRRQLHRRD	RQCGTQPKLCDY	GERVELPDLEGI
Ochotona princeps	XP_004593948	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPDLEGI
Octodon degus	XP_004645355	QRSRRQLHRRD	QQCGTQPKLCDY	GERVELPNLEGI
Peromyscus maniculatus	XP_006972083	QRSRRQLHRRD	QLCGSQPKLCDY	GERVELPNLEGI
Rattus norvegicus	EDM05655	QRSRRQLHRRD	QQCGSQPKLCDY	GQYIPLPSLQGI
Marmota marmota marmota	XP_015350926	QRSRRQLHRRD	QQCGSQPTLCDY	GEQVQLPNLEGI
Elephantulus edwardii	XP_006884677	QRSRRQLHRRD	QQCGSQPKLCDY	GQYIPLPSLQGI
Chrysochloris asiatica	XP_006833707	QRSRRQPHRKD	QQCGSQPKLCDY	GERVELPDLEGI
Orycteropus afer afer	XP_007947851	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Trichechus manatus	XP_004377957	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Loxodonta africana	XP_003414437	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Sus scrofa	XP_005657020	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Canis lupus familiaris	XP_548222	QRSRRQLHRRD	QQCGSQPKLCDY	GERVELPNLEGI
Equus asinus	XP_014716551	QRSRRQLHRRD	QQCGTQPKLCDY	GERVELPNLEGI
Dasypus novemcinctus	XP_004481303	QRSRRQLHRRD	QQCGSQPKLCDY	GEQVELPNLEGI
Chelonia mydas	XP_007055179	QRSRRQVLRRD	QQCGTQPKLCDY	GERIDLPNLEGI
Chrysemys picta bellii	XP_008165775	QRSRRQVLRRD	QQCGTQPKLCDY	GERIDLPNLEGI
Alligator mississippiensis	XP_006262811	QRSRRQVLRQD	QQCGTQPKLCDY	GERISLPNLEGI
Chaetura pelagica	XP_010000988	QRSRRQLQIRD	QQCGTQPKLCDY	GERIELPNLEGI
Calypte anna	XP_008492680	QRSRRQLLIRD	QQCGTQPKLCDY	GERIELPNLEGI
Opisthocomus hoazin	XP_009936730	QRSRRQLLIRD	QQCGTQPKLCDY	GERIELPNLEGI
Picoides pubescens	XP_009909835	QRSRRQLLIRD	QQCGTQPKLCDY	GERIELPNLEGI
Serinus canaria	XP_009092615	QRSRRQLLIRD	QQCGTQPKLCDY	GERIELPNLEGI

FIGURE 5 | Excerpts from multiple sequence alignment of DGKε sequences showing three conserved sequence polymorphisms that are specific for different groups of primates. All of the sequences were downloaded from the NCBI database and the sequence alignment to identify conserved characteristic was carried out as in earlier work (Gupta, 2016). The two sequence polymorphisms (highlighted in blue) are specific for the Catarrhini subdivision of primates, which includes humans, great apes, gibbons, and old world monkeys. The third polymorphism marked in green is specific for the *Cercopithecoidea* family, comprising of the old-world monkeys. Sequence information is shown for all primate homologs and for representative species from other vertebrate groups.

in arachidonic-acid containing PIP2, but no change in DAG or PA (Rodriguez de Turco et al., 2001). Similar studies in fibroblasts derived from these mice revealed a similar pattern (Milne et al., 2008). *In vitro* corroboration of these results with RNA interference has not been reported.

While exon 1 was unquestionably replaced by a neomycin resistance cassette in these mice (Rodriguez de Turco et al., 2001), it is important to acknowledge that absence of Dgkε protein has never been specifically demonstrated. The main reason for this omission is the poor performance of anti-murine Dgkε antibodies when used to detect endogenous Dgkε with Western blotting or tissue staining. It was also thought that even if made, this truncated version of DGKε, which would lack both C1 domains (tDGKε), would be unlikely to be functional (although, as mentioned in the Section Atypical C1 Domains, the role of these C1 domain remains unclear). Data obtained with another truncated DGKε (DGKεΔ, lacking the segment from Pro_6 to Pro_{188}) showed that such a truncated protein had no activity toward SAG (Tang, 1999). Of note, DGKεΔ activity toward other DAG substrates was not tested.

Now that work on Dgkε-null mice has potential health-related ramifications (for patients with DGKε-associated nephropathy, See Section Relationship to Disease), it is critical to determine if this model accurately reflects true Dgkε-deficiency. Indeed,

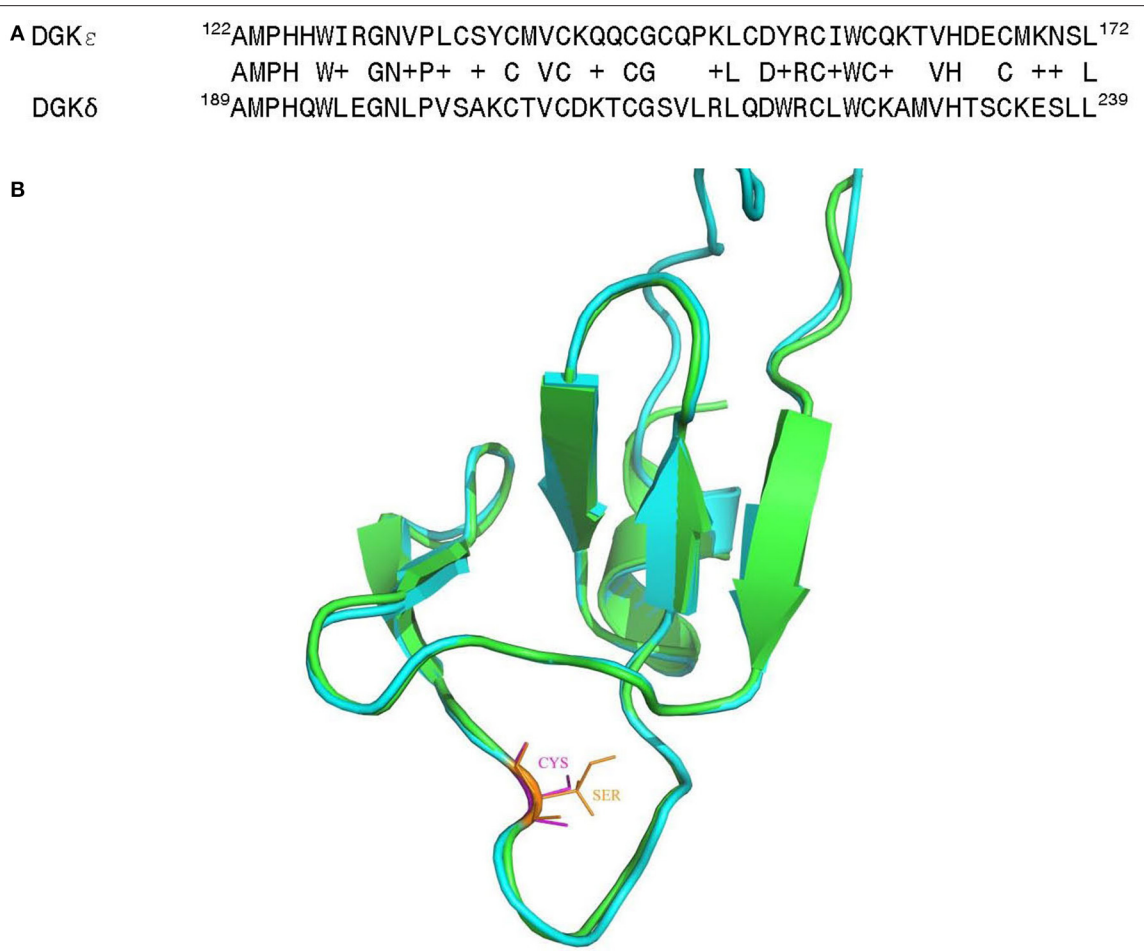


FIGURE 6 | (A) Sequence alignment of the C1B domain from human DGKε with the corresponding region of the DGKδ isoform, whose solution structure has been determined by NMR (Miyamoto et al., 2004). **(B)** Superposition of the three-dimensional (3D) structures of the C1B domain of human DGKε (shown in green) with human DGKδ (shown in cyan). Homology models of the human DGKε were built using the NMR structural information for human DGKδ (PDB ID: 1R79) (Liu et al., 2015). The first model in NMR was utilized as the template and the structure was generated using MODELLER v 9.15 (Sali and Blundell, 1993). The structural comparison of the modeled human DGKε with the DGKδ isoform was carried out using PyMol (http://www.pymol.org).

incomplete knockouts have been described before, and the methodology employed to generate early mouse models often involved deleting exon 1 (Müller, 1999). According to the Knockout Mouse Project initiative (KOMP), the critical exon for DGKε is exon 3, not exon 1. KOMP is a major NIH-sponsored initiative aimed at making thousands of knockout mice widely available (Skarnes et al., 2011). Targeting of the critical exon maximizes the likelihood of generating a true knockout because this exon must be comprised in all spliced isoforms and, when deleted, it must create a frameshift mutation.

If expressed, tDgkε produced from a putative alternative start codon would be missing the C1 domains. Since the function of these atypical C1 domains is unclear (see Section Atypical C1 Domains), the functional impact of that loss is difficult to predict. Importantly, the kinase and catalytic accessory domains would be intact. tDgkε would thus resemble bacterial forms of dgks, which lack the segment harboring the C1 domains. Just like these dgks, tDgkε could in principle actively phosphorylate many other targets besides SAG, including other types of DAG (Walsh et al., 1990). More concerning however is the possibility that tDGKε may gain the ability to phosphorylate ceramide (Schneider and Kennedy, 1973). The cells may thus be simultaneously exposed to both a loss and a gain of function. The biological consequences of such a complex system would be challenging to interpret: it remains an open question whether this is a viable model for a human disease caused by a "simple" DGKε deficiency. For these reasons, it is critical to confirm if this mouse model is indeed a true knockout.

RELATIONSHIP TO DISEASE

Atypical Hemolytic Uremic Syndrome (aHUS)

Whole-exome sequencing recently uncovered an unexpected link between homozygous mutations in the gene encoding for DGKε

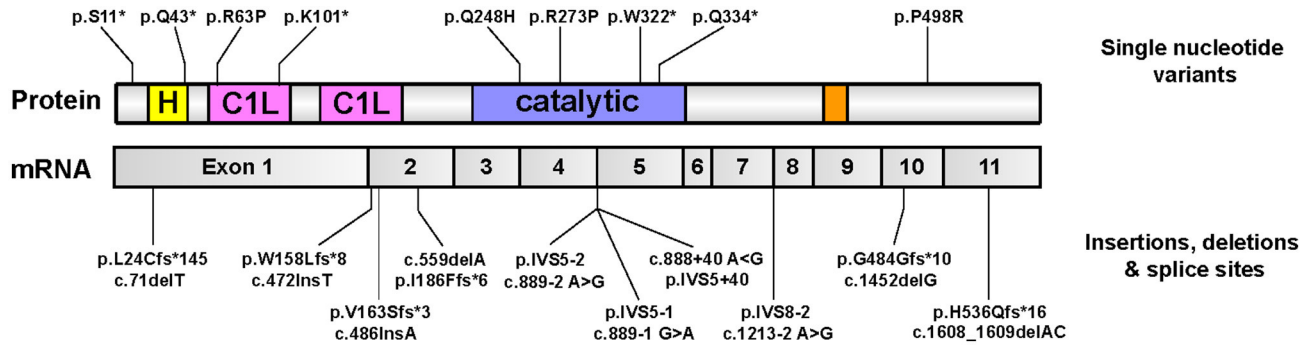


FIGURE 7 | Pathologic mutations in DGKE identified in patients with DGKE-associated nephropathy. All published missense or nonsense Dgkε mutations that are reported in the literature (Lemaire et al., 2013; Ozaltin et al., 2013; Sánchez Chinchilla et al., 2014; Westland et al., 2014; Lee et al., 2015; Mele et al., 2015; Miyata et al., 2015), as of August, 2016 are overlayed on an illustration of Dgkε and its functional domains (top). Mutations that affect splice sites or result in insertions/deletions are overlayed onto an illustration of DGKε mRNA with the boundaries of the 11 coding exons (bottom). Mutation names used adhere to the standard method promulgated by the Human Genome Variation Society (HGVS) for sequence variation nomenclature (http://varnomen.hgvs.org). Single letter codes for amino acids are used. Abbreviations used: "c.", cDNA position; fs, frameshift; IVS, intervening sequence (intron); "p.", protein position."

and DGKE-associated nephropathy (Lemaire et al., 2013; Ozaltin et al., 2013). The bulk of mutations are expected to result in complete loss-of-function (nonsense, splice site, frameshift); no clustering to any particular domain was observed (**Figure 7**). This rare condition is due to recurrent episodes of thrombosis in the kidney glomeruli microvasculature. The salient clinical findings are acute renal failure, low platelets, and hemolytic anemia. On that basis, it is hypothesized that DGKε protein must play an important role in regulating thrombosis in the human kidney. This discovery forced experts to reconsider the pathophysiologic underpinnings of aHUS (Quaggin, 2013). Of note, a small group of patients with pathogenic DGKE mutations present with clinical features that resemble more that of another glomerular disease, membranoproliferative glomerulonephritis (Ozaltin et al., 2013). Up until then, abnormal activation of the alternative complement pathway was thought to be invariably associated with nearly all forms of aHUS (Noris et al., 2012): the vast majority of aHUS patients harboring DGKE mutations exhibit no evidence of complement activation (Lemaire et al., 2013; Ozaltin et al., 2013). Only supportive measures may be offered to these patients because there are no targeted therapies.

DGKε was originally cloned from human umbilical vein endothelial cells, but mRNA expression was predominant in testes (Tang et al., 1996). The discovery of the link between DGKε and aHUS recently prompted more in-depth investigations focused on its presumed role in kidney biology. DGKε protein was shown to be expressed in three cell types that play major roles in kidney glomeruli, namely endothelial cells, podocytes, and platelets (Lemaire et al., 2013; Ozaltin et al., 2013). However, the lack of disease recurrence in patients after kidney transplantation strongly suggests that platelets, which are produced by the bone marrow, are unlikely to be central players in the disease process (Lemaire et al., 2013). The mechanism by which DGKε deficiency causes thrombosis exclusively in the kidney remains unclear. Its expression in other vascular beds has not been investigated. Quantification of key members of the PI-cycle needs to be assessed specifically in endothelial cells to determine if DGKε deficiency in this setting also leads to paucity of PIP_2 (see Section DGKε Has a Role in the PI-Cycle). Experiments done in cultured endothelial cells show that siRNA knockdown of DGKε was associated with several phenotypes: endothelial cell increased activation, increase apoptosis and decreased proliferation (Bruneau et al., 2015). It will be important to determine if DGKε-null endothelial cells display the same characteristics because the siRNA knockdown and knockout of the same gene may yield very different phenotypes (Rossi et al., 2015).

The generation of an animal model is often very useful to delineate the biology of human diseases. Since most patients are expected to have DGKε deficiency, the Dgkε-null mouse reported in 2001 would be an ideal candidate model. The original report showed that there were no major abnormalities with these animals (see Section Post-translational Modifications of DGKε Proteins; Rodriguez de Turco et al., 2001). The renal phenotype of this mouse model was recently re-evaluated in more detail: the animals developed mild signs of renal disease with age (Zhu et al., 2016). Interestingly, glomerular lesions were noted in Dgkε-null mice after exposure to doses of nephrotoxic serum that did not affect wild type littermates (Zhu et al., 2016). Mouse models of aHUS often require exposure to exogenous triggers to reveal their pathogenic potential (Pickering et al., 2006; Thurman et al., 2012; Vernon et al., 2016). Importantly, exogenous factors are also known to act as triggers in many patients with genetic forms of aHUS (Kavanagh et al., 2013). Dgkε-null mice may thus be a promising research tool to further our understanding of DGKε-associated nephropathy (assuming it is a full knockout—see Section Post-translational Modifications of DGKε Proteins).

Data from the recent report focused on the Dgkε-null mice suggests that DGKε deficiency leads to systemic inability to induce cyclooxygenase-2 (Cox-2) (Zhu et al., 2016). This enzyme, which is responsible for inducible production of prostanglandins,

was previously shown to be decreased in the brains of Dgkε-null mice after kindling (Lukiw et al., 2005). When exposed to lipopolysaccharides, wild-type macrophages increase Cox-2 mRNA and protein levels; similar inductions were observed when wild-type fibroblasts were incubated with interleukin-β (Zhu et al., 2016). Interestingly, these responses were abrogated in macrophages and fibroblasts derived from Dgkε-null mice. Another line of evidence comes from experiments with wild-type mice treated with the nephrotoxin puromycin aminonucleoside: animals invariably develop glomerular lesions that lead to proteinuria. This phenomenon was shown to be accompanied by robust inductions of Cox-2 expression and urinary prostaglandin excretion (Zhu et al., 2016). Application of the same protocol to Dgkε-null mice revealed relative protection against the proteinuric effects of this toxin, and this effect was correlated with blunted Cox-2 and prostaglandin responses (Zhu et al., 2016). How DGKε activity directly modulates Cox-2 expression has yet to be established. The relevance of these findings to patients with mutations in DGKε is unclear.

The renal phenotypes induced by the subclinical doses of nephrotoxic serum or puromycin are now the main distinctive features of the Dgkε-null mouse model when compared to control littermates. It is unclear if other organs are affected because the bulk of these new investigations were focused on the kidney. While the other Dgk-null animals have no obvious renal phenotypes at baseline [Dgkα (Olenchock et al., 2006), Dgkβ (Shirai et al., 2010), Dgkδ (Crotty et al., 2006), Dgkζ (Zhong et al., 2003), Dgkι (Regier et al., 2005), Dgkη (Isozaki et al., 2016), Dgkθ (Goldschmidt et al., 2016)] it is important to realize that none of these models were exposed to these nephrotoxins. It is therefore not possible to conclude that the renal lesions observed in treated Dgkε–null mice are specific to this animal model.

Cardiac Hypertrophy and Heart Failure

DGKε is one of the main DGK isoforms expressed in cardiac ventricles; others are DGKα and DGKζ (Takeda et al., 2001). This suggests that DGKε may play an important role in the heart. Decreased DgkE mRNA levels were observed in the hearts of rats used for modeling left ventricular hypertrophy (Yahagi et al., 2005) and myocardial infarction (Takeda et al., 2001). These results did not tease out if this reduction was a normal compensatory mechanism, or if it was an integral part of the disease processes. To start addressing this question, transgenic mice overexpressing DGKε only in the heart were generated (Niizeki et al., 2008). When subjected to two distinct protocols known to induce left ventricular hypertrophy in wild type mice, DGKε-overexpressing mice appeared to be protected (Niizeki et al., 2008). These findings translated into a substantial survival advantage: 4 weeks after the procedure, nearly 80% of DGKε-overexpressing mice were still alive, almost double that of wild type controls (Niizeki et al., 2008). Investigations of well-established biological markers of cardiac pathology corroborate these findings. Upregulation of transient receptor potential channel-6 (Kuwahara et al., 2006) and increased PKCε and PKCα translocation (Hahn et al., 2003; Song et al., 2015) were only observed in wild type mice (Niizeki et al., 2008). Taken together, these results suggest that increasing DGKε function may be a promising target to help prevent heart failure and restore cardiac function. The first step in that direction will be to determine how relevant these data are to patients with cardiac dysfunction. If substantiated, the path to DGKε-based therapy will not be straightforward since it would require tissue-specific overexpression of DGKε.

Epilepsy and Seizure Susceptibility

High expression of DGKε in brain tissue suggests that it may play an important role in this organ system (other DGKs are also high in the brain, including β, γ, and ζ) (Zhang et al., 2012). DgkE$^{-/-}$ mice exhibit higher resistance to electroconvulsive shock (ECS) when compared to control littermates (Rodriguez de Turco et al., 2001). A role for DGKε in this process was supported by phosphoinositide quantifications: while wild type mice displayed increased ECS-induced polyphosphoinositide (PIPn) degradation and 20:4 DAG formation, DgkE$^{-/-}$ mice did not (Rodriguez de Turco et al., 2001).

In a similar study, DgkE$^{-/-}$ mice displayed fewer motor seizures, fewer epileptic events, and rapid behavioral recovery following brain stimulation compared to wild type mice (Musto and Bazan, 2006). In addition, wild type mice serially exposed to multiple small seizure events (kindling) developed typical brain morphological changes associated with seizures, but these were absent in DgkE$^{-/-}$ mice (Musto and Bazan, 2006). Kindling induced upregulation of cyclooxygenase-2 (COX-2) and tyrosine hydroxylase (TH) gene expression in wild type mice but not in DgkE$^{-/-}$ mice (Lukiw et al., 2005). High COX-2 expression has been associated with recurrence of hippocampal seizures (Takemiya et al., 2003), and repeated seizures lead to increased TH levels in the brain (Ryu et al., 2000). These data thus suggest that DGKε regulates seizure susceptibility via modulation of COX-2 and TH levels in the brain. It is important to acknowledge that the relevance of these data to humans is unclear as there are no data linking aberrant DGKε function to patients with neurological conditions.

Huntington's Disease

DGKε has been identified as a promising target for treating Huntington's Disease (HD) (Zhang et al., 2012). This condition is characterized by polyglutamine expansion in the N-terminus of the Huntingtin protein (Htt) that causes significant neuronal loss in the striatum and cortex (MacDonald et al., 1993; Zhang et al., 2012). DGK inhibitor II (R59022) was identified as a promising anti-HD compound in a kinase inhibitor library screen. This *in vitro* assay was performed on mouse HD striatal cell model and the readout was the level of mutant Htt cellular toxicity (Zhang et al., 2012). More specifically, R59022 inhibited the expected increase in caspase 3 and 7 activity triggered by serum withdrawal (Zhang et al., 2012). Cells expressing mutant Htt were also found to have lower levels of PIP_n, that can be restored upon decreasing DGK activity (Zhang et al., 2012).

Pinpointing which of the 10 mammalian DGK isoforms was involved in this process required further testing since this compound is a non-specific DGK inhibitor (Sato et al., 2013). siRNA knockdown experiments carried out against the four DGK isoforms expressed in the mouse striatum showed that only DgkE

siRNA caused a decrease in caspase 3 and 7 similar to that observed with R59022 (Zhang et al., 2012). Data from two well-established *in vivo* models support the hypothesis suggesting that *in vivo,* enhanced DGKε activity plays a role in HD pathogenesis. First, expression of DgkE shRNA in a *Drosophila* HD model partially rescued the motor impairment induced by Htt (Zhang et al., 2012). Second, DgkE mRNA levels were higher in the striatum of a mouse model of HD (Htt overexpression) (Zhang et al., 2012).

FUTURE PERSPECTIVES

The unique primary structure of the smallest known isoform of mammalian DGK, as well as the specificity of DGKε for arachidonoyl-containing DAG, has been known for some time. However, recent findings have presented the possibility of further advances of knowledge in the near future. Two developments have been principally responsible for this. One is the purification of DGKε that will allow new protein structural and membrane binding studies that have not been previously possible. There has also recently been described a causal link between recessive DGKE mutations and a human disease, atypical hemolytic-uremic syndrome (aHUS) (Lemaire et al., 2013). This has given increased importance to defining the biological roles of DGKε in the normal and diseased state.

Crystal Structure

Up to this time, no isoform of a mammalian DGK has been crystallized and its structure determined. However, the crystal structure of a bacterial dgkB from *Staphylococcus aureus*, has been determined (Miller et al., 2008). The amino acid sequence shows 18% identity, with the active site being particularly well conserved (Jennings et al., 2015). A tentative model for the folding of DGKε has been made using the crystallographic structure of dgkB (Jennings et al., 2015).

Lipid Dependence of Membrane Binding

Binding studies of DGKε with liposomes of defined lipid composition have not yet been carried out because the enzyme was not available in pure form but only as membrane pellets from over-expression systems. Now that we have purified DGKε in solution we can perform such experiments. Dr. Prasanta Hota has done some initial studies in Dr. Epand's laboratory using PIP strips (Echelon Biosciences Inc., Salt Lake City, Utah). It was found that the binding to PI, phosphatidylcholine and lysophosphatidylcholine was very weak, binding to PA, phosphatidylserine, and phosphatidylethanolamine was intermediate and binding to PI with one, two, or three phosphate groups added to the inositol ring was very strong. It is known that phosphorylated forms of phosphatidylinositol are good inhibitors of DGKε (Walsh et al., 1995). Phosphatidylinositol-(4,5)-bisphosphate is a noncompetitive inhibitor with respect to SAG but a competitive inhibitor with respect to ATP (Walsh et al., 1995). This is not purely an electrostatic effect since phosphatidylinositol-trisphosphate is a weaker inhibitor of DGKε than is the diphosphate (Walsh et al., 1995). There is thus likely to be some specificity in the binding of DGKε to membranes containing anionic lipids. The effect of anionic lipids on DGKε binding to membranes has not been fully assessed.

Activity and Substrate Specificity in Membrane Bilayers

There is no reported data on the activity of DGKε using an assay system with phospholipid bilayers. All the reported enzyme activity studies with DGKε have been done with a detergent-solubilized system. Phospholipids in liposomes are arranged as bilayers, which more closely simulates their arrangement in biological membranes. Even the fundamental property of arachidonoyl substrate specificity has not been tested in a bilayer-based system. It also is not clear how membrane binding relates to enzymatic activity of DGKε. It is clear that the DAG substrate is part of the membrane and that DGKε has to bind to a membrane, at least transiently, for phosphorylation to occur. However, as indicated by our preliminary data, anionic lipids strengthen the binding of DGKε to the membrane. Why then don't anionic lipids promote the activity of DGKε as they do for other isoforms of mammalian DGK?

Development of a DGKε-Specific Inhibitor

In order to gain a better understanding of DGKε-related diseases, isoform-specific inhibitors should be identified and used in experimental work to delineate the role of DGKε in various tissues and cell types. Although there are several commercially available DGK inhibitors, such as R59022 (de Chaffoy de Courcelles et al., 1985) and R59949 (Sato et al., 2013), there are currently no isoform-specific DGKε inhibitors.

Traditionally, the activities of DGK isoforms were assessed using a micelle-based assay, which utilizes detergents to solubilize lipid components from a lipid film (Epand and Topham, 2007). The hydrated lipid film is then sonicated to produce small unilamellar vesicles. The vesicles are used in a radioactive assay to evaluate the activity of the enzyme, by measuring the transfer of ^{32}P from [γ-^{32}P]-ATP to DAG to form PA (Epand and Topham, 2007). Although this procedure is quite insensitive to non-DGK ATPase activity, the assay might not be well suited for a high-throughput system due to the extensive radioactive and extraction procedures.

Recently, an ATP-luciferase system was developed to evaluate the isoform selectivity of the R59949 and R59022 inhibitors for various DGKs (Sato et al., 2013). The assay was later optimized to study DGKα, and was used to identify a novel DGKα-specific inhibitor, CU-3 (Liu et al., 2016). Similarly, the ATP-luciferase assay can be optimized to study DGKε in a high-throughput format to identify a DGKε-specific inhibitor. Due to the fact that DGKε utilizes ATP as a phosphate donor in the conversion of DAG into PA, the concentration of ADP generated can be used as a measure of PA production and enzyme activity (Shulga et al., 2011c). Since the ATP-luciferase assay detects the ADP released in the kinase reaction, the assay must utilize purified DGKε, in order to reduce contamination with other sources of ATPase activity (Zegzouti et al., 2009).

Once a pool of candidate inhibitors has been identified using the high-throughput method, the candidates can be validated

using various assays. For example, the traditional micelle-based radioactive assay or the liposome based assay currently being developed can be used to confirm changes in enzyme activity in the presence of inhibitors (Epand and Topham, 2007). In addition, a ^{31}P nuclear magnetic resonance assay (NMR) can be used to identify phosphorous containing substrates and products and detect potential contaminants with ATPase activity (Prodeus et al., 2013). Lastly, the ATP-luciferase assay can be optimized to assess the activity of various other DGK isoforms. Once optimized, the assay would be used to the test the inhibitory potential of candidate compounds against various DGK isoforms, to assess the isoform specificity of novel inhibitors.

The development of a DGKε-specific inhibitor would be invaluable for studying the role of DGKε in various disease processes. Specifically, the isoform-specific inhibitor could be used to recapitulate physiological environments lacking DGKε. In addition, the luciferase assay holds potential as a diagnostic tool for measuring the enzyme activity of DGKε in patients (or with CRISPR-Cas9 mutated cells) with missense DGKε mutations.

Possible Modulation of DGKε's Transmembrane vs. Re-entrant Helix

The amino terminal hydrophobic segment of DGKε is highly conserved in evolution (**Figure 3**). In particular, there is an invariant proline residue at position 33 in human DGKε that is present at the same position of the aligned sequences from all DGKε from a range of organisms (Supplementary Materials). We have shown that this proline residue has an important role in determining the position of equilibrium between a transmembrane helix and a re-entrant helix (Decaffmeyer et al., 2008). This segment of the protein has no effect on the activity or specificity of DGKε using an *in vitro* assay in detergent micelles with N-terminal 40–60 residue deletion mutants. However, we believe that it is unlikely that this segment of the protein would not have a biological function. Forming a re-entrant helix would promote the positive curvature of the monolayer in which it is present. This could influence the region of the membrane to which DGKε partitions and/or modulate its interaction with other proteins. An understanding of the role of this proline residue and possibly of the interconversion between re-entrant and transmembrane helix is a theme for future investigations.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Supported by a grant from the Natural Sciences and Engineering Research Council of Canada (Grant 9848, to RE).

ACKNOWLEDGMENTS

We are grateful to Dr. Prasanta Hota, currently at the Hemvati Nandan Bahiguna Garhwal University, who performed the PIP strip binding studies and who contributed to the early stages of the purification of DGKε.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00112

REFERENCES

Abe, T., Lu, X., Jiang, Y., Boccone, C. E., Qian, S., Vattem, K. M., et al. (2003). Site-directed mutagenesis of the active site of diacylglycerol kinase alpha: calcium and phosphatidylserine stimulate enzyme activity via distinct mechanisms. *Biochem. J.* 375, 673–680. doi: 10.1042/bj20031052

Ahmed, S., Kozma, R., Lee, J., Monfries, C., Harden, N., and Lim, L. (1991). The cysteine-rich domain of human proteins, neuronal chimaerin, protein kinase C and diacylglycerol kinase binds zinc. Evidence for the involvement of a zinc-dependent structure in phorbol ester binding. *Biochem. J.* 280(Pt 1), 233–241. doi: 10.1042/bj2800233

Akiva, E., Itzhaki, Z., and Margalit, H. (2008). Built-in loops allow versatility in domain-domain interactions: lessons from self-interacting domains. *Proc. Natl. Acad. Sci. U.S.A.* 105, 13292–13297. doi: 10.1073/pnas.0801207105

Blobel, G. (1980). Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 77, 1496–1500. doi: 10.1073/pnas.77.3.1496

Bruneau, S., Néel, M., Roumenina, L. T., Frimat, M., Laurent, L., Frémeaux-Bacchi, V., et al. (2015). Loss of DGKε induces endothelial cell activation and death independently of complement activation. *Blood* 125, 1038–1046. doi: 10.1182/blood-2014-06-579953

Catzeflis, F. M., Aguilar, J. P., and Jaeger, J. J. (1992). Muroid rodents: phylogeny and evolution. *Trends Ecol. Evol.* 7, 122–126. doi: 10.1016/0169-5347(92)90146-3

Colón-González, F., and Kazanietz, M. G. (2006). C1 domains exposed: from diacylglycerol binding to protein-protein interactions. *Biochim. Biophys. Acta* 1761, 827–837. doi: 10.1016/j.bbalip.2006.05.001

Crotty, T., Cai, J., Sakane, F., Taketomi, A., Prescott, S. M., and Topham, M. K. (2006). Diacylglycerol kinase delta regulates protein kinase C and epidermal growth factor receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* 103, 15485–15490. doi: 10.1073/pnas.0604104103

Decaffmeyer, M., Shulga, Y. V., Dicu, A. O., Thomas, A., Truant, R., Topham, M. K., et al. (2008). Determination of the topology of the hydrophobic segment of mammalian diacylglycerol kinase epsilon in a cell membrane and its relationship to predictions from modeling. *J. Mol. Biol.* 383, 797–809. doi: 10.1016/j.jmb.2008.08.076

de Chaffoy de Courcelles, D. C., Roevens, P., and Van Belle, H. (1985). R 59 022, a diacylglycerol kinase inhibitor. Its effect on diacylglycerol and thrombin-induced C kinase activation in the intact platelet. *J. Biol. Chem.* 260, 15762–15770.

Dicu, A. O., Topham, M. K., Ottaway, L., and Epand, R. M. (2007). Role of the hydrophobic segment of diacylglycerol kinase epsilon. *Biochemistry* 46, 6109–6117. doi: 10.1021/bi6024726

D'Souza, K., and Epand, R. M. (2012). Catalytic activity and acyl-chain selectivity of diacylglycerol kinase epsilon are modulated by residues in and near the lipoxygenase-like motif. *J. Mol. Biol.* 416, 619–628. doi: 10.1016/j.jmb.2012.01.008

D'Souza, K., and Epand, R. M. (2014). Enrichment of phosphatidylinositols with specific acyl chains. *Biochim. Biophys. Acta* 1838, 1501–1508. doi: 10.1016/j.bbamem.2013.10.003

D'Souza, K., Kim, Y. J., Balla, T., and Epand, R. M. (2014). Distinct properties of the two isoforms of CDP-diacylglycerol synthase. *Biochemistry* 53, 7358–7367. doi: 10.1021/bi501250m

Epand, R. M. (2016). Features of the phosphatidylinositol cycle and its role in signal transduction. *J. Membr. Biol.* doi: 10.1007/s00232-016-9909-y. [Epub ahead of print].

Epand, R. M., Kam, A., Bridgelal, N., Saiga, A., and Topham, M. K. (2004). The alpha isoform of diacylglycerol kinase exhibits arachidonoyl specificity with alkylacylglycerol. *Biochemistry* 43, 14778–14783. doi: 10.1021/bi0484724

Epand, R. M., and Topham, M. K. (2007). Measurement of mammalian diacylglycerol kinase activity *in vitro* and in cells. *Methods Enzymol.* 434, 293–304. doi: 10.1016/S0076-6879(07)34016-0

Ewing, R. M., Chu, P., Elisma, F., Li, H., Taylor, P., Climie, S., et al. (2007). Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol. Syst. Biol.* 3, 89. doi: 10.1038/msb4100134

Fanani, M. L., Topham, M. K., Walsh, J. P., and Epand, R. M. (2004) Lipid modulation of the activity of diacylglycerol kinase alpha- and zeta-isoforms: activation by phosphatidylethanolamine and cholesterol. *Biochemistry* 43, 14767–14777. doi: 10.1021/bi049145z

Gantayet, A., Jegatheswaran, J., Jayakumaran, G., Topham, M. K., and Epand, R. M. (2011). Endocannabinoids and diacylglycerol kinase activity. *Biochim. Biophys. Acta* 1808, 1050–1053. doi: 10.1016/j.bbamem.2010.12.022

Gijón, M. A., Riekhof, W. R., Zarini, S., Murphy, R. C., and Voelker, D. R. (2008). Lysophospholipid acyltransferases and arachidonate recycling in human neutrophils. *J. Biol. Chem.* 283, 30235–30245. doi: 10.1074/jbc.M806194200

Glukhov, E., Shulga, Y. V., Epand, R. F., Dicu, A. O., Topham, M. K., Deber, C. M., et al. (2007). Membrane interactions of the hydrophobic segment of diacylglycerol kinase epsilon. *Biochim. Biophys. Acta* 1768, 2549–2558. doi: 10.1016/j.bbamem.2007.06.012

Goldschmidt, H. L., Tu-Sekine, B., Volk, L., Anggono, V., Huganir, R. L., and Raben, D. M. (2016). DGKθ catalytic activity is required for efficient recycling of presynaptic vesicles at excitatory synapses. *Cell Rep.* 14, 200–207. doi: 10.1016/j.celrep.2015.12.022

Goldstein, I., Yizhak, K., Madar, S., Goldfinger, N., Ruppin, E., and Rotter, V. (2013). p53 promotes the expression of gluconeogenesis-related genes and enhances hepatic glucose production. *Cancer Metab.* 1:9. doi: 10.1186/2049-3002-1-9

Gupta, R. S. (2016). Molecular signatures that are distinctive characteristics of the vertebrates and chordates and supporting a grouping of vertebrates with the tunicates. *Mol. Phylogenet. Evol.* 94, 383–391. doi: 10.1016/j.ympev.2015.09.019

Hahn, H. S., Marreez, Y., Odley, A., Sterbling, A., Yussman, M. G., Hilty, K. C., et al. (2003). Protein kinase Calpha negatively regulates systolic and diastolic function in pathological hypertrophy. *Circ. Res.* 93, 1111–1119. doi: 10.1161/01.RES.0000105087.79373.17

Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997). Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci.* 6, 477–480. doi: 10.1002/pro.5560060228

Isozaki, T., Komenoi, S., Lu, Q., Usuki, T., Tomokata, S., Matsutomo, D., et al. (2016). Deficiency of diacylglycerol kinase eta induces lithium-sensitive mania-like behavior. *J. Neurochem.* 138, 448–456. doi: 10.1111/jnc.13661

Jennings, W. (2016). *Purification and Characterization of Diacylglycerol Kinase Epsilon.* M.Sc. thesis, McMaster University, Hamilton, ON.

Jennings, W., Doshi, S., D'Souza, K., and Epand, R. M. (2015). Molecular properties of diacylglycerol kinase-epsilon in relation to function. *Chem. Phys. Lipids* 192, 100–108. doi: 10.1016/j.chemphyslip.2015.06.003

Kavanagh, D., Goodship, T. H., and Richards, A. (2013). Atypical hemolytic uremic syndrome. *Semin. Nephrol.* 33, 508–530. doi: 10.1016/j.semnephrol.2013.08.003

Kimura, T., Jennings, W., and Epand, R. M. (2016). Roles of specific lipid species in the cell and their molecular mechanism. *Prog. Lipid Res.* 62, 75–92. doi: 10.1016/j.plipres.2016.02.001

Kobayashi, N., Hozumi, Y., Ito, T., Hosoya, T., Kondo, H., and Goto, K. (2007). Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. *Eur. J. Cell Biol.* 86, 433–444. doi: 10.1016/j.ejcb.2007.05.002

Komander, D., and Rape, M. (2012). The ubiquitin code. *Annu. Rev. Biochem.* 81, 203–229. doi: 10.1146/annurev-biochem-060310-170328

Kuwahara, K., Wang, Y., McAnally, J., Richardson, J. A., Bassel-Duby, R., Hill, J. A., et al. (2006). TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. *J. Clin. Invest.* 116, 3114–3126. doi: 10.1172/JCI27702

Lee, J. M., Park, Y. S., Lee, J. H., Park, S. J., Shin, J. I., Park, Y. H., et al. (2015). Atypical hemolytic uremic syndrome: Korean pediatric series. *Pediatr. Int.* 57, 431–438. doi: 10.1111/ped.12549

Lemaire, M., Frémeaux-Bacchi, V., Schaefer, F., Choi, M., Tang, W. H., Le Quintrec, M., et al. (2013). Recessive mutations in DGKE cause atypical hemolytic-uremic syndrome. *Nat. Genet.* 45, 531–536. doi: 10.1038/ng.2590

Li, C. H., Cheng, Y. W., Liao, P. L., and Kang, J. J. (2010). Translocation of p53 to mitochondria is regulated by its lipid binding property to anionic phospholipids and it participates in cell death control. *Neoplasia* 12, 150–160. doi: 10.1593/neo.91500

Liu, K., Kunii, N., Sakuma, M., Yamaki, A., Mizuno, S., Sato, M., et al. (2016). A novel diacylglycerol kinase α-selective inhibitor, CU-3, induces cancer cell apoptosis and enhances immune response. *J. Lipid* Res. 57, 368–379. doi: 10.1194/jlr.M062794

Liu, W., Xie, Y., Ma, J., Luo, X., Nie, P., Zuo, Z., et al. (2015). IBS: an illustrator for the presentation and visualization of biological sequences. *Bioinformatics* 31, 3359–3361. doi: 10.1093/bioinformatics/btv362

Lukiw, W. J., Cui, J. G., Musto, A. E., Musto, B. C., and Bazan, N. G. (2005). Epileptogenesis in diacylglycerol kinase epsilon deficiency up-regulates COX-2 and tyrosine hydroxylase in hippocampus. *Biochem. Biophys. Res. Commun.* 338, 77–81. doi: 10.1016/j.bbrc.2005.08.109

Lung, M., Shulga, Y. V., Ivanova, P. T., Myers, D. S., Milne, S. B., Brown, H. A., et al. (2009). Diacylglycerol kinase epsilon is selective for both acyl chains of phosphatidic acid or diacylglycerol. *J. Biol. Chem.* 284, 31062–31073. doi: 10.1074/jbc.M109.050617

MacDonald, M. E., Ambrose, C. M., Duyao, M. P., Myers, R. H., Lin, C., Srinidhi, L., et al. (1993). A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 72, 971–983. doi: 10.1016/0092-8674(93)90585-E

Matsui, H., Hozumi, Y., Tanaka, T., Okada, M., Nakano, T., Suzuki, Y., et al. (2014). Role of the N-terminal hydrophobic residues of DGKepsilon in targeting the endoplasmic reticulum. *Biochim. Biophys. Acta* 1841, 1440–1450. doi: 10.1016/j.bbalip.2014.07.007

Mele, C., Lemaire, M., Iatropoulos, P., Piras, R., Bresin, E., Bettoni, S., et al. (2015). Characterization of a new *DGKE* intronic mutation in genetically unsolved cases of familial atypical hemolytic uremic syndrome. *Clin. J. Am. Soc. Nephrol.* 10, 1011–1019. doi: 10.2215/CJN.08520814

Mertins, P., Qiao, J. W., Patel, J., Udeshi, N. D., Clauser, K. R., Mani, D. R., et al. (2013). Integrated proteomic analysis of post-translational modifications by serial enrichment. *Nat. Methods* 10, 634–637. doi: 10.1038/nmeth.2518

Miller, D. J., Jerga, A., Rock, C. O., and White, S. W. (2008). Analysis of the *Staphylococcus aureus* DgkB structure reveals a common catalytic mechanism for the soluble diacylglycerol kinases. *Structure* 16, 1036–1046. doi: 10.1016/j.str.2008.03.019

Milne, S. B., Ivanova, P. T., Armstrong, M. D., Myers, D. S., Lubarda, J., Shulga, Y. V., et al. (2008). Dramatic differences in the roles in lipid metabolism of two isoforms of diacylglycerol kinase. *Biochemistry* 47, 9372–9379. doi: 10.1021/bi800492c

Miyamoto, K., Tomizawa, T., Koshiba, S., Inoue, M., Kigawa, T., and Yokoyama, S. (2004). *Solution Structure of the c1 Domain of the Human Diacylglycerol Kinase Delta.* Accession number 1R79, PDB Database. Available online at: http://www.rcsb.org/pdb/home/home.do

Miyata, T., Uchida, Y., Ohta, T., Urayama, K., Yoshida, Y., and Fujimura, Y. (2015). Atypical haemolytic uraemic syndrome in a Japanese patient with DGKE genetic mutations. *Thromb. Haemost.* 114, 862–863. doi: 10.1160/TH15-01-0007

Müller, U. (1999). Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mech. Dev.* 82, 3–21. doi: 10.1016/S0925-4773(99)00021-0

Musto, A., and Bazan, N. G. (2006). Diacylglycerol kinase epsilon modulates rapid kindling epileptogenesis. *Epilepsia* 47, 267–276. doi: 10.1111/j.1528-1167.2006.00418.x

Naguib, A., Bencze, G., Engle, D. D., Chio, I. I., Herzka, T., Watrud, K., et al. (2015). p53 mutations change phosphatidylinositol acyl chain composition. *Cell Rep.* 10, 8–19. doi: 10.1016/j.celrep.2014.12.010

Natalini, P. M., Zulian, S. E., Ilincheta de Boschero, M. G., and Giusto, N. M. (2013). Diacylglycerol kinase epsilon in bovine and rat photoreceptor cells. Light-dependent distribution in photoreceptor cells. *Exp. Eye Res.* 112, 139–150. doi: 10.1016/j.exer.2013.04.012

Neau, D. B., Gilbert, N. C., Bartlett, S. G., Boeglin, W., Brash, A. R., and Newcomer, M. E. (2009). The 1.85 A structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. *Biochemistry* 48, 7906–7915. doi: 10.1021/bi900084m

Niizeki, T., Takeishi, Y., Kitahara, T., Arimoto, T., Ishino, M., Bilim, O., et al. (2008). Diacylglycerol kinase-epsilon restores cardiac dysfunction under chronic pressure overload: a new specific regulator of Galpha(q) signaling cascade. *Am. J. Physiol. Heart Circ. Physiol.* 295, H245–H255. doi: 10.1152/ajpheart.00066.2008

Nørholm, M. H., Shulga, Y. V., Aoki, S., Epand, R. M., and von Heijne, G. (2011). Flanking residues help determine whether a hydrophobic segment adopts a monotopic or bitopic topology in the endoplasmic reticulum membrane. *J. Biol. Chem.* 286, 25284–25290. doi: 10.1074/jbc.M111.244616

Noris, M., Mescia, F., and Remuzzi, G. (2012). STEC-HUS, atypical HUS and TTP are all diseases of complement activation. *Nat. Rev. Nephrol.* 8, 622–633. doi: 10.1038/nrneph.2012.195

Olenchock, B. A., Guo, R., Carpenter, J. H., Jordan, M., Topham, M. K., Koretzky, G. A., et al. (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* 7, 1174–1181. doi: 10.1038/ni1400

Ozaltin, F., Li, B., Rauhauser, A., An, S. W., Soylemezoglu, O., Gonul, I. I., et al. (2013). DGKE variants cause a glomerular microangiopathy that mimics membranoproliferative GN. *J. Am. Soc. Nephrol.* 24, 377–384. doi: 10.1681/ASN.2012090903

Park, J. M., Park, J. H., Mun, D. G., Bae, J., Jung, J. H., Back, S., et al. (2015). Integrated analysis of global proteome, phosphoproteome, and glycoproteome enables complementary interpretation of disease-related protein networks. *Sci. Rep.* 5:18189. doi: 10.1038/srep18189

Pettitt, T. R., and Wakelam, M. J. (1999). Diacylglycerol kinase epsilon, but not zeta, selectively removes polyunsaturated diacylglycerol, inducing altered protein kinase C distribution *in vivo*. *J. Biol. Chem.* 274, 36181–36186. doi: 10.1074/jbc.274.51.36181

Pickering, M. C., Warren, J., Rose, K. L., Carlucci, F., Wang, Y., Walport, M. J., et al. (2006). Prevention of C5 activation ameliorates spontaneous and experimental glomerulonephritis in factor H-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9649–9654. doi: 10.1073/pnas.0601094103

Premont, R. T., and Gainetdinov, R. R. (2007). Physiological roles of G protein-coupled receptor kinases and arrestins. *Annu. Rev. Physiol.* 69, 511–534. doi: 10.1146/annurev.physiol.69.022405.154731

Prodeus, A., Berno, B., Topham, M. K., and Epand, R. M. (2013). The basis of the substrate specificity of the epsilon isoform of human diacylglycerol kinase is not a consequence of competing hydrolysis of ATP. *Chem. Phys. Lipids* 166, 26–30. doi: 10.1016/j.chemphyslip.2012.11.006

Quaggin, S. E. (2013). DGKE and atypical HUS. *Nat. Genet.* 45, 475–476. doi: 10.1038/ng.2622

Regier, D. S., Higbee, J., Lund, K. M., Sakane, F., Prescott, S. M., and Topham, M. K. (2005). Diacylglycerol kinase iota regulates Ras guanyl-releasing protein 3 and inhibits Rap1 signaling. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7595–7600. doi: 10.1073/pnas.0500663102

Rodriguez de Turco, E. B., Tang, W., Topham, M. K., Sakane, F., Marcheselli, V. L., Chen, C., et al. (2001). Diacylglycerol kinase epsilon regulates seizure susceptibility and long-term potentiation through arachidonoyl-inositol lipid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4740–4745. doi: 10.1073/pnas.081536298

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., et al. (2015). Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature* 524, 230–233. doi: 10.1038/nature14580

Roux, K. J., Kim, D. I., and Burke, B. (2013). BioID: a screen for protein-protein interactions. *Curr. Protoc. Protein Sci.* 74:Unit 19.23. doi: 10.1002/0471140864.ps1923s74

Ryu, J. R., Shin, C. Y., Park, K. H., Jeon, G. S., Kim, H., Kim, W., et al. (2000). Effect of repeated seizure experiences on tyrosine hydroxylase immunoreactivities in the brain of genetically epilepsy-prone rats. *Brain Res. Bull.* 53, 777–782. doi: 10.1016/S0361-9230(00)00373-7

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007). Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* 1771, 793–806. doi: 10.1016/j.bbalip.2007.04.006

Sakane, F., Kai, M., Wada, I., Imai, S., and Kanoh, H. (1996). The C-terminal part of diacylglycerol kinase alpha lacking zinc fingers serves as a catalytic domain. *Biochem. J.* 318(Pt 2), 583–590. doi: 10.1042/bj3180583

Sali, A., and Blundell, T. L. (1993). Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.* 234, 779–815. doi: 10.1006/jmbi.1993.1626

Sánchez Chinchilla, D., Pinto, S., Hoppe, B., Adragna, M., Lopez, L., Justa Roldan, M. L., et al. (2014). Complement mutations in diacylglycerol kinase-epsilon-associated atypical hemolytic uremic syndrome. *Clin. J. Am. Soc. Nephrol.* 9, 1611–1619. doi: 10.2215/CJN.01640214

Sato, M., Liu, K., Sasaki, S., Kunii, N., Sakai, H., Mizuno, H., et al. (2013). Evaluations of the selectivities of the diacylglycerol kinase inhibitors R59022 and R59949 among diacylglycerol kinase isozymes using a new non-radioactive assay method. *Pharmacology* 92, 99–107. doi: 10.1159/000351849

Sato, Y., Murakami, C., Yamaki, A., Mizuno, S., Sakai, H., and Sakane, F. (2016). Distinct 1-monoacylglycerol and 2-monoacylglycerol kinase activities of diacylglycerol kinase isozymes. *Biochim. Biophys. Acta* 1864, 1170–1176. doi: 10.1016/j.bbapap.2016.06.012

Schneider, E. G., and Kennedy, E. P. (1973). Phosphorylation of ceramide by diglyceride kinase preparations from *Escherichia coli*. *J. Biol. Chem.* 248, 3739–3741.

Shen, M. Y., and Sali, A. (2006). Statistical potential for assessment and prediction of protein structures. *Protein Sci.* 15, 2507–2524. doi: 10.1110/ps.062416606

Shindo, M., Irie, K., Masuda, A., Ohigashi, H., Shirai, Y., Miyasaka, K., et al. (2003). Synthesis and phorbol ester binding of the cysteine-rich domains of diacylglycerol kinase (DGK) isozymes. DGKgamma and DGKbeta are new targets of tumor-promoting phorbol esters. *J. Biol. Chem.* 278, 18448–18454. doi: 10.1074/jbc.M300400200

Shindo, M., Irie, K., Ohigashi, H., Kuriyama, M., and Saito, N. (2001). Diacylglycerol kinase gamma is one of the specific receptors of tumor-promoting phorbol esters. *Biochem. Biophys. Res. Commun.* 289, 451–456. doi: 10.1006/bbrc.2001.5935

Shirai, Y., Ikeda, M., and Saito, N. (2012). Regulation of diacylglycerol kinase by phosphorylation. *Adv. Biol. Regul.* 52, 239–247. doi: 10.1016/j.advenzreg.2011.09.004

Shirai, Y., Kouzuki, T., Kakefuda, K., Moriguchi, S., Oyagi, A., Horie, K., et al. (2010). Essential role of neuron-enriched diacylglycerol kinase (DGK), DGKbeta in neurite spine formation, contributing to cognitive function. *PLoS ONE* 5:e11602. doi: 10.1371/journal.pone.0011602

Shulga, Y. V., Loukov, D., Ivanova, P. T., Milne, S. B., Myers, D. S., Hatch, G. M., et al. (2013). Diacylglycerol kinase delta promotes lipogenesis. *Biochemistry* 52, 7766–7776. doi: 10.1021/bi401178y

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011a). Substrate specificity of diacylglycerol kinase-epsilon and the phosphatidylinositol cycle. *FEBS Lett.* 585, 4025–4028. doi: 10.1016/j.febslet.2011.11.016

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011b). Study of arachidonoyl specificity in two enzymes of the PI cycle. *J. Mol. Biol.* 409, 101–112. doi: 10.1016/j.jmb.2011.03.071

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011c). Regulation and functions of diacylglycerol kinases. *Chem. Rev.* 111, 6186–6208. doi: 10.1021/cr1004106

Singh, B., and Gupta, R. S. (2009). Conserved inserts in the Hsp60 (GroEL) and Hsp70 (DnaK) proteins are essential for cellular growth. *Mol. Genet. Genomics* 281, 361–373. doi: 10.1007/s00438-008-0417-3

Skarnes, W. C., Rosen, B., West, A. P., Koutsourakis, M., Bushell, W., Iyer, V., et al. (2011). A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* 474, 337–342. doi: 10.1038/nature10163

So, V., Jalan, D., Lemaire, M., Topham, M. K., Hatch, G. M., and Epand, R. M. (2016). Diacylglycerol kinase epsilon suppresses expression of P53 and glycerol kinase in mouse embryo fibroblasts. *Biochim. Biophys. Acta* 1861, 1993–1999. doi: 10.1016/j.bbalip.2016.09.021

Song, M., Matkovich, S. J., Zhang, Y., Hammer, D. J., and Dorn, G. W. (2015). Combined cardiomyocyte PKCdelta and PKCepsilon gene deletion uncovers their central role in restraining developmental and reactive heart growth. *Sci. Signal.* 8, ra39. doi: 10.1126/scisignal.aaa1855

Takeda, M., Kagaya, Y., Takahashi, J., Sugie, T., Ohta, J., Watanabe, J., et al. (2001). Gene expression and *in situ* localization of diacylglycerol kinase isozymes in normal and infarcted rat hearts: effects of captopril treatment. *Circ. Res.* 89, 265–272. doi: 10.1161/hh1501.094185

Takemiya, T., Suzuki, K., Sugiura, H., Yasuda, S., Yamagata, K., Kawakami, Y., et al. (2003). Inducible brain COX-2 facilitates the recurrence of hippocampal seizures in mouse rapid kindling. *Prostaglandins Other Lipid Mediat.* 71, 205–216. doi: 10.1016/S1098-8823(03)00040-6

Tang, W. (1999). *Identification and Gene-Targeting of a Novel Diacylglycerol Kinase Highly Selective for Arachidonate-Cont Aining Substrates*. Ph.D. dissertation, University of Utah, Salt Lake City, UT, 1–131. Available online at: https://goo.gl/zUlcSm

Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. *J. Biol. Chem.* 271, 10237–10241. doi: 10.1074/jbc.271.17.10230

Thomas, W. E., and Glomset, J. A. (1999a). Affinity purification and catalytic properties of a soluble, Ca2+-independent, diacylglycerol kinase. *Biochemistry* 38, 3320–3326. doi: 10.1021/bi982567m

Thomas, W. E., and Glomset, J. A. (1999b). Multiple factors influence the binding of a soluble, Ca^{2+}-independent, diacylglycerol kinase to unilamellar phosphoglyceride vesicles. *Biochemistry* 38, 3310–3319. doi: 10.1021/bi982566u

Thurman, J. M., Tchepeleva, S. N., Haas, M., Panzer, S., Boackle, S. A., Glogowska, M. J., et al. (2012). Complement alternative pathway activation in the autologous phase of nephrotoxic serum nephritis. *Am. J. Physiol. Renal Physiol.* 302, F1529–F1536. doi: 10.1152/ajprenal.00422.2011

Topham, M. K., and Prescott, S. M. (1999). Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* 274, 11447–11450. doi: 10.1074/jbc.274.17.11447

van Blitterswijk, W. J., and Houssa, B. (2000). Properties and functions of diacylglycerol kinases. *Cell Signal.* 12, 595–605. doi: 10.1016/S0898-6568(00)00113-3

Vernon, K. A., Ruseva, M. M., Cook, H. T., Botto, M., Malik, T. H., and Pickering, M. C. (2016). Partial complement factor H deficiency associates with C3 glomerulopathy and thrombotic microangiopathy. *J. Am. Soc. Nephrol.* 27, 1334–1342. doi: 10.1681/ASN.2015030295

Wagner, S. A., Beli, P., Weinert, B. T., Nielsen, M. L., Cox, J., Mann, M., et al. (2011). A proteome-wide, quantitative survey of *in vivo* ubiquitylation sites reveals widespread regulatory roles. *Mol. Cell. Proteomics* 10:M111. doi: 10.1074/mcp.M111.013284

Wagner, S. A., Beli, P., Weinert, B. T., Schölz, C., Kelstrup, C. D., Young, C., et al. (2012). Proteomic analyses reveal divergent ubiquitylation site patterns in murine tissues. *Mol. Cell. Proteomics* 11, 1578–1585. doi: 10.1074/mcp.M112.017905

Walsh, J. P., Fahrner, L., and Bell, R. M. (1990). sn-1,2-diacylglycerol kinase of *Escherichia coli*. Diacylglycerol analogues define specificity and mechanism. *J. Biol. Chem.* 265, 4374–4381.

Walsh, J. P., Suen, R., and Glomset, J. A. (1995). Arachidonoyl-diacylglycerol kinase. Specific *in vitro* inhibition by polyphosphoinositides suggests a mechanism for regulation of phosphatidylinositol biosynthesis. *J. Biol. Chem.* 270, 28647–28653. doi: 10.1074/jbc.270.48.28647

Wang, J., Huo, K., Ma, L., Tang, L., Li, D., Huang, X., et al. (2011). Toward an understanding of the protein interaction network of the human liver. *Mol. Syst. Biol.* 7, 536. doi: 10.1038/msb.2011.67

Westland, R., Bodria, M., Carrea, A., Lata, S., Scolari, F., Fremeaux-Bacchi, V., et al. (2014). Phenotypic expansion of DGKE-associated diseases. *J. Am. Soc. Nephrol.* 25, 1408–1414. doi: 10.1681/ASN.2013080886

Xiao, K., McClatchy, D. B., Shukla, A. K., Zhao, Y., Chen, M., Shenoy, S. K., et al. (2007). Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 12011–12016. doi: 10.1073/pnas.0704849104

Xu, D., and Zhang, Y. (2011). Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. *Biophys. J.* 101, 2525–2534. doi: 10.1016/j.bpj.2011.10.024

Yahagi, H., Takeda, M., Asaumi, Y., Okumura, K., Takahashi, R., Takahashi, J., et al. (2005). Differential regulation of diacylglycerol kinase isozymes in cardiac hypertrophy. *Biochem. Biophys. Res. Commun.* 332, 101–108. doi: 10.1016/j.bbrc.2005.04.094

Zegzouti, H., Zdanovskaia, M., Hsiao, K., and Goueli, S. A. (2009). ADP-Glo: a Bioluminescent and homogeneous ADP monitoring assay for kinases, Assay. *Drug Dev. Technol.* 7, 560–572. doi: 10.1089/adt.2009.0222

Zhang, N., Li, B., Al-Ramahi, I., Cong, X., Held, J. M., Kim, E., et al. (2012). Inhibition of lipid signaling enzyme diacylglycerol kinase epsilon attenuates mutant huntingtin toxicity. *J. Biol. Chem.* 287, 21204–21213. doi: 10.1074/jbc.M111.321661

Zhong, X. P., Hainey, E. A., Olenchock, B. A., Jordan, M. S., Maltzman, J. S., Nichols, K. E., et al. (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat. Immunol.* 4, 882–890. doi: 10.1038/ni958

Zhu, J., Chaki, M., Lu, D., Ren, C., Wang, S. S., Rauhauser, A., et al. (2016). Loss of diacylglycerol kinase epsilon in mice causes endothelial distress and impairs glomerular Cox-2 and PGE2 production. *Am. J. Physiol. Renal Physiol.* 310, F895–F908. doi: 10.1152/ajprenal.00431.2015

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 18 November 2016
doi: 10.3389/fcell.2016.00132

Arachidonoyl-Specific Diacylglycerol Kinase ε and the Endoplasmic Reticulum

*Tomoyuki Nakano[1], Hirooki Matsui[1], Toshiaki Tanaka[1], Yasukazu Hozumi[1], Ken Iseki[2], Kaneyuki Kawamae[3] and Kaoru Goto[1]**

[1] Department of Anatomy and Cell Biology, Yamagata University School of Medicine, Yamagata, Japan, [2] Department of Emergency and Critical Care Medicine, Fukushima Medical University School of Medicine, Fukushima, Japan, [3] Department of Anesthesiology, Yamagata University School of Medicine, Yamagata, Japan

The endoplasmic reticulum (ER) comprises an interconnected membrane network, which is made up of lipid bilayer and associated proteins. This organelle plays a central role in the protein synthesis and sorting. In addition, it represents the synthetic machinery of phospholipids, the major constituents of the biological membrane. In this process, phosphatidic acid (PA) serves as a precursor of all phospholipids, suggesting that PA synthetic activity is closely associated with the ER function. One enzyme responsible for PA synthesis is diacylglycerol kinase (DGK) that phosphorylates diacylglycerol (DG) to PA. DGK is composed of a family of enzymes with distinct features assigned to each isozyme in terms of structure, enzymology, and subcellular localization. Of DGKs, DGKε uniquely exhibits substrate specificity toward arachidonate-containing DG and is shown to reside in the ER. Arachidonic acid, a precursor of bioactive eicosanoids, is usually acylated at the *sn*-2 position of phospholipids, being especially enriched in phosphoinositide. In this review, we focus on arachidonoyl-specific DGKε with respect to the historical context, molecular basis of the substrate specificity and ER-targeting, and functional implications in the ER.

Keywords: diacylglycerol kinase, arachidonate, substrate specificity, endoplasmic reticulum, phosphoinositide, ER stress

OPEN ACCESS

Edited by:
Isabel Merida, Spanish National Research Council, Spain

Reviewed by:
Uwe Lendeckel, University of Greifswald, Germany
Frank Thévenod, Witten/Herdecke University, Germany

****Correspondence:***
Kaoru Goto
kgoto@med.id.yamagata-u.ac.jp

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *03 August 2016*
Accepted: *31 October 2016*
Published: *18 November 2016*

Citation:
Nakano T, Matsui H, Tanaka T, Hozumi Y, Iseki K, Kawamae K and Goto K (2016) Arachidonoyl-Specific Diacylglycerol Kinase ε and the Endoplasmic Reticulum. Front. Cell Dev. Biol. 4:132. doi: 10.3389/fcell.2016.00132

INTRODUCTION

The endoplasmic reticulum (ER), which comprises a tubular and planar network of lipid bilayer membranes (Croze and Morré, 1984), represents a specialized site of protein synthesis and subsequent folding machinery. In addition, the ER plays a central role in the synthesis and transport of major membrane phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI; Gaspar et al., 2007). In response to cellular requirements, this tubular and planar ER network extends to all regions of cell interfaces at membrane contact sites with the plasma membrane, mitochondria, and Golgi apparatus for lipid transfer, integration of metabolic pathways, and calcium homeostasis (Lagace and Ridgway, 2013). In terms of energy homeostasis, fatty acids supply a major source of energy for organisms, but they can also be toxic. When exposed to excess fatty acids, cells esterify fatty acids into neutral lipids and package them into lipid droplets (LDs). Actually, an LD is an ER-derived organelle that is necessary for the storage and mobilization

of neutral lipids in a specialized cell type: adipocyte (Martin and Parton, 2006; Brasaemle and Wolins, 2012). Under pathological conditions including nutrient and oxygen starvation, calcium depletion and altered redox status, protein folding, and lipid biosynthesis are impaired, thereby producing ER stress. Therefore, the ER integrates cellular activities of protein and lipid synthesis as well as pathological responses such as unfolding protein response (UPR; Berridge, 2002; Ron and Walter, 2007; Sano and Reed, 2013).

During the process of lipid synthesis, phosphatidic acid (PA) serves as an intermediate molecule for all phospholipids. It is therefore conceivable that PA synthetic activity is intimately involved in the ER function, and that one enzyme responsible for this activity is diacylglycerol kinase (DGK; Kanoh et al., 1990). Actually, DGK comprises a family of enzymes. Each of the isozymes exhibits a characteristic feature in terms of structural, enzymological, and morphological aspects (Goto et al., 2007; Sakane et al., 2007; Mérida et al., 2008; Topham and Epand, 2009; **Table 1**). Each member of the DGK family presents a unique subcellular localization in transfected cells and presumably plays a specific role at each site (Kobayashi et al., 2007). Of the DGKs, DGKε is unique in its substrate specificity toward arachidonate-containing DG and resides in the ER (Matsui et al., 2014). In this review, we specifically examine the functional role of DGKε in this organelle.

IDENTIFICATION OF ARACHIDONOYL DGK

Since the first discovery of DGK activity in a brain microsome fraction (Hokin and Hokin, 1959), it has been reported as distributed widely in animal tissues (Hokin and Hokin, 1963; Sastry and Hokin, 1966; Prottey and Hawthorne, 1967; Lapetina and Hawthorne, 1971; Farese et al., 1981). The DGK activity was associated with various fractions of cells, including soluble, membranous, and cytoskeletal fractions (Call and Rubert, 1973; Daleo et al., 1974). These features suggest the heterogeneity of DGK in animal tissues and cells. In this respect, Glomset group reported that cytosolic and membrane-bound DGKs in Swiss 3T3 cells show different substrate selectivity (MacDonald et al., 1988). Intriguingly, the membrane-bound DGK is unique in that it selectively catalyzes DG containing arachidonate at the *sn*-2 position (Lemaitre et al., 1990). Moreover, it is rapidly inactivated by preincubation with its preferred substrate. Generally, DGK activity is determined using several assay systems with different detergents, including octylglucoside mixed micelle assay, deoxycholate assay, and Triton X-100 assay (Walsh et al., 1994). It is particularly noteworthy that the detection of arachidonoyl-specificity depends on the assay system that is used. The substrate selectivity toward arachidonate-containing DG is most sensitive in the octylglucoside assay, but is not detected in the deoxycholate assay. The sensitive assay system together with presumed thermal lability made it difficult to purify the enzyme. Biochemical purification of this "arachidonoyl DGK" from bovine testis estimated the molecular mass as 58,000 (Walsh et al., 1994), although PCR cloning using degenerate primers succeeded in isolating the cDNA clone encoding arachidonoyl DGK, designated as DGKε (Tang et al., 1996).

MOLECULAR BASIS FOR ARACHIDONOYL SPECIFICITY OF DGKε

DGKε is the only isozyme that shows substrate specificity toward arachidonate (20:4)-containing DG. As a substrate for DGKε, *sn*-1-stearoyl-2-arachidonoyl-DG (18:0/20:4-DG) is preferred over saturated DG (*sn*-1,2-didecanoyl-DG, 10:0/10:0-DG) or monounsaturared DG (*sn*-1,2-dioleoyl-DG, 18:1/18:1-DG). It should be mentioned that DGKε prefers 18:0/20:4-DG to *sn*-1-stearoyl-2-linoleoyl-DG (18:0/18:2-DG) and *sn*-1-stearoyl-2-docosahexaenoyl-DG (18:0/22:6-DG) (Lemaitre et al., 1990; Tang et al., 1996; Shulga et al., 2011a). Therefore, it is concluded that DGKε prefers arachidonate at the *sn*-2 position. Arachidonic acid, an essential polyunsaturated fatty acid, contains four double bonds. Arachidonate is not only a major component of membrane phospholipid; it is also the precursor of bioactive molecules designated as eicosanoids, such as prostaglandins and leukotrienes that are catalyzed, respectively, by cyclooxygenase (COX) and lipoxygenase (LOX; Funk, 2001; Buczynski et al., 2009). Because these arachidonate-derivatives serve as key mediators of several pathophysiological events, free arachidonate itself should be maintained within a restricted concentration (Peters-Golden and Henderson, 2007). Under physiological conditions, arachidonate is incorporated into the *sn*-2 position of phospholipids by the enzymes arachidonoyl-CoA synthetase and lysophospholipid acyltransferases (Pérez-Chacón et al., 2009). These enzymes, together with DGKε, specifically recognize arachidonate moiety. However, how is the arachidonoyl specificity achieved?

To investigate the molecular basis of the substrate specificity of DGKε toward arachidonate, the Epand group compared amino acid sequences of the enzymes that specifically recognize this fatty acid (Shulga et al., 2011b). They identified in the catalytic domain of DGKε (aa. 421–453 in human sequence) the motif L-X(3-4)-R-X(2)-L-X(4)-G, in which -X(n)- is n residues of any amino acid. This domain, which is contained in DGKε of various species as well as phosphatidylinositol-4-phosphate-5-kinase type Iα, resembles a polyunsaturated fatty acid-recognizing domain identified in lipoxygenases (Neau et al., 2009). Mutations of the essential residues in this motif, L431I and L438I, significantly reduce arachidonoyl specificity. Furthermore, the group found a sequence similar to this LOX-like motif in non-specific isozyme DGKα, with a V656 residue instead of Leu in DGKε. They confirm that V656L mutation introduces some specificity for arachidonate-containing DG to DGKα.

TARGETING OF DGKε TO THE ER

The DGK family is localized to distinct subcellular compartments in cDNA-transfected cells, including the cytoplasm, ER, Golgi complex, actin-cytoskeleton, and nucleus (Kobayashi et al., 2007). In an early fractionation study using Swiss 3T3 cells,

TABLE 1 | Characteristic features of mammalian DGK isozymes.

	Molecular weight (kDa)	Substrate specificity and Ca^{2+}-dependency	Main tissue and cell expression	Subcellular localization in native cells	References
TYPE I					
DGKα	82	Non-specific, Ca^{2+}-dependent	T cells, brain (oligodendrocytes)	Cytoplasm, nucleus	Sakane et al., 1990; Schaap et al., 1990; Goto et al., 1992
DGKβ	90	Non-specific, Ca^{2+}-dependent	Brain (striatal neurons)	Perisynaptic membrane	Goto and Kondo, 1993; Hozumi et al., 2008
DGKγ	88	Non-specific, Ca^{2+}-dependent	Brain (cerebellar Purkinje neurons)	Golgi apparatus	Goto et al., 1994; Kai et al., 1994; Nakano et al., 2012
TYPE II					
DGKδ	130	Non-specific	Reproductive organs, leukocytes, ubiquitous	Cytoplasm	Sakane et al., 1996, 2002
DGKη	127	Non-specific	Reproductive organs, ubiquitous	Cytoplasm	Klauck et al., 1996; Murakami et al., 2003
DGKκ	142	Non-specific	Reproductive organs	Plasma membrane	Imai et al., 2005
TYPE III					
DGKε	64	sn-2-arachidonoyl (20:4) -DG-specific	Brain (neurons), ubiquitous	Endoplasmic reticulum	Lemaitre et al., 1990; Tang et al., 1996; Shulga et al., 2011a; Matsui et al., 2014
TYPE IV					
DGKζ	104	Non-specific	Brain (neurons), ubiquitous	Nucleus	Bunting et al., 1996; Goto and Kondo, 1996; Hozumi et al., 2003
DGKι	117	Non-specific	Brain (neurons), retina	Postsynaptic region of rod bipolar dendrites	Ding et al., 1998; Ito et al., 2004; Hozumi et al., 2013
TYPE V					
DGKθ	110	Non-specific	Brain (neurons), smooth muscle, and endothelial cells	Excitatory presynapses, nuclear speckles	Houssa et al., 1997; Walker et al., 2001; Tabellini et al., 2003; Goldschmidt et al., 2016

"arachidonoyl DGK activity" comigrated with that of the ER marker enzymes, together with other PI-metabolizing enzyme PI synthase (Glomset, 1996). In agreement with the biochemical data, DGKε is targeted to the ER. Because DGKε is highly insoluble, the hydrophobic region was presumed to play a key role in the ER targeting. We investigated the sequence responsible for ER targeting of DGKε (Matsui et al., 2014). Various deletion and substitution mutations of rat DGKε tagged with GFP were transfected in cells and were compared with ER markers. Results show clearly that a stretch of hydrophobic amino acid sequence 20–40 (DGKε 20–40) in the N-terminus is a determinant sequence in controlling the ER targeting of DGKε. This hydrophobic region adopts an α-helical structure of the transmembrane segment (Glukhov et al., 2007).

In this regard, a detailed modeling study suggests the possibility that this sequence structure can take two representative models of low-energy conformations, such as a long straight helix and a U-bend helix (Decaffmeyer et al., 2008). Two interchangeable structures of monotopic and bitopic nature might confer on DGKε a unique feature in relation to the ER. Changing conditions such as a redox state and pH can regulate the conformation of DGKε between these two structures, thereby affecting the relation of DGKε and the ER membrane.

The α-helical structure of the hydrophobic sequence 20–40 creates a "hydrophobic patch" composed of L22, L25, and L29 (according to the rat sequence; **Figure 1**). To test whether the hydrophobicity is critical in the ER targeting, we produced two substitution mutants: one containing less hydrophobic Ala (hydrophobic score 1.8) and the other with hydrophilic Gln (hydrophobic score -3.5), instead of wild-type Leu (hydrophobic score 3.8; (Matsui et al., 2014)). Ala substitution fragment DGKε (20–40/L22A, L25A, L29A) is targeted to the ER membrane. It is recovered in the membrane fraction, along with wild-type fragment. However, Gln substitution fragment DGKε (20–40/L22G, L25G, L29G) containing a "hydrophilic patch" is distributed diffusely in the cytoplasm and is recovered in the soluble fraction. Furthermore, full-length Ala mutant DGKε (L22A, L25A, L29A) is shown to reside in the ER whereas Gln mutant DGKε (L22G, L25G, L29G) abolishes it. These findings suggest that the hydrophobic patch composed of L22, L25, and L29 is crucially important for ER targeting of DGKε.

No general consensus sequence for ER localization, such as the ER retention signal, is found in this hydrophobic or in other regions of DGKε (Matsui et al., 2014). Therefore, details of the ER targeting mechanism of DGKε remain unclear. How does this hydrophobic patch specifically lead DGKε to the ER, instead of the other membranes such as mitochondrial membrane? The ER consists of phospholipid bilayer containing a plethora of proteins. Is the membrane or the protein of the ER recognized by the hydrophobic patch? Does the hydrophobic patch bind to some microdomain of the membrane? Because DGKε-kinase dead mutant also resides in the ER, the substrate DG and the product PA are not involved in subcellular localization of DGKε. Therefore, the current data can be summarized as follows: ER

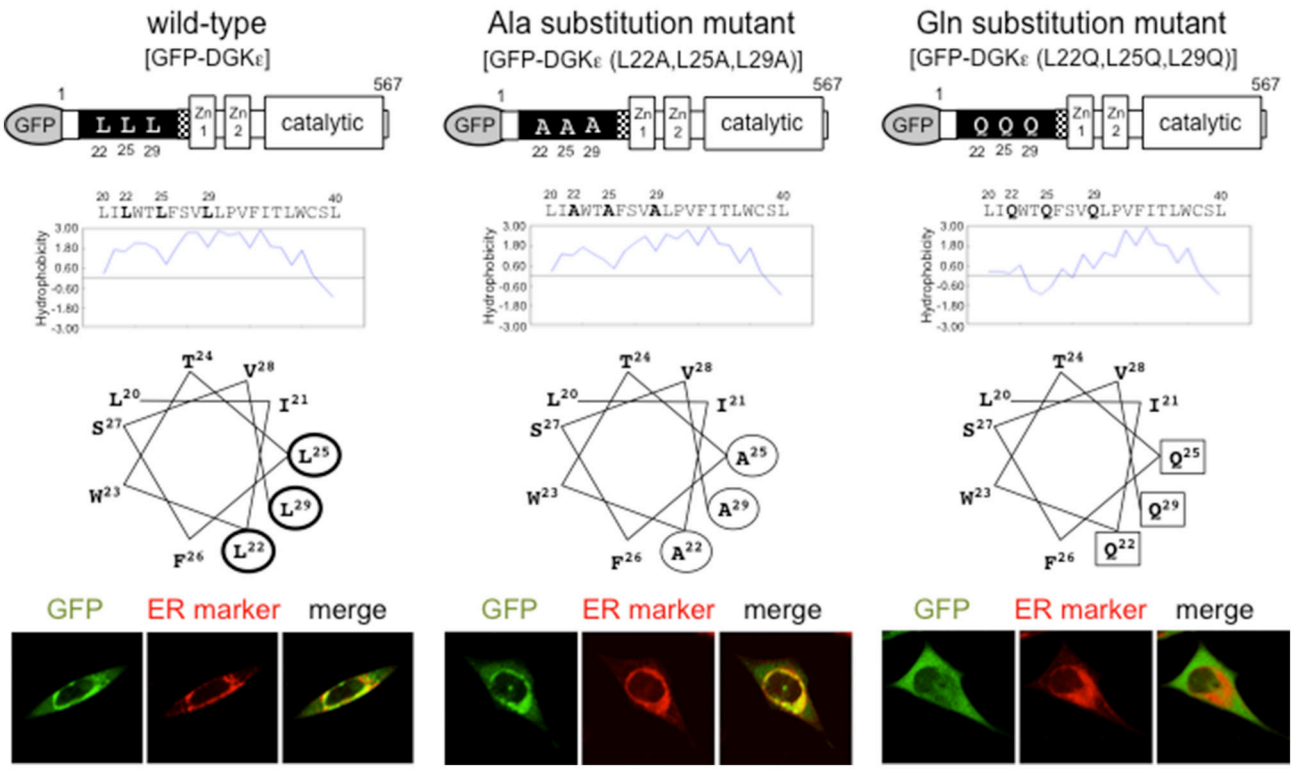


FIGURE 1 | Features of wild-type DGKε and Ala and Gln substitution mutants. Hydropathy plot analysis and helical wheel projections of the hydrophobic region of DGKε are shown. Wild-type DGKε contains highly hydrophobic residues L22, L25, L29 (thick circles), which represent a prominent hydrophobic patch. Alanine substitution mutant DGKε (L22A, L25A, L29A) have a reduced hydrophobic patch (thin circles), whereas glutamine substitution mutant DGKε (L22Q, L25Q, L29Q) includes a hydrophilic patch (squares). Immunofluorescence images of GFP for full-length wild-type and substitution mutants of DGKε together with ER marker staining in transfected HeLa cells (lower panels). Wild-type DGKε and alanine mutant DGKε (L22A, L25A, L29A) clearly colocalize with ER marker, whereas glutamine mutant DGKε (L22Q, L25Q, L29Q) shows diffuse cytoplasmic pattern. Modified from Matsui et al. (2014) with permission from Elsevier.

targeting is mediated through the N-terminal hydrophobic patch composed of L22, L25, and L29. Subsequent recognition of the arachidonoyl acyl chain of DG is achieved by a LOX-like motif in the catalytic domain of DGKε (aa. 421–453). Additional studies must be conducted to elucidate the ER targeting mechanism of DGKε.

ER STRESS

ER homeostasis is crucially important for cellular activity and survival (Ellgaard and Helenius, 2003). Stress in the ER induces the UPR, which represents a complex signaling system that controls translation and transcription in response to increased demands on the protein folding capacity of the ER for cell survival (Rutkowski and Kaufman, 2004; Koumenis and Wouters, 2006; Malhotra and Kaufman, 2007; Hetz, 2012). To meet this demand, the UPR coordinates membrane growth and phospholipid metabolism, thereby leading to ER membrane expansion and enhanced protein folding capacity (Sriburi et al., 2004). In addition to misfolding or incomplete assembly of proteins, alteration of the ER lipid composition also is shown to initiate ER stress (Devries-Seimon et al., 2005), indicating that disruption of membrane lipid homeostasis triggers directly or indirectly a mechanism to reestablish ER lipid composition (Fagone and Jackowski, 2009).

Under ER stress conditions, the glucose-regulated protein GRP78 plays a key role in UPR (Bertolotti et al., 2000). GRP78, a member of the heat shock protein 70 superfamily, serves as a major ER chaperone protein with ATPase activity. It is a key regulator of the transmembrane ER stress sensors comprised of inositol requiring enzyme 1 (IRE1), protein kinase RNA-activated (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF6) (Lee, 2014). IRE1 is a transmembrane ribonuclease that splices and activates X-box-binding protein (XBP-1) mRNA. Spliced form of XBP-1 [XBP-1(S)], together with cleaved ATF6 and ATF4, translocates to the nucleus where they induce the expression of ER stress-responsive genes (Hetz, 2012). In this regard, XBP-1(S) serves as a regulator of PC synthesis and ER membrane development (Fagone and Jackowski, 2009). PERK mediates activation of the pro-apoptotic factor C/EBP homologous protein (CHOP), thereby leading to apoptosis if the response is insufficient to reestablish homeostasis (Xu et al., 2005; Shore et al., 2011).

Therefore, the ER membrane expansion is supported by phospholipid synthesis, in which PA serves as an intermediate product. The initial step in the PA synthesis is catalyzed using a

family of glycerol 3-phosphate acyltransferases located in the ER and the outer mitochondrial membrane, followed by acyl-CoA-dependent acylation of lyso-PA to form PA (Lagace and Ridgway, 2013).

Another intermediate product DG is a precursor for PA, which is catalyzed by DGK. Therefore, DGK is intimately involved in phospholipid synthesis in the ER and presumably in the UPR. Earlier, we examined whether ER-resident DGKε participates in this process and assessed the ER stress pathways in DGKε knockdown cells under experimental ER stress conditions using tunicamycin and thapsigargin (Matsui et al., 2014). From DGKε deletion experiments conducted under ER stress conditions, we found the following: (1) The major protein chaperone GRP78 is induced to the same extent in both wild-type and DGKε-deficient cells. (2) Eukaryotic initiation factor 2α (eIF2α) is slightly, although not significantly, downregulated at the total and phosphorylated protein levels. (3) CHOP is significantly suppressed at the protein level. Analysis of cellular vulnerability, however, clearly shows that DGKε deletion reduces cell viability under ER stress conditions to some degree. Therefore, DGKε deletion seems to exert conflicting effects on apoptosis in terms of CHOP expression. In this regard, recent studies suggest that although CHOP is identified originally as a repressive member of the C/EBP family of transcription factors (Ron and Habener, 1992), it is capable of either transcriptional repression or activation, depending on the context (Oyadomari and Mori, 2004). Further studies need to be done to elucidate this point.

CONCLUDING REMARKS

Gene duplication contributes to the evolution of living creatures by expanding DNA information. *Escherichia coli* is equipped with two forms of DGK (Van Horn and Sanders, 2012; Jennings et al., 2015) whereas mammalian cells contain at least 10 isozymes. In the course of evolution, one branch of the diversified DGKs might have gained substrate specificity toward arachidonate-containing DG. Of DGs, arachidonoyl DG is phosphorylated selectively by arachidonoyl DGK to produce corresponding PA, which is further incorporated into inositol phospholipids. Multiple steps of this process are expected to enrich arachidonate in PI (Glomset, 1996). Because PIP2 is a major substrate for PLC, its enzymatic action results in the production of arachidonoyl DG. Functional implication of arachidonoyl DGK is suggested by an experimental seizure model at the organismal level. It reveals that DGKε-KO mice show lower degradation of brain PIP2 and lower accumulation of arachidonoyl-DG and free arachidonate although resting levels of PIP and PIP2 are similar between wild-type and DGKε-KO mice brains (Rodriguez de Turco et al., 2001). These findings suggest that DGKε is clearly involved in recycling PI metabolism presumably through the enrichment of arachidonate moiety. Therefore, DGKε downregulation may lead to a slowdown of PI turnover, thereby downregulating various membrane functions. In addition, it is particularly noteworthy that arachidonoyl DG also represents a substrate for another enzyme DG lipase, which cleaves *sn*-1-acyl chain to produce 2-arachidonoyl glycerol (Maejima et al., 2005). Because 2-arachidonoyl glycerol serves as an endocannabinoid for retrograde synaptic transmission, arachidonoyl DG is located at the crossroad of the two signaling cascades: DG-PA and DG-2AG pathways directed respectively by DGK and DG lipase. The mechanisms for how these signaling pathways are coordinated in parallel are just beginning to be explored.

AUTHOR CONTRIBUTIONS

TN, HM, TT, YH, KI, and KK did the experiments in the original papers and summarized the results for the mini review. TN and KG constituted and wrote the manuscript.

ACKNOWLEDGMENTS

This study is dedicated to Dr. John A. Glomset, University of Washington. Dr. Glomset reported for the first time the enzyme that preferentially phosphorylates arachidonoyl-DG and investigated the functional implication of arachidonate at the *sn*-2 position of phosphoinositide. This work was supported by Grants-in-Aid from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan [grant numbers 24390044, 26670782 (KG), 26460265 (TN), 15K20178 (HM), and 15K10530 (KK)].

REFERENCES

Berridge, M. J. (2002). The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* 32, 235–249. doi: 10.1016/S0143416002001823

Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* 2, 326–332. doi: 10.1038/35014014

Brasaemle, D. L., and Wolins, N. E. (2012). Packaging of fat: an evolving model of lipid droplet assembly and expansion. *J. Biol. Chem.* 287, 2273–2279. doi: 10.1074/jbc.R111.309088

Buczynski, M. W., Dumlao, D. S., and Dennis, E. A. (2009). Thematic review series: proteomics. An integrated omics analysis of eicosanoid biology. *J. Lipid Res.* 50, 1015–1038. doi: 10.1194/jlr.R900004-JLR200

Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Molecular cloning and characterization of a novel human diacylglycerol kinase zeta. *J. Biol. Chem.* 271, 10230–10236. doi: 10.1074/jbc.271.17.10237

Call, F. L. II, and Rubert, M. (1973). Diglyceride kinase in human platelets. *J. Lipid Res.* 14, 466–474.

Croze, E. M., and Morré, D. J. (1984). Isolation of plasma membrane, golgi apparatus, and endoplasmic reticulum fractions from single homogenates of mouse liver. *J. Cell. Physiol.* 119, 46–57. doi: 10.1002/jcp.1041190109

Daleo, G. R., Piras, M. M., and Piras, R. (1974). The presence of phospholipids and diglyceride kinase activity in microtubules from different tissues. *Biochem. Biophys. Res. Commun.* 61, 1043–1050. doi: 10.1016/0006-291X(74)90260-5

Decaffmeyer, M., Shulga, Y. V., Dicu, A. O., Thomas, A., Truant, R., Topham, M. K., et al. (2008). Determination of the topology of the hydrophobic segment of mammalian diacylglycerol kinase epsilon in a cell membrane and its relationship to predictions from modeling. *J. Mol. Biol.* 383, 797–809. doi: 10.1016/j.jmb.2008.08.076

Devries-Seimon, T., Li, Y., Yao, P. M., Stone, E., Wang, Y., Davis, R. J., et al. (2005). Cholesterol-induced macrophage apoptosis requires ER stress pathways

and engagement of the type A scavenger receptor. *J. Cell Biol.* 171, 61–73. doi: 10.1083/jcb.200502078

Ding, L., Traer, E., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1998). The cloning and characterization of a novel human diacylglycerol kinase, DGKiota. *J. Biol. Chem.* 273, 32746–32752. doi: 10.1074/jbc.273.49.32746

Ellgaard, L., and Helenius, A. (2003). Quality control in the endoplasmic reticulum. *Nat. Rev. Mol. Cell Biol.* 4, 181–191. doi: 10.1038/nrm1052

Fagone, P., and Jackowski, S. (2009). Membrane phospholipid synthesis and endoplasmic reticulum function. *J. Lipid Res.* 50, S311–S316. doi: 10.1194/jlr.R800049-JLR200

Farese, R. V., Sabir, M. A., and Larson, R. E. (1981). Effects of adrenocorticotropin and cycloheximide on adrenal diglyceride kinase. *Biochemistry* 20, 6047–6051. doi: 10.1021/bi00524a020

Funk, C. D. (2001). Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 294, 1871–1875. doi: 10.1126/science.294.5548.1871

Gaspar, M. L., Aregullin, M. A., Jesch, S. A., Nunez, L. R., Villa-García, M., and Henry, S. A. (2007). The emergence of yeast lipidomics. *Biochim. Biophys. Acta* 1771, 241–254. doi: 10.1016/j.bbalip.2006.06.011

Glomset, J. A. (1996). "A branched metabolic pathway in animal cells converts 2-monoacylglycerol into *sn*-1-stearoyl-2-arachdonoyl phosphatidylinositol and other phosphoglycerides," in *Advances in Lipobiology*, ed R. W. Cross (Greemwich, CT: JAI Press), 61–100.

Glukhov, E., Shulga, Y. V., Epand, R. F., Dicu, A. O., Topham, M. K., Deber, C. M., et al. (2007). Membrane interactions of the hydrophobic segment of diacylglycerol kinase epsilon. *Biochim. Biophys. Acta* 1768, 2549–2558. doi: 10.1016/j.bbamem.2007.06.012

Goldschmidt, H. L., Tu-Sekine, B., Volk, L., Anggono, V., Huganir, R. L., and Raben, D. M. (2016). DGKtheta catalytic activity is required for efficient recycling of presynaptic vesicles at excitatory synapses. *Cell Rep.* 14, 200–207. doi: 10.1016/j.celrep.2015.12.022

Goto, K., Funayama, M., and Kondo, H. (1994). Cloning and expression of a cytoskeleton-associated diacylglycerol kinase that is dominantly expressed in cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* 91, 13042–13046. doi: 10.1073/pnas.91.26.13042

Goto, K., Hozumi, Y., Nakano, T., Saino, S. S., and Kondo, H. (2007). Cell biology and pathophysiology of the diacylglycerol kinase family: morphological aspects in tissues and organs. *Int. Rev. Cytol.* 264, 25–63. doi: 10.1016/S0074-7696(07)64002-9

Goto, K., and Kondo, H. (1993). Molecular cloning and expression of a 90-kDa diacylglycerol kinase that predominantly localizes in neurons. *Proc. Natl. Acad. Sci. U.S.A.* 90, 7598–7602. doi: 10.1073/pnas.90.16.7598

Goto, K., and Kondo, H. (1996). A 104-kDa diacylglycerol kinase containing ankylin-like repeats localizes in the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11196–11201. doi: 10.1073/pnas.93.20.11196

Goto, K., Watanabe, M., Kondo, H., Yuasa, H., Sakane, F., and Kanoh, H. (1992). Gene cloning, sequence, expression and in situ localization of 80kDa diacylglycerol kinase specific to oligodendrocyte of rat brain. *Mol. Brain Res.* 16, 75–87.

Hetz, C. (2012). The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat. Rev. Mol. Cell Biol.* 13, 89–102. doi: 10.1038/nrm3270

Hokin, L. E., and Hokin, M. R. (1963). Diglyceride kinase and other pathways for phosphatidic acid synthesis in the erythrocyte membrane. *Biochim. Biophys. Acta* 67, 470–484. doi: 10.1016/0926-6569(63)90263-3

Hokin, M. R., and Hokin, L. E. (1959). The synthesis of phosphatidic acid from diglyceride and adenosine triphosphate in extracts of brain microsomes. *J. Biol. Chem.* 234, 1381–1386.

Houssa, B., Schaap, D., van der Val, J., Goto, K., Kondo, H., Yamakawa, A., et al. (1997). Cloning of a novel human diacylglycerol kinase (DGKtheta) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping Ras-associating domain. *J. Biol. Chem.* 272, 10422–10428. doi: 10.1074/jbc.272.16.10422

Hozumi, Y., Fukaya, M., Adachi, N., Saito, N., Otani, K., Kondo, H., et al. (2008). Diacylglycerol kinase beta accumulates on the perisynaptic site of medium spiny neurons in the striatum. *Eur. J. Neurosci.* 28, 2409–2422. doi: 10.1111/j.1460-9568.2008.06547.x

Hozumi, Y., Ito, T., Nakano, T., Nakagawa, T., Aoyagi, M., Kondo, H., et al. (2003). Nuclear localization of diacylglycerol kinase ζ in neurons. *Eur. J. Neurosci.* 18, 1448–1457. doi: 10.1046/j.1460-9568.2003.02871.x

Hozumi, Y., Matsui, H., Sakane, F., Watanabe, M., and Goto, K. (2013). Distinct Expression and Localization of Diacylglycerol Kinase Isozymes in Rat Retina. *J. Histochem. Cytochem.* 61, 462–476. doi: 10.1369/0022155413483574

Imai, S., Kai, M., Yasuda, S., Kanoh, H., and Sakane, F. (2005). Identification and characterization of a novel human type II diacylglycerol kinase, DGKκ. *J. Biol. Chem.* 280, 39870–39881. doi: 10.1074/jbc.M500669200

Ito, T., Hozumi, Y., Sakane, F., Saino-Saito, S., Kanoh, H., Aoyagi, M., et al. (2004). Cloning and characterization of diacylglycerol kinase iota splice variants in rat brain. *J. Biol. Chem.* 279, 23317–23326. doi: 10.1074/jbc.M312976200

Jennings, W., Doshi, S., D'Souza, K., and Epand, R. M. (2015). Molecular properties of diacylglycerol kinase-epsilon in relation to function. *Chem. Phys. Lipids* 192, 100–108. doi: 10.1016/j.chemphyslip.2015.06.003

Kai, M., Sakane, F., Imai, S., Wada, I., and Kanoh, H. (1994). Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells. *J. Biol. Chem.* 269, 18492–18498.

Kanoh, H., Yamada, K., and Sakane, F. (1990). Diacylglycerol kinase: a key modulator of signal transduction? *Trends Biochem. Sci.* 15, 47–50. doi: 10.1016/0968-0004(90)90172-8

Klauck, T. M., Xu, X., Mousseau, B., and Jaken, S. (1996). Cloning and characterization of a glucocorticoid-induced diacylglycerol kinase. *J. Biol. Chem.* 271, 19781–19788. doi: 10.1074/jbc.271.33.19781

Kobayashi, N., Hozumi, Y., Ito, T., Hosoya, T., Kondo, H., and Goto, K. (2007). Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. *Eur. J. Cell Biol.* 86, 433–444. doi: 10.1016/j.ejcb.2007.05.002

Koumenis, C., and Wouters, B. G. (2006). "Translating" tumor hypoxia: unfolded protein response (UPR)-dependent and UPR-independent pathways. *Mol. Cancer Res.* 4, 423–436. doi: 10.1158/1541-7786.MCR-06-0150

Lagace, T. A., and Ridgway, N. D. (2013). The role of phospholipids in the biological activity and structure of the endoplasmic reticulum. *Biochim. Biophys. Acta* 1833, 2499–2510. doi: 10.1016/j.bbamcr.2013.05.018

Lapetina, E. G., and Hawthorne, J. N. (1971). The diglyceride kinase of rat cerebral cortex. *Biochem. J.* 122, 171–179. doi: 10.1042/bj1220171

Lee, A. S. (2014). Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. *Nat. Rev. Cancer* 14, 263–276. doi: 10.1038/nrc3701

Lemaitre, R. N., King, W. C., MacDonald, M. L., and Glomset, J. A. (1990). Distribution of distinct arachidonoyl-specific and non-specific isoenzymes of diacylglycerol kinase in baboon (*Papio cynocephalus*) tissues. *Biochem. J.* 266, 291–299. doi: 10.1042/bj2660291

MacDonald, M. L., Mack, K. F., Williams, B. W., King, W. C., and Glomset, J. A. (1988). A membrane-bound diacylglycerol kinase that selectively phosphorylates arachidonoyl-diacylglycerol. Distinction from cytosolic diacylglycerol kinase and comparison with the membrane-bound enzyme from *Escherichia coli*. *J. Biol. Chem.* 263, 1584–1592.

Maejima, T., Oka, S., Hashimotodani, Y., Ohno-Shosaku, T., Aiba, A., Wu, D., et al. (2005). Synaptically driven endocannabinoid release requires Ca^{2+}-assisted metabotropic glutamate receptor subtype 1 to phospholipase Cbeta4 signaling cascade in the cerebellum. *J. Neurosci.* 25, 6826–6835. doi: 10.1523/JNEUROSCI.0945-05.2005

Malhotra, J. D., and Kaufman, R. J. (2007). The endoplasmic reticulum and the unfolded protein response. *Semin. Cell Dev. Biol.* 18, 716–731. doi: 10.1016/j.semcdb.2007.09.003

Martin, S., and Parton, R. G. (2006). Lipid droplets: a unified view of a dynamic organelle. *Nat. Rev. Mol. Cell Biol.* 7, 373–378. doi: 10.1038/nrm1912

Matsui, H., Hozumi, Y., Tanaka, T., Okada, M., Nakano, T., Suzuki, Y., et al. (2014). Role of the N-terminal hydrophobic residues of DGKepsilon in targeting the endoplasmic reticulum. *Biochim. Biophys. Acta* 1842, 1440–1450. doi: 10.1016/j.bbalip.2014.07.007

Mérida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* 409, 1–18. doi: 10.1042/BJ20071040

Murakami, T., Sakane, F., Imai, S., Houkin, K., and Kanoh, H. (2003). Identification and characterization of two splice variants of human diacylglycerol kinase eta. *J. Biol. Chem.* 278, 34364–34372. doi: 10.1074/jbc.M301542200

Nakano, T., Hozumi, Y., Goto, K., and Wakabayashi, I. (2012). Involvement of diacylglycerol kinase gamma in modulation of iNOS synthesis in Golgi apparatus of vascular endothelial cells. *Naunyn Schmiedebergs Arch. Pharmacol.* 385, 787–795. doi: 10.1007/s00210-012-0760-0

Neau, D. B., Gilbert, N. C., Bartlett, S. G., Boeglin, W., Brash, A. R., and Newcomer, M. E. (2009). The 1.85 Å structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. *Biochemistry* 48, 7906–7915. doi: 10.1021/bi900084m

Oyadomari, S., and Mori, M. (2004). Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* 11, 381–389. doi: 10.1038/sj.cdd.4401373

Pérez-Chacón, G., Astudillo, A. M., Balgoma, D., Balboa, M. A., and Balsinde, J. (2009). Control of free arachidonic acid levels by phospholipases A2 and lysophospholipid acyltransferases. *Biochim. Biophys. Acta* 1791, 1103–1113. doi: 10.1016/j.bbalip.2009.08.007

Peters-Golden, M., and Henderson, W. R. Jr. (2007). Leukotrienes. *N. Engl. J Med.* 357, 1841–1854. doi: 10.1056/NEJMra071371

Prottey, C., and Hawthorne, J. N. (1967). The biosynthesis of phosphatidic acid and phosphatidylinositol in mammalian pancreas. *Biochem. J.* 105, 379–392. doi: 10.1042/bj1050379

Rodriguez de Turco, E. B., Tang, W., Topham, M. K., Sakane, F., Marcheselli, V. L., Chen, C., et al. (2001). Diacylglycerol kinase epsilon regulates seizure susceptibility and long-term potentiation through arachidonoyl-inositol lipid signaling. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4740–4745. doi: 10.1073/pnas.081536298

Ron, D., and Habener, J. F. (1992). CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* 6, 439–453. doi: 10.1101/gad.6.3.439

Ron, D., and Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. *Nat. Rev. Mol. Cell Biol.* 8, 519–529. doi: 10.1038/nrm2199

Rutkowski, D. T., and Kaufman, R. J. (2004). A trip to the ER: coping with stress. *Trends Cell Biol.* 14, 20–28. doi: 10.1016/j.tcb.2003.11.001

Sakane, F., Imai, S., Kai, M., Wada, I., and Kanoh, H. (1996). Molecular cloning of a novel diacylglycerol kinase isozyme with a pleckstrin homology domain and a C-terminal tail similar to those of the EPH family of protein tyrosine kinase. *J. Biol. Chem.* 271, 8394–8401. doi: 10.1074/jbc.271.14.8394

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007). Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* 1771, 793–806. doi: 10.1016/j.bbalip.2007.04.006

Sakane, F., Imai, S., Yamada, K., Murakami, T., Tsushima, S., and Kanoh, H. (2002). Alternative splicing of the human diacylglycerol kinase delta gene generates two isoforms differing in their expression patterns and in regulatory functions. *J. Biol. Chem.* 277, 43519–43526. doi: 10.1074/jbc.M206895200

Sakane, F., Yamada, K., Kanoh, H., Yokoyama, C., and Tanabe, T. (1990). Porcine diacylglycerol kinase sequence has zinc finger and E–F handmotifs. *Nature* 344, 345–348. doi: 10.1038/344345a0

Sano, R., and Reed, J. C. (2013). ER stress-induced cell death mechanisms. *Biochim. Biophys. Acta* 1833, 3460–3470. doi: 10.1016/j.bbamcr.2013.06.028

Sastry, P. S., and Hokin, L. E. (1966). Studies on the role of phospholipids in phagocytosis. *J. Biol. Chem.* 241, 3354–3361.

Schaap, D., de Widt, J., van der Wal, J., Vandekerckhove, J., van Damme, J., Gussow, D., et al. (1990). Purification, cDNA-cloning and expression of human diacylglycerol kinase. *FEBS Lett.* 275, 151–158. doi: 10.1016/0014-5793(90)81461-V

Shore, G. C., Papa, F. R., and Oakes, S. A. (2011). Signaling cell death from the endoplasmic reticulum stress response. *Curr. Opin. Cell Biol.* 23, 143–149. doi: 10.1016/j.ceb.2010.11.003

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011a). Substrate specificity of diacylglycerol kinase-epsilon and the phosphatidylinositol cycle. *FEBS Lett.* 585, 4025–4028. doi: 10.1016/j.febslet.2011.11.016

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011b). Study of arachidonoyl specificity in two enzymes of the PI cycle. *J. Mol. Biol.* 409, 101–112. doi: 10.1016/j.jmb.2011.03.071

Sriburi, R., Jackowski, S., Mori, K., and Brewer, J. W. (2004). XBP1: a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. *J. Cell Biol.* 167, 35–41. doi: 10.1083/jcb.200406136

Tabellini, G., Bortul, R., Santi, S., Riccio, M., Baldini, G., Cappellini, A., et al. (2003). Diacylglycerol kinase-θ is localized in the speckle domains of the nucleus. *Exp. Cell Res.* 287, 143–154. doi: 10.1016/S0014-4827(03)00115-0

Tang, W., Bunting, M., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. *J. Biol. Chem.* 271, 10237–10241. doi: 10.1074/jbc.271.17.10230

Topham, M. K., and Epand, R. M. (2009). Mammalian diacylglycerol kinases: molecular interactions and biological functions of selected isoforms. *Biochim. Biophys. Acta* 1790, 416–424. doi: 10.1016/j.bbagen.2009.01.010

Van Horn, W. D., and Sanders, C. R. (2012). Prokaryotic diacylglycerol kinase and undecaprenol kinase. *Annu. Rev. Biophys.* 41, 81–101. doi: 10.1146/annurev-biophys-050511-102330

Walker, A. J., Draeger, A., Houssa, B., van Blitterswijk, W. J., Ohanian, V., and Ohanian, J. (2001). Diacylglycerol kinase theta is translocated and phosphoinositide 3-kinase-dependently activated by noradrenaline but not angiotensin II in intact small arteries. *Biochem. J.* 353, 129–137. doi: 10.1042/bj3530129

Walsh, J. P., Suen, R., Lemaitre, R. N., and Glomset, J. A. (1994). Arachidonoyl-diacylglycerol kinase from bovine testis. Purification and properties. *J. Biol. Chem.* 269, 21155–21164.

Xu, C., Bailly-Maitre, B., and Reed, J. C. (2005). Endoplasmic reticulum stress: cell life and death decisions. *J. Clin. Invest.* 115, 2656–2664. doi: 10.1172/JCI26373

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 14 September 2016
doi: 10.3389/fcell.2016.00101

DGK-θ: Structure, Enzymology, and Physiological Roles

*Becky Tu-Sekine, Hana L. Goldschmidt † and Daniel M. Raben **

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

Diacylglycerol kinases (DGKs) are a family of enzymes that catalyze the ATP-dependent phosphorylation of diacylglycerol (DAG) to phosphatidic acid (PtdOH). The recognition of the importance of these enzymes has been increasing ever since it was determined that they played a role in the phosphatidylinositol (PtdIns) cycle and a number of excellent reviews have already been written [(see van Blitterswijk and Houssa, 2000; Kanoh et al., 2002; Mérida et al., 2008; Tu-Sekine and Raben, 2009, 2011; Shulga et al., 2011; Tu-Sekine et al., 2013) among others]. We now know there are ten mammalian DGKs that are organized into five classes. DGK-θ is the lone member of the Type V class of DGKs and remains as one of the least studied. This review focuses on our current understanding of the structure, enzymology, regulation, and physiological roles of this DGK and suggests some future areas of research to understand this DGK isoform.

Keywords: diacylglycerol kinase, phosphatidic acid, regulation, synaptic vesicle cycle, interfacial enzymology

OPEN ACCESS

Edited by:
Isabel Merida, Spanish National Research Council, Spain

Reviewed by:
Rosely Oliveira Godinho, Federal University of São Paulo, Brazil
Matthew Topham, University of Utah, USA

****Correspondence:***
Daniel M. Raben
draben@jhmi.edu

†Present Address:
Hana Goldschmidt, The Solomon H. Snyder Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *24 June 2016*
Accepted: *29 August 2016*
Published: *14 September 2016*

Citation:
Tu-Sekine B, Goldschmidt HL and Raben DM (2016) DGK-θ: Structure, Enzymology, and Physiological Roles. Front. Cell Dev. Biol. 4:101. doi: 10.3389/fcell.2016.00101

STRUCTURE

Arguably, our lack of knowledge about the three dimensional structure of mammalian DGKs remains as one of our most difficult challenges. Currently, our knowledge of the structural features of DGKs is essentially limited to the primary sequence of these enzymes (**Figure 1**). As noted in many excellent reviews (for example, see van Blitterswijk and Houssa, 2000; Kanoh et al., 2002; Mérida et al., 2008; Shulga et al., 2011) DGK-θ's major distinguishing feature in this regard is that it possess three, instead of two, C1 domains. Like other DGKs, the C1 domains of DGK-θ is homologous to PKC C1A and C1C domains with an extended region closest to the presumed catalytic domain near the C terminus. Despite the temptation to assume these domains bind DAG, their precise role remains unclear. Such caution is supported by the observation that the C1 domains of DGK-θ does not bind phorbol esters (Shindo et al., 2003). Further, the structural studies of Hurley and colleagues showed clear differences between these domains and those found in PKCs (Hurley et al., 1997). As suggested previously (Shulga et al., 2011), these data suggest that these DGK-θ domains may bind other lipids or participate in protein-protein interactions. Such a notion is supported by the evidence showing the C1 domain of another DGK, DGKζ, mediates the interactions with β-arrestins (Nelson et al., 2007) and Rac1 (Yakubchyk et al., 2005). We should note, that there is some evidence that this domain may be involved in membrane association in response to activation of some G-protein coupled receptors (van Baal et al., 2005).

Another feature of DGK-θ is the presence of a sequence with homology to Ras binding domains which is termed the Ras-Association (RA) domain (Houssa et al., 1997). It is important to note, however, that an *in silico* analyses based on binding energies predicts that the RA domain of DGK-θ probably does not bind Ras (Kiel et al., 2005). It is interesting, in this regard, that DGK-θ has been shown to bind a different small GTPase, active (GTP-bound) RhoA, and this association inhibits the kinase activity (Houssa et al., 1997). As with the C1 domains, however, it is not clear that RhoA

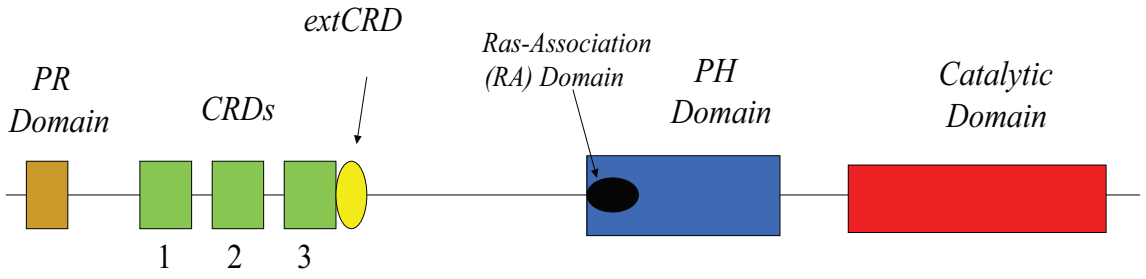


FIGURE 1 | Linear domain structure of DGK-θ.

binds specifically to this domain either and it is equally unclear as to whether RhoA is a regulator under various physiological conditions (see Regulation below).

Another major distinguishing feature of DGK-θ is the presence of a proline/glycine-rich region near its N-terminus. It is interesting to speculate that this region, may bind to proteins containing an SH3 domain given the presence of a pXPXXP motif (Yu et al., 1994). Further, a splice form of DGKδ, DGKδ2, also contains a proline-rich domain which is believed to play a role in regulating its membrane association in response to certain stimuli (Takeuchi et al., 2012). While these data are interesting, the precise role for the proline/glycine-rich domain in DGK-θ has not been fully examined.

The above clearly highlights the need for a greater understanding of the structural features of DGK-θ as well as other DGKs. Much of our structural understanding of these enzymes comes from studies of two prokaryotic DGKs. The first is a DGK found in the gram-negative organism Escherichia coli. This enzyme is different from the mammalian enzymes in that in contains three membrane spanning domains and functions as a trimer (see Van Horn and Sanders, 2012). Another DGK, found in gram-positive organisms such as *Staphylococcus aureus*, is a soluble enzyme with 15–18% homology to human DGKs (Miller et al., 2008). The structural studies of this enzyme showed the active site appears to form at a homodimer interface. Further, the data suggest a putative DAG substrate recognition loop that may provide an important difference between DGKs and related enzymes such as YegS and NAD kinases that are not bona fide DGKs (Bakali et al., 2007; Nichols et al., 2007; see Miller et al., 2008). Importantly, there is a clear need for clear structural studies on eukaryotic enzymes to determine not only the catalytic site, but also the structure of likely regulatory domains, such as the N-terminus, which are not present in the prokaryotic enzymes.

Progress in understanding the structure of eukaryotic DGKs including DGK-θ has been hampered by our inability to purify a sufficient amount of enzyme in a uniform, monodisperse solution that would be amenable to classic methodologies such as NMR and X-ray crystallography. So far, only the SAM (sterile alpha motif) domain of DGKδ1 has been determined by x-ray crystallography at a resolution of 2.9 angstrom (Harada et al., 2008). This study indicated that oligomerization of the SAM domain in a head to tail orientation leads to the cytosolic sequestration, and inhibition, of this enzyme. In addition to this structure, a solution structure of the C1 domain of DGKδ1, and although a structure of DGKα has been deposited in a structure database, it has not yet been published. We are clearly in need of new and creative approaches to determine the structure of DGK-θ and other DGK isoforms.

ENZYMOLOGY

DGK-θ belongs to a class of enzymes referred to as interfacial enzymes. These enzymes are generally soluble and have at least one insoluble substrate that is present in membranes or lipid aggregates. As a result, the enzymology of these enzymes, including DGK-θ, cannot be approached as is done for soluble enzymes with soluble substrates. Binding to membranes is often independent of catalysis and enzyme and substrate approach each other via diffusion within a membrane or lipid aggregate. Further complicating the analyses is the amount of time the enzyme resides on the interface, i.e., the enzyme's processivity, which may vary with the composition or architecture of the interface. We recently published a protocol (Tu-Sekine and Raben, submitted) that outlines an approach to the enzymology of these enzymes.

Analyses of DGK-θ present in cytosolic lysates illuminated two interesting aspects of its regulation (Tu-Sekine et al., 2006, 2007; Tu-Sekine and Raben, 2010). First, the enzyme is sensitive to both the bulk as well as surface concentration of the DAG substrate (Tu-Sekine et al., 2007). Second, the apparent Km (Km(app)) of the enzyme in these lysates was dependent on prior treatment of the cells. When DGK-θ was overexpressed in fibroblasts, the Km(app) of the enzyme for diacylglycerol (DAG) was increased in lysates obtained from mitogen-induced cells. The Km(app) then decreased with increasing bulk concentration of DAG. Additionally, the apparent Vmax (Vmax(app)) was increased in response to increases in the product, PtdOH (Tu-Sekine et al., 2007). It is noteworthy that the activation of DGK-θ in response to PtdOH occurs at 6-fold lower surface and bulk concentrations of DAG than observed when the activation occurs in response to phosphatidyserine (PtdSer). Importantly, the significant changes in the K_M and V_{max} values were observed only when physiological levels of both PtdOH and PtdSer were reached (Tu-Sekine et al., 2007).

To fully understand the enzymology, studies must be conducted on purified enzymes. One of the surprising discoveries when isolated DGK-θ was analyzed was that the enzyme is not fully active when purified. Full activity was observed when the purified enzyme was assayed in the presence of proteins rich in polybasic residues. In the presence of such polybasic cofactors, which directly interact with the enzyme, the apparent K(m) of the enzyme for DAG at the membrane surface (K(m)((surf))) was decreased These cofactors also decreased the Km for ATP. Furthermore, the cofactors synergistically increase the activity 10- to 30-fold in the presence of acidic phospholipids which were known to enhance enzyme activity (below).

There has been considerable interest in gaining a complete understanding of the enzymology and catalytic mechanism of DGK-θ and related kinases. In 2002, an interesting review appeared that highlighted the possibility that the similarity in phosphate-donor-binding sites may lead to a similar phosphorylation mechanism for four enzymes; DGKs, sphingosine kinases, NAD kinases, and phosphofructo-6-kinase (Labesse et al., 2002). Clearly, a confirmation of this hypothesisfor DGKs requires a comprehensive understanding of their structures. For example, it is possible that DGK-θ and other DGKs, act as a dimer similar to that suggested for DGKB in *Staphylococcus aureus* (Miller et al., 2008). Indeed, this may be a common paradigm for lipid kinases as another lipid kinase, sphingosine kinase 1, has also been suggested to function as a dimer (see Adams et al., 2016). As with enzymes in general, a complete understanding of DGK-θ's enzymology and catalytic mechanism will also provide critical information for the design of specific DGK-θ inhibitors.

REGULATION

As noted above, DGK-θ is very sensitive to the bulk and surface concentration of the DAG substrate. This provides a mechanism for regulating the enzyme in response to increases in DAG that accompany many signaling pathways. Additionally, as noted, DGK-θ activity is increased in response to PtdSer as well as PtdOH. Taken together, the data suggest that when DAG is generated in a signaling pathway, the Km(app) decreases and as the DAG concentration and PtdOH concentrations increase, the enzyme becomes more efficient. This could help explain the very transient nature of DAG increases in signaling pathways.

In addition to variations in membrane lipids, there are strong data supporting a role for protein-protein interactions that regulate DGK-θ activity. In 1999, Houssa et al. reported that activated (GTP-bound) RhoA associated with DGK-θ and inhibited its activity. Consistent with these data, Nurrish and colleagues have provided some fascinating data showing the homolog of DGK-θ in *C. elegans*, DGK-1, is regulated by RhoA and to modulate acetylcholine release at neuromuscular junctions (Nurrish et al., 1999; McMullan et al., 2006). As indicated above, there is a Ras-associating (RA) domain within the PH domain of DGK-θ and it has been tempting to speculate that this region was responsible for RhoA binding. Los et al., however, showed that RhoA bound to the C-terminal portion of the catalytic domain although they did not completely rule out another interaction with the RA domain (Los et al., 2004). While the precise RhoA binding regions have not been fully identified, a potential important role for these interactions is given support from the observations that other DGKs may show similar associations. For example, Tolias et al. presented evidence that a DGK associated with Rac1 (Tolias et al., 1998). This enzyme, however, is unlikely to be DGK-θ as another study showed DGK-θ does not associate with Rac1 or cdc42 (Houssa et al., 1999).

The role of phosphorylation in regulating DGK-θ is unclear. Using lysates of Cos-7 cells, PKC-ε has been shown to phosphorylate DGK-θ *in vitro* and studies using A431 cells suggested that this phosphorylation may promote membrane association (van Baal et al., 2005). Akt has also been shown to associate with and stimulate DGK-θ catalytic activity. Consistent with this, PI-3 kinase regulates the translocation and activation of DGK-θ in rat mesenteric arteries although, importantly, the mechanism of this regulation is unclear and may not involve DGK-θ phosphorylation (Walker et al., 2001; Clarke et al., 2007). Additional studies are clearly required to define the precise role of DGK-θ phosphorylation in membrane association and catalytic activation.

As indicated above, full activation of DGK-θ requires an acidic phospholipid and a polybasic cofactor. Additional analyses showed that while the acidic phospholipids recruit polybasic cofactors to the surface of artificial membranes, they do not affect the membrane association of DGK-θ suggesting interfacial association and catalytic activity are independently regulated. The data provide support for a model (**Figure 2**) in which DGK-θ exists as an auto-inhibited enzyme and that full interfacial activity requires a triad of enzyme, acidic lipid, and basic protein (Tu-Sekine et al., 2007). The enzyme is also likely to be modulated by the presence of RhoA or another small GTPase under physiological conditions. Importantly, the polybasic protein that represents the physiological regulator of DGK-θ in various tissues has/have not been identified and represents an important challenge in the field.

PHYSIOLOGICAL ROLES

Regulation of Gene Expression

DGK-θ shows a broad tissue distribution with a regulated expression profile (Ohanian and Ohanian, 2001; Topham, 2006; Ueda et al., 2013). These studies has shown it is present in various tissues that include brain, intestine, liver, kidney, and small arteries and our understanding of its physiological roles are beginning to emerge. One of the physiological roles identified for DGK-θ was as a modulator of gene expression (Li et al., 2007). Consistent with this, DGK-θ has been found to localize in the nucleus in some cells (Bregoli et al., 2001, 2002; Tabellini et al., 2003, 2004). In two immortalized neuronal cell lines (PC-12 and N2a) the enzyme has been found in speckle domains of the nucleus within a subpopulation of cells (Tabellini et al., 2003). As speckle domains are known to contain splicing factors, these data suggest that DGK-θ may play a role in RNA processing. In support of this notion, NGF (nerve growth factor) induces

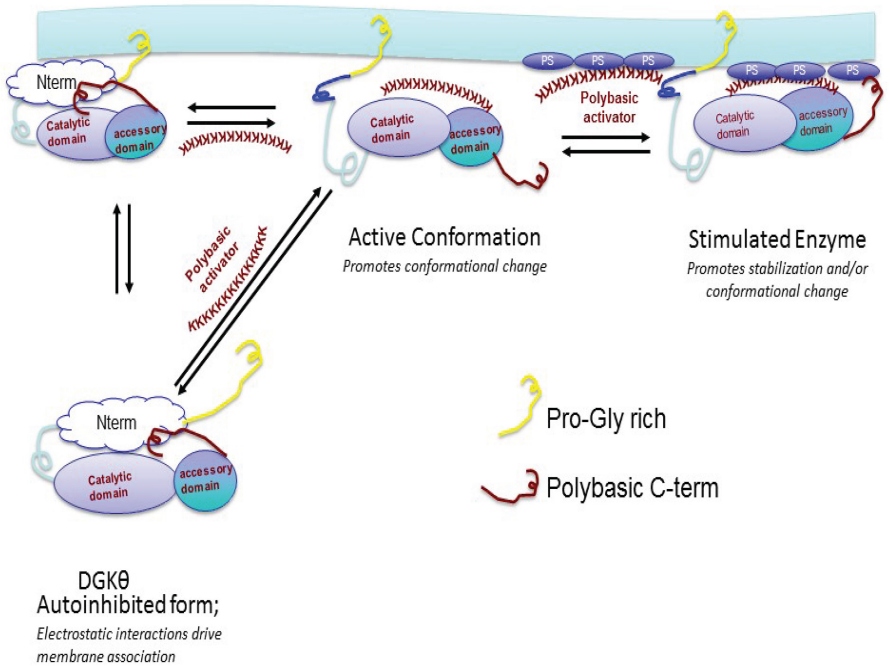


FIGURE 2 | Proposed model for DGK-θ membrane association and activation. In this model, DGK-θ exists in an auto-inhibited state which can bind to membranes in the presence or absence of a protein activator containing a polybasic region. Following membrane binding, full activation is achieved when a triad complex forms composed of enzyme, polybasic protein, and phospholipid activator.

an increase in the level and activity of DGK-θ in the nucleus (Tabellini et al., 2003). Some of the most compelling evidence supporting a role for DGK-θ in modulating gene expression has been provided by Sewer and colleagues (Li et al., 2007; Cai and Sewer, 2013; Cai et al., 2014). They show DGK-θ plays a role in the activation of the nuclear receptor steroidogenic factor 1 (SF-1). Knock down of DGK-θ, but not DGK-ζ, or expression of a kinase-dead mutant of DGK-θ abrogated agonist-induced expression of SF-1-mediated genes (Li et al., 2007). This presents another exciting area for future investigations to illuminate the role of DGK-θ-mediated nuclear PtdOH production.

Regulation of Synaptic Cycling and Neurotransmission

Nine of the ten known mammalian DGKs are expressed in neurons (see Tu-Sekine and Raben, 2011; Ishisaka and Hara, 2014). This isoform has been shown to be expressed throughout the brain with the strongest expression found in the hippocampus and cerebellar cortex (Houssa et al., 1997; Tu-Sekine and Raben, 2011; Ueda et al., 2013; Ishisaka and Hara, 2014). Recently, interest in the roles and regulation of DGKs in the mammalian central nervous system (CNS) has grown (Tu-Sekine and Raben, 2011; Ishisaka and Hara, 2014). However, as all ten DGK isoforms are abundant in this tissue, determination of unique cellular functions for each DGK isoform has been difficult. DGK-θ is expressed early during brain during development (Ueda et al., 2013) and subsequently maintained through adulthood where it has been detected in the adult nervous system (Houssa et al., 1997; Tu-Sekine and Raben, 2011; Ueda et al., 2013; Goldschmidt et al., 2016). Initial studies implicating a role for DGK-θ in synapse function came from elegant work on the *C. elegans* homolog of DGK-θ, DGK-1 (Nurrish et al., 1999; McMullan et al., 2006). Nurrish et al. showed that loss-of-function mutations in DGK-1 potentiated acetylcholine at neuromuscular junctions. This increase appears to be due to hyper-stimulation of the DAG-binding vesicle-priming protein unc-13 (Nurrish et al., 1999; Miller et al., 1999; and see Kanoh et al., 2002; Mérida et al., 2008). Based on these data, we speculated DGK-θ might regulate a similar function at mammalian central synapses (Goldschmidt et al., 2016). We found DGK-θ localized to excitatory synapses where its kinase activity promotes retrieval of synaptic vesicles following neuronal activity. Acute depletion of DGK-θ using shRNAs or significantly impaired the recycling kinetics of SVs. The same effect was observed in neurons derived from mice in which DGK-θ was genetically ablated although the mice were overtly normal. Importantly, the SV defect could only be rescued expression of enzymatically active DGK-θ, but not a kinase-dead enzyme. The data presented not only establish a role for DGK-θ kinase activity in SV cycling, but further analyses showed DGK-θ is likely to be important for synaptic transmission when sustained neuronal activity is required (Goldschmidt et al., 2016).

Pathophysiological Roles

At the present time, there is very little data regarding the pathophysiological role of DGK-θ. As noted earlier, this enzyme has been shown to be regulated in mesenteric arteries. The data suggests that this enzyme may play a role in regulating blood pressure in response to noradrenalin but not angiotensin II (Walker et al., 2001; Clarke et al., 2007). Clearly, this could have important implications for treating hypertension or pathophysiological conditions in which hypertension is a complicating factor. Other data suggest that RhoA-mediated inhibition of DGK-θ mediates ischemia/reperfusion injury in liver by allowing for the elevation of DAG (Baldanzi et al., 2010). Given the potential role for DGK-θ in modulating SF1 (above), it is tempting to speculate that this enzyme may play a role in diseases associated with this transcription factor (El-Khairi and Achermann, 2012). Our recent studies suggest that DGK-θ may play a critical role during sustained or intense neuronal stimulation (Goldschmidt et al., 2016) and therefore, may play a key role in modulating seizures. Taken together, while there are intriguing data regarding the physiologivcal and pathophysiological roles of DGK-θ additional studies are clearly needed.

FUTURE DIRECTIONS

While we have learned quite a bit about DGK-θ, there is clearly much more to be discovered. Understanding the structure of DGK-θ will provide essential insights into its catalytic mechanism and regulation. Developing new strategies to elucidate the structure of this enzyme, and identification of physiologically relevant polybasic regulators will be key in our understanding of this enzyme. Such data will be critical in our efforts to develop specific inhibitors for this isozyme which are at present not available. The importance of such inhibitors will be highlighted as we learn more about the various physiological roles of DGK-θ. For example, if DGK-θ is indeed critical during intense neuronal stimulation, a specific inhibitor may be useful in the treatment pathophysiological conditions in which such stimulation plays an important role (e.g., epilepsy). Conversely, the enzyme may play a role under physiological conditions where rapid SV cycling is essential such as ribbon synapses of some sensory neurons. The future of research on DGK-θ promises to be exciting and impactful.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

DR is funded by the National Institutes of Health RO1NS077923.

REFERENCES

Adams, D. R., Pyne, S., and Pyne, N. J. (2016). Sphingosine kinases: emerging structure-function insights. *Trends Biochem. Sci.* 41, 395–409. doi: 10.1016/j.tibs.2016.02.007

Bakali, H. M., Herman, M. D., Johnson, K. A., Kelly, A. A., Wieslander, A., Hallberg, B. M., et al. (2007). Crystal structure of YegS, a homologue to the mammalian diacylglycerol kinases, reveals a novel regulatory metal binding site. *J. Biol. Chem.* 282, 19644–19652. doi: 10.1074/jbc.M604852200

Baldanzi, G., Alchera, E., Imarisio, C., Gaggianesi, M., Dal Ponte, C., Nitti, M., et al. (2010). Negative regulation of diacylglycerol kinase theta mediates adenosine-dependent hepatocyte preconditioning. *Cell Death Differ.* 17, 1059–1068. doi: 10.1038/cdd.2009.210

Bregoli, L., Baldassare, J. J., and Raben, D. M. (2001). Nuclear diacylglycerol kinase-θ is activated in response to α-Thrombin. *J. Biol. Chem.* 276, 23288–23295. doi: 10.1074/jbc.M101501200

Bregoli, L., Tu-Sekine, B., and Raben, D. M. (2002). DGK and nuclear signaling: nuclear diacylglycerol kinases in IIC9 cells. *Adv. Enzyme Regul.* 42, 213–226. doi: 10.1016/S0065-2571(01)00032-2

Cai, K., Lucki, N. C., and Sewer, M. B. (2014). Silencing diacylglycerol kinase-theta expression reduces steroid hormone biosynthesis and cholesterol metabolism in human adrenocortical cells. *Biochim. Biophys. Acta* 1841, 552–562. doi: 10.1016/j.bbalip.2013.12.005

Cai, K., and Sewer, M. B. (2013). cAMP-stimulated transcription of DGKtheta requires steroidogenic factor 1 and sterol regulatory element binding protein 1. *J. Lipid Res.* 54, 2121–2132. doi: 10.1194/jlr.M035634

Clarke, C. J., Ohanian, V., and Ohanian, J. (2007). Norepinephrine and endothelin activate diacylglycerol kinases in caveolae/rafts of rat mesenteric arteries: agonist-specific role of PI3-kinase. *Am. J. Physiol. Heart Circ. Physiol.* 292, H2248–H2256. doi: 10.1152/ajpheart.01170.2006

El-Khairi, R., and Achermann, J. C. (2012). Steroidogenic factor-1 and human disease. *Semin. Reprod. Med.* 30, 374–381. doi: 10.1055/s-0032-1324720

Goldschmidt, H. L., Tu-Sekine, B., Volk, L., Anggono, V., Huganir, R. L., and Raben, D. M. (2016). DGKtheta catalytic activity is required for efficient recycling of presynaptic vesicles at excitatory synapses. *Cell Rep.* 14, 200–207. doi: 10.1016/j.celrep.2015.12.022

Harada, B. T., Knight, M. J., Imai, S., Qiao, F., Ramachander, R., Sawaya, M. R., et al. (2008). Regulation of enzyme localization by polymerization: polymer formation by the SAM domain of diacylglycerol kinase delta1. *Structure* 16, 380–387. doi: 10.1016/j.str.2007.12.017

Houssa, B., de Widt, J., Kranenburg, O., Moolenaar, W. H., and van Blitterswijk, W. J. (1999). Diacylglycerol kinase theta binds to and is negatively regulated by active RhoA. *J. Biol. Chem.* 274, 6820–6822.

Houssa, B., Schaap, D., van der Wal, J., Goto, K., Kondo, H., Yamakawa, A., et al. (1997). Cloning of a novel human diacylglycerol kinase (DGKtheta) containing three cysteine-rich domains, a proline-rich region, and a pleckstrin homology domain with an overlapping Ras-associating domain. *J. Biol. Chem.* 272, 10422–10428.

Hurley, J. H., Newton, A. C., Parker, P. J., Blumberg, P. M., and Nishizuka, Y. (1997). Taxonomy and function of C1 protein kinase C homology domains. *Protein Sci.* 6, 477–480. doi: 10.1002/pro.5560060228

Ishisaka, M., and Hara, H. (2014). The roles of diacylglycerol kinases in the central nervous system: review of genetic studies in mice. *J. Pharmacol. Sci.* 124, 336–343. doi: 10.1254/jphs.13R07CR

Kanoh, H., Yamada, K., and Sakane, F. (2002). Diacylglycerol kinases: emerging downstream regulators in cell signaling systems. *J. Biochem. (Tokyo).* 131, 629–633. doi: 10.1093/oxfordjournals.jbchem.a003144

Kiel, C., Wohlgemuth, S., Rousseau, F., Schymkowitz, J., Ferkinghoff-Borg, J., Wittinghofer, F., et al. (2005). Recognizing and defining true Ras binding domains II: *in silico* prediction based on homology modelling and energy calculations. *J. Mol. Biol.* 348, 759–775. doi: 10.1016/j.jmb.2005.02.046

Labesse, G., Douguet, D., Assairi, L., and Gilles, A. M. (2002). Diacylglyceride kinases, sphingosine kinases and NAD kinases: distant relatives of

6-phosphofructokinases. *Trends Biochem. Sci.* 27, 273–275. doi: 10.1016/S0968-0004(02)02093-5

Li, D., Urs, A. N., Allegood, J., Leon, A., Merrill, A. H. Jr., and Sewer, M. B. (2007). Cyclic AMP-stimulated interaction between steroidogenic factor 1 and diacylglycerol kinase theta facilitates induction of CYP17. *Mol. Cell Biol.* 27, 6669–6685. doi: 10.1128/MCB.00355-07

Los, A. P., van Baal, J., de Widt, J., Divecha, N., and van Blitterswijk, W. J. (2004). Structure-activity relationship of diacylglycerol kinase theta. *Biochim. Biophys. Acta* 1636, 169–174. doi: 10.1016/j.bbalip.2003.11.008

McMullan, R., Hiley, E., Morrison, P., and Nurrish, S. J. (2006). Rho is a presynaptic activator of neurotransmitter release at pre-existing synapses in *C. elegans*. *Genes Dev.* 20, 65–76. doi: 10.1101/gad.359706

Mérida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* 409, 1–18. doi: 10.1042/BJ20071040

Miller, D. J., Jerga, A., Rock, C. O., and White, S. W. (2008). Analysis of the *Staphylococcus aureus* DgkB structure reveals a common catalytic mechanism for the soluble diacylglycerol kinases. *Structure* 16, 1036–1046. doi: 10.1016/j.str.2008.03.019

Miller, K. G., Emerson, M. D., and Rand, J. B. (1999). Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in *C. elegans* [see comments]. *Neuron* 24, 323–333. doi: 10.1016/S0896-6273(00)80847-8

Nelson, C. D., Perry, S. J., Regier, D. S., Prescott, S. M., Topham, M. K., and Lefkowitz, R. J. (2007). Targeting of diacylglycerol degradation to M1 muscarinic receptors by beta-arrestins. *Science* 315, 663–666. doi: 10.1126/science.1134562

Nichols, C. E., Lamb, H. K., Lockyer, M., Charles, I. G., Pyne, S., Hawkins, A. R., et al. (2007). Characterization of *Salmonella typhimurium* YegS, a putative lipid kinase homologous to eukaryotic sphingosine and diacylglycerol kinases. *Proteins* 68, 13–25. doi: 10.1002/prot.21386

Nurrish, S., Ségalat, L., and Kaplan, J. M. (1999). Serotonin inhibition of synaptic transmission: galpha(0) decreases the abundance of UNC-13 at release sites. *Neuron* 24, 231–242. doi: 10.1016/S0896-6273(00)80835-1

Ohanian, J., and Ohanian, V. (2001). Lipid second messenger regulation: the role of diacylglycerol kinases and their relevance to hypertension. *J. Hum. Hypertens.* 15, 93–98. doi: 10.1038/sj.jhh.1001139

Shindo, M., Irie, K., Masuda, A., Ohigashi, H., Shirai, Y., Miyasaka, K., et al. (2003). Synthesis and phorbol ester binding of the cysteine-rich domains of diacylglycerol kinase (DGK) isozymes. DGKgamma and DGKbeta are new targets of tumor-promoting phorbol esters. *J. Biol. Chem.* 278, 18448–18454. doi: 10.1074/jbc.M300400200

Shulga, Y. V., Topham, M. K., and Epand, R. (2011). M. Regulation and functions of diacylglycerol kinases. *Chem. Rev.* 111, 6186–6208. doi: 10.1021/cr1004106

Tabellini, G., Billi, A. M., Falà, F., Cappellini, A., Evagelisti, C., Manzoli, L., et al. (2004). Nuclear diacylglycerol kinase-theta is activated in response to nerve growth factor stimulation of PC12 cells. *Cell Signal.* 16, 1263–1271. doi: 10.1016/j.cellsig.2004.03.018

Tabellini, G., Bortul, R., Santi, S., Riccio, M., Baldini, G., Cappellini, A., et al. (2003). Diacylglycerol kinase-theta is localized in the speckle domains of the nucleus. *Exp. Cell Res.* 287, 143–154. doi: 10.1016/S0014-4827(03)00115-0

Takeuchi, M., Sakiyama, S., Usuki, T., Sakai, H., and Sakane, F. (2012). Diacylglycerol kinase delta1 transiently translocates to the plasma membrane in response to high glucose. *Biochim. Biophys. Acta* 1823, 2210–2216. doi: 10.1016/j.bbamcr.2012.08.019

Tolias, K. F., Couvillon, A. D., Cantley, L. C., and Carpenter, C. L. (1998). Characterization of a Rac1- and RhoGDI-associated lipid kinase signaling complex. *Mol. Cell Biol.* 18, 762–770. doi: 10.1128/mcb.18.2.762

Topham, M. K. (2006). Signaling roles of diacylglycerol kinases. *J. Cell Biochem.* 97, 474–484. doi: 10.1002/jcb.20704

Tu-Sekine, B., Goldschmidt, H., Petro, E., and Raben, D. M. (2013). Diacylglycerol kinase theta: regulation and stability. *Adv. Biol. Regul.* 53, 118–126. doi: 10.1016/j.jbior.2012.09.007

Tu-Sekine, B., Ostroski, M., and Raben, D. M. (2006). Analysis of two diacylglycerol kinase activities in mixed micelles. *Adv. Enzyme Regul.* 46, 12–24. doi: 10.1016/j.advenzreg.2006.01.018

Tu-Sekine, B., Ostroski, M., and Raben, D. M. (2007). Modulation of diacylglycerol kinase theta activity by alpha-thrombin and phospholipids. *Biochemistry* 46, 924–932. doi: 10.1021/bi061170c

Tu-Sekine, B., and Raben, D. M. (2009). Regulation of DGK-theta. *J. Cell Physiol.* 220, 548–552. doi: 10.1002/jcp.21813

Tu-Sekine, B., and Raben, D. M. (2010). Characterization of cellular DGK-theta. *Adv. Enzyme Regul.* 50, 81–94. doi: 10.1016/j.advenzreg.2009.10.031

Tu-Sekine, B., and Raben, D. M. (2011). Regulation and roles of neuronal diacylglycerol kinases: a lipid perspective. *Crit. Rev. Biochem. Mol. Biol.* 46, 353–364. doi: 10.3109/10409238.2011.577761

Ueda, S., Tu-Sekine, B., Yamanoue, M., Raben, D. M., and Shirai, Y. (2013). The expression of diacylglycerol kinase theta during the organogenesis of mouse embryos. *BMC Dev. Biol.* 13:35. doi: 10.1186/1471-213X-13-35

van Baal, J., de Widt, J., Divecha, N., and van Blitterswijk, W. J. (2005). Translocation of diacylglycerol kinase theta from cytosol to plasma membrane in response to activation of G protein-coupled receptors and protein kinase C. *J. Biol. Chem.* 280, 9870–9878. doi: 10.1074/jbc.M409301200

van Blitterswijk, W. J., and Houssa, B. (2000). Properties and functions of diacylglycerol kinases. *Cell Signal.* 12, 595–605. doi: 10.1016/S0898-6568(00)00113-3

Van Horn, W. D., and Sanders, C. R. (2012). Prokaryotic diacylglycerol kinase and undecaprenol kinase. *Annu. Rev. Biophys.* 41, 81–101. doi: 10.1146/annurev-biophys-050511-102330

Walker, A. J., Draeger, A., Houssa, B., van Blitterswijk, W. J., Ohanian, V., and Ohanian, J. (2001). Diacylglycerol kinase θ is translocated and phosphoinositide 3-kinase-dependently activated by noradrenaline but not angiotensin II in intact small arteries. *Biochem. J.* 353(Pt 1), 129–137. doi: 10.1042/bj3530129

Yakubchyk, Y., Abramovici, H., Maillet, J. C., Daher, E., Obagi, C., Parks, R. J., et al. (2005). Regulation of neurite outgrowth in N1E-115 cells through PDZ-mediated recruitment of diacylglycerol kinase zeta. *Mol. Cell Biol.* 25, 7289–7302. doi: 10.1128/MCB.25.16.7289-7302.2005

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. *Cell* 76, 933–945. doi: 10.1016/0092-8674(94)90367-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 10 November 2016
doi: 10.3389/fcell.2016.00130

Diacylglycerol Kinases in T Cell Tolerance and Effector Function

Shelley S. Chen[1], *Zhiming Hu*[1,2] *and Xiao-Ping Zhong*[1,3,4]*

[1] Division of Allergy and Immunology, Department of Pediatrics, Duke University Medical Center, Durham, NC, USA, [2] Institute of Biotherapy, School of Biotechnology, Southern Medical University, Guangzhou, China, [3] Department of Immunology, Duke University Medical Center, Durham, NC, USA, [4] Hematologic Malignancies and Cellular Therapies Program, Duke Cancer Institute, Duke University Medical Center, Durham, NC, USA

Diacylglycerol kinases (DGKs) are a family of enzymes that regulate the relative levels of diacylglycerol (DAG) and phosphatidic acid (PA) in cells by phosphorylating DAG to produce PA. Both DAG and PA are important second messengers cascading T cell receptor (TCR) signal by recruiting multiple effector molecules, such as RasGRP1, PKCθ, and mTOR. Studies have revealed important physiological functions of DGKs in the regulation of receptor signaling and the development and activation of immune cells. In this review, we will focus on recent progresses in our understanding of two DGK isoforms, α and ζ, in CD8 T effector and memory cell differentiation, regulatory T cell development and function, and invariant NKT cell development and effector lineage differentiation.

Keywords: diacylglycerol kinase, regulatory T cells, invariant NKT cells

OPEN ACCESS

Edited by:
Fumio Sakane,
Chiba University, Japan

Reviewed by:
Kaoru Goto,
Yamagata University School of Medicine, Japan
Antonia Avila-Flores,
Centro Nacional de Biotecnologia - CSIC, Spain

***Correspondence:**
Xiao-Ping Zhong
Xiaoping.zhong@duke.edu

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *31 August 2016*
Accepted: *27 October 2016*
Published: *10 November 2016*

Citation:
Chen SS, Hu Z and Zhong X-P (2016) Diacylglycerol Kinases in T Cell Tolerance and Effector Function. Front. Cell Dev. Biol. 4:130. doi: 10.3389/fcell.2016.00130

INTRODUCTION

Diacylglycerol (DAG) and phosphatidic acid (PA) are two key lipid second messengers that facilitate efficient receptor-mediated signaling in immune cells along with many other cells. They regulate numerous intracellular signaling molecules to control cell differentiation, proliferation, survival, and function. Following T cell receptor (TCR) engagement, DAG is produced through the activation of Phospholipase Cγ1 (PLCγ1), which hydrolyzes membrane phosphatidylinositol bisphosphate (PIP_2) to DAG and inositol trisphosphate (IP3). DAG, together with other signal events, recruits downstream effector molecules to the membrane through their C1 domains and allosterically activates these effectors, with protein kinase Cθ (PKCθ), Ras guanyl–releasing protein 1 (RasGRP1), protein kinase D (PKD), Munc13s, and chimaerins being important for T cell development and/or function (Krishna and Zhong, 2013a; Merida et al., 2015).

DAG plays an important role in recruiting PKCθ to the plasma membrane and immune synapse in T cells (Diaz-Flores et al., 2003; Carrasco and Merida, 2004). The activation of PKCθ leads to TCR-mediated NF-κB and mammalian/mechanistic target of rapamycin complex 1 (mTORC1) activation in T cells (Sun et al., 2000; Isakov and Altman, 2002; Hamilton et al., 2014), which affects key processes, including T cell activation and survival (Manicassamy et al., 2006; Hayashi and Altman, 2007), IL-2 production (Werlen et al., 1998), T_H2 responses (Cannons et al., 2004; Marsland et al., 2004), T_H17 responses (Kwon et al., 2012), invariant NKT (*i*NKT) cell development and activation (Schmidt-Supprian et al., 2004; Fang et al., 2012), and Treg development (Gupta et al., 2008; Barnes et al., 2009; Medoff et al., 2009).

Ras guanyl–releasing protein 1 (RasGRP1) is another downstream molecule that is recruited to the cytoplasm membrane by DAG (Jones et al., 2002; Carrasco and Merida, 2004). RasGRP1

promotes activation of Ras by exchanging GDP for GTP, leading to the activation of the RAF1-MEK1/2-ERK1/2 pathway (Ebinu et al., 1998; Dower et al., 2000; Roose et al., 2005). Additionally, RasGRP1-Ras-Erk1/2 pathway functions upstream for TCR-induced mTORC1, mTORC2, and PI3K activation in T cells (Gorentla et al., 2011). RasGRP1 plays an essential role in conventional αβ T cell development (Dower et al., 2000; Fuller et al., 2012), particularly for the selection of thymocytes that express weak TCR signals (Priatel et al., 2002) and for early iNKT cell development (Shen et al., 2011a). While RasGRP1 appears dispensable for overall γδT cell development, it ensures IL-17 expressing γδT17 lineage differentiation and TCR-induced γδT cell activation (Chen et al., 2012). More recently, it was also found that RasGRP1, together with RasGRP3, promotes early thymic precursor generation (Golec et al., 2016). Additionally, RasGRP1 may play a role in promoting antigen-induced CD8 cell expansion by lowering the threshold of T cell activation (Priatel et al., 2010).

PKDs are recruited by both DAG and DAG-activated PKCs. Upon stimulation, inactive PKDs translocate from the cytosol to the plasma membrane in response to membrane DAG production, where they are then activated by novel PKCs (Rozengurt et al., 2005; Spitaler et al., 2006). PKDs have been shown to exert different effects on VDJ recombination at the TCRβ locus and on CD4 and CD8 expression during T cell development based on their localization at the cytosol or plasma membrane (Marklund et al., 2003; Spitaler et al., 2006). Additionally, PKD2 acts as a sensitive digital amplifier of TCR engagement, enabling CD8 T cells to match the production of inflammatory cytokines to the quality and quantity of TCR ligands (Navarro et al., 2014).

Munc13 proteins are mammalian homologs of the *C. elegans* Unc13, which are important for neurotransmitter secretion (Brose and Rosenmund, 2002). Munc13-1, Munc13-2, and Munc13-3 isoforms bind to DAG with high affinity. The Munc13-4 isoform lacks a C1 domain (Koch et al., 2000; Shirakawa et al., 2004), but it is involved in granule maturation and exocytosis in NK cells and cytotoxic T lymphocytes (CTLs) (Feldmann et al., 2003; Menager et al., 2007), phagosomal maturation, and the killing of intracellular bacteria in neutrophils (Johnson et al., 2011; Monfregola et al., 2012). Deficiency of Munc13-4 causes primary immune deficiency in patients (Feldmann et al., 2003; Cichocki et al., 2014).

Chimaerins possess Rac-specific GTPase Activating Protein (GAP) activity (Caloca et al., 1999; Yang and Kazanietz, 2007). Chimaerin isoforms α2 and β2 are expressed at different levels in T cells and have been shown to translocate to the immune synapse and to both participate in TCR signaling and receive regulation from it (Caloca et al., 2008; Siliceo and Merida, 2009). Chimaerins have been found to inhibit TCR-mediated NFAT activation and DAG-dependent actin polymerization to regulate T cell adhesion and chemotaxis (Siliceo et al., 2006).

Phosphatidic acid (PA) is produced both by the activity of DAG kinases (DGKs) and by the phospholipase D (PLD) family of enzymes in T cells. DGKs phosphorylate DAG to convert it to PA, while PLDs mediate the hydrolysis of phosphatidylcholine (Jenkins and Frohman, 2005; Zhong et al., 2008). The removal of PA is mediated by lipins, which can turn off PA-mediated signaling through dephosphorylation, and they have been shown to regulate mast cell function in the immune system (Csaki and Reue, 2010; Shin et al., 2013b). Intracellular levels of PA change dynamically in response to environmental stimuli (Wang et al., 2006). The downstream effector molecules of PA include a multitude of kinases, such as mTOR (Chen and Fang, 2002), phosphatidylinositol-4-phosphate 5-kinase (PIP5K) (Galandrini et al., 2005; Jarquin-Pardo et al., 2007; Micucci et al., 2008; Cockcroft, 2009; Yoon et al., 2011), spingosine kinase (SPHK ½), RAF1 (Ghosh et al., 1996; Shome et al., 1997; Rizzo et al., 1999, 2000; Andresen et al., 2002), and other molecules, such as Src homology region 2 domain-containing phosphatase 1 (SHP1) (Frank et al., 1999), kinase suppressor of Ras 1 (KSR1, a scaffolding protein that interacts with several components of the Raf-MEK-ERK cascade) (Morrison, 2001; Kraft et al., 2008), and Sos, another guanine nucleotide exchange factor for Ras activation (Zhao et al., 2007). Both PLD and DGK-derived PA has been shown to directly activate mTOR in non-T cells (Chen and Fang, 2002; Avila-Flores et al., 2005). In these cells, PA can also activate mTOR indirectly via ERK (Winter et al., 2010), but such a mechanism has not been examined in T cells. In T cells, DGKα and ζ mainly inhibit TCR-induced mTOR signaling by negative control of DAG-mediated RasGRP1 and likely PKCθ activation (Gorentla et al., 2011; Hamilton et al., 2014). However, DGK-derived PA has been shown to promote T cell maturation in the thymus (Guo et al., 2008) and to regulate innate immune responses (Liu et al., 2007). Future studies should determine the direct downstream of the effector(s) of PA that mediate its functions in these immune cells.

The diverse and important functions of DAG—and PA-mediated signaling suggest their levels must be tightly controlled temporally and spatially. DGKs switch from DAG-mediated signals to PA-mediated signals to dynamically regulate downstream pathways in response to the engagement of the TCR and many other receptors (Merida et al., 2008; Cai et al., 2009; Zhong et al., 2011). In mammals, there are ten DGK isoforms encoded by different genes, some of which also contain splicing variants, adding complexity to this family of enzymes. All DGKs contain a kinase domain and at least two cysteine-rich C1 domains but differ in the homology of their other structural domains as well as their interaction with other biomolecules. Based on their structural distinction and homology, DGKs are classified into five types that may differ in subcellular localization, function, and regulation. The existence of multiple isoforms poses a significant challenge in studying the physiological roles of any specific isoforms in cellular development and functions due to functional redundancies, a fact demonstrated in conventional αβ T cell and iNKT cell development in mice deficient in both DGKα and DGKζ (Guo et al., 2008; Shen et al., 2011b). Of these ten isoforms, DGKα and DGKζ as well as DGKδ are the major isoforms expressed in T cells (Zhong et al., 2002; Olenchock et al., 2006a; Sakane et al., 2007). Both DGKα and ζ have been found to regulate multiple signaling pathways downstream from the TCR (Zhong et al., 2002, 2003; Sanjuan et al., 2003; Baldanzi et al., 2011; Gharbi et al., 2011; Gorentla et al., 2011), such as the RasGRP1-Ras-Erk1/2 pathway, the PKCθ-IKK-NFκB pathway,

mTOR signaling (Gorentla et al., 2011), and MAP kinase-interacting serine/threonine kinase (Mnk) 1 and 2 signaling (Gorentla et al., 2013). They control T cell development (Outram et al., 2002; Guo et al., 2008; Almena et al., 2013), activation and anergy (Zhong et al., 2003; Olenchock et al., 2006a; Zha et al., 2006; Baldanzi et al., 2011), survival (Baldanzi et al., 2011; Ruffo et al., 2016), secretion (Alonso et al., 2007, 2011; Chauveau et al., 2014), and effector function (Shin et al., 2012; Yang et al., 2016b). Besides T cells, DGKζ also regulates the development, survival, and function of mast cells (Olenchock et al., 2006b), B cells (Wheeler et al., 2013), dendritic cells and macrophages (Liu et al., 2007), osteoclasts (Zamani et al., 2015), and NK cells (Yang et al., 2016a). Extensive reviews about DGKs in immune cells have been published recently (Merida et al., 2008, 2015; Zhong et al., 2008; Krishna and Zhong, 2013b). Here, we will focus on recent literature concerning DGKs in T cell tolerance, iNKT cell development and function, and CD8 T cell-mediated antimicrobial and antitumor immunity.

DGKα AND DGKζ IN T CELL TOLERANCE

Clonal deletion of highly self-reactive T cells in the thymic medulla, generation of properly functioning regulatory T cells (Treg), and T cell anergy are among the most important mechanisms of T cell tolerance that prevent autoimmune diseases (Metzger and Anderson, 2011; Xing and Hogquist, 2012). Although DGKα and ζ synergistically promote T cell maturation from the $CD4^+CD8^+$ double positive (DP) to the $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive (SP) stage, no direct evidence has implicated DGKα and ζ in interference with negative selection in establishing central tolerance (Guo et al., 2008).

Regulatory T cells generated in the thymus (tTregs) dominantly suppress T cells and other immune cells to prevent autoimmune diseases. However, they also negatively regulate antitumor and antipathogen immune responses. tTregs are derived from CD4 SP thymocytes in the thymic medulla after relatively strong but transient TCR-MHC/peptide engagement and signaling (Mahmud et al., 2014; Li and Rudensky, 2016). They express Foxp3, a key transcription factor that is critical for their development, maintenance, and function. TCR signaling is not only essential for tTreg generation but also required for tTreg homeostasis and function (Kim et al., 2009; Delpoux et al., 2014; Levine et al., 2014; Vahl et al., 2014). Multiple DAG-mediated signaling pathways are involved in tTreg development and function, indicated by the impaired tTreg development and function in mice deficient in either RasGRP1-Ras or PKCθ-IKK-NFκB signaling. Both NFκB and AP1 are involved in transcriptional activation of Foxp3 expression and possibly in regulating other tTreg properties (Schmidt-Supprian et al., 2004; Willoughby et al., 2007; Chen et al., 2008; Gupta et al., 2008; Barnes et al., 2009; Medoff et al., 2009). Both the percentage and number of tTregs in the $CD4^+$ population are increased in DGKζ-deficient (but not DGKα-deficient) thymocytes and splenocytes, compared to wild-type (WT) controls (**Table 1**). Additionally, $Foxp3^-CD25^+$ cells within the CD4 SP thymocytes are increased in a DGKζ-deficient thymus, suggesting that DGKζ negatively controls early tTreg development. The inhibitory effect of DGKζ on tTreg development is found to be dependent on its negative control of the NFκB/c-Rel and RasGRP1-Ras-Erk pathways (Joshi et al., 2013; Schmidt et al., 2013). Of note are reports that DGKα and ζ manifest differential effects on TNFα-induced NFκB activation in tumor cells and fibroblasts, with DGKα positively regulating PKCζ-mediated p65/RelA at serine 311 residue (Yanagisawa et al., 2007; Kai et al., 2009), while DGKζ inhibits TNFα-induced NFκB activation via decreasing NFkB phosphorylation at Ser468/536, its nuclear localization, and its association with CBP (Tsuchiya et al., 2015). It would be interesting to investigate whether such mechanisms also operate in T cells or downstream of TCR to contribute to DGKα and ζ function in tTreg differentiation. It also remains unclear if DGKα and ζ act redundantly or synergistically to control Treg differentiation and function.

T cell anergy is a form of peripheral tolerance whereby T cells that recognize self-antigens in the absence of co-stimulatory signals are rendered functionally inactive (Schwartz, 2003; Powell, 2006; Fathman and Lineberry, 2007; Chappert and Schwartz, 2010; Kalekar et al., 2016). In anergic T cells, DAG-mediated signaling, including Ras/Erk1/2, NFκB, and mTOR activation, is diminished, while Ca^{++}-mediated signaling and NFAT are selectively elevated or unhindered (Powell, 2006; Chappert and Schwartz, 2010; Xie et al., 2012; **Figure 1**). Both DGKα and ζ are expressed at higher levels in anergic T cells than in activated T cells (Macian et al., 2002; Olenchock et al., 2006a; Zha et al., 2006). Deficiency of either DGKα or ζ or inhibition of DGK activity contributes T cell resistance to anergic induction (Olenchock et al., 2006a; Zha et al., 2006), while overexpression of DGKα promotes T cell anergy (Zha et al., 2006). Because DAG and IP3 are produced at an equimolar ratio by PLCγ1 from PIP2, the elevated DGKα and ζ expression in anergic T cells may shift the equilibrium of IP3 and DAG toward the predominance of IP3-Ca^{++}-NFAT signaling over DAG signaling and subsequent AP1 induction. NFAT forms a NFAT/AP1 dimer to promote T cell activation, but it also functions as a monomer to induce transcription of anergy-promoting molecules, such as Cbl-b and TRAIL (Macian et al., 2002; Wu et al., 2006). It is postulated that elevated DGK activity may lead to NFAT monomer predominance over NFAT/AP1 dimer for anergy induction (Zhong et al., 2008; Krishna and Zhong, 2013a), although experimental evidence has not yet been presented.

An important issue is how DGKα and ζ expression is regulated. The transcription factor early growth response gene 2 (Egr2) is upregulated in anergic T cells and plays an important role in T cell anergy (Zheng et al., 2012). It binds directly to both *Dgka* and *Dgkz* promoters to increase the expression of these genes as well as several other anergy-promoting genes (Zheng et al., 2012, 2013). Another transcription factor, Foxo1, also directly promotes *Dgka* transcription (Martinez-Moreno et al., 2012). Foxo1 function, which is regulated by its subcellular localization between the cytosol and nuclei, is sequestered in the cytosolic compartment following Akt-mediated phosphorylation, which prevents it from association with target genes. In naïve or unstimulated T cells, nuclear Foxo1

TABLE 1 | Comparison of DGKα$^{-/-}$, DGKζ$^{-/-}$, and DGKα$^{-/-}$ζ$^{-/-}$ mice.

		DGKζ$^{-/-}$	DGKα$^{-/-}$	DGKα$^{-/-}$ζ$^{-/-}$	References
T cell development	Positive selection	Not affected	Not affected	Severe decreases of CD4 SP and CD8 SP thymocytes	Zhong et al., 2003; Olenchock et al., 2006a; Guo et al., 2008
	Negative selection	Not affected	Not affected	Not affected	Guo et al., 2008
Regulatory T cell	Foxp3$^-$CD25$^+$ CD4$^+$SP thymocytes	Increased frequencies	Increased but less obvious than DGKζ$^{-/-}$	Not reported	Joshi et al., 2013; Schmidt et al., 2013
	Foxp3$^+$ Treg	Increased in thymus and spleen	Not increased	Not reported	Schmidt et al., 2013
	Suppressive function (*in vitro*)	Enhanced	Not obviously changed	Not reported	Schmidt et al., 2015
iNKT cells	iNKT cell numbers	Not affected	Not affected	Severely decreased	Shen et al., 2011b
	iNKT17 cell	Decreased in numbers due to extrinsic mechanisms	Not reported	Not reported	Wu et al., 2013
CD8 T cells	Primary responses to pathogens	Enhanced expansion and cytokine production in response to LCMV	Less obvious expansion than DGKζ$^{-/-}$ but similar enhanced cytokine production in response to LCMV	Severely impaired in migration, expansion, and cytokine production in response to LM-Ova	Zhong et al., 2003; Shin et al., 2012; Yang et al., 2016b
	Memory responses	Decreased formation; impaired in expansion, enhanced IFNγ and TNFα production in recall responses to LCMV	Decreased formation; impaired in expansion (more severe than DGKζ$^{-/-}$), enhanced IFNγ but not TNFα production in recall responses to LCMV	Impaired formation and maintenance; Decreased expansion but enhanced IFNγ and TNFα production in recall response to LM-Ova	Shin et al., 2012; Yang et al., 2016b
	Sensitivity to TGF-β	Decreased	Not reported	Not reported	Arumugam et al., 2015
	Anti-tumor immunity-OT1 T cells	Enhanced expansion and effector function; Enhanced tumor control	Not reported	Not reported	Riese et al., 2011, 2013
	Anti-tumor immunity-Meso-CAR T cells	Enhanced effector function	Enhanced effector function	Stronger effector function than DGKα or ζ single deficiency; Better tumor control	Riese et al., 2013

activates *Dgka* expression. TCR engagement in the presence of CD28 costimulation induces strong PI3K/Akt activation, which may reduce nuclear Foxo1 and subsequent DGKα expression to ensure full T cell activation and avoidance of anergy (Martinez-Moreno et al., 2012). DGKζ expression has also been found to be regulated by microRNA. Two conserved sequences that match to the miR-34a seed sequence are located in the coding region and 3′ untranslated region (3′ UTR) of *Dgkz*. miR-34a expression is greatly upregulated in activated T cells. miR-34a directly represses DGKζ expression through targeting both *Dgkz* 3′ UTR and the coding region to promote T cell activation (Shin et al., 2013a).

DGKS IN INKT CELL DEVELOPMENT AND FUNCTION

Invariant NKT (iNKT) cells express the invariant Vα14Jα18 TCR, which recognizes lipid antigens presented by MHC class I-like CD1d molecules (Kawano et al., 1997; Mendiratta et al., 1997; Gapin et al., 2001). They are derived from a unique innate-like lymphoid cell lineage and can rapidly respond to agonist stimulation in both innate and adaptive immune responses via production of cytokines, such as IL-4, IL-17, IL-10, IL-13, IFNγ, and TNFα (Bendelac et al., 2007; Coquet et al., 2008; Godfrey et al., 2010; Milpied et al., 2011; Brennan et al., 2013; Salio et al., 2014). iNKT cells participate in host defense against microbial infection, antitumor immunity, and many diseases, such as allergies, asthma, graft-vs.-host disease, and obesity (Osman et al., 2000; Terashima et al., 2008; Van Kaer et al., 2013; Berzins and Ritchie, 2014).

Based on surface CD24, CD44, and NK1.1 expression, iNKT cells are traditionally defined by four developmental stages in the thymus: stage 0 (CD24$^+$CD44$^-$NK1.1$^-$), stage 1 (CD24$^-$CD44$^-$NK1.1$^-$), stage 2 (CD24$^-$CD44$^+$NK1.1$^-$), and stage 3 (CD24$^-$CD44$^+$NK1.1$^+$) (Bendelac et al., 2007; Godfrey et al., 2010; **Figure 2**). Recently, iNKT cells have also been defined into multiple terminally differentiated effector lineages, such as IFN-γ-producing iNKT1, IL-4-producing iNKT2, and IL-17-producing iNKT17 lineage (Matsuda et al., 2006; Michel et al., 2007, 2008). In addition, IL-10-producing iNKT10, T follicular helper (Tfh)-like iNKT cells (iNKT$_{FH}$), and regulatory T cell (Treg)-like iNKT cells have also recently been described (Chang et al., 2012; Tonti et al., 2012; Sag et al., 2014; Lynch et al., 2015; Rampuria and Lang, 2015). iNKT1 and iNKT17 cells mostly reside in the CD44$^+$NK1.1$^+$ and the CD44$^+$NK1.1$^-$ICOS$^+$

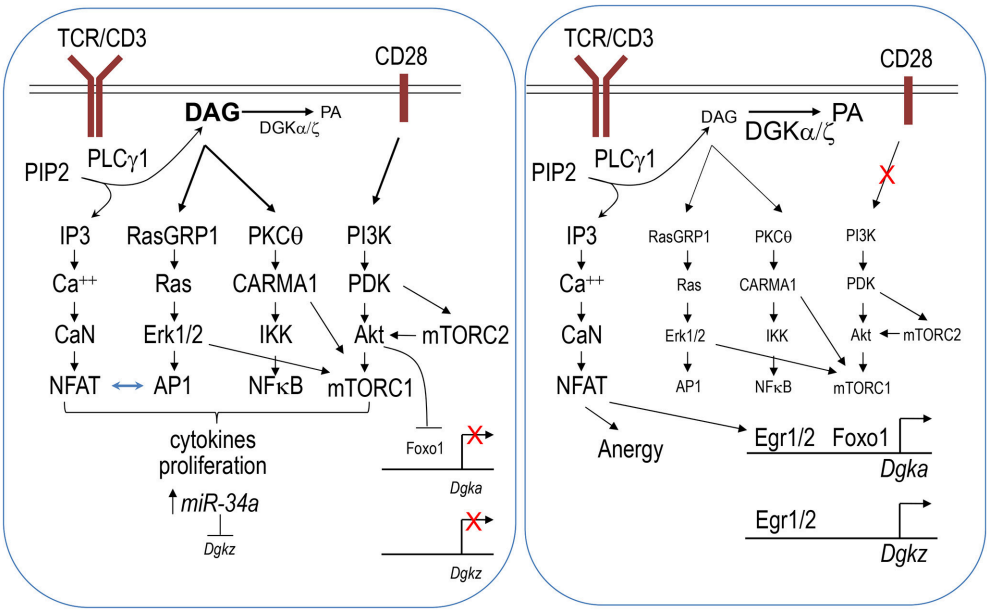


FIGURE 1 | DGKα and DGKζ in T cell activation and anergy. Engagement of the TCR in the presence of co-stimulation results in strong activation of the PI3K-PDK1-Akt pathway (**left panel**). This pathway leads to mTORC2 signaling. Together with activation of the RasGRP1/Ras-Erk1/2 and PKCθ-CARMA1 pathways, they leads to mTORC1 activation. mTORC2 also promotes Akt activation via phosphorylation. Activated Akt phosphorylates Foxo1, leading to it sequestration in the cytosol and failure to activate DGKα transcription. In activated T cells, miR-34a is upregulated, which in turn downregulates DGKζ expression. Decreased DGKα and ζ expression leads to strong DAG-mediated signaling including increases of AP-1 and NFκB activity. AP-1 associates with NFAT to promote T cell activation. At the same time, AP-1 reduces monomeric NFAT to prevent it from inducing anergy promoting molecules. Strong DAG signaling together with IP3-CaN (calcineurin)-NFAT signaling allows full activation of T cells. In contrast, engagement of TCR in the absence of co-stimulation decreases PI3K-Akt-mTOR signaling, leading to increased nuclear Foxo1 and DGKα transcription (**right panel**). miR-34a mediated repression of DGKζ might also be lost under anergy inducing conditions. Increased DGKα and ζ expression may lead to a skewed balance between IP3 and DAG toward strong or selective Ca^{++}-NFAT signaling and induction of Egr1/2, which further induce transcription of DGKα and DGKζ as well as other anergy promoting molecules. Selective IP3-Ca^{++}-NFAT signaling in the presence of weak DAG-mediated signaling induces T cells to enter an anergic state.

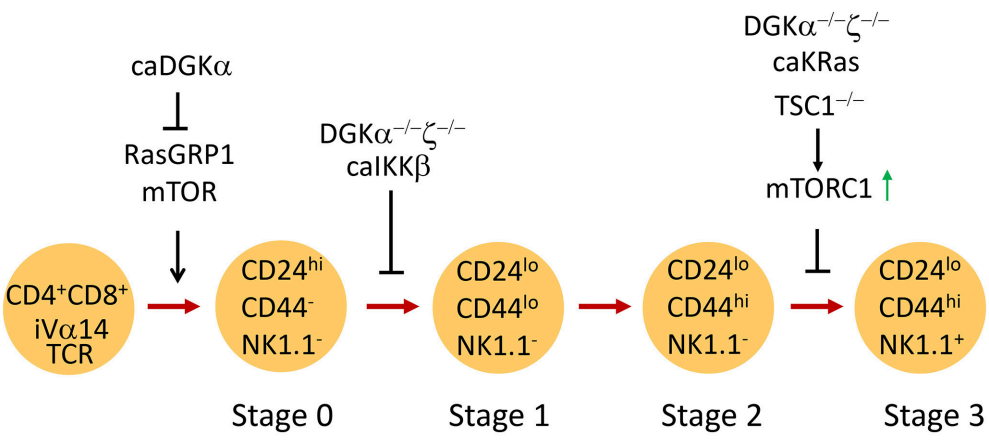


FIGURE 2 | Regulation of iNKT cell development by DGKα and DGKζ. $CD4^+CD8^+$ DP thymocytes expressing the *i*Vα14TCR undergo positive selection to become iNKT cells. RasGRP1/mTOR signaling is critical for generation of stage 0 iNKT cells. Constitutive DGKα inhibits iNKT generation possibly by inhibiting RasGRP1/Erk1/2 activation. DGKα and ζ double deficiency or overactivation of IKKβ causes similar blockade of early iNKT cell development. Overactivation of mTORC1 due to TSC1 deficiency leads to blockade of iNKT terminal maturation. DGKα and ζ double deficiency or expression of a constitutively active KRas also results in impaired iNKT terminal maturation, correlated with elevated mTORC1 activation.

populations, respectively (Watarai et al., 2012; Constantinides and Bendelac, 2013; Lee et al., 2013; Wu et al., 2014b).

Both the RasGRP1-Ras-Erk1/2 and PKCθ-IKK-NFκB pathways have been shown to play important roles in iNTK cell development (Yang et al., 2015). Although it was initially thought that Ras and Erk1/2 activation were dispensable for iNKT cell ontogeny, two recent studies have provided evidence that the RasGRP1-Ras-Mek1/2-Erk1/2 pathway is critical for early iNKT cell development (Hu et al., 2011; Shen et al., 2011a). In RasGRP1-deficient mice, stage 0 iNKT cells as well as total iNKT cell count are significantly decreased, suggesting defective positive selection (Shen et al., 2011a). In concordance with these observations, mice expressing dominant negative Ras in developing thymocytes demonstrated iNKT cell developmental defects (Hu et al., 2011). The RasGRP1-Ras-Erk1/2 pathway activates mTORC1 and mTORC2 signaling as well as Mnk1/2 in developing thymocytes (Gorentla et al., 2011, 2013). Both mTORC1 and mTORC2, but not Mnk1/2, are important for early iNKT cell development (Gorentla et al., 2013; Shin et al., 2014; Wei et al., 2014; Zhang et al., 2014; Prevot et al., 2015), revealing a RasGRP1-Ras-Erk1/2-mTOR signal cascade in iNKT cells for their development. mTORC1, but not mTORC2, promotes PLZF nuclear localization, which may ensure iNKT cell maturation in stage 1 and differentiation to cytokine-producing cells (Shin et al., 2014; Prevot et al., 2015). In iNKT cells, both the DAG and the SLAM (signaling lymphocytic-activation molecule)-SAP (SLAM adaptor protein)-FynT pathway are involved in PKCθ and subsequent NFκB activation. The PKCθ -IKK-NFκB pathway is essential in the ontogeny of iNKT cells, at least in part by increasing expression of antiapoptotic proteins, such as Bcl-xL (Elewaut et al., 2003; Sivakumar et al., 2003; Schmidt-Supprian et al., 2004; Stanic et al., 2004; Chung et al., 2005; Nichols et al., 2005; Pasquier et al., 2005; Griewank et al., 2007; Fang et al., 2012), but it is independent of CARMA1 and Malt1 (Mucosa-associated lymphoid tissue lymphoma translocation protein 1) (Medoff et al., 2009). CARMA1 contributes to TCR-induced mTORC1 activation in T cells (Hamilton et al., 2014). Given the minimal requirement of CARMA1 for iNKT cell development, it would be interesting to determine if TCR-induced mTORC1 activation in iNKT cells would be independent of CARMA1.

Emerging evidence demonstrates that tight regulation of DAG-mediated signaling by DGK activity is critical for the development of iNKT cells. Elevated DGKα activity brought about by expressing a membrane-targeted caDGKα in thymocytes under the control of the proximal Lck promoter caused reduced Erk1/2 activation in thymocytes and a 50% decrease of thymic iNKT cells (Almena et al., 2013). Germline deletion of either DGKα or ζ did not significantly alter iNKT cell numbers in mice. However, simultaneous ablation of both enzymes resulted in a drastic decrease in the number of iNKT cells in the thymus and in peripheral lymphoid organs (Shen et al., 2011b), correlated with prolonged DAG accumulation, elevated Ras-Erk1/2 and PKCθ-IKK signaling, and enhanced activation of both mTORC1 and mTORC2 activities in DP thymocytes (Guo et al., 2008; Gorentla et al., 2011). In DGKα and ζ double knockout mice, there was a decrease in the number of stage 1 to stage 3 iNKT cells. Stage 0 iNKT cells were not examined. The remaining iNKT cells in these mice were mostly $CD44^{+}NK1.1^{-}$ stage 2 cells, suggesting that DGKα and ζ promote both early and terminal iNKT cell maturation (Shen et al., 2011b). Interestingly, expression of constitutive active (CA) IKKβ in developing thymocytes caused a severe reduction in the number of stage 1–3 iNKT cells. Thus, DGKα and ζ double deficiency may cause dysregulation of the PKCθ-IKK-NFκB pathway, leading to early iNKT cell developmental blockage. Different from CA-IKKβ, expression of CA-KRas in thymocytes caused a selective blockage of the transition from stage 2 to 3 of iNKT cells and was associated with decreased T-bet expression (Shen et al., 2011b). Because CA-KRas and DGKα and ζ double deficiency caused elevated mTORC1 signaling (Gorentla et al., 2011) and overactivation of mTORC1 in the absence of TSC1 also resulted in a similar iNKT cell terminal maturation defect (Wu et al., 2014b), DGKα and ζ may synergistically promote iNKT cell terminal maturation at least in part by preventing overactivation of the RasGRP1-Ras-Erk1/2-mTORC1 signaling cascade.

The role of DGKs in iNKT effector functions, however, is less clear. DAG-mediated signaling pathways play important roles in T cell activation, effector lineage differentiation, and tolerance (Chen et al., 2012). They are thus expected to be important in iNKT activation and function. For example, PKCθ is essential for iNKT-mediated liver inflammation (Fang et al., 2012). In germline DGKζ-deficient mice, iNKT17, but not iNKT1 cell number, was selectively decreased. Interestingly, iNKT-17 defects caused by DGKζ deficiency can be corrected in chimeric mice reconstituted with mixed WT and DGKζ-deficient bone marrow cells, suggesting that DGKζ controls iNKT-17 differentiation via an extrinsic mechanism (Wu et al., 2013). Future investigation should define the type of cells that provide such a DGKζ-regulated extrinsic control of iNKT-17 development. Additionally, mTORC1 deficient iNKT cells are defective in activation and are not able to inflict liver damage (Shin et al., 2014). Overactivation of mTORC1 due to TSC1 deficiency shapes iNKT cell effector lineage fates and contributes to their resistance to anergy and enhanced antitumor immunity (Wu et al., 2014a,b). Given the ability of DGKs in regulating mTOR and PKCθ signaling, future studies should determine if DGKs intrinsically regulate iNKT cell functions and effector lineage differentiation under steady state and in various pathologic conditions.

DGKα AND ζ IN CD8 T CELL-MEDIATED ANTIPATHOGEN IMMUNE RESPONSES

CD8 T cells play important roles in immune responses against pathogens, particularly intracellular pathogens. Upon microbial infection, naïve CD8 T cells are activated after engagement of their TCRs with pathogen-derived peptides presented by antigen-presenting cells. They massively expand and differentiate into cytotoxic T cells that are equipped to kill pathogen-infected target cells and secrete proinflammatory cytokines. A typical antigen-specific CD8 T cell-mediated response includes an expansion phase in which CD8 cells proliferate rapidly and differentiate into effector cells, a contraction phase in which 90–95% of effector

CD8 cells die due to apoptosis, and a memory maintenance phase in which the remaining 5–10% of cells are retained as fast-responding memory cells (Williams et al., 2006; Harty and Badovinac, 2008; Zhang and Bevan, 2011). During the expansion phase, effector CD8 T cells differentiate into short-lived effector cells (SLECs, $CD127^{low}KLRG1^{hi}$) and memory precursor effector cells (MPECs, $CD127^{hi}KLRG1^{low}$) (Kaech et al., 2003; Sarkar et al., 2008). SLECs produce high levels of cytokines but are prone to death, while MPECs have high potential to differentiate to long-lived memory cells.

Engagement of the TCR on naïve CD8 T cells provides a critical signal that initiates their activation and expansion. TCR signal strength and quality regulate both the magnitude of expansion and the effector fates of CD8 T cells (Zehn et al., 2009; Iborra et al., 2013; Marchingo et al., 2014; Fulton et al., 2015) through the Ras-Erk1/2-AP1 and PKCθ-IKK-NFκB signaling pathways (Sun et al., 2000; Priatel et al., 2002; Zhong et al., 2008; Merida et al., 2015). An initial study found that DGKζ-deficient mice mounted an enhanced antiviral immune response following lymphocytic choriomeningitis virus (LCMV) infection. These mice showed enhanced expansion of viral-specific effector CD4 and CD8 T cells that contained higher percentages of IFNγ-producing cells 7 days after LCMV infection, which resulted in a quicker clearance of the virus than in WT mice (Zhong et al., 2003). A subsequent study further revealed that DGKα and ζ differentially regulate effector and memory CD8 T cell differentiation. While a deficiency of either DGKα or ζ resulted in enhanced effector CD8 T cell expansion, it slightly decreased memory CD8 T cell formation and response to LCMV infection, which correlated with elevated mTORC1 signaling in these cells (Shin et al., 2012).

Although deficiency of either DGKα or ζ enhances antiviral immune responses, DGKα and ζ double deficiency actually caused severe impairment of CD8 T cell-mediated responses to *Listeria monocytogenes* (LM) infection (Yang et al., 2016b). In an ovalbumin (OVA) specific OT1 TCR transgenic model and newly generated floxed DGKζ conditional-deficient mice where DGKα and ζ activity can be selectively deleted in naïve and memory CD8 T cells, it was found that ablation of both DGKα and ζ, but not of the individual DGKα or ζ isoform, impaired primary CD8 T cell responses (**Table 1**). At the earliest hours after LM-OVA infection, DGKα and ζ double deficient CD8 T cells expressed decreased levels of chemokine receptors CCR4, CCR5, and CXCR3 and showed impaired migration to the draining lymph nodes (dLNs). Cells that migrated to the dLNs were compromised in their proliferative ability due to not yet defined mechanism(s). In contrast to this *in vivo* setting, DGKα and ζ double deficient CD8 T cells proliferated more vigorously than WT controls *in vitro* following antigen stimulation, suggesting that the defect in proliferation was not due to intrinsic defects. It would be interesting to determine if DGKα and ζ are involved in regulating T cell/APC engagement for initiation of T cell activation. As a consequence of impaired expansion of DGKα and ζ double deficient CD8 T cells during primary immune responses, formation of memory cells was severely decreased as well. In addition, DGKα and ζ double deficiency compromised memory CD8 T cell function in homeostasis. Ablation of DGKα and ζ in preformed memory CD8 T cells accelerated the decline of these cells due to increased death and decreased homeostatic proliferative renewal (Yang et al., 2016b).

In DGKα and ζ double deficient CD8 T cells, TCR-induced NFκB nuclear localization was surprisingly diminished, although nuclear NFκB was elevated before stimulation (Yang et al., 2016b). A similar situation was also observed in T cells expressing a constitutive active IKKβ. CD8 T cells expressing a constitutive active IKKβ are defective in expansion *in vivo* following LM-OVA infection and are impaired in TCR-induced nuclear NFκB translocation (Krishna et al., 2012). It is likely, then, that elevated DAG levels may lead to an increase of basal activation of the PKCθ-IKK-NFκB pathway, which may trigger a negative feedback inhibition for TCR-induced activation of this pathway. Further studies should illustrate the exact negative feedback mechanism caused by DGKα and ζ double deficiency and by overactivation of IKKβ.

One consequence of decreased NFκB activation in DGKα and ζ double deficient CD8 T cells was decreased miR-155 expression and, subsequently, increased SOCS1 expression (Yang et al., 2016b). miR-155 promotes expansion of effector CD8 T cells and generation of memory CD8 T cells by targeting SOCS1 expression to ensure signaling from the common γ (γc) chain cytokine receptors (Dudda et al., 2013; Gracias et al., 2013). Common γ chain receptor signaling is known to be critical for CD8 effector and memory responses (Becker et al., 2002; Kieper et al., 2002; Carrio et al., 2004; Bachmann et al., 2007; Cui and Kaech, 2010; Sandau et al., 2010; Feau et al., 2011; Boyman and Sprent, 2012; Van Der Windt et al., 2012; Starbeck-Miller et al., 2014; Cui et al., 2015); SOCS1 negatively controls signaling from these γc-chain cytokine receptors (Cornish et al., 2003). Overexpression of miR-155 restored signaling from these receptors in DGKα and ζ double deficient CD8 T cells and partially corrected their defective responses. The data identified a DGK-NFκB-miR-155-SOCS1 axis that bridges TCR and γc-chain cytokine signaling for robust CD8 T-cell primary and memory responses to bacterial infection (Yang et al., 2016b).

DGKα AND ζ REGULATE CD8 T CELL AND CAR-T CELL MEDIATED ANTITUMOR IMMUNITY

A tumor microenvironment suppresses T cell mediated antitumor immunity, rendering tumor-infiltrating T cells hyporesponsive or anergic (Abe and Macian, 2013; Crespo et al., 2013). DGKζ-deficient CD8 T cells contain elevated antitumor immunity. DGKζ-deficient mice subcutaneously injected with the EL-4 thymoma had reduced tumor burdens and increased tumor-specific proliferative CD8 effector T cells compared to WT controls (Riese et al., 2011, 2013). Both increased Erk1/2 activation and decreased sensitivity to the suppressive cytokine TGF-β in DGKζ-deficient CD8 T cells may be responsible for stronger activation and antitumor immunity (Arumugam et al., 2015).

Recently, chimeric antigen receptor (CAR) T cells (CAR-T cells) have demonstrated superior activity in tumor control and,

in some cases, tumor eradication (Fesnak et al., 2016). However, CAR-T cells have manifested limited efficacy for solid tumors in that they are subjected to suppression by the local tumor environment and may become hyporesponsive or anergic. Such hyporesponsive or anergic tumor-infiltrating T cells or CAR-T cells show decreased Ras/Erk activation but elevated DGKα and ζ levels (Moon et al., 2014). Both type 1 and type 2 DGK inhibitors are capable of reversing such hyporesponsiveness in tumor-infiltrating CAR-T cells *ex vivo*, leading to increased cytotoxicity (Moon et al., 2014). Consistent with this finding, genetic ablation of DGKα, ζ, or both DGKα and ζ enhanced CD8 T cells transduced with a mesoCAR, a CAR with high affinity to the human tumor antigen mesothelin. DGKα and ζ single or double deficient mesoCAR-T cells produced elevated IFNγ production and demonstrated stronger antitumor cytotoxicity than WT controls, which correlated with reduced sensitivity to TGFβ and increased expression of FasL and TRAIL, ligands for the death receptors FAS and TRAIL-RI/RII. Importantly, DGK-deficient mesoCAR-T cells controlled mesothelioma *in vivo* better than WT controls (Riese et al., 2013). The enhancement of CAR-T function by DGKα and ζ double deficiency sharply contrasts with the defective anti-LM responses of DGKα and ζ double deficient CD8 T cells, suggesting differential requirements of DAG-mediated signaling downstream of CARs and TCR and for CAR-T and conventional CD8 T cell activation.

SUMMARY

Over the past few years, our understanding of the DGK family of enzymes in immune cells has been significantly advanced. DGKα and ζ act individually to negatively control T cell activation, effector CD8 T cell differentiation and function during antimicrobial and antitumor immune responses, and tTreg generation. DGKα and ζ also manifest functional redundancy in promoting conventional αβ T cell and iNKT cell development and in enhancing CAR-T cell function. The unexpected severe impairment of CD8 T cell-mediated immune responses to microbial infection in the absence of both DGKα and ζ underscores the importance of fine-tuning DAG levels and also suggests potential negative feedback mechanisms triggered by deregulated DAG-mediated signaling. Defining such mechanisms should shed additional light on the regulation of DAG-mediated signaling pathways. Additional efforts are also needed to illustrate the underlying mechanisms of differential effects of DGKα and ζ double deficiency on CD8 T cells during antitumor and antipathogen immune responses. While DGKα and ζ perform similar or redundant functions, a more prominent role of DGKζ than DGKα in certain aspects of T cell biology, such as effector CD8 T cell differentiation and Treg, development has been noted (**Table 1**); however, determinants of such differences between DGKα and ζ remain unclear. The drastic differences observed between DGKα and ζ double and single deficient CD8 T cells during immune responses beg for development of DGK isoform-specific inhibitors. Such inhibitors used individually or in combination may provide great advantages over pan-DGK inhibitors in modulating immune responses for therapeutic purposes in different disease settings to minimize undesirable side effects. Key elements, such as transcription factors, microRNAs, and posttranslational modifications that control the dynamic individual and synergistic functions of DGK isoforms in T cells are beginning to be appreciated and require further exploration for better understanding of their physiological importance and the development of novel strategies enabling selective modulation of DGK α and ζ expression and activities for treating autoimmune diseases, viral infections, and cancer.

AUTHOR CONTRIBUTIONS

SC, ZH, and X-PZ are involved in preparation of the manuscript.

ACKNOWLEDGMENTS

This work is supported by the National Institutes of Health (R01AI079088 and R01AI101206) for X-PZ.

REFERENCES

Abe, B. T., and Macian, F. (2013). Uncovering the mechanisms that regulate tumor-induced T-cell anergy. *Oncoimmunol* 2:e22679. doi: 10.4161/onci.22679

Almena, M., Andrada, E., Liebana, R., and Merida, I. (2013). Diacylglycerol metabolism attenuates T-cell receptor signaling and alters thymocyte differentiation. *Cell Death Dis.* 4:e912. doi: 10.1038/cddis.2013.396

Alonso, R., Mazzeo, C., Mérida, I., and Izquierdo, M. (2007). A new role of diacylglycerol kinase α on the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes. *Biochimie* 89, 213–221. doi: 10.1016/j.biochi.2006.07.018

Alonso, R., Mazzeo, C., Rodriguez, M. C., Marsh, M., Fraile-Ramos, A., Calvo, V., et al. (2011). Diacylglycerol kinase α regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of Fas ligand-containing exosomes in T lymphocytes. *Cell Death Differ.* 18, 1161–1173. doi: 10.1038/cdd.2010.184

Andresen, B. T., Rizzo, M. A., Shome, K., and Romero, G. (2002). The role of phosphatidic acid in the regulation of the Ras/MEK/Erk signaling cascade. *FEBS Lett.* 531, 65–68. doi: 10.1016/S0014-5793(02)03483-X

Arumugam, V., Bluemn, T., Wesley, E., Schmidt, A. M., Kambayashi, T., Malarkannan, S., et al. (2015). TCR signaling intensity controls $CD8^+$ T cell responsiveness to TGF-β. *J. Leukoc. Biol.* 98, 703–712. doi: 10.1189/jlb.2HIMA1214-578R

Avila-Flores, A., Santos, T., Rincón, E., and Mérida, I. (2005). Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. *J. Biol. Chem.* 280, 10091–10099. doi: 10.1074/jbc.M412296200

Bachmann, M. F., Wolint, P., Walton, S., Schwarz, K., and Oxenius, A. (2007). Differential role of IL-2R signaling for $CD8^+$ T cell responses in acute and chronic viral infections. *Eur. J. Immunol.* 37, 1502–1512. doi: 10.1002/eji.200637023

Baldanzi, G., Pighini, A., Bettio, V., Rainero, E., Traini, S., Chianale, F., et al. (2011). SAP-mediated inhibition of diacylglycerol kinase α regulates TCR-induced diacylglycerol signaling. *J. Immunol.* 187, 5941–5951. doi: 10.4049/jimmunol.1002476

Barnes, M. J., Krebs, P., Harris, N., Eidenschenk, C., Gonzalez-Quintial, R., Arnold, C. N., et al. (2009). Commitment to the regulatory T cell lineage requires CARMA1 in the thymus but not in the periphery. *PLoS Biol.* 7:e51. doi: 10.1371/journal.pbio.1000051

Becker, T. C., Wherry, E. J., Boone, D., Murali-Krishna, K., Antia, R., Ma, A., et al. (2002). Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. *J. Exp. Med.* 195, 1541–1548. doi: 10.1084/jem.20020369

Bendelac, A., Savage, P. B., and Teyton, L. (2007). The biology of NKT cells. *Annu. Rev. Immunol.* 25, 297–336. doi: 10.1146/annurev.immunol.25.022106.141711

Berzins, S. P., and Ritchie, D. S. (2014). Natural killer T cells: drivers or passengers in preventing human disease? *Nat. Rev. Immunol.* 14, 640–646. doi: 10.1038/nri3725

Boyman, O., and Sprent, J. (2012). The role of interleukin-2 during homeostasis and activation of the immune system. *Nat. Rev. Immunol.* 12, 180–190. doi: 10.1038/nri3156

Brennan, P. J., Brigl, M., and Brenner, M. B. (2013). Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat. Rev. Immunol.* 13, 101–117. doi: 10.1038/nri3369

Brose, N., and Rosenmund, C. (2002). Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *J. Cell Sci.* 115, 4399–4411. doi: 10.1242/jcs.00122

Cai, J., Abramovici, H., Gee, S. H., and Topham, M. K. (2009). Diacylglycerol kinases as sources of phosphatidic acid. *Biochim. Biophys. Acta* 1791, 942–948. doi: 10.1016/j.bbalip.2009.02.010

Caloca, M. J., Delgado, P., Alarcón, B., and Bustelo, X. R. (2008). Role of chimaerins, a group of Rac-specific GTPase activating proteins, in T-cell receptor signaling. *Cell. Signal.* 20, 758–770. doi: 10.1016/j.cellsig.2007.12.015

Caloca, M. J., Garcia-Bermejo, M. L., Blumberg, P. M., Lewin, N. E., Kremmer, E., Mischak, H., et al. (1999). β2-chimaerin is a novel target for diacylglycerol: binding properties and changes in subcellular localization mediated by ligand binding to its C1 domain. *Proc. Natl. Acad. Sci. U.S.A.* 96, 11854–11859. doi: 10.1073/pnas.96.21.11854

Cannons, J. L., Yu, L. J., Hill, B., Mijares, L. A., Dombroski, D., Nichols, K. E., et al. (2004). SAP regulates T(H)2 differentiation and PKC-θ-mediated activation of NF-κB1. *Immunity* 21, 693–706. doi: 10.1016/j.immuni.2004.09.012

Carrasco, S., and Merida, I. (2004). Diacylglycerol-dependent binding recruits PKCθ and RasGRP1 C1 domains to specific subcellular localizations in living T lymphocytes. *Mol. Biol. Cell* 15, 2932–2942. doi: 10.1091/mbc.E03-11-0844

Carrio, R., Bathe, O. F., and Malek, T. R. (2004). Initial antigen encounter programs $CD8^+$ T cells competent to develop into memory cells that are activated in an antigen-free, IL-7- and IL-15-rich environment. *J. Immunol.* 172, 7315–7323. doi: 10.4049/jimmunol.172.12.7315

Chang, P. P., Barral, P., Fitch, J., Pratama, A., Ma, C. S., Kallies, A., et al. (2012). Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat. Immunol.* 13, 35–43. doi: 10.1038/ni.2166

Chappert, P., and Schwartz, R. H. (2010). Induction of T cell anergy: integration of environmental cues and infectious tolerance. *Curr. Opin. Immunol.* 22, 552–559. doi: 10.1016/j.coi.2010.08.005

Chauveau, A., Le Floc'h, A., Bantilan, N. S., Koretzky, G. A., and Huse, M. (2014). Diacylglycerol kinase α establishes T cell polarity by shaping diacylglycerol accumulation at the immunological synapse. *Sci. Signal.* 7:ra82. doi: 10.1126/scisignal.2005287

Chen, J., and Fang, Y. (2002). A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. *Biochem. Pharmacol.* 64, 1071–1077. doi: 10.1016/S0006-2952(02)01263-7

Chen, X., Priatel, J. J., Chow, M. T., and Teh, H. S. (2008). Preferential development of CD4 and CD8 T regulatory cells in RasGRP1-deficient mice. *J. Immunol.* 180, 5973–5982. doi: 10.4049/jimmunol.180.9.5973

Chen, Y., Ci, X., Gorentla, B., Sullivan, S. A., Stone, J. C., Zhang, W., et al. (2012). Differential requirement of RasGRP1 for γδ T cell development and activation. *J. Immunol.* 189, 61–71. doi: 10.4049/jimmunol.1103272

Chung, B., Aoukaty, A., Dutz, J., Terhorst, C., and Tan, R. (2005). Signaling lymphocytic activation molecule-associated protein controls NKT cell functions. *J. Immunol.* 174, 3153–3157. doi: 10.4049/jimmunol.174.6.3153

Cichocki, F., Schlums, H., Li, H., Stache, V., Holmes, T., Lenvik, T. R., et al. (2014). Transcriptional regulation of Munc13-4 expression in cytotoxic lymphocytes is disrupted by an intronic mutation associated with a primary immunodeficiency. *J. Exp. Med.* 211, 1079–1091. doi: 10.1084/jem.20131131

Cockcroft, S. (2009). Phosphatidic acid regulation of phosphatidylinositol 4-phosphate 5-kinases. *Biochim. Biophys. Acta* 1791, 905–912. doi: 10.1016/j.bbalip.2009.03.007

Constantinides, M. G., and Bendelac, A. (2013). Transcriptional regulation of the NKT cell lineage. *Curr. Opin. Immunol.* 25, 161–167. doi: 10.1016/j.coi.2013.01.003

Coquet, J. M., Chakravarti, S., Kyparissoudis, K., Mcnab, F. W., Pitt, L. A., Mckenzie, B. S., et al. (2008). Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11287–11292. doi: 10.1073/pnas.0801631105

Cornish, A. L., Chong, M. M., Davey, G. M., Darwiche, R., Nicola, N. A., Hilton, D. J., et al. (2003). Suppressor of cytokine signaling-1 regulates signaling in response to interleukin-2 and other γ c-dependent cytokines in peripheral T cells. *J. Biol. Chem.* 278, 22755–22761. doi: 10.1074/jbc.M303021200

Crespo, J., Sun, H., Welling, T. H., Tian, Z., and Zou, W. (2013). T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr. Opin. Immunol.* 25, 214–221. doi: 10.1016/j.coi.2012.12.003

Csaki, L. S., and Reue, K. (2010). Lipins: multifunctional lipid metabolism proteins. *Annu. Rev. Nutr.* 30, 257–272. doi: 10.1146/annurev.nutr.012809.104729

Cui, G., Staron, M. M., Gray, S. M., Ho, P. C., Amezquita, R. A., Wu, J., et al. (2015). IL-7-Induced Glycerol Transport and TAG Synthesis Promotes Memory $CD8^+$ T Cell Longevity. *Cell* 161, 750–761. doi: 10.1016/j.cell.2015.03.021

Cui, W., and Kaech, S. M. (2010). Generation of effector $CD8^+$ T cells and their conversion to memory T cells. *Immunol. Rev.* 236, 151–166. doi: 10.1111/j.1600-065X.2010.00926.x

Delpoux, A., Yakonowsky, P., Durand, A., Charvet, C., Valente, M., Pommier, A., et al. (2014). TCR signaling events are required for maintaining CD4 regulatory T cell numbers and suppressive capacities in the periphery. *J. Immunol.* 193, 5914–5923. doi: 10.4049/jimmunol.1400477

Díaz-Flores, E., Siliceo, M., Martínez, A. C., and Mérida, I. (2003). Membrane translocation of protein kinase Cθ during T lymphocyte activation requires phospholipase C-γ-generated diacylglycerol. *J. Biol. Chem.* 278, 29208–29215. doi: 10.1074/jbc.M303165200

Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., et al. (2000). RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat. Immunol.* 1, 317–321. doi: 10.1038/80799

Dudda, J. C., Salaun, B., Ji, Y., Palmer, D. C., Monnot, G. C., Merck, E., et al. (2013). MicroRNA-155 is required for effector $CD8^+$ T cell responses to virus infection and cancer. *Immunity* 38, 742–753. doi: 10.1016/j.immuni.2012.12.006

Ebinu, J. O., Bottorff, D. A., Chan, E. Y., Stang, S. L., Dunn, R. J., and Stone, J. C. (1998). RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science* 280, 1082–1086. doi: 10.1126/science.280.5366.1082

Elewaut, D., Shaikh, R. B., Hammond, K. J., De Winter, H., Leishman, A. J., Sidobre, S., et al. (2003). NIK-dependent RelB activation defines a unique signaling pathway for the development of Vα 14i NKT cells. *J. Exp. Med.* 197, 1623–1633. doi: 10.1084/jem.20030141

Fang, X., Wang, R., Ma, J., Ding, Y., Shang, W., and Sun, Z. (2012). Ameliorated ConA-induced hepatitis in the absence of PKC-theta. *PLoS ONE* 7:e31174. doi: 10.1371/journal.pone.0031174

Fathman, C. G., and Lineberry, N. B. (2007). Molecular mechanisms of $CD4^+$ T-cell anergy. *Nat. Rev. Immunol.* 7, 599–609. doi: 10.1038/nri2131

Feau, S., Arens, R., Togher, S., and Schoenberger, S. P. (2011). Autocrine IL-2 is required for secondary population expansion of $CD8^+$ memory T cells. *Nat. Immunol.* 12, 908–913. doi: 10.1038/ni.2079

Feldmann, J., Callebaut, I., Raposo, G., Certain, S., Bacq, D., Dumont, C., et al. (2003). Munc13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* 115, 461–473. doi: 10.1016/S0092-8674(03)00855-9

Fesnak, A. D., June, C. H., and Levine, B. L. (2016). Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat. Rev. Cancer* 16, 566–581. doi: 10.1038/nrc.2016.97

Frank, C., Keilhack, H., Opitz, F., Zschörnig, O., and Böhmer, F. D. (1999). Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry* 38, 11993–12002. doi: 10.1021/bi982586w

Fuller, D. M., Zhu, M., Song, X., Ou-Yang, C. W., Sullivan, S. A., Stone, J. C., et al. (2012). Regulation of RasGRP1 function in T cell development

and activation by its unique tail domain. *PLoS ONE* 7:e38796. doi: 10.1371/journal.pone.0038796

Fulton, R. B., Hamilton, S. E., Xing, Y., Best, J. A., Goldrath, A. W., Hogquist, K. A., et al. (2015). The TCR's sensitivity to self peptide-MHC dictates the ability of naive $CD8^+$ T cells to respond to foreign antigens. *Nat. Immunol.* 16, 107–117. doi: 10.1038/ni.3043

Galandrini, R., Micucci, F., Tassi, I., Cifone, M. G., Cinque, B., Piccoli, M., et al. (2005). Arf6: a new player in FcgammaRIIIA lymphocyte-mediated cytotoxicity. *Blood* 106, 577–583. doi: 10.1182/blood-2004-10-4100

Gapin, L., Matsuda, J. L., Surh, C. D., and Kronenberg, M. (2001). NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat. Immunol.* 2, 971–978. doi: 10.1038/ni710

Gharbi, S. I., Rincon, E., Avila-Flores, A., Torres-Ayuso, P., Almena, M., Cobos, M. A., et al. (2011). Diacylglycerol kinase ζ controls diacylglycerol metabolism at the immunological synapse. *Mol. Biol. Cell* 22, 4406–4414. doi: 10.1091/mbc.E11-03-0247

Ghosh, S., Strum, J. C., Sciorra, V. A., Daniel, L., and Bell, R. M. (1996). Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. *J. Biol. Chem.* 271, 8472–8480. doi: 10.1074/jbc.271.14.8472

Godfrey, D. I., Stankovic, S., and Baxter, A. G. (2010). Raising the NKT cell family. *Nat. Immunol.* 11, 197–206. doi: 10.1038/ni.1841

Golec, D. P., Henao Caviedes, L. M., and Baldwin, T. A. (2016). RasGRP1 and RasGRP3 Are required for efficient generation of early thymic progenitors. *J. Immunol.* 197, 1743–1753. doi: 10.4049/jimmunol.1502107

Gorentla, B. K., Krishna, S., Shin, J., Inoue, M., Shinohara, M. L., Grayson, J. M., et al. (2013). Mnk1 and 2 are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis. *J. Immunol.* 190, 1026–1037. doi: 10.4049/jimmunol.1200026

Gorentla, B. K., Wan, C. K., and Zhong, X. P. (2011). Negative regulation of mTOR activation by diacylglycerol kinases. *Blood* 117, 4022–4031. doi: 10.1182/blood-2010-08-300731

Gracias, D. T., Stelekati, E., Hope, J. L., Boesteanu, A. C., Doering, T. A., Norton, J., et al. (2013). The microRNA miR-155 controls $CD8^+$ T cell responses by regulating interferon signaling. *Nat. Immunol.* 14, 593–602. doi: 10.1038/ni.2576

Griewank, K., Borowski, C., Rietdijk, S., Wang, N., Julien, A., Wei, D. G., et al. (2007). Homotypic interactions mediated by Slamf1 and Slamf6 receptors control NKT cell lineage development. *Immunity* 27, 751–762. doi: 10.1016/j.immuni.2007.08.020

Guo, R., Wan, C. K., Carpenter, J. H., Mousallem, T., Boustany, R. M., Kuan, C. T., et al. (2008). Synergistic control of T cell development and tumor suppression by diacylglycerol kinase α and ζ. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11909–11914. doi: 10.1073/pnas.0711856105

Gupta, S., Manicassamy, S., Vasu, C., Kumar, A., Shang, W., and Sun, Z. (2008). Differential requirement of PKC-θ in the development and function of natural regulatory T cells. *Mol. Immunol.* 46, 213–224. doi: 10.1016/j.molimm.2008.08.275

Hamilton, K. S., Phong, B., Corey, C., Cheng, J., Gorentla, B., Zhong, X., et al. (2014). T cell receptor-dependent activation of mTOR signaling in T cells is mediated by Carma1 and MALT1, but not Bcl10. *Sci. Signal.* 7:ra55. doi: 10.1126/scisignal.2005169

Harty, J. T., and Badovinac, V. P. (2008). Shaping and reshaping $CD8^+$ T-cell memory. *Nat. Rev. Immunol.* 8, 107–119. doi: 10.1038/nri2251

Hayashi, K., and Altman, A. (2007). Protein kinase C theta (PKCθ): a key player in T cell life and death. *Pharmacol. Res.* 55, 537–544. doi: 10.1016/j.phrs.2007.04.009

Hu, T., Gimferrer, I., Simmons, A., Wiest, D., and Alberola-Ila, J. (2011). The Ras/MAPK pathway is required for generation of iNKT cells. *PLoS ONE* 6:e19890. doi: 10.1371/journal.pone.0019890

Iborra, S., Ramos, M., Arana, D. M., Lazaro, S., Aguilar, F., Santos, E., et al. (2013). N-ras couples antigen receptor signaling to Eomesodermin and to functional $CD8^+$ T cell memory but not to effector differentiation. *J. Exp. Med.* 210, 1463–1479. doi: 10.1084/jem.20112495

Isakov, N., and Altman, A. (2002). protein kinase cθ in T cell activation. *Annu. Rev. Immunol.* 20, 761–794. doi: 10.1146/annurev.immunol.20.100301.064807

Jarquin-Pardo, M., Fitzpatrick, A., Galiano, F. J., First, E. A., and Davis, J. N. (2007). Phosphatidic acid regulates the affinity of the murine phosphatidylinositol 4-phosphate 5-kinase-Iβ for phosphatidylinositol-4-phosphate. *J. Cell. Biochem.* 100, 112–128. doi: 10.1002/jcb.21027

Jenkins, G. M., and Frohman, M. A. (2005). Phospholipase D: a lipid centric review. *Cell. Mol. Life Sci.* 62, 2305–2316. doi: 10.1007/s00018-005-5195-z

Johnson, J. L., Hong, H., Monfregola, J., Kiosses, W. B., and Catz, S. D. (2011). Munc13-4 restricts motility of Rab27a-expressing vesicles to facilitate lipopolysaccharide-induced priming of exocytosis in neutrophils. *J. Biol. Chem.* 286, 5647–5656. doi: 10.1074/jbc.M110.184762

Jones, D. R., Sanjuan, M. A., Stone, J. C., and Merida, I. (2002). Expression of a catalytically inactive form of diacylglycerol kinase α induces sustained signaling through RasGRP. *FASEB J.* 16, 595–597. doi: 10.1096/fj.01-0762fje

Joshi, R. P., Schmidt, A. M., Das, J., Pytel, D., Riese, M. J., Lester, M., et al. (2013). The ζ isoform of diacylglycerol kinase plays a predominant role in regulatory T cell development and TCR-mediated ras signaling. *Sci. Signal.* 6:ra102. doi: 10.1126/scisignal.2004373

Kaech, S. M., Tan, J. T., Wherry, E. J., Konieczny, B. T., Surh, C. D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4, 1191–1198. doi: 10.1038/ni1009

Kai, M., Yasuda, S., Imai, S., Toyota, M., Kanoh, H., and Sakane, F. (2009). Diacylglycerol kinase α enhances protein kinase Cζ-dependent phosphorylation at Ser311 of p65/RelA subunit of nuclear factor-κB. *FEBS Lett.* 583, 3265–3268. doi: 10.1016/j.febslet.2009.09.017

Kalekar, L. A., Schmiel, S. E., Nandiwada, S. L., Lam, W. Y., Barsness, L. O., Zhang, N., et al. (2016). $CD4^+$ T cell anergy prevents autoimmunity and generates regulatory T cell precursors. *Nat. Immunol.* 17, 304–314. doi: 10.1038/ni.3331

Kawano, T., Cui, J., Koezuka, Y., Toura, I., Kaneko, Y., Motoki, K., et al. (1997). CD1d-restricted and TCR-mediated activation of $V_\alpha 14$ NKT cells by glycosylceramides. *Science* 278, 1626–1629. doi: 10.1126/science.278.5343.1626

Kieper, W. C., Tan, J. T., Bondi-Boyd, B., Gapin, L., Sprent, J., Ceredig, R., et al. (2002). Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype $CD8^+$ T cells. *J. Exp. Med.* 195, 1533–1539. doi: 10.1084/jem.20020067

Kim, J. K., Klinger, M., Benjamin, J., Xiao, Y., Erle, D. J., Littman, D. R., et al. (2009). Impact of the TCR signal on regulatory T cell homeostasis, function, and trafficking. *PLoS ONE* 4:e6580. doi: 10.1371/journal.pone.0006580

Koch, H., Hofmann, K., and Brose, N. (2000). Definition of Munc13-homology-domains and characterization of a novel ubiquitously expressed Munc13 isoform. *Biochem. J.* 349, 247–253. doi: 10.1042/bj3490247

Kraft, C. A., Garrido, J. L., Fluharty, E., Leiva-Vega, L., and Romero, G. (2008). Role of phosphatidic acid in the coupling of the ERK cascade. *J. Biol. Chem.* 283, 36636–36645. doi: 10.1074/jbc.M804633200

Krishna, S., Xie, D., Gorentla, B., Shin, J., Gao, J., and Zhong, X. P. (2012). Chronic activation of the kinase IKKβ impairs T cell function and survival. *J. Immunol.* 189, 1209–1219. doi: 10.4049/jimmunol.1102429

Krishna, S., and Zhong, X. (2013a). Role of diacylglycerol kinases in T cell development and function. *Crit. Rev. Immunol.* 33, 97–118.

Krishna, S., and Zhong, X. P. (2013b). Regulation of lipid signaling by diacylglycerol kinases during T cell development and function. *Front. Immunol.* 4:178. doi: 10.3389/fimmu.2013.00178

Kwon, M. J., Ma, J., Ding, Y., Wang, R., and Sun, Z. (2012). Protein kinase C-θ promotes Th17 differentiation via upregulation of Stat3. *J. Immunol.* 188, 5887–5897. doi: 10.4049/jimmunol.1102941

Lee, Y. J., Holzapfel, K. L., Zhu, J., Jameson, S. C., and Hogquist, K. A. (2013). Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nat. Immunol.* 14, 1146–1154. doi: 10.1038/ni.2731

Levine, A. G., Arvey, A., Jin, W., and Rudensky, A. Y. (2014). Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* 15, 1070–1078. doi: 10.1038/ni.3004

Li, M. O., and Rudensky, A. Y. (2016). T cell receptor signalling in the control of regulatory T cell differentiation and function. *Nat. Rev. Immunol.* 16, 220–233. doi: 10.1038/nri.2016.26

Liu, C. H., Machado, F. S., Guo, R., Nichols, K. E., Burks, A. W., Aliberti, J. C., et al. (2007). Diacylglycerol kinase ζ regulates microbial recognition

and host resistance to Toxoplasma gondii. *J. Exp. Med.* 204, 781–792. doi: 10.1084/jem.20061856

Lynch, L., Michelet, X., Zhang, S., Brennan, P. J., Moseman, A., Lester, C., et al. (2015). Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nat. Immunol.* 16, 85–95. doi: 10.1038/ni.3047

Macian, F., Garcia-Cozar, F., Im, S. H., Horton, H. F., Byrne, M. C., and Rao, A. (2002). Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* 109, 719–731. doi: 10.1016/S0092-8674(02)00767-5

Mahmud, S. A., Manlove, L. S., Schmitz, H. M., Xing, Y., Wang, Y., Owen, D. L., et al. (2014). Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat. Immunol.* 15, 473–481. doi: 10.1038/ni.2849

Manicassamy, S., Gupta, S., Huang, Z., and Sun, Z. (2006). Protein kinase C-θ-mediated signals enhance $CD4^+$ T cell survival by up-regulating Bcl-xL. *J. Immunol.* 176, 6709–6716. doi: 10.4049/jimmunol.176.11.6709

Marchingo, J. M., Kan, A., Sutherland, R. M., Duffy, K. R., Wellard, C. J., Belz, G. T., et al. (2014). T cell signaling. Antigen affinity, costimulation, and cytokine inputs sum linearly to amplify T cell expansion. *Science* 346, 1123–1127. doi: 10.1126/science.1260044

Marklund, U., Lightfoot, K., and Cantrell, D. (2003). Intracellular location and cell context-dependent function of protein kinase D. *Immunity* 19, 491–501. doi: 10.1016/S1074-7613(03)00260-7

Marsland, B. J., Soos, T. J., Spath, G., Littman, D. R., and Kopf, M. (2004). Protein kinase Cθ is critical for the development of *in vivo* T helper (Th)2 cell but not Th1 cell responses. *J. Exp. Med.* 200, 181–189. doi: 10.1084/jem.20032229

Martinez-Moreno, M., Garcia-Lievana, J., Soutar, D., Torres-Ayuso, P., Andrada, E., Zhong, X. P., et al. (2012). FoxO-dependent regulation of diacylglycerol kinase α gene expression. *Mol. Cell. Biol.* 32, 4168–4180. doi: 10.1128/MCB.00654-12

Matsuda, Y., Toda, M., Kato, T., Kuribayashi, K., and Kakimi, K. (2006). Fulminant liver failure triggered by therapeutic antibody treatment in a mouse model. *Int. J. Oncol.* 29, 1119–1125. doi: 10.3892/ijo.29.5.1119

Medoff, B. D., Sandall, B. P., Landry, A., Nagahama, K., Mizoguchi, A., Luster, A. D., et al. (2009). Differential requirement for CARMA1 in agonist-selected T-cell development. *Eur. J. Immunol.* 39, 78–84. doi: 10.1002/eji.200838734

Menager, M. M., Menasche, G., Romao, M., Knapnougel, P., Ho, C. H., Garfa, M., et al. (2007). Secretory cytotoxic granule maturation and exocytosis require the effector protein hMunc13-4. *Nat. Immunol.* 8, 257–267. doi: 10.1038/ni1431

Mendiratta, S. K., Martin, W. D., Hong, S., Boesteanu, A., Joyce, S., and Van Kaer, L. (1997). CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6, 469–477. doi: 10.1016/S1074-7613(00)80290-3

Merida, I., Andrada, E., Gharbi, S. I., and Avila-Flores, A. (2015). Redundant and specialized roles for diacylglycerol kinases α and ζ in the control of T cell functions. *Sci. Signal.* 8:re6. doi: 10.1126/scisignal.aaa0974

Merida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* 409, 1–18. doi: 10.1042/BJ20071040

Metzger, T. C., and Anderson, M. S. (2011). Control of central and peripheral tolerance by Aire. *Immunol. Rev.* 241, 89–103. doi: 10.1111/j.1600-065X.2011.01008.x

Michel, M. L., Keller, A. C., Paget, C., Fujio, M., Trottein, F., Savage, P. B., et al. (2007). Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J. Exp. Med.* 204, 995–1001. doi: 10.1084/jem.20061551

Michel, M. L., Mendes-Da-Cruz, D., Keller, A. C., Lochner, M., Schneider, E., Dy, M., et al. (2008). Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 19845–19850. doi: 10.1073/pnas.0806472105

Micucci, F., Capuano, C., Marchetti, E., Piccoli, M., Frati, L., Santoni, A., et al. (2008). PI5KI-dependent signals are critical regulators of the cytolytic secretory pathway. *Blood* 111, 4165–4172. doi: 10.1182/blood-2007-08-108886

Milpied, P., Massot, B., Renand, A., Diem, S., Herbelin, A., Leite-De-Moraes, M., et al. (2011). IL-17-producing invariant NKT cells in lymphoid organs are recent thymic emigrants identified by neuropilin-1 expression. *Blood* 118, 2993–3002. doi: 10.1182/blood-2011-01-329268

Monfregola, J., Johnson, J. L., Meijler, M. M., Napolitano, G., and Catz, S. D. (2012). MUNC13-4 protein regulates the oxidative response and is essential for phagosomal maturation and bacterial killing in neutrophils. *J. Biol. Chem.* 287, 44603–44618. doi: 10.1074/jbc.M112.414029

Moon, E. K., Wang, L. C., Dolfi, D. V., Wilson, C. B., Ranganathan, R., Sun, J., et al. (2014). Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor-transduced human T cells in solid tumors. *Clin. Cancer Res.* 20, 4262–4273. doi: 10.1158/1078-0432.CCR-13-2627

Morrison, D. K. (2001). KSR: a MAPK scaffold of the Ras pathway? *J. Cell Sci.* 114, 1609–1612.

Navarro, M. N., Feijoo-Carnero, C., Arandilla, A. G., Trost, M., and Cantrell, D. A. (2014). Protein kinase D2 is a digital amplifier of T cell receptor-stimulated diacylglycerol signaling in naive $CD8^+$ T cells. *Sci. Signal.* 7:ra99. doi: 10.1126/scisignal.2005477

Nichols, K. E., Hom, J., Gong, S. Y., Ganguly, A., Ma, C. S., Cannons, J. L., et al. (2005). Regulation of NKT cell development by SAP, the protein defective in XLP. *Nat. Med.* 11, 340–345. doi: 10.1038/nm1189

Olenchock, B. A., Guo, R., Carpenter, J. H., Jordan, M., Topham, M. K., Koretzky, G. A., et al. (2006a). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* 7, 1174–1181. doi: 10.1038/ni1400

Olenchock, B. A., Guo, R., Silverman, M. A., Wu, J. N., Carpenter, J. H., Koretzky, G. A., et al. (2006b). Impaired degranulation but enhanced cytokine production after FcεRI stimulation of diacylglycerol kinase ζ-deficient mast cells. *J. Exp. Med.* 203, 1471–1480. doi: 10.1084/jem.20052424

Osman, Y., Kawamura, T., Naito, T., Takeda, K., Van Kaer, L., Okumura, K., et al. (2000). Activation of hepatic NKT cells and subsequent liver injury following administration of α-galactosylceramide. *Eur. J. Immunol.* 30, 1919–1928. doi: 10.1002/1521-4141(200007)30:7<1919::AID-IMMU1919>3.0.CO;2-3

Outram, S. V., Crompton, T., Merida, I., Varas, A., and Martinez, A. C. (2002). Diacylglycerol kinase α activity promotes survival of $CD4^+CD8^+$ double positive cells during thymocyte development. *Immunology* 105, 391–398. doi: 10.1046/j.1365-2567.2002.01385.x

Pasquier, B., Yin, L., Fondaneche, M. C., Relouzat, F., Bloch-Queyrat, C., Lambert, N., et al. (2005). Defective NKT cell development in mice and humans lacking the adapter SAP, the X-linked lymphoproliferative syndrome gene product. *J. Exp. Med.* 201, 695–701. doi: 10.1084/jem.20042432

Powell, J. D. (2006). The induction and maintenance of T cell anergy. *Clin. Immunol.* 120, 239–246. doi: 10.1016/j.clim.2006.02.004

Prevot, N., Pyaram, K., Bischoff, E., Sen, J. M., Powell, J. D., and Chang, C. H. (2015). Mammalian target of rapamycin complex 2 regulates invariant NKT cell development and function independent of promyelocytic leukemia zinc-finger. *J. Immunol.* 194, 223–230. doi: 10.4049/jimmunol.1401985

Priatel, J. J., Chen, X., Huang, Y. H., Chow, M. T., Zenewicz, L. A., Coughlin, J. J., et al. (2010). RasGRP1 regulates antigen-induced developmental programming by naive CD8 T cells. *J. Immunol.* 184, 666–676. doi: 10.4049/jimmunol.0803521

Priatel, J. J., Teh, S. J., Dower, N. A., Stone, J. C., and Teh, H. S. (2002). RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity* 17, 617–627. doi: 10.1016/S1074-7613(02)00451-X

Rampuria, P., and Lang, M. L. (2015). CD1d-dependent expansion of NKT follicular helper cells *in vivo* and *in vitro* is a product of cellular proliferation and differentiation. *Int. Immunol.* 27, 253–263. doi: 10.1093/intimm/dxv007

Riese, M. J., Grewal, J., Das, J., Zou, T., Patil, V., Chakraborty, A. K., et al. (2011). Decreased diacylglycerol metabolism enhances ERK activation and augments CD8+ T cell functional responses. *J. Biol. Chem.* 286, 5254–5265. doi: 10.1074/jbc.M110.171884

Riese, M. J., Wang, L. C., Moon, E. K., Joshi, R. P., Ranganathan, A., June, C. H., et al. (2013). Enhanced effector responses in activated CD8+ T cells deficient in diacylglycerol kinases. *Cancer Res.* 73, 3566–3577. doi: 10.1158/0008-5472.CAN-12-3874

Rizzo, M. A., Shome, K., Vasudevan, C., Stolz, D. B., Sung, T. C., Frohman, M. A., et al. (1999). Phospholipase D and its product, phosphatidic acid, mediate agonist-dependent raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem.* 274, 1131–1139. doi: 10.1074/jbc.274.2.1131

Rizzo, M. A., Shome, K., Watkins, S. C., and Romero, G. (2000). The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. *J. Biol. Chem.* 275, 23911–23918. doi: 10.1074/jbc.M001553200

Roose, J. P., Mollenauer, M., Gupta, V. A., Stone, J., and Weiss, A. (2005). A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol. Cell. Biol.* 25, 4426–4441. doi: 10.1128/MCB.25.11.4426-4441.2005

Rozengurt, E., Rey, O., and Waldron, R. T. (2005). Protein kinase D signaling. *J. Biol. Chem.* 280, 13205–13208. doi: 10.1074/jbc.R500002200

Ruffo, E., Malacarne, V., Larsen, S. E., Das, R., Patrussi, L., Wulfing, C., et al. (2016). Inhibition of diacylglycerol kinase α restores restimulation-induced cell death and reduces immunopathology in XLP-1. *Sci. Transl. Med.* 8:321ra327. doi: 10.1126/scitranslmed.aad1565

Sag, D., Krause, P., Hedrick, C. C., Kronenberg, M., and Wingender, G. (2014). IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J. Clin. Invest.* 124, 3725–3740. doi: 10.1172/JCI72308

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007). Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* 1771, 793–806. doi: 10.1016/j.bbalip.2007.04.006

Salio, M., Silk, J. D., Jones, E. Y., and Cerundolo, V. (2014). Biology of CD1- and MR1-restricted T cells. *Annu. Rev. Immunol.* 32, 323–366. doi: 10.1146/annurev-immunol-032713-120243

Sandau, M. M., Kohlmeier, J. E., Woodland, D. L., and Jameson, S. C. (2010). IL-15 regulates both quantitative and qualitative features of the memory CD8 T cell pool. *J. Immunol.* 184, 35–44. doi: 10.4049/jimmunol.0803355

Sanjuan, M. A., Pradet-Balade, B., Jones, D. R., Martinez, A. C., Stone, J. C., Garcia-Sanz, J. A., et al. (2003). T cell activation *in vivo* targets diacylglycerol kinase α to the membrane: a novel mechanism for Ras attenuation. *J. Immunol.* 170, 2877–2883. doi: 10.4049/jimmunol.170.6.2877

Sarkar, S., Kalia, V., Haining, W. N., Konieczny, B. T., Subramaniam, S., and Ahmed, R. (2008). Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J. Exp. Med.* 205, 625–640. doi: 10.1084/jem.20071641

Schmidt, A. M., Lu, W., Sindhava, V. J., Huang, Y., Burkhardt, J. K., Yang, E., et al. (2015). Regulatory T cells require TCR signaling for their suppressive function. *J. Immunol.* 194, 4362–4370. doi: 10.4049/jimmunol.1402384

Schmidt, A. M., Zou, T., Joshi, R. P., Leichner, T. M., Pimentel, M. A., Sommers, C. L., et al. (2013). Diacylglycerol kinase ζ limits the generation of natural regulatory T cells. *Sci. Signal.* 6:ra101. doi: 10.1126/scisignal.2004411

Schmidt-Supprian, M., Tian, J., Grant, E. P., Pasparakis, M., Maehr, R., Ovaa, H., et al. (2004). Differential dependence of $CD4^+CD25^+$ regulatory and natural killer-like T cells on signals leading to NF-κB activation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4566–4571. doi: 10.1073/pnas.0400885101

Schwartz, R. H. (2003). T cell anergy. *Annu. Rev. Immunol.* 21, 305–334. doi: 10.1146/annurev.immunol.21.120601.141110

Shen, S., Chen, Y., Gorentla, B. K., Lu, J., Stone, J. C., and Zhong, X. P. (2011a). Critical roles of RasGRP1 for invariant NKT cell development. *J. Immunol.* 187, 4467–4473. doi: 10.4049/jimmunol.1003798

Shen, S., Wu, J., Srivatsan, S., Gorentla, B. K., Shin, J., Xu, L., et al. (2011b). Tight regulation of diacylglycerol-mediated signaling is critical for proper invariant NKT cell development. *J. Immunol.* 187, 2122–2129. doi: 10.4049/jimmunol.1100495

Shin, J., O'brien, T. F., Grayson, J. M., and Zhong, X. P. (2012). Differential regulation of primary and memory CD8 T cell immune responses by diacylglycerol kinases. *J. Immunol.* 188, 2111–2117. doi: 10.4049/jimmunol.1102265

Shin, J., Wang, S., Deng, W., Wu, J., Gao, J., and Zhong, X. P. (2014). Mechanistic target of rapamycin complex 1 is critical for invariant natural killer T-cell development and effector function. *Proc. Natl. Acad. Sci. U.S.A.* 111, E776–E783. doi: 10.1073/pnas.1315435111

Shin, J., Xie, D., and Zhong, X. P. (2013a). MicroRNA-34a enhances T cell activation by targeting diacylglycerol kinase ζ. *PLoS ONE* 8:e77983. doi: 10.1371/journal.pone.0077983

Shin, J., Zhang, P., Wang, S., Wu, J., Guan, Z., and Zhong, X. P. (2013b). Negative control of mast cell degranulation and the anaphylactic response by the phosphatase lipin1. *Eur. J. Immunol.* 43, 240–248. doi: 10.1002/eji.201242571

Shirakawa, R., Higashi, T., Tabuchi, A., Yoshioka, A., Nishioka, H., Fukuda, M., et al. (2004). Munc13-4 is a GTP-Rab27-binding protein regulating dense core granule secretion in platelets. *J. Biol. Chem.* 279, 10730–10737. doi: 10.1074/jbc.M309426200

Shome, K., Vasudevan, C., and Romero, G. (1997). ARF proteins mediate insulin-dependent activation of phospholipase D. *Curr. Biol.* 7, 387–396. doi: 10.1016/S0960-9822(06)00186-2

Siliceo, M., Garcia-Bernal, D., Carrasco, S., Diaz-Flores, E., Coluccio Leskow, F., Teixido, J., et al. (2006). β2-chimaerin provides a diacylglycerol-dependent mechanism for regulation of adhesion and chemotaxis of T cells. *J. Cell Sci.* 119, 141–152. doi: 10.1242/jcs.02722

Siliceo, M., and Merida, I. (2009). T cell receptor-dependent tyrosine phosphorylation of β2-chimaerin modulates its Rac-GAP function in T cells. *J. Biol. Chem.* 284, 11354–11363. doi: 10.1074/jbc.M806098200

Sivakumar, V., Hammond, K. J., Howells, N., Pfeffer, K., and Weih, F. (2003). Differential requirement for Rel/nuclear factor κB family members in natural killer T cell development. *J. Exp. Med.* 197, 1613–1621. doi: 10.1084/jem.20022234

Spitaler, M., Emslie, E., Wood, C. D., and Cantrell, D. (2006). Diacylglycerol and protein kinase D localization during T lymphocyte activation. *Immunity* 24, 535–546. doi: 10.1016/j.immuni.2006.02.013

Stanic, A. K., Bezbradica, J. S., Park, J. J., Van Kaer, L., Boothby, M. R., and Joyce, S. (2004). Cutting edge: the ontogeny and function of Va14Ja18 natural T lymphocytes require signal processing by protein kinase Cθ and NF-κB. *J. Immunol.* 172, 4667–4671. doi: 10.4049/jimmunol.172.8.4667

Starbeck-Miller, G. R., Xue, H. H., and Harty, J. T. (2014). IL-12 and type I interferon prolong the division of activated CD8 T cells by maintaining high-affinity IL-2 signaling *in vivo*. *J. Exp. Med.* 211, 105–120. doi: 10.1084/jem.20130901

Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J., Gandhi, L., et al. (2000). PKC-θ is required for TCR-induced NF-κB activation in mature but not immature T lymphocytes. *Nature* 404, 402–407. doi: 10.1038/35006090

Terashima, A., Watarai, H., Inoue, S., Sekine, E., Nakagawa, R., Hase, K., et al. (2008). A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity. *J. Exp. Med.* 205, 2727–2733. doi: 10.1084/jem.20080698

Tonti, E., Fedeli, M., Napolitano, A., Iannacone, M., Von Andrian, U. H., Guidotti, L. G., et al. (2012). Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of $CD4^+$ T cell help. *J. Immunol.* 188, 3217–3222. doi: 10.4049/jimmunol.1103501

Tsuchiya, R., Tanaka, T., Hozumi, Y., Nakano, T., Okada, M., Topham, M. K., et al. (2015). Downregulation of diacylglycerol kinase ζ enhances activation of cytokine-induced NF-κB signaling pathway. *Biochim. Biophys. Acta* 1853, 361–369. doi: 10.1016/j.bbamcr.2014.11.011

Vahl, J. C., Drees, C., Heger, K., Heink, S., Fischer, J. C., Nedjic, J., et al. (2014). Continuous T cell receptor signals maintain a functional regulatory T cell pool. *Immunity* 41, 722–736. doi: 10.1016/j.immuni.2014.10.012

Van Der Windt, G. J., Everts, B., Chang, C. H., Curtis, J. D., Freitas, T. C., Amiel, E., et al. (2012). Mitochondrial respiratory capacity is a critical regulator of $CD8^+$ T cell memory development. *Immunity* 36, 68–78. doi: 10.1016/j.immuni.2011.12.007

Van Kaer, L., Parekh, V. V., and Wu, L. (2013). Invariant natural killer T cells as sensors and managers of inflammation. *Trends Immunol.* 34, 50–58. doi: 10.1016/j.it.2012.08.009

Wang, X., Devaiah, S. P., Zhang, W., and Welti, R. (2006). Signaling functions of phosphatidic acid. *Prog. Lipid Res.* 45, 250–278. doi: 10.1016/j.plipres.2006.01.005

Watarai, H., Sekine-Kondo, E., Shigeura, T., Motomura, Y., Yasuda, T., Satoh, R., et al. (2012). Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines. *PLoS Biol.* 10:e1001255. doi: 10.1371/journal.pbio.1001255

Wei, J., Yang, K., and Chi, H. (2014). Cutting edge: discrete functions of mTOR signaling in invariant NKT cell development and NKT17 fate decision. *J. Immunol.* 193, 4297–4301. doi: 10.4049/jimmunol.1402042

Werlen, G., Jacinto, E., Xia, Y., and Karin, M. (1998). Calcineurin preferentially synergizes with PKC-θ to activate JNK and IL-2 promoter in T lymphocytes. *EMBO J.* 17, 3101–3111. doi: 10.1093/emboj/17.11.3101

Wheeler, M. L., Dong, M. B., Brink, R., Zhong, X. P., and Defranco, A. L. (2013). Diacylglycerol kinase ζ limits B cell antigen receptor-dependent activation of ERK signaling to inhibit early antibody responses. *Sci. Signal.* 6, ra91. doi: 10.1126/scisignal.2004189

Williams, M. A., Holmes, B. J., Sun, J. C., and Bevan, M. J. (2006). Developing and maintaining protective CD8$^+$ memory T cells. *Immunol. Rev.* 211, 146–153. doi: 10.1111/j.0105-2896.2006.00389.x

Willoughby, J. E., Costello, P. S., Nicolas, R. H., Robinson, N. J., Stamp, G., Powrie, F., et al. (2007). Raf signaling but not the ERK effector SAP-1 is required for regulatory T cell development. *J. Immunol.* 179, 6836–6844. doi: 10.4049/jimmunol.179.10.6836

Winter, J. N., Fox, T. E., Kester, M., Jefferson, L. S., and Kimball, S. R. (2010). Phosphatidic acid mediates activation of mTORC1 through the ERK signaling pathway. *Am. J. Physiol. Cell Physiol.* 299, C335–C344. doi: 10.1152/ajpcell.00039.2010

Wu, J., Shen, S., Yang, J., Xia, Z., and Zhong, X. P. (2013). Diacylglycerol kinase zeta positively controls the development of iNKT-17 cells. *PLoS ONE* 8:e75202. doi: 10.1371/journal.pone.0075202

Wu, J., Shin, J., Xie, D., Wang, H., Gao, J., and Zhong, X. P. (2014a). Tuberous sclerosis 1 promotes invariant NKT cell anergy and inhibits invariant NKT cell-mediated antitumor immunity. *J. Immunol.* 192, 2643–2650. doi: 10.4049/jimmunol.1302076

Wu, J., Yang, J., Yang, K., Wang, H., Gorentla, B., Shin, J., et al. (2014b). iNKT cells require TSC1 for terminal maturation and effector lineage fate decisions. *J. Clin. Invest.* 124, 1685–1698. doi: 10.1172/JCI69780

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A. D., Stroud, J. C., et al. (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 126, 375–387. doi: 10.1016/j.cell.2006.05.042

Xie, D. L., Wu, J., Lou, Y. L., and Zhong, X. P. (2012). Tumor suppressor TSC1 is critical for T-cell anergy. *Proc. Natl. Acad. Sci. U.S.A.* 109, 14152–14157. doi: 10.1073/pnas.1119744109

Xing, Y., and Hogquist, K. A. (2012). T-cell tolerance: central and peripheral. *Cold Spring Harb. Perspect. Biol.* 4, 1–15. doi: 10.1101/cshperspect.a006957

Yanagisawa, K., Yasuda, S., Kai, M., Imai, S., Yamada, K., Yamashita, T., et al. (2007). Diacylglycerol kinase α suppresses tumor necrosis factor-alpha-induced apoptosis of human melanoma cells through NF-κB activation. *Biochim. Biophys. Acta* 1771, 462–474. doi: 10.1016/j.bbalip.2006.12.008

Yang, C., and Kazanietz, M. G. (2007). Chimaerins: GAPs that bridge diacylglycerol signalling and the small G-protein Rac. *Biochem. J.* 403, 1–12. doi: 10.1042/BJ20061750

Yang, E., Singh, B. K., Paustian, A. M., and Kambayashi, T. (2016a). Diacylglycerol Kinase ζ Is a Target To Enhance NK Cell Function. *J. Immunol.* 197, 934–941. doi: 10.4049/jimmunol.1600581

Yang, J., Zhang, P., Krishna, S., Wang, J., Lin, X., Huang, H., et al. (2016b). Unexpected positive control of NFκB and miR-155 by DGKα and ζ ensures effector and memory CD8$^+$ T Cell differentiation. *Oncotarget* 23, 33744–33764. doi: 10.18632/oncotarget.8164

Yang, W., Gorentla, B., Zhong, X. P., and Shin, J. (2015). mTOR and its tight regulation for iNKT cell development and effector function. *Mol. Immunol.* 68, 536–545. doi: 10.1016/j.molimm.2015.07.022

Yoon, M. S., Sun, Y., Arauz, E., Jiang, Y., and Chen, J. (2011). Phosphatidic acid activates mammalian target of rapamycin complex 1 (mTORC1) kinase by displacing FK506 binding protein 38 (FKBP38) and exerting an allosteric effect. *J. Biol. Chem.* 286, 29568–29574. doi: 10.1074/jbc.M111.262816

Zamani, A., Decker, C., Cremasco, V., Hughes, L., Novack, D. V., and Faccio, R. (2015). Diacylglycerol kinase ζ (DGKζ) is a critical regulator of bone homeostasis via modulation of c-fos levels in osteoclasts. *J. Bone Miner. Res.* 30, 1852–1863. doi: 10.1002/jbmr.2533

Zehn, D., Lee, S. Y., and Bevan, M. J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. *Nature* 458, 211–214. doi: 10.1038/nature07657

Zha, Y., Marks, R., Ho, A. W., Peterson, A. C., Janardhan, S., Brown, I., et al. (2006). T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α. *Nat. Immunol.* 7, 1166–1173. doi: 10.1038/ni1394

Zhang, L., Tschumi, B. O., Corgnac, S., Ruegg, M. A., Hall, M. N., Mach, J. P., et al. (2014). Mammalian target of rapamycin complex 1 orchestrates invariant NKT cell differentiation and effector function. *J. Immunol.* 193, 1759–1765. doi: 10.4049/jimmunol.1400769

Zhang, N., and Bevan, M. J. (2011). CD8$^+$ T cells: foot soldiers of the immune system. *Immunity* 35, 161–168. doi: 10.1016/j.immuni.2011.07.010

Zhao, C., Du, G., Skowronek, K., Frohman, M. A., and Bar-Sagi, D. (2007). Phospholipase D2-generated phosphatidic acid couples EGFR stimulation to Ras activation by Sos. *Nat. Cell Biol.* 9, 706–712. doi: 10.1038/ncb1594

Zheng, Y., Zha, Y., Driessens, G., Locke, F., and Gajewski, T. F. (2012). Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy *in vitro* and *in vivo*. *J. Exp. Med.* 209, 2157–2163. doi: 10.1084/jem.20120342

Zheng, Y., Zha, Y., Spaapen, R. M., Mathew, R., Barr, K., Bendelac, A., et al. (2013). Egr2-dependent gene expression profiling and ChIP-Seq reveal novel biologic targets in T cell anergy. *Mol. Immunol.* 55, 283–291. doi: 10.1016/j.molimm.2013.03.006

Zhong, X. P., Guo, R., Zhou, H., Liu, C., and Wan, C. K. (2008). Diacylglycerol kinases in immune cell function and self-tolerance. *Immunol. Rev.* 224, 249–264. doi: 10.1111/j.1600-065X.2008.00647.x

Zhong, X. P., Hainey, E. A., Olenchock, B. A., Jordan, M. S., Maltzman, J. S., Nichols, K. E., et al. (2003). Enhanced T cell responses due to diacylglycerol kinase ζ deficiency. *Nat. Immunol.* 4, 882–890. doi: 10.1038/ni958

Zhong, X. P., Hainey, E. A., Olenchock, B. A., Zhao, H., Topham, M. K., and Koretzky, G. A. (2002). Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase ζ. *J. Biol. Chem.* 277, 31089–31098. doi: 10.1074/jbc.M203818200

Zhong, X. P., Shin, J., Gorentla, B. K., O'brien, T., Srivatsan, S., Xu, L., et al. (2011). Receptor signaling in immune cell development and function. *Immunol. Res.* 49, 109–123. doi: 10.1007/s12026-010-8175-9

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

REVIEW
published: 07 September 2016
doi: 10.3389/fcell.2016.00096

The Immunomodulatory Functions of Diacylglycerol Kinase ζ

Brenal K. Singh and Taku Kambayashi *

Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

The generation of diacylglycerol (DAG) is critical for promoting immune cell activation, regulation, and function. Diacylglycerol kinase ζ (DGKζ) serves as an important negative regulator of DAG by enzymatically converting DAG into phosphatidic acid (PA) to shut down DAG-mediated signaling. Consequently, the loss of DGKζ increases DAG levels and the duration of DAG-mediated signaling. However, while the enhancement of DAG signaling is thought to augment immune cell function, the loss of DGKζ can result in both immunoactivation and immunomodulation depending on the cell type and function. In this review, we discuss how different immune cell functions can be selectively modulated by DGKζ. Furthermore, we consider how targeting DGKζ can be potentially beneficial for the resolution of human diseases by either promoting immune responses important for protection against infection or cancer or dampening immune responses in immunopathologic conditions such as allergy and septic shock.

Keywords: diacylglycerol, diacylglycerol kinase, signal transduction, immunomodulation, phospholipase C, extracellular signal-regulated kinase, phosphatidic acid

OPEN ACCESS

Edited by:
Isabel Merida, Spanish National Research Council, Spain

Reviewed by:
Narayanan Parameswaran, Michigan State University, USA
Kaoru Goto, Yamagata University School of Medicine, Japan

***Correspondence:**
Taku Kambayashi
taku.kambayashi@uphs.upenn.edu

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *01 July 2016*
Accepted: *22 August 2016*
Published: *07 September 2016*

Citation:
Singh BK and Kambayashi T (2016) The Immunomodulatory Functions of Diacylglycerol Kinase ζ. Front. Cell Dev. Biol. 4:96. doi: 10.3389/fcell.2016.00096

INTRODUCTION

Diacylglycerol (DAG) is a key secondary lipid messenger for transducing signals downstream of many receptors expressed by hematopoietic cells. DAG has shown to be important in driving the activation, proliferation, migration, and effector function of adaptive and innate immune cells. The generation of DAG can be accomplished by the activation of various cell-surface receptors, including G_q-mediated G-protein coupled receptors (GPCR)s (e.g., muscarinic and histamine receptors) and immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors [e.g., T cell receptor (TCR), FcεRI] (Topham and Prescott, 1999; Kambayashi and Koretzky, 2007; Smith-Garvin et al., 2009; Wright et al., 2013). The activation of these receptors results in the formation of proximal signaling complexes that are critical for the activation of phospholipase C (PLC). PLC activation leads to enzymatic cleavage of phosphoinositol 4,5-bisphosphate (PIP_2) into DAG and inositol 1,4,5-triphosphate (IP_3) (Imboden and Stobo, 1985). While IP_3 mobilizes Ca^{2+}, DAG activates the NF-κB and extracellular regulated kinase (ERK) pathways through protein kinase C (PKC) and RasGRP, respectively, to promote cell function (Tognon et al., 1998; Coudronniere et al., 2000; Dower et al., 2000; Sun et al., 2000; Wang et al., 2004; Quann et al., 2011). Consequently, the levels of DAG must be tightly regulated to control the magnitude and duration of the responses generated.

Diacylglycerol kinases (DGK) regulate DAG signaling by phosphorylating DAG and converting it into phosphatidic acid (PA) (Topham and Prescott, 1999; Joshi and Koretzky, 2013). The loss of DGKs increases DAG levels and the duration of DAG-mediated signaling. One might expect that elevated DAG levels would lead to general enhancement of effector responses. However, the

enhancement of DAG signaling through the loss of DGKs can lead to either hyperactivation or hyporesponsiveness depending on the cell type and the type of response. There are 10 different isoforms comprising five different classes of DGKs, each of which control different cellular functions based on their distinct structural motifs and subcellular localization (Bunting et al., 1996; Goto and Kondo, 1996; Topham and Prescott, 1999; Kobayashi et al., 2007; Joshi and Koretzky, 2013). The three major isoforms that are abundantly expressed in lymphoid tissues are DGKα, DGKδ, and DGKζ (Shulga et al., 2011). In particular, mice that lack the zeta (ζ) isoform of DGK, which is highly expressed in hematopoietic cells, display profound effects on the functional behavior of various cell types. In the present review, we will focus on how DGKζ plays both negative and positive roles in immune responses mounted by different cell types.

NEGATIVE REGULATION OF EFFECTOR RESPONSES BY DGKζ

CD4$^+$ and CD8$^+$ Conventional T Cells

DGKζ serves as a critical negative regulator of DAG signaling downstream of the TCR and can modulate the strength of TCR signaling. Early experiments using the immortalized Jurkat T cell line showed that overexpression of DGKζ inhibits TCR signaling by reducing the levels of active GTP-bound Ras and, consequently, diminishing ERK activation (Zhong et al., 2002). Furthermore, the overexpression of DGKζ was associated with decreased AP-1 transcription factor activity and CD69 expression (an early T cell activation marker) following TCR stimulation, both of which are regulated by the Ras-ERK pathway. Importantly, Ca^{2+} flux was normal regardless of DGKζ overexpression, suggesting that DGKζ selectively regulated DAG-mediated signaling pathways downstream of the TCR. Further biochemical analysis through the use of a kinase dead DGKζ mutant revealed that the enzymatic activity of DGKζ was critical for its inhibitory effects on TCR signaling.

To test the physiological role of DGKζ in T cells, Zhong et al. generated DGKζ knockout mice (Zhong et al., 2003). Initial phenotypic analysis showed that DGKζ KO mice contained similar frequencies and numbers of CD4$^+$ and CD8$^+$ T cells in secondary lymphoid organs and displayed no obvious defects in lymphoid architecture or cellularity. Furthermore, thymic development as analyzed by the number and frequency of CD4 single-positive (SP), CD8 SP, double-positive (DP), and double-negative (DN) thymocytes in DGKζ-deficient mice was similar to wild-type (WT) mice. However, upon TCR stimulation, naïve DGKζ KO CD4$^+$ and CD8$^+$ T cells displayed enhanced upregulation of activation markers CD25 and CD69 and increased proliferation compared to WT T cells. The increased expression of activation markers was associated with enhanced phosphorylation of ERK but normal induction of non-DAG mediated pathways including Ca^{2+} flux and JNK activation. Importantly, bypassing TCR activation with a DAG analog, phorbol-12-myristate-13-acetate (PMA), abolished differences in activation between DGKζ KO and WT T cells, suggesting that the hyperactivation of DGKζ KO T cells was secondary to defective regulation of DAG.

In agreement with enhanced TCR signaling, DGKζ KO T cells also display improved anti-viral responses (Zhong et al., 2003). DGKζ KO mice infected with LCMV Armstrong showed enhanced viral-specific T cell responses as evidenced by decreased viral titers at day 7 following infection. This effect correlated with an increased number of total and CD44^{hi}CD62L^{lo} effector CD8$^+$ T cells in the spleen. Furthermore, LCMV-infected DGKζ KO mice exhibited increased number of IFNγ-producing CD8$^+$ and CD4$^+$ T cells when restimulated with LCMV-specific peptides, suggesting that DGKζ KO T cells displayed enhanced effector function following LCMV infection.

Similarly, DGKζ-deficient mice also exhibit enhanced anti-tumor responses. DGKζ KO mice subcutaneously injected with OVA-expressing EL4 T cell lymphoma, had significantly reduced tumor mass compared to their WT counterparts (Riese et al., 2011). This effect was accompanied by an increased number of total and antigen-specific tumor-infiltrating CD44^{hi}CD8$^+$ T cells. To show that T cells were responsible for the enhanced anti-tumor effect by DGKζ deficiency, DGKζ KO and WT OVA-specific OT-I T cells were adoptively transferred into naïve recipient WT mice. Mice receiving DGKζ KO compared to WT OVA-specific OT-I T cells also exhibited lower tumor mass upon challenge with OVA-expressing EL4 cells. Isolation of tumor-infiltrating OT-I cells revealed that the loss of DGKζ increased the frequency of CD44^{hi} and IL-2-producing OT-I cells in a cell-intrinsic manner. In addition to preventing tumor engraftment, DGKζ deficiency also improves tumor rejection of established tumors, as the adoptive transfer of DGKζ KO but not WT OT-I effector T cells into tumor-bearing mice significantly reduced tumor burden (Riese et al., 2013). Thus, DGKζ could represent a novel target for enhancing anti-tumor responses in adoptive immunotherapy. This could also be applied to engineered T cells that express chimeric antigen receptors (CAR) directed against the tumor, as DGKζ deficiency was also shown to promote CAR T cell-mediated anti-tumor responses (Riese et al., 2013). How DGKζ deficiency augments anti-tumor responses is unclear. Although DGKζ KO CD8$^+$ T cells display increased cytokine production and increased proliferation, their cytotoxic function is comparable to WT CD8$^+$ T cells (Riese et al., 2011). Nevertheless, these studies demonstrate that DGKζ serves to constrain T cell activation and anti-viral and anti-tumor T cell responses. Thus, inhibition of DGKζ might provide a therapeutic opportunity to enhance immune-mediated viral and tumor clearance.

It is possible that DGKζ is physiologically important for limiting over-activation and inducing anergy in inappropriately activated T cells. The expression level of DGKζ can be controlled depending on the type of stimulation the T cell receives. T cells that are stimulated through their TCR and co-stimulatory molecules downregulate DGKζ transcript levels, thereby allowing appropriately activated T cells to become fully activated (Macian et al., 2002; Olenchock et al., 2006a; Zha et al., 2006). In contrast, T cells that receive TCR stimulation alone in the absence of co-stimulation do not downregulate DGKζ levels, potentially leading to attenuated DAG-mediated signaling and

decreased activation. Consistent with this notion, DGKζ KO T cells resist anergy induction when activated by TCR alone in the absence of co-stimulatory signals (Olenchock et al., 2006a). In addition to TCR-mediated regulation, DGKζ might also be regulated by environmental cytokines. In particular, IL-33 has been shown to up-regulate DGKζ in cardiomyocytes following stimulation (Rui et al., 2012). Although it is unknown if IL-33 can upregulate DGKζ in immune cells, it is tantalizing to speculate that cytokine signaling can affect the TCR responsiveness of T cells by regulating DGKζ levels.

NK Cells

NK cells are cytotoxic members of the innate lymphoid cell (ILC) family and play an important role in protection against viral infection and clearance of tumors (Artis and Spits, 2015). Unlike their adaptive counterparts ($CD8^+$ T cells), they do not possess a somatically-rearranged antigen receptor but rather express a variety of activating receptors specific for ligands displayed on virally-infected, stressed, or transformed cells (Lanier, 2008). NK cell activating receptors can be categorized into three main families based on the signaling adaptors used to relay downstream activation signals. These families include SAP-dependent (e.g., 2B4), ITAM-dependent (e.g., CD16), or DAP10-dependent (e.g., NKG2D) receptors (McVicar et al., 1998; Wu et al., 1999; Chen et al., 2006; Lanier, 2008). The activation of any of these three families of receptors relies on proximal signaling complexes involving SLP-76, which subsequently leads to the activation of PLCγ and the production of DAG (Wu and Koretzky, 2004; Tassi et al., 2005; May et al., 2013). In addition to these activating receptors, NK cells express an assortment of inhibitory receptors, many of which bind to MHC class I alleles and negatively regulate activating receptor signaling by the recruitment of phosphatases such as SHP-1 and SHIP (Binstadt et al., 1996; Lanier, 2008).

NK cell activation is determined by the net balance of the activating and inhibitory inputs that the NK cell receives through its receptors. For example, NK cells are activated when neoplastic cells upregulate ligands such as RAE-1 or MICA, which are recognized by the activating receptor NKG2D (Jung et al., 2012). Likewise, NK cells are activated through disinhibition when tumor cells lose MHC class I, a process known as missing self recognition (Kärre et al., 1986). Since SHP-1 and SHIP negatively regulate activating receptor signaling, one might predict that the loss of these molecules would boost the effector function of NK cells. Surprisingly, however, SHP-1 and SHIP deficiency in NK cells renders them less functional than their WT counterparts (Lowin-Kropf et al., 2000; Viant et al., 2014; Gumbleton et al., 2015). One explanation of this seemingly paradoxical finding is that NK cells continuously adjust their responsiveness to activating stimuli in their local environment, a phenomenon known as tuning (Höglund and Brodin, 2010). Thus, NK cells that chronically lack inhibitory signals, such as in SHP-1 or SHIP deficiency, require more stimulation to achieve their threshold of activation (Lowin-Kropf et al., 2000; Viant et al., 2014; Gumbleton et al., 2015). While NK cell tuning may protect the host from NK cell-mediated immunopathology, this process can hamper important effector responses against chronic viral infections or tumors.

Although the molecular mechanism of NK cell tuning is unknown, stimulation of NK cells with PMA and a calcium ionophore, ionomycin, can bypass the hyporesponsiveness of SHP-1 and SHIP KO NK cells (Viant et al., 2014; Gumbleton et al., 2015). These data suggest that the tuning process is proximal to PLCγ-mediated production of DAG. Thus, we speculated that NK cells may not be able to tune their responsiveness in response to enhanced DAG-mediated signaling by DGKζ deficiency. Indeed, we recently demonstrated that DGKζ KO NK cells are hyperfunctional compared to WT NK cells (Yang et al., 2016). DGKζ KO NK cells displayed increased cytokine production and cytotoxicity following stimulation through ITAM, SAP, and DAP10-dependent activating receptors. In contrast, IFNγ production by DGKζ KO and WT NK cells was similar following stimulation with IL-12 and IL-18, which utilize a DAG-independent signaling pathway, suggesting that the loss of DGKζ selectively augmented NK cell responsiveness to DAG-dependent stimuli. Like T cells, the hyperfunctionality of DGKζ KO NK cells was dependent on enhanced ERK signaling. Importantly, DGKζ KO mice cleared the NK cell-sensitive RMA-S tumor more efficiently than WT mice. Thus, the inactivation of negative regulators distal to PLCγ such as DGKζ might prove therapeutically useful in enhancing NK cell function.

B Cells

B cells comprise the second arm of the adaptive immune system and are critical for the generation of protective antibody responses during infection. The induction of antibody production results from the stimulation of the somatically rearranged B cell receptor (BCR) by cognate antigen (McHeyzer-Williams and McHeyzer-Williams, 2005; Kurosaki et al., 2010). Similar to the TCR, activation of the BCR leads to downstream biochemical cascades that ultimately result in the generation of DAG through PLCγ and, subsequently, the activation of ERK (Hashimoto et al., 2000; Saijo et al., 2003). ERK has been shown to play multiple roles during B cell responses, including the promotion of B cell survival, proliferation, and differentiation into antibody-secreting plasma cells (Richards et al., 2001; Coughlin et al., 2005; Yasuda et al., 2011). Furthermore, attenuation of ERK activation has been shown to important during B cell development, since ERK signals decrease as B cells progress from the immature transitional stage to mature follicular B cells (Yasuda et al., 2008; Gross et al., 2009; Rowland et al., 2010).

Given the role of ERK in these B cell processes, controlling the levels of BCR-induced DAG through DGKζ might be important in regulating B cell development, activation, and antibody secretion capabilities. For example, mRNA transcripts of DGKζ are upregulated as B cells progress from early transitional to the mature follicular stage, which is associated with decreased ERK activation (Wheeler et al., 2013). Accordingly, the loss of DGKζ only affected ERK activation and IκBα degradation in the follicular but not early immature transitional B cell pool in response to BCR stimulation. Importantly, the augmentation of

BCR-induced activation in DGKζ KO follicular B cells was seen even under less optimal BCR activation conditions, suggesting that DGKζ might control the BCR activation threshold in these cells.

The effects of DGKζ on B cell signaling threshold translate to functional consequences on B cell effector responses. BCR stimulation of purified DGKζ KO splenic B cells *in vitro* led to increased expression of CD69 and enhanced proliferation compared to WT B cells. DGKζ KO mice displayed enhanced antibody responses to T-independent and T-dependent antigens (Wheeler et al., 2013). The heightened antibody response by DGKζ-deficiency was accompanied by increased antigen-specific expansion of both germinal center (GC) B cells and plasma cells. These results demonstrate that regulation of DAG-dependent ERK activation by DGKζ is critical for selectively controlling the activation threshold of mature B cells to limit their activation.

THE IMMUNOMODULATORY ROLE OF DGKζ

We have so far described how the loss or inhibition of DGKζ can lead to increased immune responses against viruses or cancer. As DGKζ is a negative regulator of DAG-mediated signaling, it is conceivable that immune responses would be enhanced in the absence of DGKζ. However, DGKζ deficiency may also lead to dampening or regulation of immune responses. In the sections below, we will discuss how the absence of DGKζ can direct and indirectly suppress or modulate rather than enhance immune responses.

Regulatory T Cells

Regulatory T cells (Tregs) are a key subset of T cells that display suppressive function and are important for the regulation of adaptive immune responses. Tregs are governed by the master transcription factor, forkhead box P3 (Foxp3), and exert their immunosuppressive function via the production of immunoregulatory cytokines and through cell contact dependent mechanisms (Josefowicz et al., 2012). Loss of function mutations in the *Foxp3* gene, as seen in Scurfy mice and humans with immune dysregulation, polyendocrinopathy, and X-linked lymphoproliferative disease (IPEX), leads to lethal systemic autoimmunity early in life, highlighting the importance of Tregs in inducing immunotolerance against self antigens (Chatila et al., 2000; Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001).

T cells that strongly recognize self antigens are deleted during thymic development in a process known as negative selection. Specifically, T cells that receive strong TCR signals in the thymus, implying overt self reactivity, undergo apoptosis. As an alternative fate, strong TCR stimulation in developing thymocytes can also lead to Treg differentiation (Josefowicz et al., 2012). Thus, we hypothesized that enhancement of TCR-mediated DAG signaling by DGKζ deficiency in developing thymocytes may increase Treg generation. Indeed, the loss of DGKζ resulted in a significant increase in Treg development in the thymus in a cell-intrinsic manner (Schmidt et al., 2013). DAG-mediated signaling leads to the activation of the NF-κB (through activation of PKC) and ERK pathways. One NF-κB family member, c-Rel, was previously shown to be important for inducing Foxp3 expression in thymocytes (Long et al., 2009; Ruan et al., 2009). Although Treg generation in DGKζ KO mice was reduced in the absence of c-Rel, there was still residual Tregs in the thymus, suggesting that c-Rel was only partially responsible for the increased generation of Tregs in DGKζ KO mice (Schmidt et al., 2013). In fact, ERK activation appeared to be more important in the enhancement of Treg generation in DGKζ KO mice. Using an *in vitro* Treg development assay, we found that the inhibition of ERK phosphorylation by a MEK inhibitor led to decreased Treg generation in a dose-dependent manner, whereby the level of phosphorylated ERK (pERK) directly correlated to the magnitude of Treg generation. Importantly, Treg generation was also increased in sevenmaker mice (Sharp et al., 1997), which express a gain of function ERK mutation that leads to increased resistance to dephosphorylation of active pERK, suggesting that the selective enhancement of the ERK pathway alone is sufficient to increase Treg generation.

In addition to Treg generation in the thymus, TCR signaling plays an important role in the function of Tregs. Although some Treg function may be preserved in the absence of TCR signaling, we demonstrated that Tregs lacking SLP-76 cannot suppress TCR-driven proliferation of conventional T cells (Schmidt et al., 2015). Furthermore, Tregs with a Y→F mutation at tyrosine 145 (Y145F) of SLP-76, which leads to defective PLCγ activation, also display attenuated suppressive function, suggesting that PLCγ is important for Treg function. Consistent with this notion, Tregs lacking DGKζ display significantly increased suppression of TCR-driven conventional T cell proliferation compared to WT Tregs. Together, these data demonstrate that DGKζ limit Treg generation and function. Thus, DGKζ deficiency may indirectly lead to the suppression of immune responses through Tregs.

Mast Cells

Mast cells are critical mediators in type 2 immune responses involved in protection against helminthes and in pathologic responses in asthma and allergy (Locksley, 2010; Voehringer, 2013). A key feature of mast cell function is the immediate release of pre-formed inflammatory mediators such as histamine, cytokines, and proteases in a process called degranulation. In addition, mast cells produce arachidonic acid metabolites and cytokines in a protracted manner (Voehringer, 2013). One major stimulus for the release of these inflammatory mediators is crosslinking of FcεRI, the high affinity receptor for the Fc region of immunoglobulin E (Kinet, 1999).

The interaction of allergens with IgE-FcεRI complexes results in formation of signaling complexes that converge on the activation of PLCγ (Atkinson et al., 1992; Schneider et al., 1992). PLCγ and subsequent PKC activation have been shown to be critical in controlling mast cell degranulation, suggesting that controlling the levels of DAG might be important for regulating this process (Nechushtan et al., 2000; Wang et al., 2000; Leitges

et al., 2002; Wen et al., 2002). Indeed, the loss of DGKζ in FcεRI-stimulated mast cells leads to increased DAG levels, along with enhancement of downstream DAG-dependent signals, including RasGTP and ERK (Olenchock et al., 2006b). Accordingly, DGKζ deficiency leads to enhanced mast cell production of IL-6 following FcεRI stimulation.

Intriguingly, however, FcεRI-stimulated DGKζ KO mast cells display impaired degranulation and are resistant to local skin anaphylaxis (Olenchock et al., 2006b). The differential effect of DGKζ deficiency on mast function (the hypersecretion of IL-6 vs. decreased degranulation) may be explained by the negative feedback of DAG on PLCγ activation in mast cells. The elevation of DAG by DGKζ deficiency appears to negatively regulate the phosphorylation and subsequent activity of PLCγ. Thus, although DAG accumulates, the production of IP_3, and hence Ca^{2+} flux is attenuated in DGKζ KO mast cells. As degranulation responses are highly dependent on elevation of intracellular Ca^{2+} levels, this may cause a differential effect on degranulation and cytokine production by mast cells (Ozawa et al., 1993; Olenchock et al., 2006b). Thus, as opposed to T cells and NK cells, DGKζ exerts both activating and inhibitory effects on mast cell functional responses.

Macrophages and Dendritic Cells

Macrophages and dendritic cells (DC) play a key role in bridging the adaptive and innate immune responses (Medzhitov, 2001; Janeway and Medzhitov, 2002; Akira and Takeda, 2004). Toll-like receptors (TLR) serve as an important mechanism for equipping macrophages and DCs with the ability to recognize the presence of pathogenic infection and, subsequently, instruct adaptive immune cells on the type of response needed to effectively clear the infection. TLRs can signal through either MyD88 and/or TRIF to induce activation of the NF-κB and ERK pathways (Akira and Takeda, 2004). While TLR activation does not generally lead to PLCγ activation, DAG has been shown to be induced in macrophages following stimulation with LPS (TLR4 agonist) and lipopeptide (TLR2 agonist) (Monick et al., 1999; Zhang et al., 2001a,b). Furthermore, inhibition of PLC or PLD reduced cytokine production and nitric oxide formation by macrophages following TLR stimulation, suggesting that control of DAG levels through DGK might be important in regulating TLR-mediated responses.

Interestingly, modulation of DAG levels by the loss of DGKζ resulted in impairment rather than enhancement of cytokine production by macrophages and DCs in response to TLR stimulation. Specifically, in a developmentally independent manner, bone marrow derived macrophages (BMMΦ) and splenic DCs produced substantially less IL-12p40 and TNFα following *in vitro* stimulation through a variety of TLR agonists (Liu et al., 2007). This paradoxical finding may be explained by the role of DGK in converting DAG into PA. Biochemical analysis revealed that the loss of DGKζ resulted in selective elevation of the PI3K-Akt pathway but no difference in activation of the ERK or NK-κB pathways following TLR stimulation. Activation of the PI3K pathway has been shown to negatively regulate TLR stimulation (Fukao et al., 2002; Guha and Mackman, 2002; Martin et al., 2005) and chemical inhibition of the PI3K restored LPS-induced IL-12p40 production from DGKζ KO BMMΦs (Liu et al., 2007). Intriguingly, the addition of PA also restored LPS-induced IL-12p40 production, suggesting that the cytokine production defect in DGKζ KO DCs and macrophages may be due to reduced PA rather than elevated DAG levels. Exactly how PA rescues TLR-induced cytokine production is unknown, but one possible mechanism is through the recruitment of SHP-1 to negatively regulate PI3K activation (Cuevas et al., 1999; Frank et al., 1999; Zhang et al., 2003).

Defective cytokine production was also observed *in vivo* following intraperitoneal injection of TLR agonists, which correlated with enhanced survival of DGKζ KO mice after LPS-induced septic shock (Liu et al., 2007). However, while DGKζ KO mice were protected from TLR-mediated pathology, the loss of DGKζ conferred susceptibility to *Toxoplasma gondii*. DGKζ KO mice infected with *T. gondii* displayed decreased serum IL-12p40 and IFNγ levels compared to WT mice. Furthermore, IFNγ production by DGKζ KO splenocytes isolated at day 15 and 30 post-infection was significantly attenuated following restimulation with *T. gondii* antigen STAg. Intriguingly, total $CD4^+$ and $CD8^+$ T cell numbers were similar between WT and DGKζ KO mice following infection with the frequency of $CD44^+CD62^{lo}$ effector T cells higher in infected DGKζ KO mice. As TLR-induced IL-12p40 production and the subsequent induction of a Th1 response are critical for protection against *T. gondii* infection, the impairment of immune responses against *T. gondii* by DGKζ KO mice could be secondary to a defect in macrophage and DC-derived cytokines that drive Th1 responses.

THE ROLE OF OTHER DGK ISOFORMS ON DGKζ-REGULATED IMMUNE FUNCTION

So far, we have discussed isoform-specific regulation of immune function by DGKζ, however it is possible that the loss of DGKζ has other functional consequences that might be masked by redundant functions of other DGK isoforms. Indeed, DGKα has been shown to display some redundant function with DGKζ during conventional T and invariant NKT cell (iNKT) development. While singly-deficient DGKα KO and DGKζ KO mice display no gross defects in thymic T cell development, mice deficient in both DGKα and DGKζ (DGKαζ DKO) have significant reductions in CD4 and CD8 SP populations in the thymus due to a cell-intrinsic block in positive selection from the DP to SP stage (Guo et al., 2008). Interestingly, the addition of PA to fetal thymic organ cultures could partially restore T cell maturation defect in DGKαζ DKO thymocytes, suggesting that DGKα and DGKζ regulate T cell development partly through redundant production of PA.

Similarly, the development of iNKT cells is intact in mice singly-deficient for either DGKα or DGKζ (Shen et al., 2011). However, the loss of both DGKα and DGKζ results in a complete impairment of iNKT cell maturation in the thymus, spleen, and liver at both early and terminal stages in a cell-intrinsic

manner. Selective enhancement of the ERK pathway through the expression of a constitutively active K-ras resulted in a significant reduction in mature iNKT cells due a block in Stage II to Stage III maturation of iNKT precursors. Furthermore, augmented activation of the NF-κB pathway through the expression of a constitutively active IKKβ also resulted in an impairment in iNKT maturation at both early and terminal stages of development. These results suggest that DGKα and DGKζ play redundant roles in the regulation of iNKT maturation by controlling DAG-mediated activation of the ERK and NF-κB pathways.

In addition to controlling innate and conventional T cell development, DGKα has also been shown to promote T cell anergy in conjunction with DGKζ. Overexpression of either DGKα or DGKζ in Jurkat T cells induces an anergic-like state that is highlighted by reduced DAG-dependent TCR signals without the impairment of calcium flux (Olenchock et al., 2006a). Similar to DGKζ KO T cells, T cells deficient in DGKα resist anergy induction when activated through their TCR in the absence of costimulation and during superantigen-induced activation. Furthermore, pharmacological inhibition of DGKα in DGKζ-deficient T cells can further enhance proliferation and IL-2 production in response to anergy-inducing conditions, suggesting that both DGKα and DGKζ contribute to anergy induction in inappropriately activated T cells through the synergistic regulation of TCR-induced DAG-mediated signaling.

While DGKα and DGKζ can share similar functions, DGKα does not simply compensate for all DGKζ-regulated functions. For example, unlike DGKζ KO mice, DGKα-deficient mice do not display an enhancement in Treg generation in the thymus or hyperfunctional NK cell responses, thus emphasizing that the regulation of these processes by DGKs is isoform-specific and unique to DGKζ (Joshi et al., 2013; Yang et al., 2016). The independent and redundant roles of DGKα and other DGK isoforms on DGKζ-regulated functions in other immune cells remain unexplored.

CONCLUDING REMARKS

As a negative regulator of DAG-mediated signaling, one might predict that the loss of DGKζ would universally lead to immune activation. Interestingly, however, the inhibition of DGKζ does not only enhance but also suppresses selective immune responses (**Figure 1**). Thus, although DGKζ may represent a drug target for enhancing cytotoxic T cell responses against cancer, it may also simultaneously serve as a target for the treatment of allergic responses or septic shock. In addition to activation or inhibition of immune responses, DGKζ may also play a role in modulating the immune response. For example, it has been shown that the differentiation of $CD4^+$ T cells into Th1 and Th2 subsets may be dependent on TCR signal strength, whereby strong TCR-induced ERK signals favor Th1 over Th2 differentiation (Yamane et al.,

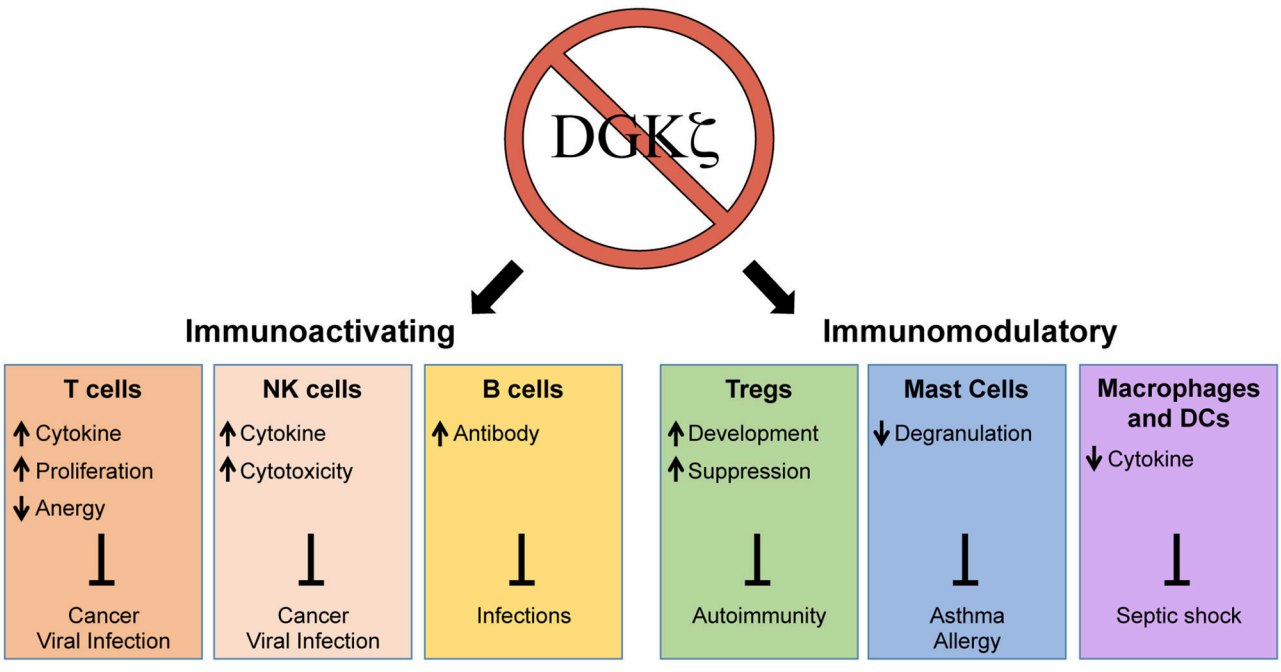


FIGURE 1 | Enhancement and suppression of selective immune functions through the inhibition of DGKζ. The loss of DGKζ can result in activating or modulatory effects on the immune response that can be therapeutically beneficial for the resolution or prevention of a variety of human diseases. The inhibition of DGKζ has positive effects on T cells, NK cells, and B cells, which could promote immunity against cancer and infections. In contrast, the inhibition of DGKζ can suppress immune responses by augmenting Treg development, inhibiting mast cell degranulation, and attenuating macrophage/DC cytokine release. These effects could be beneficial in treatment of autoimmunity, asthma/allergy, and septic shock.

2005). Thus, it is possible that DGKζ controls Th1 vs. Th2 lineage commitment by regulating DAG-mediated ERK activation in T cells. Although the impact of DGKζ deficiency on Th2 responses is unknown, DGKζ KO T cells display heightened Th1-driven anti-viral and anti-tumor responses. Thus, it would be interesting to test whether the loss of DGKζ impairs protection against Th2-inducing helminth infections or beneficially promotes protection against Th2-mediated diseases such as asthma. The precise delineation of how DGKζ controls the outcome of immune responses will yield insight into how DGKζ could be targeted for the treatment of various immune-mediated disorders.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

This work was supported by grants from the Translational Center of Excellence in Hematological Malignancies of the Abramson Cancer Center and by National Institutes of Health Grants R01HL107589 and R01HL111501.

REFERENCES

Akira, S., and Takeda, K. (2004). Toll-like receptor signalling. *Nat. Rev. Immunol.* 4, 499–511. doi: 10.1038/nri1391

Artis, D., and Spits, H. (2015). The biology of innate lymphoid cells. *Nature* 517, 293–301. doi: 10.1038/nature14189

Atkinson, T. P., Kaliner, M. A., and Hohman, R. J. (1992). Phospholipase C-gamma 1 is translocated to the membrane of rat basophilic leukemia cells in response to aggregation of IgE receptors. *J. Immunol.* 148, 2194–2200.

Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., et al. (2001). The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27, 20–21. doi: 10.1038/83713

Binstadt, B. A., Brumbaugh, K. M., Dick, C. J., Scharenberg, A. M., Williams, B. L., Colonna, M., et al. (1996). Sequential involvement of Lck and SHP-1 with MHC-recognizing receptors on NK Cells inhibits FcR-initiated tyrosine kinase activation. *Immunity* 5, 629–638. doi: 10.1016/S1074-7613(00)80276-9

Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S.-A., et al. (2001). Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27, 68–73. doi: 10.1038/83784

Bunting, M., Tang, W., Zimmerman, G. A., McIntyre, T. M., and Prescott, S. M. (1996). Molecular cloning and characterization of a novel human diacylglycerol kinase zeta. *J. Biol. Chem.* 271, 10230–10236. doi: 10.1074/jbc.271.17.10237

Chatila, T. A., Blaeser, F., Ho, N., Lederman, H. M., Voulgaropoulos, C., Helms, C., et al. (2000). JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. *J. Clin. Invest.* 106, R75–R81. doi: 10.1172/JCI11679

Chen, R., Latour, S., Shi, X., and Veillette, A. (2006). Association between SAP and FynT: inducible SH3 domain-mediated interaction controlled by engagement of the SLAM receptor. *Mol. Cell. Biol.* 26, 5559–5568. doi: 10.1128/MCB.00357-06

Coudronniere, N., Villalba, M., Englund, N., and Altman, A. (2000). NF-κB activation induced by T cell receptor/CD28 costimulation is mediated by protein kinase C-θ. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3394–3399. doi: 10.1073/pnas.060028097

Coughlin, J. J., Stang, S. L., Dower, N. A., and Stone, J. C. (2005). RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. *J. Immunol.* 175, 7179–7184. doi: 10.4049/jimmunol.175.11.7179

Cuevas, B., Lu, Y., Watt, S., Kumar, R., Zhang, J., Siminovitch, K. A., et al. (1999). SHP-1 regulates Lck-induced phosphatidylinositol 3-kinase phosphorylation and activity. *J. Biol. Chem.* 274, 27583–27589. doi: 10.1074/jbc.274.39.27583

Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., et al. (2000). RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat. Immunol.* 1, 317–321. doi: 10.1038/79766

Frank, C., Keilhack, H., Opitz, F., Zschörnig, O., and Böhmer, F. D. (1999). Binding of phosphatidic acid to the protein-tyrosine phosphatase SHP-1 as a basis for activity modulation. *Biochemistry* 38, 11993–12002. doi: 10.1021/bi982586w

Fukao, T., Tanabe, M., Terauchi, Y., Ota, T., Matsuda, S., Asano, T., et al. (2002). PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol.* 3, 875–881. doi: 10.1038/ni825

Goto, K., and Kondo, H. (1996). A 104-kDa diacylglycerol kinase containing ankyrin-like repeats localizes in the cell nucleus. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11196–11201. doi: 10.1073/pnas.93.20.11196

Gross, A. J., Lyandres, J. R., Panigrahi, A. K., Prak, E. T. L., and DeFranco, A. L. (2009). Developmental acquisition of the Lyn–CD22–SHP-1 inhibitory pathway promotes B cell tolerance. *J. Immunol.* 182, 5382–5392. doi: 10.4049/jimmunol.0803941

Guha, M., and Mackman, N. (2002). The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *J. Biol. Chem.* 277, 32124–32132. doi: 10.1074/jbc.M203298200

Gumbleton, M., Vivier, E., and Kerr, W. G. (2015). SHIP1 intrinsically regulates NK cell signaling and education, resulting in tolerance of an MHC class I-mismatched bone marrow graft in mice. *J. Immunol.* 194, 2847–2854. doi: 10.4049/jimmunol.1402930

Guo, R., Wan, C. K., Carpenter, J. H., Mousallem, T., Boustany, R. M., Kuan, C. T., et al. (2008). Synergistic control of T cell development and tumor suppression by diacylglycerol kinase alpha and zeta. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11909–11914. doi: 10.1073/pnas.0711856105

Hashimoto, A., Takeda, K., Inaba, M., Sekimata, M., Kaisho, T., Ikehara, S., et al. (2000). Cutting edge: essential role of phospholipase C-γ 2 in B cell development and function. *J. Immunol.* 165, 1738–1742. doi: 10.4049/jimmunol.165.4.1738

Höglund, P., and Brodin, P. (2010). Current perspectives of natural killer cell education by MHC class I molecules. *Nat. Rev. Immunol.* 10, 724–734. doi: 10.1038/nri2835

Imboden, J. B., and Stobo, J. D. (1985). Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol phosphates and releases calcium ions from intracellular stores. *J. Exp. Med.* 161, 446–456.

Janeway, C. A. Jr., and Medzhitov, R. (2002). Innate immune recognition. *Annu. Rev. Immunol.* 20, 197–216. doi: 10.1146/annurev.immunol.20.083001.084359

Josefowicz, S. Z., Lu, L. F., and Rudensky, A. Y. (2012). Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* 30, 531–564. doi: 10.1146/annurev.immunol.25.022106.141623

Joshi, R. P., and Koretzky, G. A. (2013). Diacylglycerol kinases: regulated controllers of T cell activation, function, and development. *Int. J. Mol. Sci.* 14, 6649–6673. doi: 10.3390/ijms14046649

Joshi, R. P., Schmidt, A. M., Das, J., Pytel, D., Riese, M. J., Lester, M., et al. (2013). The ζ isoform of diacylglycerol kinase plays a predominant role in regulatory T cell development and TCR-mediated ras signaling. *Sci. Signal.* 6:ra102. doi: 10.1126/scisignal.2004373

Jung, H., Hsiung, B., Pestal, K., Procyk, E., and Raulet, D. H. (2012). RAE-1 ligands for the NKG2D receptor are regulated by E2F transcription factors, which control cell cycle entry. *J. Exp. Med.* 209, 2409–2422. doi: 10.1084/jem.20120565

Kambayashi, T., and Koretzky, G. A. (2007). Proximal signaling events in Fc epsilon RI-mediated mast cell activation. *J. Allergy Clin. Immunol.* 119, 544–552; quiz 553–544. doi: 10.1016/j.jaci.2007.01.017

Kärre, K., Ljunggren, H. G., Piontek, G., and Kiessling, R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature* 319, 675–678.

Kinet, J. P. (1999). The high-affinity IgE receptor (FcεRI): from physiology to pathology. *Annu. Rev. Immunol.* 17, 931–972. doi: 10.1146/annurev.immunol.17.1.931

Kobayashi, N., Hozumi, Y., Ito, T., Hosoya, T., Kondo, H., and Goto, K. (2007). Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. *Eur. J. Cell Biol.* 86, 433–444. doi: 10.1016/j.ejcb.2007.05.002

Kurosaki, T., Shinohara, H., and Baba, Y. (2010). B cell signaling and fate decision. *Annu. Rev. Immunol.* 28, 21–55. doi: 10.1146/annurev.immunol.021908.132541

Lanier, L. L. (2008). Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* 9, 495–502. doi: 10.1038/ni1581

Leitges, M., Gimborn, K., Elis, W., Kalesnikoff, J., Hughes, M. R., Krystal, G., et al. (2002). Protein Kinase C- Is a negative regulator of antigen-induced mast cell degranulation. *Mol. Cell. Biol.* 22, 3970–3980. doi: 10.1128/MCB.22.12.3970-3980.2002

Liu, C.-H. H., Machado, F. S., Guo, R., Nichols, K. E., Burks, A. W., Aliberti, J. C., et al. (2007). Diacylglycerol kinase zeta regulates microbial recognition and host resistance to *Toxoplasma gondii*. *J. Exp. Med.* 204, 781–792. doi: 10.1084/jem.20061856

Locksley, R. M. (2010). Asthma and allergic inflammation. *Cell* 140, 777–783. doi: 10.1016/j.cell.2010.03.004

Long, M., Park, S. G., Strickland, I., Hayden, M. S., and Ghosh, S. (2009). Nuclear factor-κB modulates regulatory T cell development by directly regulating expression of Foxp3 transcription factor. *Immunity* 31, 921–931. doi: 10.1016/j.immuni.2009.09.022

Lowin-Kropf, B., Kunz, B., Beermann, F., and Held, W. (2000). Impaired natural killing of MHC class I-deficient targets by NK cells expressing a catalytically inactive form of SHP-1. *J. Immunol.* 165, 1314–1321. doi: 10.4049/jimmunol.165.3.1314

Macian, F., Garcia-Cozar, F., Im, S. H., Horton, H. F., Byrne, M. C., and Rao, A. (2002). Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* 109, 719–731. doi: 10.1016/S0092-8674(02)00767-5

Martin, M., Rehani, K., Jope, R. S., and Michalek, S. M. (2005). Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* 6, 777–784. doi: 10.1038/ni1221

May, R. M., Okumura, M., Hsu, C. J., Bassiri, H., Yang, E., Rak, G., et al. (2013). Murine natural killer immunoreceptors use distinct proximal signaling complexes to direct cell function. *Blood* 121, 3135–3146. doi: 10.1182/blood-2012-12-474361

McHeyzer-Williams, L. J., and McHeyzer-Williams, M. G. (2005). Antigen-specific memory B cell development. *Annu. Rev. Immunol.* 23, 487–513. doi: 10.1146/annurev.immunol.23.021704.115732

McVicar, D. W., Taylor, L. S., Gosselin, P., Willette-Brown, J., Mikhael, A. I., Geahlen, R. L., et al. (1998). DAP12-mediated signal transduction in natural killer cells. *A dominant role for the Syk protein-tyrosine kinase. J. Biol. Chem.* 273, 32934–32942.

Medzhitov, R. (2001). Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* 1, 135–145. doi: 10.1038/35100529

Monick, M. M., Carter, A. B., Gudmundsson, G., Mallampalli, R., Powers, L. S., and Hunninghake, G. W. (1999). A phosphatidylcholine-specific phospholipase C regulates activation of p42/44 mitogen-activated protein kinases in lipopolysaccharide-stimulated human alveolar macrophages. *J. Immunol.* 162, 3005–3012.

Nechushtan, H., Leitges, M., Cohen, C., Kay, G., and Razin, E. (2000). Inhibition of degranulation and interleukin-6 production in mast cells derived from mice deficient in protein kinase Cβ. *Blood* 95, 1752.

Olenchock, B. A., Guo, R., Carpenter, J. H., Jordan, M., Topham, M. K., Koretzky, G. A., et al. (2006a). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* 7, 1174–1181. doi: 10.1038/ni1400

Olenchock, B. A., Guo, R., Silverman, M. A., Wu, J. N., Carpenter, J. H., Koretzky, G. A., et al. (2006b). Impaired degranulation but enhanced cytokine production after Fc epsilonRI stimulation of diacylglycerol kinase zeta-deficient mast cells. *J. Exp. Med.* 203, 1471–1480. doi: 10.1084/jem.20052424

Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F., et al. (1993). Ca(2+)-dependent and Ca(2+)-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca2+ and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* 268, 1749-1756.

Quann, E. J., Liu, X., Altan-Bonnet, G., and Huse, M. (2011). A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat. Immunol.* 12, 647–654. doi: 10.1038/ni.2033

Richards, J. D., Dave, S. H., Chou, C. H., Mamchak, A. A., and DeFranco, A. L. (2001). Inhibition of the MEK/ERK signaling pathway blocks a subset of B cell responses to antigen. *J. Immunol.* 166, 3855–3864. doi: 10.4049/jimmunol.166.6.3855

Riese, M. J., Grewal, J., Das, J., Zou, T., Patil, V., Chakraborty, A. K., et al. (2011). Decreased diacylglycerol metabolism enhances ERK activation and augments CD8+ T cell functional responses. *J. Biol. Chem.* 286, 5254–5265. doi: 10.1074/jbc.M110.171884

Riese, M. J., Wang, L.-C. S. C., Moon, E. K., Joshi, R. P., Ranganathan, A., June, C. H., et al. (2013). Enhanced effector responses in activated CD8+ T cells deficient in diacylglycerol kinases. *Cancer Res.* 73, 3566–3577. doi: 10.1158/0008-5472.CAN-12-3874

Rowland, S. L., DePersis, C. L., Torres, R. M., and Pelanda, R. (2010). Ras activation of Erk restores impaired tonic BCR signaling and rescues immature B cell differentiation. *J. Exp. Med.* 207, 607–621. doi: 10.1084/jem.20091673

Ruan, Q., Kameswaran, V., Tone, Y., Li, L., Liou, H. C., Greene, M. I., et al. (2009). Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. *Immunity* 31, 932–940. doi: 10.1016/j.immuni.2009.10.006

Rui, T., Zhang, J., Xu, X., Yao, Y., Kao, R., and Martin, C. M. (2012). Reduction in IL-33 expression exaggerates ischaemia/reperfusion-induced myocardial injury in mice with diabetes mellitus. *Cardiovasc. Res.* 94, 370–378. doi: 10.1093/cvr/cvs015

Saijo, K., Schmedt, C., Su, I. H. H., Karasuyama, H., Lowell, C. A., Reth, M., et al. (2003). Essential role of Src-family protein tyrosine kinases in NF-κB activation during B cell development. *Nat. Immunol.* 4, 274–279. doi: 10.1038/ni893

Schmidt, A. M., Lu, W., Sindhava, V. J., Huang, Y., Burkhardt, J. K., Yang, E., et al. (2015). Regulatory T cells require TCR signaling for their suppressive function. *J. Immunol.* 194, 4362–4370. doi: 10.4049/jimmunol.1402384

Schmidt, A. M., Zou, T., Joshi, R. P., Leichner, T. M., Pimentel, M. A., Sommers, C. L., et al. (2013). Diacylglycerol kinase ζ limits the generation of natural regulatory T cells. *Sci. Signal.* 6:ra101. doi: 10.1126/scisignal.2004411

Schneider, H., Cohen-Dayag, A., and Pecht, I. (1992). Tyrosine phosphorylation of phospholipase C(γ1) couples the Fcε receptor mediated signal to mast cells secretion. *Int. Immunol.* 4, 447–453. doi: 10.1093/intimm/4.4.447

Sharp, L. L., Schwarz, D. A., Bott, C. M., Marshall, C. J., and Hedrick, S. M. (1997). The influence of the MAPK pathway on T cell lineage commitment. *Immunity* 7, 609–618. doi: 10.1016/S1074-7613(00)80382-9

Shen, S., Wu, J., Srivatsan, S., Gorentla, B. K., Shin, J., Xu, L., et al. (2011). Tight regulation of diacylglycerol-mediated signaling is critical for proper invariant NKT cell development. *J. Immunol.* 187, 2122–2129. doi: 10.4049/jimmunol.1100495

Shulga, Y. V., Topham, M. K., and Epand, R. M. (2011). Regulation and functions of diacylglycerol kinases. *Chem. Rev.* 111, 6186–6208. doi: 10.1021/cr1004106

Smith-Garvin, J. E., Koretzky, G. A., and Jordan, M. S. (2009). T cell activation. *Annu. Rev. Immunol.* 27, 591–619. doi: 10.1146/annurev.immunol.021908.132706

Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J., Gandhi, L., et al. (2000). PKC-[θ] is required for TCR-induced NF-[κ]B activation in mature but not immature T lymphocytes. *Nature* 404, 402–407. doi: 10.1038/35006090

Tassi, I., Presti, R., Kim, S., Yokoyama, W. M., Gilfillan, S., and Colonna, M. (2005). Phospholipase C-γ2 is a critical signaling mediator for murine NK cell activating receptors. *J. Immunol.* 175, 749–754. doi: 10.4049/jimmunol.175.2.749

Tognon, C. E., Kirk, H. E., Passmore, L. A., Whitehead, I. P., Der, C. J., and Kay, R. J. (1998). Regulation of RasGRP via a phorbol ester-responsive C1 domain. *Mol. Cell. Biol.* 18, 6995–7008. doi: 10.1128/MCB.18.12.6995

Topham, M. K., and Prescott, S. M. (1999). Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* 274, 11447–11450. doi: 10.1074/jbc.274.17.11447

Viant, C., Fenis, A., Chicanne, G., Payrastre, B., Ugolini, S., and Vivier, E. (2014). SHP-1-mediated inhibitory signals promote responsiveness and anti-tumour functions of natural killer cells. *Nat. Commun.* 5, 5108. doi: 10.1038/ncomms6108

Voehringer, D. (2013). Protective and pathological roles of mast cells and basophils. *Nat. Rev. Immunol.* 13, 362–375. doi: 10.1038/nri3427

Wang, D., Feng, J., Wen, R., Marine, J. C., Sangster, M. Y., Parganas, E., et al. (2000). Phospholipase Cγ2 is essential in the functions of B cell and several Fc receptors. *Immunity* 13, 25–35. doi: 10.1016/S1074-7613(00)00005-4

Wang, D., Matsumoto, R., You, Y., Che, T., Lin, X. Y., Gaffen, S. L., et al. (2004). CD3/CD28 costimulation-induced NF-κB activation is mediated by recruitment of protein kinase C-θ, Bcl10, and IκB kinase β to the immunological synapse through CARMA1. *Mol. Cell. Biol.* 24, 164–171. doi: 10.1128/MCB.24.1.164-171.2003

Wen, R., Jou, S.-T. T., Chen, Y., Hoffmeyer, A., and Wang, D. (2002). Phospholipase C-γ2 is essential for specific functions of Fcε R and Fcγ R. *J. Immunol.* 169, 6743–6752. doi: 10.4049/jimmunol.169.12.6743

Wheeler, M. L., Dong, M. B., Brink, R., Zhong, X. -P. P., and DeFranco, A. L. (2013). Diacylglycerol kinase ζ limits B cell antigen receptor-dependent activation of ERK signaling to inhibit early antibody responses. *Sci. Signal.* 6:ra91. doi: 10.1126/scisignal.2004189

Wildin, R. S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J. L., Buist, N., et al. (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat. Genet.* 27, 18–20. doi: 10.1038/83707

Wright, D. B., Tripathi, S., Sikarwar, A., Santosh, K. T., Perez-Zoghbi, J., Ojo, O. O., et al. (2013). Regulation of GPCR-mediated smooth muscle contraction: implications for asthma and pulmonary hypertension. *Pulm. Pharmacol. Ther.* 26, 121–131. doi: 10.1016/j.pupt.2012.06.005

Wu, J. N., and Koretzky, G. A. (2004). The SLP-76 family of adapter proteins. *Semin. Immunol.* 16, 379–393. doi: 10.1016/j.smim.2004.08.018

Wu, J., Song, Y., Bakker, A. B., Bauer, S., Spies, T., Lanier, L. L., et al. (1999). An activating immunoreceptor complex formed by NKG2D and DAP10. *Science* 285, 730–732. doi: 10.1126/science.285.5428.730

Yamane, H., Zhu, J., and Paul, W. E. (2005). Independent roles for IL-2 and GATA-3 in stimulating naive CD4+ T cells to generate a Th2-inducing cytokine environment. *J. Exp. Med.* 202, 793–804. doi: 10.1084/jem.20051304

Yang, E., Singh, B. K., Paustian, A. M., and Kambayashi, T. (2016). Diacylglycerol Kinase ζ Is a Target To Enhance NK Cell Function. *J. Immunol.* 197, 934–941. doi: 10.4049/jimmunol.1600581

Yasuda, T., Kometani, K., Takahashi, N., Imai, Y., Aiba, Y., and Kurosaki, T. (2011). ERKs induce expression of the transcriptional repressor Blimp-1 and subsequent plasma cell differentiation. *Sci. Signal.* 4, ra25. doi: 10.1126/scisignal.2001592

Yasuda, T., Sanjo, H., Pagès, G., Kawano, Y., Karasuyama, H., Pouysségur, J., et al. (2008). Erk kinases link pre-B cell receptor signaling to transcriptional events required for early B cell expansion. *Immunity* 28, 499–508. doi: 10.1016/j.immuni.2008.02.015

Zha, Y., Marks, R., Ho, A. W., Peterson, A. C., Janardhan, S., Brown, I., et al. (2006). T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-α. *Nat. Immunol.* 7, 1166–1173. doi: 10.1038/ni1394

Zhang, F., Zhao, G., and Dong, Z. (2001a). Phosphatidylcholine-specific phospholipase C and D in stimulation of RAW264.7 mouse macrophage-like cells by lipopolysaccharide. *Int. Immunopharmacol.* 1, 1375–1384.

Zhang, F., Zhao, G., and Dong, Z. (2001b). Phosphatidylcholine-specific phospholipase C regulates activation of RAW264.7 macrophage-like cells by lipopeptide JBT3002. *J. Leukoc. Biol.* 69, 1060–1066.

Zhang, Z., Jimi, E., and Bothwell, A. L. M. (2003). Receptor activator of NF-κ B ligand stimulates recruitment of SHP-1 to the complex containing TNFR-associated factor 6 that regulates osteoclastogenesis. *J. Immunol.* 171, 3620–3626. doi: 10.4049/jimmunol.171.7.3620

Zhong, X.-P. P., Hainey, E. A., Olenchock, B. A., Jordan, M. S., Maltzman, J. S., Nichols, K. E., et al. (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat. Immunol.* 4, 882–890. doi: 10.1038/ni958

Zhong, X.-P. P., Hainey, E. A., Olenchock, B. A., Zhao, H., Topham, M. K., and Koretzky, G. A. (2002). Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase zeta. *J. Biol. Chem.* 277, 31089–31098. doi: 10.1074/jbc.M203818200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

PERSPECTIVE
published: 03 March 2017
doi: 10.3389/fcell.2017.00016

DGK-α: A Checkpoint in Cancer-Mediated Immuno-Inhibition and Target for Immunotherapy

Elfriede Noessner *

Immunoanalytics Core Facility and Research Group Tissue Control of Immunocytes, Helmholtz Zentrum München, München, Germany

Immunotherapy is moving to the forefront of cancer treatments owing to impressive durable responses achieved with checkpoint blockade antibodies and adoptive T-cell therapy. Still, improvements are necessary since, overall, only a small percentage of patients benefit from current therapies. Here, I summarize evidence that DGK-α may represent an immunological checkpoint suppressing the activity of cytotoxic immunocytes in the tumor microenvironment. DGK-inhibitors can restore the antitumor function of tumor-suppressed adaptive and innate cytotoxic immunocytes. The activity of DGK-inhibitors lays downstream of current checkpoint blockade antibodies. Thus, synergistic effects are expected from combination strategies. Moreover, DGK-inhibitors may permit a double-strike attack on tumor cells as DGK-inhibition may not only re-instate immunological tumor attack but also may harm tumor cells directly by interfering with oncogenic survival pathways. Together, DGK-inhibitors have very promising characteristics and may be beneficially included into the armamentarium of cancer immunotherapeutics.

Keywords: tumor-infiltrating lymphocytes, renal cell carcinoma, diacylglycerol kinase, immunotherapy, anergy, human tumor

OPEN ACCESS

Edited by:
Isabel Merida, Centro Nacional de Biotecnología (CSIC), Spain

Reviewed by:
Kamalakannan Rajasekaran, Genentech Foundation, USA
Pedro A. Lazo, Instituto de Biología Molecular y Celular del Cancer (CSIC), Spain

***Correspondence:**
Elfriede Noessner
noessner@helmholtz-muenchen.de

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *05 January 2017*
Accepted: *17 February 2017*
Published: *03 March 2017*

Citation:
Noessner E (2017) DGK-α: A Checkpoint in Cancer-Mediated Immuno-Inhibition and Target for Immunotherapy. Front. Cell Dev. Biol. 5:16. doi: 10.3389/fcell.2017.00016

IMMUNOTHERAPY, APPROACHING FOREFRONT OF CANCER THERAPY, SEARCHING FOR COLLABORATION

Immunotherapy is moving to the forefront of cancer therapies with an increasing assortment of approaches being evaluated and approved for clinical application (Callahan et al., 2016; Papaioannou et al., 2016). Recent data document measurable improvement in patient outcome and, in several cases, induction of durable responses even in patients with far advanced disease that proved refractory to available therapies. The new therapies indicate a paradigmatic shift in cancer therapy in that tumor cells are no longer direct therapeutic target, but instead, therapies are directed toward the cells of the immune system restoring their ability to recognize and destroy tumor cells.

The immune system is ideally equipped to fight cancer as its components continuously travel throughout the body providing surveillance; immune cells can be specifically activated against tumors, which express antigen and are often immunogenic; and they can protect against tumor relapse owing to their ability to acquire specific and long-lasting memory. Yet, tumors escape from immune surveillance due to immunoediting (Dunn et al., 2004) and the development of immune cell dysfunctions (Frey and Monu, 2006; Gajewski et al., 2006). The new cancer immunotherapies became possible through a deeper understanding of the regulatory mechanisms that govern an effective immune response and technological advances in T-cell cultivation, engineering and antigen identification.

The most advanced immunotherapeutic protocols to date include (**Figure 1**): (i) adoptive T-cell therapies, using *ex-vivo*-expanded autologous T cells isolated from tumor tissue (TILs) or autologous T cells engineered with therapeutic T-cell receptors (TCRs) or chimeric antigen receptors (CARs) recognizing tumor-expressed antigens; (ii) vaccination using tumor antigens or tumor antigen-presenting dendritic cells to stimulate the patient's immune system to generate tumor-reactive T cells *in situ*; and (iii) antibody-based therapies blocking immune checkpoints that would naturally elicit negative signals that hold back T cells to prevent autoimmune attack (Pardoll, 2012; Sharma and Allison, 2015).

While vaccination has yet to yield measurable clinical response (van der Burg et al., 2016), high and often long-lasting response rates are achieved with adoptive TIL therapy (Rosenberg and Restifo, 2015) and CD19-directed CAR-T cell therapy (Fesnak et al., 2016; Park et al., 2016). Yet, despite its high promises, adoptive T-cell therapy still faces significant hurdles to become one of the mainstay cancer therapies: TIL therapy is limited to tumor entities from which sufficient TILs can be procured [mainly melanoma and renal cell cancer (RCC)] and TCR- or CAR-T-cell therapy requires the knowledge of tumor-specific antigens to which T cells can be safely directed without harming vital organs. Currently, CAR-therapy is restricted to leukemia and lymphoma that express CD19 as targetable antigens. Treatment of solid tumors is explored, such as glioblastoma expressing a mutant form of the epidermal growth factor (EGFRvIII) or adenocarcinoma expressing cancer-associated glycoforms of mucin (Newick et al., 2016; Posey et al., 2016). Moreover, safety issues need to be resolved since serious adverse effects have been reported in TCR- and CAR-therapy trials (Gross and Eshhar, 2016).

Contrasting the currently limited application of adoptive T-cell therapy, immunotherapy with checkpoint blockade antibodies has achieved exciting results across a wide variety of cancer entities, not limited to commonly assumed immunogenic tumors such as melanoma or RCC, but also in lung cancer, bladder cancer or head and neck cancer. Three checkpoint blockade antibodies are currently in the clinic. One targets the cytotoxic T-lymphocyte-associated protein (CTLA)-4 (Postow et al., 2015; Sharma and Allison, 2015), which is an intrinsic negative regulator of T-cell activation during T-cell priming. The other two antibodies target the programmed death (PD) pathway through binding to the PD-1 protein or its ligand PD-L1.

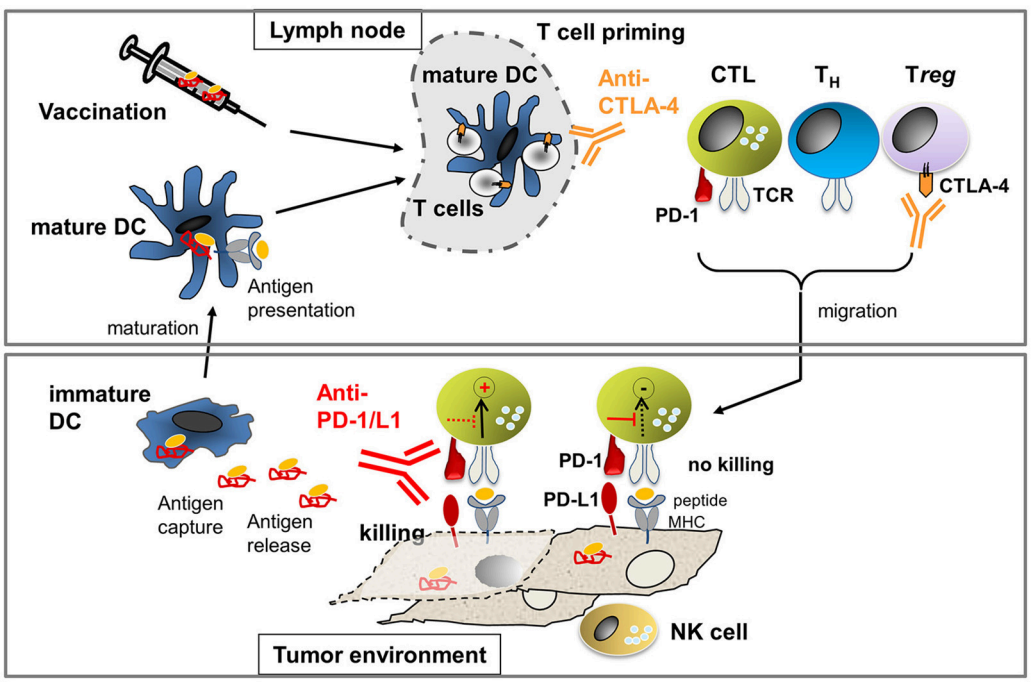


FIGURE 1 | Processes to activate T cell immunity against cancer: In the lymph node, T cell priming occurs through mature dendritic cells (DCs) presenting tumor-derived antigens. The number of arising T helper (T_H) cells and cytotoxic T cells (CTL) is limited through cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4). Antibodies against CTLA-4 allow for activation of more T cells due to the amelioration of negative signals during T-cell priming. In addition, anti-CTLA-4 inhibits T*reg* which express high levels of CTLA-4 constitutively. Activated T cells migrate into the tumor milieu where they engage with tumor cells expressing peptide-MHC that can be recognized by the T cell receptor (TCR). TCR-pMHC interaction will activate tumor cell killing processes unless suppression occurs through concomitant PD-1/PD-L1 interaction. Killing of tumor cells can occur if the negative signaling is blocked through anti-PD-1 or anti-PD-L1 antibodies. NK cells can recognize tumor cells that express low or no MHC and, thus, cooperate with CTL to prevent tumor escape. If tumor cell killing occurs, antigen is released which can be taken up by immature DCs. Immature DCs can mature to mature DCs which then present antigen to T cells in the lymph node, leading to the generation of new tumor-reactive T cells. If the natural process of antigen presentation does not occur (efficiently), therapeutic vaccination using *ex vivo* generated antigen-loaded DCs or peptides may be applied.

The PD-1/PD-L1 checkpoint is an extrinsic "off" signal that is operative in peripheral tissues turning off T-cell function to help control local inflammatory responses and maintain self-tolerance. Impressive durable responses have been observed using anti-CTLA-4 and anti-PD-1 resulting in their approval for the treatment of several cancers (Callahan et al., 2016). Yet, it has to be recognized that, overall, only a minority of patients experience substantial clinical benefit (around 15–40% depending on the tumor entity) (Sunshine and Taube, 2015; Ribas and Hu-Lieskovan, 2016). Improvements are necessary to unleash the full potential of immunotherapy and to potentially offer benefit to patients whose tumors are refractory to current therapies.

DIACYLGLYCEROL KINASE ALPHA: A CHECKPOINT THAT NEGATIVELY REGULATES T-CELL FUNCTION AND CURBS THE ACTIVITY OF CD8-T AND NK CELLS IN THE TUMOR MICROENVIRONMENT

T cells, in particular T_H1/T_C1-polarized lymphocytes, are important players in the antitumor response. Not only is their abundance associated with good prognosis in many tumor types (Fridman et al., 2012), they are also required for therapeutic response to checkpoint blockade therapy (Herbst et al., 2014; Tumeh et al., 2014). NK cells are innate cytotoxic lymphocytes appreciated for their ability to lyse virally infected cells as well as tumors. They play a complementary role to CD8-T cells as they recognize tumors which are resistant to T-cell killing due to downregulation or loss of MHC-class I molecules (refs. in Prinz et al., 2014). In some tumor types, such as RCC, they appear to play a prominent role as their number is predictive of good prognosis while that of CD8-T cells is not (Nakano et al., 2001; Eckl et al., 2012).

While the value of the natural immune infiltrate in tumors is appreciated, it has to be recognized that the natural immune response is not sufficient to control tumor progression in most cases. Various mechanisms are known that contribute to tumor immune escape ranging from ignorance to active suppression (Frey and Monu, 2006; Gajewski et al., 2006). One major hurdle is the inhibition of T-cell function in the tumor milieu. The suppressive quality of the tumor environment not only impacts the natural immune cell infiltrate but also curbs the efficacy of adoptive therapy, as even highly functional *in vitro* engineered CAR-T cell become unresponsive in solid tumor milieus (Janicki et al., 2008; Imai et al., 2009; Moon et al., 2014). Recognized mechanisms are T-cell exhaustion characterized by high expression of co-inhibitory receptors (PD-1, LAG-3, TIM-3) (Wherry et al., 2007; Pardoll, 2012), division arrest (Beyer et al., 2009), or effector phase inhibition due to disruption of TCR-signaling events (Frey and Monu, 2006).

We have analyzed the dysfunctional state of CD8-T and NK cells in human clear cell RCC (ccRCC) and observed a signature of anergy (Prinz et al., 2012, 2014). We found that TILs were non-responsive to stimulation, with much lower degranulation (measured by the appearance of CD107 protein on the cell surface), poor cytolytic activity (measured by chromium release assay) and low cytokine production compared to CD8-T cells and NK cells isolated from the non-tumor inflicted pole of the kidney (NILs) or from peripheral blood (PBLs). Mechanistically, no differences were seen activating proximal signaling molecules (Lck, ZAP70 or PLCγ) between TILs and NILs or PBLs; however, deficits in activating distal signaling molecules were evident. Identified key features included high levels of diacylglycerol kinase-α (DGK-α), low basal phosphorylation of the extracellular signal-regulated kinase (ERK) as well as reduced stimulation-induced phosphorylation of ERK, c-Jun N-terminal kinase (JNK) and AKT/protein kinase B. These features were caused by the tumor microenvironment as they were not observed in CD8-NILs or NK-NILs, and these lymphocytes were functionally active. The signature was similar to that previously described for anergic $CD4^-$T cells (Macian et al., 2002; Zheng et al., 2008).

DGKs are appreciated as physiologic regulator of T- and NK-cell development, differentiation and function, through their activity to regulate the levels of DAG and phosphatidic acid (PA), which are important second messengers in the TCR-signaling cascade. The rasGRP/ERK pathway, activated by DAG, is crucial for MTOC-polarization, the delivery of lytic granules to the immunologic synapse (Quann et al., 2009) and the subsequent lytic attack on target cells. Cytotoxicity and production of IFN-γ, controlled among others by the ERK-pathway, are the most important effector activities required for tumor rejection. Thus, control of the ERK-pathway ultimately determines a T- and NK-cell's antitumor activity.

In experimental systems, overexpression of DGK led to a general attenuation of TCR-signaling as a direct result of decreased RasGRP1/Ras/ERK-pathway activation. Moreover, it has been shown that DGK-α and DGK-ζ, the abundant isoforms in T cells, are necessary for establishment of anergy (Zhong et al., 2008; Merida et al., 2015; Chen et al., 2016). Together, these experimental findings support our interpretation that T cells and also NK cells in the human RCC environment are anergic, showing overexpression of DGK-α, blunted ERK signaling and unresponsiveness to stimulation. Observing an anergic signature in TILs of ccRCCs is not unexpected since ccRCC is an epithelial tumor and, thus, largely devoid of co-stimulatory ligands, representing the classical anergy-inducing environment. Still, we do not rule out that additional mechanisms such as exhaustion or tolerance mediated through surface expressed co-inhibitory molecules such as PD-1, also contribute to functional unresponsiveness of TILs (**Figure 2A**). Rather, the causes of functional unresponsiveness in the tumor milieu are likely multifactorial. This is exemplified in an adoptive transfer experiment using CAR-T cells (Moon et al., 2014): in the tumor microenvironment, CAR-T cells rapidly became hypofunctional with identified upregulation of intrinsic T-cell inhibitory enzymes (DGK-α, DGK-ζ, SHP-1) as well as

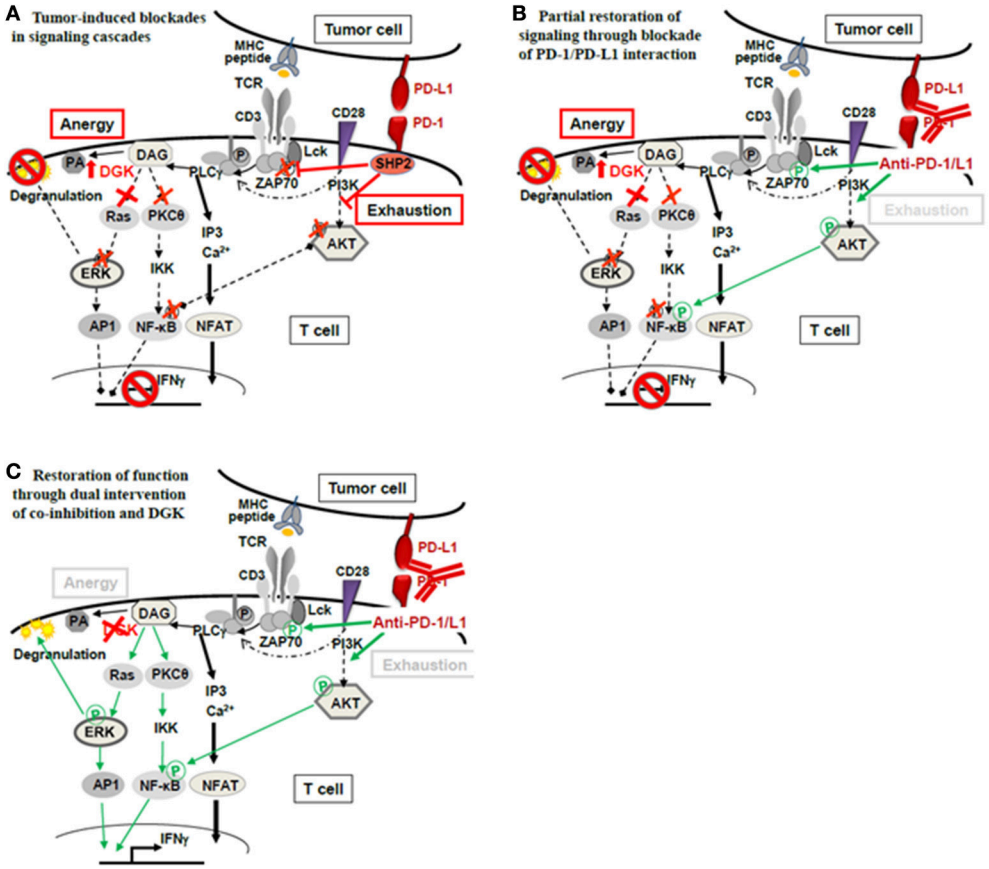


FIGURE 2 | Theoretical concept of combined application of checkpoint blockade therapy and DGK-inhibition. (A) Functional unresponsiveness of TILs in the tumor milieu may have different mutually non-exclusive causes: (i) Ligation of PD-1 on T cells by tumor expressed PD-L1 may cause recruitment of phosphatase SHP2 and subsequent dephosphorylation of the TCR proximal signal transmitter Lck as well as attenuation of AKT-signaling. Consequently, signals initiated by TCR-peptide/MHC recognition intended to activate T-cell effector function (degranulation leading to lysis of target cells as well as IFNγ) are interrupted. The signal interruption through PD-1/PD-L1 classically occurs as a consequence of T-cell exhaustion. (ii) Anergy is another mechanism of T-cell silencing. The underlying cause is upregulated diacylglycerol kinase (DGK), in T cells mainly DGK-α and DGK-ζ. DGKs metabolize diacylglycerol (DAG) to phosphatidic acid (PA) lowering DAG levels which are necessary to activate TCR distal signaling through Ras/ERK. The ERK pathway is critically import for the degranulation process that delivers lytic proteins into the target cell for target cell death. **(B)** De-blocking the exhaustion pathway through checkpoint antibodies (anti-PD-1/PD-L1) releases the proximal brake at the TCR-associated molecules (Lck- and AKT-phosphorylation); however, distal brakes through DGK may still be active (blocked ERK pathway and attenuated PKC- and NFκB-activation) preventing full activation of the T cell's antitumor functions (degranulation, IFNγ). **(C)** Combined treatment with checkpoint antibodies and DGK-inhibitor may be required to open the signaling cascade fully, allowing effector function.

expression of surface co-inhibitory receptors (PD-1, LAG-3, TIM-3, 2B4).

REVERSAL OF TUMOR-INDUCED SUPPRESSION AND RESTORATION OF T- AND NK-CELL ACTIVITY THROUGH DGK-INHIBITION

IL-2 is a well-known growth factor for T and NK cells and has a history in RCC immunotherapy, achieving tumor control in a subgroup of patients (McDermott, 2009). IL-2 is known to regulate DGK-α and to restore functional responsiveness of anergic CD4-T cells (Macian et al., 2002). We could show that IL-2 restored *in vivo*-repressed cytokine secretion and cytotoxicity of CD8-TILs and NK-TILs. In CD8-TILs, functional recovery occurred concomitantly with a decrease in DGK-α and an increase in basal and stimulation-induced phosphorylation of key signaling proteins (ERK, AKT). In NK-TILs, IL-2 also restored activity; here, no change in DGK-α protein was observed suggesting direct regulation of ERK-phosphorylation, which is in accordance to published literature (Kondadasula et al., 2008).

We used the commercial DGK-inhibitor R-59022 and were able to document restored degranulation of CD8-TILs and NK-TILs, and, concomitantly, stronger ERK-phosphorylation, thus linking DGK-α to suppressed ERK-phosphorylation and inhibited degranulation. Of note is that the level of degranulation of TILs in the presence of DGK-inhibition was not higher than that observed with NK-NILs or CD8-NILs indicating

that DGK-inhibition can restore suppressed degranulation but does not augment degranulation beyond an NK or T cell's intrinsic response efficacy. This finding helps alleviate concerns about potentially unleashing undesirable autoimmunity through DGK-inhibition, which is an important issue when considering potential targeting of DGK in a clinical setting.

DGK-INHIBITORS TO COLLABORATE FOR EFFECTIVE CANCER IMMUNOTHERAPY

DGKs, expressed in T and NK cells, are attractive targets for immunotherapy considering their physiologic function in regulating strength and duration of signaling cascades important for T- and NK-cell function. Observing that DGK-α and DKG-ζ are exploited by cancer cells to suppress the activity of cytotoxic immunocytes in the tumor microenvironment encourages the idea that DGK-inhibitors might enrich the current cancer immunotherapy armamentarium. Indeed, in experimental settings, T- and NK-cell activity can be enhanced and anergy development can be prevented through deletion or inhibition of DGK-α or DGK-ζ (Riese et al., 2011, 2013, 2016; Martínez-Moreno et al., 2012; Yang et al., 2016). Our results with TILs from human RCC further suggest that DGK-inhibition may not only prevent development of unresponsiveness but may also be able to restore activity of suppressed immune cells. Importantly, DGK-α inhibition can restore the function of CD8-T cells and NK cells. NK cells can destroy tumor cells with low or no MHC-class I proteins that may develop as escape variants after successful T-cell therapy and can be the cause of treatment failure. Development of escape variants may well be prevented in therapeutic settings that activates and maintains NK-cell function conjointly to the activation of a T-cell response (Fruci et al., 2013).

The multifactorial nature of tumor-induced unresponsiveness necessitates the application of multiple means to fully unleash the power of immunotherapy. Anergy has to be recognized as part of the inactivation process and, DGKs as its mediators, should be considered as an additional checkpoint controlling T-cell and NK-cell function, in addition to the currently appreciated co-inhibitory checkpoints (PD-1, LAG-3, TIM-3, CTLA-4) (**Figure 2A**). The classical co-inhibition molecules interrupt the signaling cascade at proximal steps (Lck, ZAP70, PI3K/AKT), while anergy-associated blockades are located further downstream. Thus, when co-inhibition is therapeutically alleviated through anti-PD-1/PD-L1 or anti-CTLA-4, signaling will still be halted by paucity of DAG through high DGKs (**Figure 2B**). Thus, if T-cell non-responsiveness (also) involves high DGK, it is expected that releasing distal signaling blockades, i.e., through DGK-inhibition, is required in addition to checkpoint blockade therapy to fully reverse T-cell suppression (**Figure 2C**). Currently, the combined application of checkpoint antibodies and DGK-inhibition is a theoretical concept and awaits supportive data from experimental models.

DGK-inhibition may further improve immunotherapy considering that CAR-T cells lacking DGK-ζ were found to be resistant to the suppressive cytokine TGF-ß (Riese et al., 2013; Arumugam et al., 2015). The molecular basis for the cross-talk between the two signaling cascades remains to be resolved. One explanation could be a digital conversion of ERK-phosphorylation to function, whereby function is enabled if ERK-phosphorylation is above a certain threshold (outlined also by Prinz et al., 2012). Higher ERK-phosphorylation reached through DGK-inhibition may enable T cells to maintain phospho-ERK levels above the threshold required for function in the presence of other suppressive signals.

FURTHER CONSIDERATIONS FOR THE DEVELOPMENT OF DGK-INHIBITION FOR IMMUNOTHERAPY

DGK-inhibition has promising feature for immunotherapy. T and NK cells express two isoforms, DGK-α and DGK-ζ, which both regulate effector-lymphocyte function through controlling DAG-abundance. Will it thus suffice to inhibit only one isoform to help T and NK cells maintain function in the tumor milieu, or is the inhibition of both required?

DGK-α and DGK-ζ activities are comprehensively discussed in recent reviews, and thus are only briefly touched here (Merida et al., 2015; Chen et al., 2016; Singh and Kambayashi, 2016). As specific inhibitors of DGK-ζ are not available, the issue which one of the isoforms or whether both should be preferably inhibited to support T and NK cells in the tumor microenvironment cannot be adequately addressed at the moment. Data generated with knock-out mice are of limited information concerning effects of DGKs in the effector phase of the immune response since disturbances in the development, in particular observed development of hyporesponsive NK cells and altered development of regulatory T cells (T*regs*), may obscure effects that DGK-inhibition might have when applied to the already developed immune system. Inhibition experiments need to be performed using immune cells from the tumor environment, since here the immune escape processes are manifested that are the target of immunotherapy. For clinical extrapolation, experiments need to utilize human immune cells as significant differences exist, previously discussed by us and others (Prinz et al., 2012; Moon et al., 2014). Anergy-inducing conditions might arise much more frequently in humans, since CD8-T cell effector differentiation causes CD28 loss in humans (but not in mouse) which deprives human CD8-T cells from receiving co-stimulation. This may moderate the extent to which DGK-α or DGK-ζ participates in the regulation of DAG-mediated pathways in human and mouse models.

Different structural designs of DGK-α and DKG-ζ and accordingly different modes of activation allow some speculation as to which isoform possibly contributes more to the regulation of DAG-mediated signals in a specific situation. DGK-α, containing a calcium-binding EF-hand motif, is activated through Ca^{2+} ions, in addition to Lck-mediated phosphorylation, while DKG-ζ, lacking the calcium-binding EF motif, is not responsive to calcium signals and is activated through protein kinase C (PKC)-mediated phosphorylation. In physiologic situation where TCR-activation occurs concomitantly with co-stimulation, DKG-ζ may play the dominant role. In situations, however, where

the co-stimulatory pathway is not provided, DGK-α will be disproportionally activated through Ca-induced conformational changes and Lck-dependent phosphorylation. TCR-stimulation without co-stimulation commonly occurs during effector phase activation of CD8-T cells in epithelial tissue or carcinomas due to the paucity of CD28 on human CD8-T effector cells and the lack of co-stimulatory ligands in epithelial tissues. Evidence for this scenario is seen in TILs of RCC that failed to activate the AKT pathway after TCR-stimulation while Lck- and PLCγ-activation occurred normally. Thus, the necessary signals (Lck, Ca^{2+}) for DGK-α-activation are provided, with ensuing depletion of DAG and attenuation of effector activity. Extending on this, one might speculate that DGK-α is more relevant isoform to be targeted in cancer immunotherapy.

However, DKG-ζ was not analyzed in TILs due to the lack of specific reagents. Results from adoptive transfer studies using human CAR-T cells showed that DGK-α and DKG-ζ were upregulated in hyporesponsive CAR-T cells recovered from the tumor milieu of human xenografts (Moon et al., 2014). Here again DGK-ζ specific inhibition was not performed; thus, it remains to be addressed to which extent DKG-ζ overexpression contributed to the hypofunctional state of the CAR-T cells.

Considering DGK-inhibition for cancer immunotherapy attention should also be given to possible effects on other immune cells in the tumor microenvironment and the cancer cells themselves. DGK-α and DGK-ζ have effects on macrophages, dendritic cells and T*reg*s (Singh and Kambayashi, 2016). In macrophages and dendritic cells, DKG-ζ deficiency was found to be associated with impaired secretion of inflammatory IL-12 and TNF and impaired Th1-responses. Both isoforms were reported to inhibit the suppressive activity of T*reg*s. Considering the required function of myeloid cells and T*reg*s in antitumor response, DGK-inhibition (independent of isoform) may not yield desirable outcome. However, it has to be noted that none of the experiments were performed with tumor-educated cell types, thus the outcome of DGK-inhibition on antitumor immunity of myeloid cells and T*regs* remains an open question.

Concerning effects on cancer cells, contrasting outcomes are reported for DGK-α and DKG-ζ in the regulation of the NFκB-pathway, with DGK-α providing activation and DGK-ζ being inhibitory under inflammatory conditions (Tsuchiya et al., 2015). In such scenario, DGK-α inhibition would be a preferable intervention. Along this line, another study reported suppression of oncogenic survival pathways through DGK-α inhibition causing tumor cell death *in vitro* and reducing tumor growth in mice (Dominguez et al., 2013).

Collectively, DGK-inhibition has promising feature for cancer immunotherapy on multiple levels, re-invigorating T and NK cells for tumor cell attack, possibly making them resistant to TGF-ß suppression, and also weakening tumor cells directly. As DGKs and co-inhibitory surface proteins (PD-1, CTLA-4) control different steps in the signaling cascade, it is expected that DGK-inhibition will combine beneficially with current checkpoint blockade therapies or other immunotherapies. Further development is needed in the field of specific DGK-inhibitors (Sakane et al., 2016) and side-by-side comparisons of DGK-α and DGK-ζ inhibition to delineate the specific contribution that each of the isoforms might have in the restoration or maintenance of immune cell function in the tumor environment.

ETHICS STATEMENT

The human material was collected as part of the surgical procedure and only left-over material was used for research. The patients gave informed consent that their material may be used for research. The material for research was given anonymously; therefore, the study is considered ethically unobjectionable.

AUTHOR CONTRIBUTIONS

EN wrote the article and performed the literature search.

ACKNOWLEDGMENTS

Funding was provided through the Deutsche Forschungsgemeinschaft SFB-TR36, Deutsche Krebshilfe (110370) and the Erich und Gertrud von Roggenbuck Stiftung (218-15).

REFERENCES

Arumugam, V., Bluemn, T., Wesley, E., Schmidt, A. M., Kambayashi, T., Malarkannan, S., et al. (2015). TCR signaling intensity controls $CD8^+$ T cell responsiveness to TGF-ß. *J. Leukoc. Biol.* 98, 703–712. doi: 10.1189/jlb.2HIMA1214-578R

Beyer, M., Karbach, J., Mallmann, M. R., Zander, T., Eggle, D., Classen, S., et al. (2009). Cancer vaccine enhanced, non-tumor-reactive $CD8^+$ T cells exhibit a distinct molecular program associated with "division arrest anergy". *Cancer. Res.* 69, 4346–4354. doi: 10.1158/0008-5472.CAN-08-3796

Callahan, M. K., Postow, M. A., and Wolchok, J. D. (2016). Targeting T Cell Co-receptors for Cancer Therapy. *Immunity* 44, 1069–1078. doi: 10.1016/j.immuni.2016.04.023

Chen, S. S., Hu, Z., and Zhong, X. P. (2016). Diacylglycerol kinases in T cell tolerance and effector function. *Front. Cell Dev. Biol.* 4:130. doi: 10.3389/fcell.2016.00130

Dominguez, C. L., Floyd, D. H., Xiao, A., Mullins, G. R., Kefas, B. A., Xin, W., et al. (2013). Diacylglycerol kinase α is a critical signaling node and novel therapeutic target in glioblastoma and other cancers. *Cancer Discov.* 3, 782–797. doi: 10.1158/2159-8290.CD-12-0215

Dunn, G. P., Old, L. J., and Schreiber, R. D. (2004). The three Es of cancer immunoediting. *Annu. Rev. Immunol.* 22, 329–360. doi: 10.1146/annurev.immunol.22.012703.104803

Eckl, J., Buchner, A., Prinz, P. U., Riesenberg, R., Siegert, S. I., Kammerer, R., et al. (2012). Transcript signature predicts tissue NK cell content and defines renal cell carcinoma subgroups independent of TNM staging. *J. Mol. Med.* 90, 55–66. doi: 10.1007/s00109-011-0806-7

Fesnak, A. D., June, C. H., and Levine, B. L. (2016). Engineered T cells: the promise and challenges of cancer immunotherapy. *Nat. Rev. Cancer* 16, 566–581. doi: 10.1038/nrc.2016.97

Frey, A. B., and Monu, N. (2006). Effector-phase tolerance: another mechanism of how cancer escapes antitumor immune response. *J. Leukoc. Biol.* 79, 652–662. doi: 10.1189/jlb.1105628

Fridman, W. H., Pagès, F., Sautès-Fridman, C., and Galon, J. (2012). The immune contexture in human tumours: impact on clinical outcome. *Nat. Rev. Cancer* 12, 298–306. doi: 10.1038/nrc3245

Fruci, D., Lo Monaco, E., Cifaldi, L., Locatelli, F., Tremante, E., Benevolo, M., et al. (2013). T and NK cells: two sides of tumor immunoevasion. *J. Transl. Med.* 11:30. doi: 10.1186/1479-5876-11-30

Gajewski, T. F., Meng, Y., Blank, C., Brown, I., Kacha, A., Kline, J., et al. (2006). Immune resistance orchestrated by the tumor microenvironment. *Immunol. Rev.* 213, 131–145. doi: 10.1111/j.1600-065X.2006.00442.x

Gross, G., and Eshhar, Z. (2016). Therapeutic potential of T cell chimeric antigen receptors (CARs) in cancer treatment: counteracting off-tumor toxicities for safe CAR T cell therapy. *Annu. Rev. Pharmacol. Toxicol.* 56, 59–83. doi: 10.1146/annurev-pharmtox-010814-124844

Herbst, R. S., Soria, J. C., Kowanetz, M., Fine, G. D., Hamid, O., Gordon, M. S., et al. (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 515, 563–567. doi: 10.1038/nature14011

Imai, N., Ikeda, H., Tawara, I., and Shiku, H. (2009). Tumor progression inhibits the induction of multifunctionality in adoptively transferred tumor-specific $CD8^+$ T cells. *Eur. J. Immunol.* 39, 241–253. doi: 10.1002/eji.200838824

Janicki, C. N., Jenkinson, S. R., Williams, N. A., and Morgan, D. J. (2008). Loss of CTL function among high-avidity tumor-specific $CD8^+$ T cells following tumor infiltration. *Cancer Res.* 68, 2993–3000. doi: 10.1158/0008-5472.CAN-07-5008

Kondadasula, S. V., Varker, K. A., Lesinski, G. B., Benson, D. M. Jr., Lehman, A., Olencki, T., et al. (2008). Activation of extracellular signaling regulated kinase in natural killer cells and monocytes following IL-2 stimulation *in vitro* and in patients undergoing IL-2 immunotherapy: analysis via dual parameter flow-cytometric assay. *Cancer Immunol. Immunother.* 57, 1137–1149. doi: 10.1007/s00262-007-0444-7

Macian, F., Garcia-Cozar, F., Im, S. H., Horton, H. F., Byrne, M. C., and Rao, A. (2002). Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* 109, 719–731. doi: 10.1016/S0092-8674(02)00767-5

Martínez-Moreno, M., García-Liévana, J., Soutar, D., Torres-Ayuso, P., Andrada, E., Zhong, X. P., et al. (2012). FoxO-dependent regulation of diacylglycerol kinase α gene expression. *Mol. Cell. Biol.* 32, 4168–4180. doi: 10.1128/MCB.00654-12

McDermott, D. F. (2009). Immunotherapy of metastatic renal cell carcinoma. *Cancer* 115, 2298–2305. doi: 10.1002/cncr.24236

Merida, I., Andrada, E., Gharbi, S. I., and Avila-Flores, A. (2015). Redundant and specialized roles for diacylglycerol kinases a and z in the control of T cell functions. *Sci. Signal.* 8:re6. doi: 10.1126/scisignal.aaa0974

Moon, E. K., Wang, L. C., Dolfi, D. V., Wilson, C. B., Ranganathan, R., Sun, J., et al. (2014). Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor-transduced human T cells in solid tumors. *Clin. Cancer Res.* 20, 4262–4273. doi: 10.1158/1078-0432.CCR-13-2627

Nakano, O., Sato, M., Naito, Y., Suzuki, K., Orikasa, S., Aizawa, M., et al. (2001). Proliferative activity of intratumoral $CD8^+$ T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res.* 61, 5132–5136.

Newick, K., O'Brien, S., Moon, E., and Albelda, S. M. (2016). CAR T cell therapy for solid tumors. *Annu. Rev. Med.* 68, 139–152. doi: 10.1146/annurev-med-062315-120245

Papaioannou, N. E., Beniata, O. V., Vitsos, P., Tsitsilonis, O., and Samara, P. (2016). Harnessing the immune system to improve cancer therapy. *Ann. Transl. Med.* 4:261. doi: 10.21037/atm.2016.04.01

Pardoll, D. M. (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* 12, 252–264. doi: 10.1038/nrc3239

Park, J. H., Geyer, M. B., and Brentjens, R. J. (2016). CD19-targeted CAR T-cell therapeutics for hematologic malignancies: interpreting clinical outcomes to date. *Blood* 127, 3312–3320. doi: 10.1182/blood-2016-02-629063

Posey, A. D. Jr., Schwab, R. D., Boesteanu, A. C., Steentoft, C., Mandel, U., Engels, B., et al. (2016). Engineered CAR T cells targeting the cancer-associated Tn-glycoform of the membrane mucin MUC1 control adenocarcinoma. *Immunity* 44, 1444–1454. doi: 10.1016/j.immuni.2016.05.014

Postow, M. A., Callahan, M. K., and Wolchok, J. D. (2015). Immune checkpoint blockade in cancer therapy. *J. Clin. Oncol.* 33, 1974–1982. doi: 10.1200/JCO.2014.59.4358

Prinz, P. U., Mendler, A. N., Brech, D., Masouris, I., Oberneder, R., and Noessner, E. (2014). NK-cell dysfunction in human renal carcinoma reveals diacylglycerol kinase as key regulator and target for therapeutic intervention. *Int. J. Cancer* 135, 1832–1841. doi: 10.1002/ijc.28837

Prinz, P. U., Mendler, A. N., Masouris, I., Durner, L., Oberneder, R., and Noessner, E. (2012). High DGK-α and disabled MAPK pathways cause dysfunction of human tumor-infiltrating $CD8^+$ T cells that is reversible by pharmacologic intervention. *J. Immunol.* 188, 5990–6000. doi: 10.4049/jimmunol.1103028

Quann, E. J., Merino, E., Furuta, T., and Huse, M. (2009). Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat. Immunol.* 10, 627–635. doi: 10.1038/ni.1734

Ribas, A., and Hu-Lieskovan, S. (2016). What does PD-L1 positive or negative mean? *J. Exp. Med.* 213, 2835–2840. doi: 10.1084/jem.20161462

Riese, M. J., Grewal, J., Das, J., Zou, T., Patil, V., Chakraborty, A. K., et al. (2011). Decreased diacylglycerol metabolism enhances ERK activation and augments $CD8^+$ T cell functional responses. *J. Biol. Chem.* 286, 5254–5265. doi: 10.1074/jbc.M110.171884

Riese, M. J., Moon, E. K., Johnson, B. D., and Albelda, S. M. (2016). Diacylglycerol kinases (DGKs): novel targets for improving T cell activity in cancer. *Front. Cell Dev. Biol.* 4:108. doi: 10.3389/fcell.2016.00108

Riese, M. J., Wang, L. C., Moon, E. K., Joshi, R. P., Ranganathan, A., June, C. H., et al. (2013). Enhanced effector responses in activated $CD8^+$ T cells deficient in diacylglycerol kinases. *Cancer Res.* 73, 3566–3577. doi: 10.1158/0008-5472.CAN-12-3874

Rosenberg, S. A., and Restifo, N. P. (2015). Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* 348, 62–68. doi: 10.1126/science.aaa4967

Sakane, F., Mizuno, S., and Komenoi, S. (2016). Diacylglycerol kinases as emerging potential drug targets for a variety of diseases: an update. *Front. Cell Dev. Biol.* 4:82. doi: 10.3389/fcell.2016.00082

Sharma, P., and Allison, J. P. (2015). The future of immune checkpoint therapy. *Science* 348, 56–61. doi: 10.1126/science.aaa8172

Singh, B. K., and Kambayashi, T. (2016). The immunomodulatory functions of diacylglycerol kinase zeta. *Front. Cell Dev. Biol.* 4:96. doi: 10.3389/fcell.2016.00096

Sunshine, J., and Taube, J. M. (2015). PD-1/PD-L1 inhibitors. *Curr. Opin. Pharmacol.* 23, 32–38. doi: 10.1016/j.coph.2015.05.011

Tsuchiya, R., Tanaka, T., Hozumi, Y., Nakano, T., Okada, M., Tophamc, M. K., et al. (2015). Downregulation of diacylglycerol kinase ζ enhances activation of cytokine-induced NF-κB signaling pathway. *Biochim. Biophys. Acta* 1853, 361–369. doi: 10.1016/j.bbamcr.2014.11.011

Tumeh, P. C., Harview, C. L., Yearley, J. H., Shintaku, I. P., Taylor, E. J., Robert, L., et al. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 515, 568–571. doi: 10.1038/nature13954

van der Burg, S. H., Arens, R., Ossendorp, F., van Hall, T., and Melief, C. J. (2016). Vaccines for established cancer: overcoming the challenges posed by immune evasion. *Nat. Rev. Cancer* 16, 219–233. doi: 10.1038/nrc.2016.16

Wherry, E. J., Ha, S. J., Kaech, S. M., Haining, W. N., Sarkar, S., Kalia, V., et al. (2007). Molecular signature of $CD8^+$ T cell exhaustion during chronic viral infection. *Immunity* 27, 670–684. doi: 10.1016/j.immuni.2007.09.006

Yang, E., Singh, B. K., Paustian, A. M., and Kambayashi, T. (2016). Diacylglycerol kinase ζ is a target to enhance NK cell function. *J. Immunol.* 197, 934–941. doi: 10.4049/jimmunol.1600581

Zheng, Y., Zha, Y., and Gajewski, T. F. (2008). Molecular regulation of T-cell anergy. *EMBO Rep.* 9, 50–55. doi: 10.1038/sj.embor.7401138

Zhong, X. P., Guo, R., Zhou, H., Liu, C., and Wan, C. K. (2008). Diacylglycerol kinases in immune cell function and self-tolerance. *Immunol. Rev.* 224, 249–264. doi: 10.1111/j.1600-065X.2008.00647.x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Cell and Developmental Biology

MINI REVIEW
published: 17 October 2016
doi: 10.3389/fcell.2016.00108

Diacylglycerol Kinases (DGKs): Novel Targets for Improving T Cell Activity in Cancer

Matthew J. Riese[1,2], Edmund K. Moon[3], Bryon D. Johnson[4] and Steven M. Albelda[3]**

[1] Division of Hematology/Oncology, Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, USA, [2] Blood Center of Wisconsin, Blood Research Institute, Milwaukee, WI, USA, [3] Division of Pulmonary, Allergy, and Critical Care, Department of Medicine, University of Pennsylvania, Philadelphia, PA, USA, [4] Division of Hematology/Oncology/Transplant, Department of Pediatrics, Medical College of Wisconsin, Milwaukee, WI, USA

Diacylglycerol kinases (DGKs) are a family of enzymes that catalyze the metabolism of diacylglycerol (DAG). Two isoforms of DGK, DGKα, and DGKζ, specifically regulate the pool of DAG that is generated as a second messenger after stimulation of the T cell receptor (TCR). Deletion of either isoform in mouse models results in T cells bearing a hyperresponsive phenotype and enhanced T cell activity against malignancy. Whereas, DGKζ appears to be the dominant isoform in T cells, rationale exists for targeting both isoforms individually or coordinately. Additional work is needed to rigorously identify the molecular changes that result from deletion of DGKs in order to understand how DAG contributes to T cell activation, the effect of DGK inhibition in human T cells, and to rationally develop combined immunotherapeutic strategies that target DGKs.

Keywords: diacylglycerol, diacylglycerol kinase, immunotherapy, CD8+ T cell, T cell receptor

OPEN ACCESS

Edited by:
Isabel Merida, Spanish National Research Council, Spain

Reviewed by:
Narayanan Parameswaran, Michigan State University, USA
Ignacio Rubio, University of Jena, Germany
Gianluca Baldanzi, Università Piemonte Orientale, Italy

****Correspondence:***
Matthew J. Riese
matthew.riese@bcw.edu
Steven M. Albelda
albelda@mail.med.upenn.edu

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *28 July 2016*
Accepted: *14 September 2016*
Published: *17 October 2016*

Citation:
Riese MJ, Moon EK, Johnson BD and Albelda SM (2016) Diacylglycerol Kinases (DGKs): Novel Targets for Improving T Cell Activity in Cancer. Front. Cell Dev. Biol. 4:108. doi: 10.3389/fcell.2016.00108

DIACYLGLYCEROL KINASES IN T CELL RECEPTOR SIGNAL TRANSDUCTION

Diacylglycerol kinases (DGKs) represent a family of enzymes that catalyze phosphorylation of the membrane lipid sn-1,2 diacylglycerol (DAG) to form phosphatidic acid (PA) (Eichmann and Lass, 2015). In T cells, DAG is formed downstream of the T cell receptor (TCR) after activation of the gamma 1 isoform of phospholipase C (PLCγ1) and cleavage of phosphatidylinositol 4,5-biphosphate (PIP_2) into DAG and an additional second messenger, inositol 1,4,5-triphosphate (IP_3) (Krishna and Zhong, 2013). Whereas, IP_3 is important in facilitating release of calcium from the endoplasmic reticulum, DAG interacts with other proteins important in TCR signal transduction, such as Protein kinase C (predominantly θ isoform in T cells, but also isoforms ε and η; Quann et al., 2011) and the Ras activating protein RasGRP1 (Krishna and Zhong, 2013). Biochemically, targeting the activity of DGKs in T cells, either by germline deletion, or with chemical inhibitors, results in enhanced and sustained signaling downstream of T cells, as assessed by prolonged phosphorylation of downstream molecules, such as extracellular signal-related kinases 1/2 (ERK1/2; Zhong et al., 2003; Olenchock et al., 2006; Riese et al., 2011). Although, three isoforms of DGK are known to be present within T cells (DGKα, DGKδ, and DGKζ), only two, DGKα and DGKζ, are thought to play an important role in facilitating DAG metabolism downstream of the TCR (Joshi and Koretzky, 2013). The function of DGKδ is unknown in T cells; its role in facilitating metabolic flexibility between lipid and carbohydrate utilization suggest that it may regulate pools of DAG unrelated to TCR signaling (Chibalin et al., 2008). The signaling

changes resulting from the absence of DGKα or DGKζ alter the transcriptional program of activated T cells. For instance, generation of the transcription factor AP1, which is dependent on Ras/ERK signaling, is decreased in stimulated Jurkat T cells overexpressing DGKζ (Zhong et al., 2002). Similarly, NF-κB (nuclear factor kappa-ligh-chain-enhancer of activated B cells), a critical transcription factor activated downstream of PKCθ in T cells, is present at increased levels after stimulation of DGKζ-deficient lymphocytes as compared to DGKζ-replete cells (Schmidt et al., 2013), though other data suggests that the regulation of NF-κB may, in some instances, be positively regulated by DGKs (Yang et al., 2016).

The change in activation of transcription factors in stimulated T cells after manipulation of DGK activity correlates with changes in T cell activation markers and function. For instance, overexpression of DGKα or DGKζ in Jurkat T cells results in decreased expression of the activation marker CD69 after stimulation through the TCR complex (Sanjuán et al., 2001; Zhong et al., 2002), and overexpression of DGKα induces a state of decreased functional activity resembling an anergy-like state (Zha et al., 2006). In contrast, deletion of DGKα or DGKζ results in T cells with enhanced production of effector cytokines, such as IL2 and IFNγ, and enhanced proliferation (Zhong et al., 2003; Olenchock et al., 2006). Inhibition of DGKα also allows T cells to overcome TCR signaling defects present in human X-linked lymphoproliferative disease (XLP-1) resulting from the loss of SAP [signaling lymphocytic activation molecule (SLAM)-associated protein (Baldanzi et al., 2011; Ruffo et al., 2016)], along with uncontrolled effector T cell expansion after exposure to Epstein Barr virus (EBV) characteristic of this disease (Ruffo et al., 2016).

TARGETING DIACYLGLYCEROL KINASES TO ENHANCE T CELL ANTI-TUMOR ACTIVITY

Given the enhanced functional activity conferred by loss of DGKs in T cells, our group and others have tested the hypothesis that these proteins may serve as useful targets for enhancing T cell anti-tumor activity. Recently, strategies to target negative regulators of T cells to enhance their anti-tumor activity have been successfully translated from basic science studies into clinical care (Byrne et al., 2015; Sharma and Allison, 2015; Shin and Ribas, 2015; Callahan et al., 2016). Although, antibodies directed against CTLA-4 and PD-1 are the most prominent examples of therapies that have generated clinical responses in human malignancy, there is significant interest in identifying additional inhibitory regulators of T cells to combine with existing approaches and to use in instances where blockade of PD-1 and other immune checkpoints is ineffective (Restifo et al., 2016). We focused our studies on DGKζ, since that enzyme appears to represent the dominant isoform in T cells, based on a direct comparison of TCR signal strength between T cells deficient in either DGKα or DGKζ (Joshi et al., 2013). Using an EL4-ova subcutaneous model system to permit tracking of immune responses, we observed that DGKζ$^{-/-}$ mice had an increased frequency of tumor rejection, along with a trend toward increased number of tumor-specific CD8^{+} T cells (Riese et al., 2011). Additionally, we demonstrated that adoptively transferred naïve (Riese et al., 2011) or activated (Riese et al., 2013) tumor-specific effector T cells displayed increased activation by tumor and resultant inhibition of tumor growth. While these studies relied on strong-antigen driven tumor models, it is likely that loss of DGKs also enhances T cell anti-tumor activity in tumors with low-grade antigens, since DAG-mediated activation of RasGRP1 regulates the threshold for T cell activation (Das et al., 2009), and earlier studies implicated a role for Ras in Jurkat T cell activation mediated by low grade TCR stimulus (Perez de Castro et al., 2004).

DGKα has also been evaluated as a potential target to improve T cell activity against tumor, based on the observation that DGKα is upregulated in certain inhibited T cell conditions, such as anergy (Zha et al., 2006), and that DGKα is upregulated in tumor-infiltrating lymphocytes in human renal cell carcinoma (Prinz et al., 2012). For instance, our own study using adoptive transfer of CAR (chimeric antigen receptor)-T cells demonstrated similar increases in efficacy (compared to wild type T cells) between T cells deficient in DGKα or DGKζ in the treatment of murine mesothelioma (Riese et al., 2013). Additionally, a study testing the importance of DGKα in glioblastoma multiforme (GBM) cells found that concurrent administration of the relatively non-specific DGKα inhibitor R59022 resulted in decreased growth of intracranially injected GBM tumors. Although, a preponderance of evidence suggested that the decreased tumor growth in this model resulted from inhibition of DGKα within the tumor cells, modulation of immune activity was not assessed, and could have been contributory (Dominguez et al., 2013). DGKs also play a role in limiting the activity of NK cells isolated from tumors in patients with renal cell carcinoma, since the addition of either IL-2 or DGK inhibitor (R59022) to culture media improves the impaired function of tumor-associated NK cells (Prinz et al., 2014). Recently, a more specific inhibitor for DGKα has been developed that may be useful to extend these studies into additional tumor models (Liu et al., 2016). Although a direct comparison has not to-date been performed comparing tumor growth in DGKα$^{-/-}$ and DGKζ$^{-/-}$ mice, future studies will undoubtedly provide additional comparisons between the two genotypes with respect to T cell anti-tumor immunity.

MECHANISM OF ENHANCED T CELL ANTI-TUMOR ACTIVITY IN DGKζ-DEFICIENT T CELLS

Enhanced anti-tumor activity observed in DGKζ-deficient T cells was initially thought to result from increased cytokine production generated after TCR stimulation; however, it is clear that intrinsic insensitivity to inhibitory signals in the tumor microenvironment is also an important determinant. In broad terms, T cell inhibitory factors can be broadly separated into two groups: those that inhibit T cells by directly inhibiting proximal TCR signal transduction, and those that inhibit T cells independent of attenuation of TCR signaling. Examples

of immunosuppressant pathways that facilitate direct inhibition of TCR signaling include PD-1 (Chemnitz et al., 2004; Parry et al., 2005; Yokosuka et al., 2012), Lag3 (Okazaki et al., 2011), Prostglandin E_2 (PGE_2) (Wehbi and Taskén, 2016), adenosine 2A receptor (Linnemann et al., 2009; Linden and Cekic, 2012), and as we have recently identified, TGFβ (Arumugam et al., 2015; Newman et al., 2016). Whereas, PD-1 and TGFβ inhibit TCR signaling by directly or indirectly recruiting inhibitory tyrosine phosphatases, such as SHP-1 or SHP-2 (Src homology region 2 domain-containing phosphatase-1/2) to the cell surface, adenosine 2A, and PGE_2 receptors activate protein kinase A (PKA) leading to Csk-mediated inhibition of the proximal activating tyrosine kinase Lck (Newick et al., 2016). In contrast, Lag3 appears to oppose TCR signaling via a KIEELE motif that acts through an unclear mechanism (Freeman and Sharpe, 2012). In either case, these inhibitory factors act to oppose activation events facilitated by proximal tyrosine protein kinases, such as Lck or Zap-70, that are responsible for initiating TCR signal cascades and are upstream of DAG generation. In contrast are inhibitory factors that act predominantly by inhibiting T cells independent of TCR activation and DAG generation. These include a subset of immune checkpoint receptors, such as Tim3 (Jones et al., 2008; Lee et al., 2011), and CTLA-4. Although some reports have suggested direct proximal inhibition of TCR mediated by CTLA-4 (Lee et al., 1998), CTLA-4 more likely functions predominantly as a sink to sequester CD80 and CD86, the ligands of the co-stimulatory molecule CD28 (Green et al., 1994; Walunas et al., 1996; van der Merwe et al., 1997; Collins et al., 2002). T cells deficient in DGKs may demonstrate differential sensitivity to inhibition mediated by dependently or independently of TCR signaling (**Figure 1**, **Table 1**). For instance, a test of numerous T cell inhibitory pathways revealed that, in contrast to wild type T cells, T cells lacking DGKζ demonstrate reduced inhibition of IFNγ production in the presence of high concentrations of TGFβ, Prostaglandin E_2 (PGE_2) or adenosine (Riese et al., 2013). This suggests that one can predict, in broad terms, how DGK-deficient T cells will respond to various immunosuppressive pathways within the tumor microenvironment, in that one would anticipate DGK-deficient T cells to be insensitive to inhibitors that directly attenuate TCR signaling, such as PD-1 or Lag3, but sensitive to inhibitory pathways that do not directly interfere with TCR signaling, such as Tim3 or CTLA-4. This model also predicts which immune targets might be optimally paired with agents that block DGKs, such that one would anticipate little synergy with direct regulators of TCR signaling, but potential excellent synergy with TCR-independent inhibitors. Experiments are currently ongoing to test these hypotheses.

POTENTIAL ISSUES ARISING FROM TARGETING DGKS

The diverse set of cellular functions governed by DAG and PA, including signal transduction (Mérida et al., 2008), lipid biogenesis (Shulga et al., 2013), and membrane trafficking (Cho and Stahelin, 2005), presents challenges for broadly targeting DGK activity therapeutically, especially since the enzymatic specificity of the 10 mammalian DGKs is highly conserved, with the exception of DGKε (Jennings et al., 2015). Thus, isolation of a compound with specificity toward an individual isoform may prove difficult, a problem exacerbated by the paucity of structural information available about eukaryotic DGKs. Although, a prokaryotic form of DGK has been solved structurally (Li et al., 2013), it is sufficiently divergent from eukaryotic isoforms that it provides little value in predicting active site topography of eukaryotic DGKs. For instance, prokaryotic DGKA is not limited to enzymatic activity against lipids, but can also catalyze reactions using glycerol and water as substrates for phosphorylation (Ullrich et al., 2011). Therefore, approaches to target non-catalytic domains of DGKζ may provide the best means to achieve isoform specificity. The structural domains of DGKζ have been well defined, and include a C1 domain, a MARCKS domain, an ankyrin repeat domain, and a C-terminal PDZ-binding domain (Joshi and Koretzky, 2013). Of these domains, only the MARCKS domain and ankyrin repeat domain are unique to DGKζ among the 10 DGK family members (Joshi and Koretzky, 2013). Furthermore, only the MARCKS domain, a substrate for serine/threonine phosphorylation by PKCα (Topham et al., 1998), is required for DGKζ function (Santos et al., 2002). Thus, targeting the MARCKS domain may be an effective strategy for therapeutic targeting of DGKζ, in a manner that confers specificity.

Apart from concerns with isotype specificity, therapeutic targeting of DGKα and DGKζ could result in deleterious "on-target" effects, such as enhanced cellular proliferation and autoimmunity. As expected, mice with deletions of both DGKα and DGKζ generate T cells with enhanced TCR signaling downstream of DAG, as well as more potent effector functions after *in vitro* stimulation relative to single knockouts or wild type T cells (Guo et al., 2008; Riese et al., 2013). However, the activated level of Ras/Erk signal transduction in double knockout mice (DKO) results in thymic lymphomagenesis (Guo et al., 2008). This pro-malignant potential of enhanced DAG signaling is consistent with data from human patients with T cell acute lymphoblastic leukemia (T-ALL), in which RasGRP1, the Ras activating protein activated by DAG, has been found to be frequently overexpressed (Hartzell et al., 2013). Thus, caution must be used if DGK germline deletion (e.g., using CRISPR-based approaches) is used as a means to target DGKs in adoptive T cell therapies. Generating auto-immune disease may also be an issue when targeting DGKs. Although mice deficient in DGKα, DGKζ, or both do not develop overt autoimmunity, it is likely that the mice would develop enhanced T cell responses in autoimmune models such as experimental autoimmune encephalitis (EAE), especially given the role DGKs play in limiting activation of Mnk1/2-mediated activation and development of encephalitis in the EAE model (Gorentla et al., 2013). A possible explanation for the lack of overt autoimmunity in DGKζ or DKO mice may be the concurrent enhanced generation of natural T regs in these mice (Joshi et al., 2013), although this remains speculative, and raises the additional consideration that thymic development of T cells may be impacted by manipulation of DGK activity. For instance, constitutive expression of a membrane-bound form of DGKα

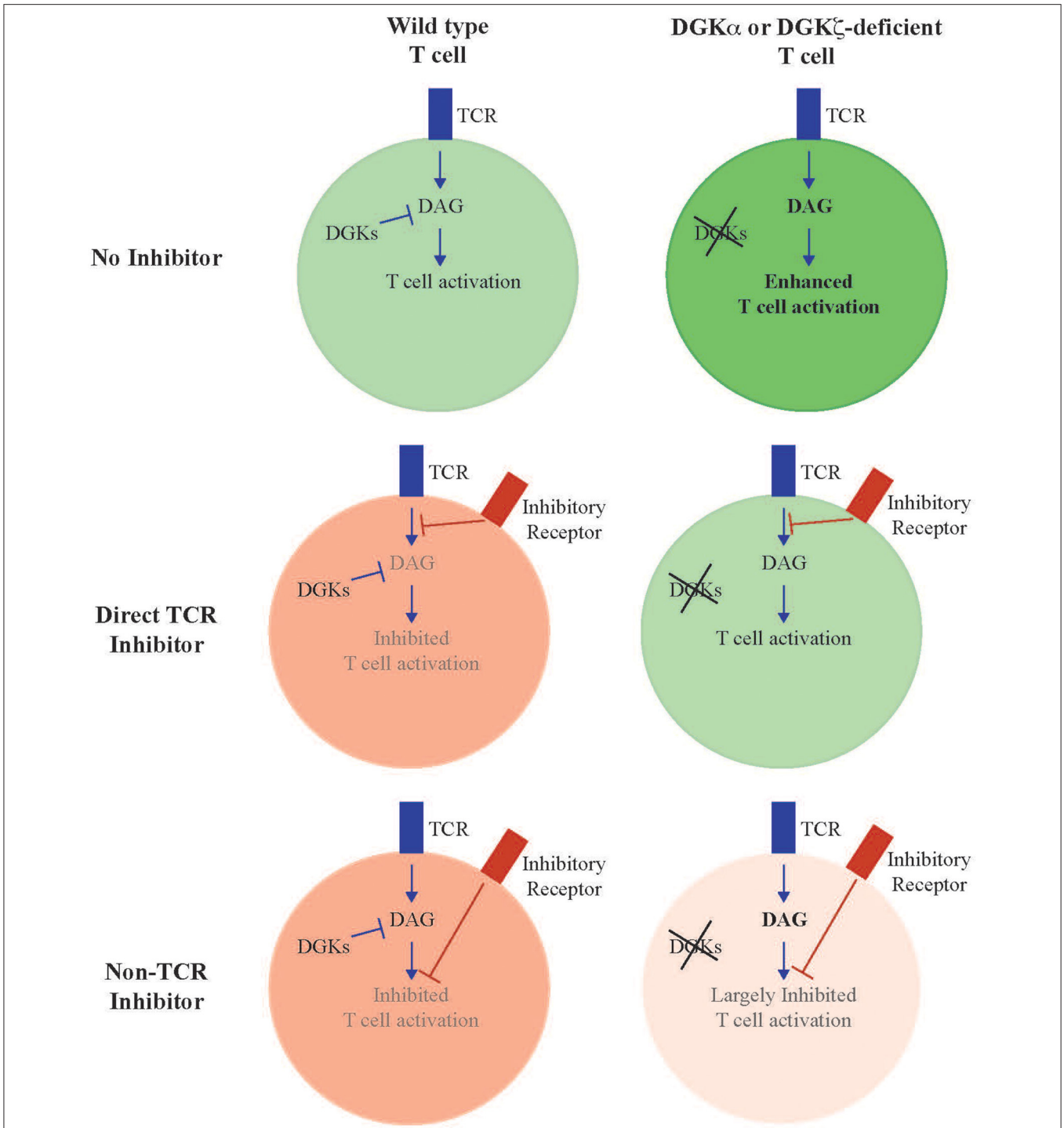


FIGURE 1 | Model of enhanced T cell activity in DGK-deficient T cells. DGKα and DGKζ function to metabolize DAG generated downstream of TCR activation. Deletion of either or both isoforms results in T cells with enhanced TCR signal transduction downstream of DAG and resultant enhanced effector functions ("No inhibitor"). Loss of DGK confers resistance to inhibitory pathways that function through direct interference of TCR signal transduction, since these inhibitory events occur proximal to generation of DAG ("Direct TCR Inhibitor"). We predict that T cells deficient in DGKs remain sensitive to inhibitory pathways that mediate their effect independent of interference with TCR signal transduction ("Non-TCR Inhibitor"), or for pathways that inhibit TCR signaling at rate limiting steps downstream of DAG formation.

TABLE 1 | Relative insensitivity to inhibitory stimuli of CD8+ T cells.

Inhibitory stimuli/receptor	Inhibitor-mediated fold ↓ in IFNγ by CD8+ T cell		Does inhibitory pathway directly impact TCR signaling?	References
	WT	DGKζ$^{-/-}$		
TGFβ	35x	1x	Yes	Arumugam et al., 2015
PGE_2	2x	1.5x	Yes	Riese et al., 2013
Adenosine	2x	1.2x	Yes	Riese et al., 2013
PD-1	4x	Unknown	Yes	Parry et al., 2005
Lag3	2x	Unknown	Yes	Hannier et al., 1998; Huang et al., 2015
Tim-3	None	Unknown	No	Lee et al., 2011
CTLA-4	2x	Unknown	Uncertain	Krummel and Allison, 1995; Walunas and Bluestone, 1998

leads to accumulation of immature CD8+ "single positive" T cells within the thymus in addition to peripheral lymphopenia (Almena et al., 2013), and DKO mice demonstrate a severe impairment in thymic development of invariant NKT cells, a subset of innate T cells (Shen et al., 2011). Ongoing experiments are needed to test whether DGK-deficient mice will develop more severe disease than wild type counterparts after autoimmune challenges, and to determine whether subtle alterations are present in the thymic development of other conventional T cell subsets.

CONCLUSIONS

As immunotherapeutic approaches come to the forefront of cancer treatment, there is an increased need to evaluate proteins and molecules that inhibit the immune system, especially in T cells. Diacylglycerol kinases should warrant a high degree of consideration in these targeting strategies, ideally with the development of small molecule inhibitors. In the meanwhile, it may be advantageous to move forward with gene manipulation in adoptive cellular therapies. The ability to very specifically target DGKs within only the transferred T cells will minimize systemic side effects. It will also be possible to include suicide genes to enable destruction of the transferred T cells, should it become necessary. A better understanding of the changes that result from acute and long-term targeting of DGKs should help discern the effectiveness of this strategy both alone, and in combination with other therapies designed to induce immune cell activation.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the manuscript and contributed writing to the paper.

FUNDING

Funding provided by NIH K08 CA151893 (MR), NIH K08 CA163941 (EM), American Cancer Society (MR), Kathy Duffey Fogerty Family Foundation (MR), HRHM Program of MACC Fund (BJ).

ACKNOWLEDGMENTS

The authors thank Subramaniam Malarkannan (Medical College of Wisconsin and Blood Center of Wisconsin, Milwaukee, WI) for critical review of the manuscript.

REFERENCES

Almena, M., Andrada, E., Liebana, R., and Merida, I. (2013). Diacylglycerol metabolism attenuates T-cell receptor signaling and alters thymocyte differentiation. *Cell Death Dis.* 4, e912. doi: 10.1038/cddis.2013.396

Arumugam, V., Bluemn, T., Wesley, E., Schmidt, A. M., Kambayashi, T., Malarkannan, S., et al. (2015). TCR signaling intensity controls CD8+ T cell responsiveness to TGF-β. *J. Leukoc. Biol.* 98, 703–712. doi: 10.1189/jlb.2HIMA1214-578R

Baldanzi, G., Pighini, A., Bettio, V., Rainero, E., Traini, S., Chianale, F., et al. (2011). SAP-mediated inhibition of diacylglycerol kinase α regulates TCR-induced diacylglycerol signaling. *J. Immunol.* 187, 5941–5951. doi: 10.4049/jimmunol.1002476

Byrne, K. T., Vonderheide, R. H., Jaffee, E. M., and Armstrong, T. D. (2015). Special conference on tumor immunology and immunotherapy: a new chapter. *Cancer Immunol. Res.* 3, 590–597. doi: 10.1158/2326-6066.CIR-15-0106

Callahan, M. K., Postow, M. A., and Wolchok, J. D. (2016). Targeting T cell co-receptors for cancer therapy. *Immunity* 44, 1069–1078. doi: 10.1016/j.immuni.2016.04.023

Chemnitz, J. M., Parry, R. V., Nichols, K. E., June, C. H., and Riley, J. L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J. Immunol.* 173, 945–954.

Chibalin, A. V., Leng, Y., Vieira, E., Krook, A., Björnholm, M., Long, Y. C., et al. (2008). Downregulation of diacylglycerol kinase delta contributes to hyperglycemia-induced insulin resistance. *Cell* 132, 375–386. doi: 10.1016/j.cell.2007.12.035

Cho, W., and Stahelin, R. V. (2005). Membrane-protein interactions in cell signaling and membrane trafficking. *Annu. Rev. Biophys. Biomol. Struct.* 34, 119–151. doi: 10.1146/annurev.biophys.33.110502.133337

Collins, A. V., Brodie, D. W., Gilbert, R. J. C., Iaboni, A., Manso-Sancho, R., Walse, B., et al. (2002). The interaction properties of costimulatory molecules revisited. *Immunity* 17, 201–210.

Das, J., Ho, M., Zikherman, J., Govern, C., Yang, M., Weiss, A., et al. (2009). Digital signaling and hysteresis characterize ras activation in lymphoid cells. *Cell* 136, 337–351. doi: 10.1016/j.cell.2008.11.051

Dominguez, C. L., Floyd, D. H., Xiao, A., Mullins, G. R., Kefas, B. A., Xin, W., et al. (2013). Diacylglycerol kinase α is a critical signaling node and novel therapeutic target in glioblastoma and other cancers. *Cancer Discov.* 3, 782–797. doi: 10.1158/2159-8290.CD-12-0215

Eichmann, T. O., and Lass, A. (2015). DAG tales: the multiple faces of diacylglycerol–stereochemistry, metabolism, and signaling. *Cell. Mol. Life Sci.* 72, 3931–3952. doi: 10.1007/s00018-015-1982-3

Freeman, G. J., and Sharpe, A. H. (2012). A new therapeutic strategy for malaria: targeting T cell exhaustion. *Nat. Immunol.* 13, 113–115. doi: 10.1038/ni.2211

Gorentla, B. K., Krishna, S., Shin, J., Inoue, M., Shinohara, M. L., Grayson, J. M., et al. (2013). Mnk1 and 2 are dispensable for T cell development and activation but important for the pathogenesis of experimental autoimmune encephalomyelitis. *J. Immunol.* 190, 1026–1037. doi: 10.4049/jimmunol.1200026

Green, J. M., Noel, P. J., Sperling, A. I., Walunas, T. L., Gray, G. S., Bluestone, J. A., et al. (1994). Absence of B7-dependent responses in CD28-deficient mice. *Immunity* 1, 501–508.

Guo, R., Wan, C.-K., Carpenter, J. H., Mousallem, T., Boustany, R.-M. N., Kuan, C.-T., et al. (2008). Synergistic control of T cell development and tumor suppression by diacylglycerol kinase alpha and zeta. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11909–11914. doi: 10.1073/pnas.0711856105

Hannier, S., Tournier, M., Bismuth, G., and Triebel, F. (1998). CD3/TCR complex-associated lymphocyte activation gene-3 molecules inhibit CD3/TCR signaling. *J. Immunol.* 161, 4058–4065.

Hartzell, C., Ksionda, O., Lemmens, E., Coakley, K., Yang, M., Dail, M., et al. (2013). Dysregulated RasGRP1 responds to cytokine receptor input in T cell leukemogenesis. *Sci. Signal.* 6, ra21. doi: 10.1126/scisignal.2003848

Huang, R. Y., Eppolito, C., Lele, S., Shrikant, P., Matsuzaki, J., and Odunsi, K. (2015). LAG3 and PD1 co-inhibitory molecules collaborate to limit CD8+ T cell signaling and dampen antitumor immunity in a murine ovarian cancer model. *Oncotarget* 6, 27359–27377. doi: 10.18632/oncotarget.4751

Jennings, W., Doshi, S., D'Souza, K., and Epand, R. M. (2015). Molecular properties of diacylglycerol kinase-epsilon in relation to function. *Chem. Phys. Lipids* 192, 100–108. doi: 10.1016/j.chemphyslip.2015.06.003

Jones, R. B., Ndhlovu, L. C., Barbour, J. D., Sheth, P. M., Jha, A. R., Long, B. R., et al. (2008). Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J. Exp. Med.* 205, 2763–2779. doi: 10.1084/jem.20081398

Joshi, R. P., and Koretzky, G. A. (2013). Diacylglycerol kinases: regulated controllers of T cell activation, function, and development. *Int. J. Mol. Sci.* 14, 6649–6673. doi: 10.3390/ijms14046649

Joshi, R. P., Schmidt, A. M., Das, J., Pytel, D., Riese, M. J., Lester, M., et al. (2013). The ζ isoform of diacylglycerol kinase plays a predominant role in regulatory T cell development and TCR-mediated ras signaling. *Sci. Signal.* 6, ra102. doi: 10.1126/scisignal.2004373

Krishna, S., and Zhong, X. (2013). Role of diacylglycerol kinases in T cell development and function. *Crit. Rev. Immunol.* 33, 97–118.

Krummel, M. F., and Allison, J. P. (1995). CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med.* 182, 459–465.

Lee, J., Su, E. W., Zhu, C., Hainline, S., Phuah, J., Moroco, J. A., et al. (2011). Phosphotyrosine-dependent coupling of Tim-3 to T-cell receptor signaling pathways. *Mol. Cell. Biol.* 31, 3963–3974. doi: 10.1128/MCB.05297-11

Lee, K. M., Chuang, E., Griffin, M., Khattri, R., Hong, D. K., Zhang, W., et al. (1998). Molecular basis of T cell inactivation by CTLA-4. *Science* 282, 2263–2266.

Li, D., Lyons, J. A., Pye, V. E., Vogeley, L., Aragão, D., Kenyon, C. P., et al. (2013). Crystal structure of the integral membrane diacylglycerol kinase. *Nature* 497, 521–524. doi: 10.1038/nature12179

Linden, J., and Cekic, C. (2012). Regulation of lymphocyte function by adenosine. *Arterioscler. Thromb. Vasc. Biol.* 32, 2097–2103. doi: 10.1161/ATVBAHA.111.226837

Linnemann, C., Schildberg, F. A., Schurich, A., Diehl, L., Hegenbarth, S. I., Endl, E., et al. (2009). Adenosine regulates CD8 T-cell priming by inhibition of membrane-proximal T-cell receptor signalling. *Immunology* 128, e728–e737. doi: 10.1111/j.1365-2567.2009.03075.x

Liu, K., Kunii, N., Sakuma, M., Yamaki, A., Mizuno, S., Sato, M., et al. (2016). A novel diacylglycerol kinase α-selective inhibitor, CU-3, induces cancer cell apoptosis and enhances immune response. *J. Lipid Res.* 57, 368–379. doi: 10.1194/jlr.M062794

Mérida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* 409, 1–18. doi: 10.1042/BJ20071040

Newick, K., O'Brien, S., Sun, J., Kapoor, V., Maceyko, S., Lo, A., et al. (2016). Augmentation of CAR T-cell trafficking and anti-tumor efficacy by blocking protein kinase A. *Localization* 4, 541–551. doi: 10.1158/2326-6066.CIR-15-0263

Newman, D. K., Fu, G., Adams, T., Arumugam, V., Bluemn, T., and Riese, M. J. (2016). An unexpected role for PECAM-1 in TGF beta-mediated inhibition of T cell function. *Sci. Signal.* 9, 1–12.

Okazaki, T., Okazaki, I.-M., Wang, J., Sugiura, D., Nakaki, F., Yoshida, T., et al. (2011). PD-1 and LAG-3 inhibitory co-receptors act synergistically to prevent autoimmunity in mice. *J. Exp. Med.* 208, 395–407. doi: 10.1084/jem.20100466

Olenchock, B. A., Guo, R., Carpenter, J. H., Jordan, M., Topham, M. K., Koretzky, G. A., et al. (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* 7, 1174–1181. doi: 10.1038/ni1400

Parry, R. V., Chemnitz, J. M., Frauwirth, K. A., Lanfranco, A. R., Braunstein, I., Kobayashi, S. V., et al. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Mol. Cell. Biol.* 25, 9543–9553. doi: 10.1128/MCB.25.21.9543-9553.2005

Perez de Castro, I., Bivona, T. G., Philips, M. R., and Pellicer, A. (2004). Ras activation in Jurkat T cells following low-grade stimulation of the T-cell receptor is specific to N-Ras and occurs only on the Golgi apparatus. *Mol. Cell. Biol.* 24, 3485–3496.

Prinz, P. U., Mendler, A. N., Brech, D., Masouris, I., Oberneder, R., and Noessner, E. (2014). NK-cell dysfunction in human renal carcinoma reveals diacylglycerol kinase as key regulator and target for therapeutic intervention. *Int. J. Cancer* 135, 1832–1841. doi: 10.1002/ijc.28837

Prinz, P. U., Mendler, A. N., Masouris, I., Durner, L., Oberneder, R., and Noessner, E. (2012). High DGK-α and disabled MAPK pathways cause dysfunction of human tumor-infiltrating CD8+ T cells that is reversible by pharmacologic intervention. *J. Immunol.* 188, 5990–6000. doi: 10.4049/jimmunol.1103028

Quann, E. J., Liu, X., Altan-Bonnet, G., and Huse, M. (2011). A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat. Immunol.* 12, 647–654. doi: 10.1038/ni.2033

Restifo, N. P., Smyth, M. J., and Snyder, A. (2016). Acquired resistance to immunotherapy and future challenges. *Nat. Rev. Cancer* 16, 121–126. doi: 10.1038/nrc.2016.2

Riese, M. J., Grewal, J., Das, J., Zou, T., Patil, V., Chakraborty, A. K., et al. (2011). Decreased diacylglycerol metabolism enhances ERK activation and augments CD8+ T cell functional responses. *J. Biol. Chem.* 286, 5254–5265. doi: 10.1074/jbc.M110.171884

Riese, M. J., Wang, L.-C. S., Moon, E. K., Joshi, R. P., Ranganathan, A., June, C. H., et al. (2013). Enhanced effector responses in activated CD8+ T cells deficient in diacylglycerol kinases. *Cancer Res.* 73, 3566–3577. doi: 10.1158/0008-5472.CAN-12-3874

Ruffo, E., Malacarne, V., Larsen, S. E., Das, R., Patrussi, L., Wülfing, C., et al. (2016). Inhibition of diacylglycerol kinase α restores restimulation-induced cell death and reduces immunopathology in XLP-1. *Sci. Transl. Med.* 8, 321ra7. doi: 10.1126/scitranslmed.aad1565

Sanjuán, M. A., Jones, D. R., Izquierdo, M., and Mérida, I. (2001). Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. *J. Cell Biol.* 153, 207–220.

Santos, T., Carrasco, S., Jones, D. R., Mérida, I., and Eguinoa, A. (2002). Dynamics of diacylglycerol kinase zeta translocation in living T-cells. Study of the structural domain requirements for translocation and activity. *J. Biol. Chem.* 277, 30300–30309. doi: 10.1074/jbc.M200999200

Schmidt, A. M., Zou, T., Joshi, R. P., Leichner, T. M., Pimentel, M. A., Sommers, C. L., et al. (2013). Diacylglycerol kinase ζ limits the generation of natural regulatory T cells. *Sci. Signal.* 6, ra101. doi: 10.1126/scisignal.2004411

Sharma, P., and Allison, J. P. (2015). The future of immune checkpoint therapy. *Science* 348, 56–61. doi: 10.1126/science.aaa8172

Shen, S., Wu, J., Srivatsan, S., Gorentla, B. K., Shin, J., Xu, L., et al. (2011). Tight regulation of diacylglycerol-mediated signaling is critical for proper invariant NKT cell development. *J. Immunol.* 187, 2122–2129. doi: 10.4049/jimmunol.1100495

Shin, D. S., and Ribas, A. (2015). The evolution of checkpoint blockade as a cancer therapy: what"s here, what"s next? *Curr. Opin. Immunol.* 33, 23–35. doi: 10.1016/j.coi.2015.01.006

Shulga, Y. V., Loukov, D., Ivanova, P. T., Milne, S. B., Myers, D. S., Hatch, G. M., et al. (2013). Diacylglycerol kinase delta promotes lipogenesis. *Biochemistry* 52, 7766–7776. doi: 10.1021/bi401178y

Topham, M. K., Bunting, M., Zimmerman, G. A., McIntyre, T. M., Blackshear, P. J., and Prescott, S. M. (1998). Protein kinase C regulates the nuclear localization of diacylglycerol kinase-zeta. *Nature* 394, 697–700. doi: 10.1038/29337

Ullrich, S. J., Hellmich, U. A., Ullrich, S., and Glaubitz, C. (2011). Interfacial enzyme kinetics of a membrane bound kinase analyzed by real-time MAS-NMR. *Nat. Chem. Biol.* 7, 263–270. doi: 10.1038/nchembio.543

van der Merwe, P. A., Bodian, D. L., Daenke, S., Linsley, P., and Davis, S. J. (1997). CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J. Exp. Med.* 185, 393–403.

Walunas, T. L., Bakker, C. Y., and Bluestone, J. A. (1996). CTLA-4 ligation blocks CD28-dependent T cell activation. *J. Exp. Med.* 183, 2541–2550.

Walunas, T. L., and Bluestone, J. A. (1998). CTLA-4 regulates tolerance induction and T cell differentiation *in vivo. J. Immunol.* 187, 3466–3474.

Wehbi, V. L., and Taskén, K. (2016). Molecular mechanisms for cAMP-mediated immunoregulation in T cells - role of anchored protein Kinase A signaling units. *Front. Immunol.* 7:222. doi: 10.3389/fimmu.2016.00222

Yang, J., Zhang, P., Krishna, S., Wang, J., Lin, X., Huang, H., et al. (2016). Unexpected positive control of NFκB and miR-155 by DGKα and ζ ensures effector and memory CD8+ T cell differentiation. *Oncotarget.* 7, 33744–33764. doi: 10.18632/oncotarget.8164

Yokosuka, T., Takamatsu, M., Kobayashi-Imanishi, W., Hashimoto-Tane, A., Azuma, M., and Saito, T. (2012). Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *J. Exp. Med.* 209, 1201–1217. doi: 10.1084/jem.20112741

Zha, Y., Marks, R., Ho, A. W., Peterson, A. C., Janardhan, S., Brown, I., et al. (2006). T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. *Nat. Immunol.* 7, 1166–1173. doi: 10.1038/ni1394

Zhong, X.-P., Hainey, E. A., Olenchock, B. A., Jordan, M. S., Maltzman, J. S., Nichols, K. E., et al. (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat. Immunol.* 4, 882–890. doi: 10.1038/ni958

Zhong, X.-P., Hainey, E. A., Olenchock, B. A., Zhao, H., Topham, M. K., and Koretzky, G. A. (2002). Regulation of T cell receptor-induced activation of the Ras-ERK pathway by diacylglycerol kinase zeta. *J. Biol. Chem.* 277, 31089–31098. doi: 10.1074/jbc.M203818200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

MR and SA are co-holders of a pending patent application on targeting DGKs for adoptive cellular therapies.

frontiers in Cell and Developmental Biology

REVIEW
published: 29 November 2016
doi: 10.3389/fcell.2016.00140

Diacylglycerol Kinases: Shaping Diacylglycerol and Phosphatidic Acid Gradients to Control Cell Polarity

Gianluca Baldanzi[1,2]*, *Valentina Bettio*[1,2], *Valeria Malacarne*[1,3] and *Andrea Graziani*[1,3]

[1] Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy, [2] Institute for Research and Cure of Autoimmune Diseases, Novara, Italy, [3] Division of Experimental Oncology, School of Medicine, University Vita e Salute San Raffaele, Milan, Italy

Diacylglycerol kinases (DGKs) terminate diacylglycerol (DAG) signaling and promote phosphatidic acid (PA) production. Isoform specific regulation of DGKs activity and localization allows DGKs to shape the DAG and PA gradients. The capacity of DGKs to constrain the areas of DAG signaling is exemplified by their role in defining the contact interface between T cells and antigen presenting cells: the immune synapse. Upon T cell receptor engagement, both DGK α and ζ metabolize DAG at the immune synapse thus constraining DAG signaling. Interestingly, their activity and localization are not fully redundant because DGKζ activity metabolizes the bulk of DAG in the cell, whereas DGKα limits the DAG signaling area localizing specifically at the periphery of the immune synapse. When DGKs terminate DAG signaling, the local PA production defines a new signaling domain, where PA recruits and activates a second wave of effector proteins. The best-characterized example is the role of DGKs in protrusion elongation and cell migration. Indeed, upon growth factor stimulation, several DGK isoforms, such as α, ζ, and γ, are recruited and activated at the plasma membrane. Here, local PA production controls cell migration by finely modulating cytoskeletal remodeling and integrin recycling. Interestingly, DGK-produced PA also controls the localization and activity of key players in cell polarity such as aPKC, Par3, and integrin β1. Thus, T cell polarization and directional migration may be just two instances of the general contribution of DGKs to the definition of cell polarity by local specification of membrane identity signaling.

Keywords: immune synapse, migration, lipid domain, cell polarity, localization

OPEN ACCESS

Edited by:
Pedro M. Fernández-Salguero, University of Extremadura, Spain

Reviewed by:
Kamalakannan Rajasekaran, Genentech Foundation, USA
Joaquin Teixidó, Consejo Superior Investigaciones Científicas, Spain

***Correspondence:**
Gianluca Baldanzi
gianluca.baldanzi@med.uniupo.it

Specialty section:
This article was submitted to Signaling, a section of the journal Frontiers in Cell and Developmental Biology

Received: *27 August 2016*
Accepted: *14 November 2016*
Published: *29 November 2016*

Citation:
Baldanzi G, Bettio V, Malacarne V and Graziani A (2016) Diacylglycerol Kinases: Shaping Diacylglycerol and Phosphatidic Acid Gradients to Control Cell Polarity. Front. Cell Dev. Biol. 4:140. doi: 10.3389/fcell.2016.00140

MEMBRANE IDENTITY AND CELL POLARITY

The compartmentalization of plasma membrane proteins is a common characteristic of eukaryotic cells and provides the base for the establishment of signaling domains (Spira et al., 2012). Local changes in lipid distribution also contribute to plasma membrane heterogeneity. However, the actual size and stability of lipid domains in cell membranes is still debatable (Carquin et al., 2016). The presence of specific lipids in different cellular compartments, including the plasma membrane, is required for the localized recruitment of effector proteins like actin regulators, protein kinases, and small GTPases (Lemmon, 2008).

The contribution of phosphoinositides (PIs) to cell organization has been extensively characterized; specific PIs play a major role in determining the subcellular identity of membranes

(Di Paolo and De Camilli, 2006). The balance between Phosphatidylinositol-trisphosphate ($PI_{3,4,5}P3$) (Di Paolo and De Camilli, 2006; Lemmon, 2008; Sánchez-Madrid and Serrador, 2009) and Phosphatidylinositol-bisphosphate ($PI_{4,5}P2$) (Di Paolo and De Camilli, 2006; Sánchez-Madrid and Serrador, 2009) is specifically involved in the generation and maintenance of cell polarity in a variety of experimental systems. In migrating leukocytes as well as in Dictyostelium cells, a $PI_{3,4,5}P3 \rightarrow PI_{4,5}P2$ gradient identifies the leading edge of the cell compared to the uropod at the rear (Sánchez-Madrid and Serrador, 2009). Similarly, in neurons, $PI_{3,4,5}P3$ is enriched at the tip of the growing axon (Shi et al., 2003). In apical/basal polarized epithelial cells, $PI_{4,5}P2$ accumulates at the apical domain whereas the basolateral membranes are enriched in $PI_{3,4,5}P3$ (Gassama-Diagne et al., 2006). In all these cases, local lipid enrichment results from the spatial segregation of $PI_{3,4,5}P3$ generation by PI3Ks and its metabolism by PTEN activity (Funamoto et al., 2002; Lacalle et al., 2004; Martin-Belmonte et al., 2007; Leslie et al., 2008). Once established, the uneven distribution of PIs promotes cell polarization by recruiting specific effectors.

Similar to $PI_{3,4,5}P3$ and $PI_{4,5}P2$, both DAG and PA are: (i) second messengers that recruit a set of interacting proteins (Mérida et al., 2008), (ii) kept in balance by the combined action of PA phosphatases and DGKs (Sakane et al., 2007) and (iii) enriched in specific domains of the plasma membrane where they recruit specific effectors.

In quiescent cells grown on bi-dimensional surfaces, PA is present in relevant amounts with prominent distribution at the free edges compared with that at cell-cell contacts (Nishioka et al., 2010). Further PA production by PLD and DGKs is observed upon receptor triggering, with highest levels at the nascent lamellipodia (Nishioka et al., 2010). Herein, PA binding proteins such as aPKC (Chianale et al., 2010; Rainero et al., 2014) or Nir2 (Kim et al., 2013), drive cytoskeletal remodeling and protrusion elongation. Similarly, PA participates in the recruitment of the Rac activator, DOCK1, during dorsal ruffle formation in fibroblasts (Sanematsu et al., 2013), as well as in the localization and activation of the Rac-RhoGAP complex during neurite outgrowth (Kurooka et al., 2011). Moreover, DAG is locally produced by the activity of extracellular regulated phospholipase C (PLC) on $PI_{4,5}P2$ (Kadamur and Ross, 2013) and by the PA phosphatase activity at both the plasma membrane and in the intracellular organelles (Brindley et al., 2009). The resulting DAG production is essential to many biological systems such as the immune synapse, the neuronal synapse, and phagocytosis (Almena and Mérida, 2011).

PA and DAG gradients are somehow different in migrating cells. Indeed, low PA levels have been found at the leading edge of spontaneously migrating HeLa cells compared to those at the trailing edge (Ferraz-Nogueira et al., 2014), whereas DAG is symmetrically enriched at the front of migrating cells (Nishioka et al., 2008). Similarly, PA is strongly decreased at the apical domain of polarized epithelial cells, whereas DAG is lightly enriched (Gerl et al., 2012). These data demonstrate the existence of PA and DAG enriched domains that contribute to cellular asymmetry and thus suggest a putative role of DGKs in the control of cell polarity. Accordingly, while in E. coli DGK is a transmembrane protein that phosphorylates multiple lipids (Van Horn and Sanders, 2012), mammalian DGKs are soluble enzymes recruited on demand at specific cellular locations where they act on specific DAG pools (Kobayashi et al., 2007). The relevance of targeting to specific membrane domains is evidenced by the presence of multiple domains controlling membrane association, in the N terminal part of all DGKs, apart from DGKε (Mérida et al., 2008).

Here, we will discuss some well-characterized examples of the contribution of DGK activity to the generation and maintenance of lipid signaling domains in polarized cells.

DGKα AND ζ AT THE IMMUNOLOGICAL SYNAPSE

The contact zone between the T cell and the antigen-presenting cell (APC) is a specialized structure described as the immunological synapse (IS) (Monks et al., 1998; Grakoui et al., 1999). The IS has a well-defined spatial organization where supramolecular activation clusters (SMACs) are arranged in radial symmetry to form a "bull's eye" shape (Monks et al., 1998). The more distal zone (dSMAC) is CD45-enriched and is characterized by active actin movements resembling the sensory lamellipodia of epithelial cells (Dustin et al., 2010). This is followed by a peripheral zone (pSMAC) enriched in adhesion molecules such as LFA-1 (lymphocyte function-associated antigen-1, integrin αLβ2), and VLA4 (Very Late Antigen-4, integrin α4β1) and the associated talin that resemble adhesive lamella (Mittelbrunn et al., 2004). In the central part (cSMAC), coactivators (e.g., CD28) and kinases (LCK, Fyn) are enriched, but endocytosis also occurs, resembling that in uropods of migrating cells. The cSMAC is also the site of secretion of cytokines, cytolytic agents, and exosomes into the synapse (Dustin, 2014).

Upon antigen stimulation, T cell receptor (TCR) microclusters form at the IS periphery and move toward the cSMAC where they encounter the endocytic sorting machinery and are internalized. IS formation drives the polarization of the entire T cell, with the translocation of the microtubule organizing center (MTOC) between the IS and the nucleus, and the establishment of the uropod, a membrane zone enriched in signaling molecules at the opposite end of the T cell (Serrador et al., 1999). Both IS formation and repositioning of the MTOC are key events during the killing of a cognate target cell by cytotoxic T lymphocytes (CTLs). Cytotoxic granules move along microtubules and the granule content is released between the CTL and the target cell, where perforin and granzymes co-operate to induce rapid death of the target cell by apoptosis (de Saint Basile et al., 2010).

Unstimulated T cells display uniform distribution of DAG at the plasma membrane, whereas after T cell activation, a DAG gradient is established at the center of the IS by the activity of TCR-activated PLCγ (Spitaler et al., 2006) and by the combined action of PLD and PA-phosphatases (Mor et al., 2007). This DAG is essential for the recruitment of downstream DAG-dependent effectors such as conventional PKC (cPKCs), PKD,

and RasGRP1, which promote the downstream T cell responses (Spitaler et al., 2006). As recently reviewed by Merida et al., rapid DAG metabolism occurs at the IS (Mérida et al., 2015). Indeed both DGKα and DGKζ are translocated to the proximal and distal poles of the T cell during IS formation (Joshi et al., 2013) and both DGKα and DGKζ are recruited to the TCR complex (Gerl et al., 2012). Despite a substantial overlap in localization upon TCR triggering and their common function as negative regulators of TCR-downstream signaling (Zhong et al., 2003; Olenchock et al., 2006), the roles of DGKα and DGKζ do not seem fully redundant. Indeed, TCR triggers DGKζ phosphorylation on the myristoylated alanine-rich C-kinase substrate (MARKS) domain by PKC (Gharbi et al., 2011). Upon phosphorylation, DGKζ spreads among the entire immunological synapse, where it contributes to DAG metabolism (Gharbi et al., 2011; Joshi et al., 2013). Conversely, DGKα is selectively recruited to the periphery of the IS in a PI3Kδ dependent manner (Chauveau et al., 2014). Membrane-associated DGKα is phosphorylated on Y_{335} and is activated by Lck and Ca^{2+} with a timing that overlaps with PLCγ-phosphorylation (Sanjuán et al., 2001; Merino et al., 2008). *In vitro* experiments show that membrane-localized DGKα in T cells displays a substantial overlap with the F-actin ring surrounding the central DAG bulk, where DGKα plays a specific role in restricting the DAG domain. Indeed, in WT and DGKζ$^{-/-}$ T cells, the DAG probe C1δ-GFP was localized within this F-actin ring, whereas in DGKα$^{-/-}$ cells, DAG distribution appeared substantially broader (Chauveau et al., 2014). Thus, DGKα contributes to polarity determination by constraining DAG accumulation into the cSMAC, while DGKζ plays a general function in reducing the intensity of TCR-downstream signaling (Chauveau et al., 2014).

The DGKα-mediated shaping of DAG gradient at the immune synapse is required for T cell polarization as DGKα$^{-/-}$ cells show partial impairment in TCR-promoted MTOC re-localization and polarized secretion (Quann et al., 2009; Alonso et al., 2011; Chauveau et al., 2014). Absence of DGK activity closely resembles T cell treatment with DAG analogs, such as phorbol esters, which completely abrogate MTOC reorientation toward the IS (Quann et al., 2009). The relevance of DGKα in T cell polarization is less evident when assayed in conjugates between antigen presenting B cells and Jurkat T cells. In these IS, DGKα inhibition does not perturb MTOC and F-actin polarization, but significantly affects DAG accumulation at the IS, suggesting that some polarization events also occur in the absence of localized DAG signaling (Ruffo et al., 2016). A striking example of the functional relevance of DGKα in the control of T cell polarization is the X-linked lymphoproliferative disease 1 (XLP-1). XLP-1 is a primary immunodeficiency due to defects in signaling lymphocytic activation molecule (SLAM)–associated protein (SAP), an adaptor protein that modulates TCR-induced signaling. We have demonstrated that the SLAM-SAP signaling axis negatively regulates DGKα activity in T cells (Baldanzi et al., 2011a). In XLP-1 SAP is mutated or absent and results in constitutive DGKα activity that blunts the DAG dependent TCR signaling (Dustin et al., 2010). Interestingly, SAP deficient cells not only show partial impairment of TCR signaling, but also have specific defects in novel PKC (nPKC) recruitment at the IS (Cannons et al., 2004, 2010a), thus reducing the IS stability ad effector functions (Cannons et al., 2010b; Zhao et al., 2012). These defects are due to excessive DGKα activity, as silencing or inhibiting DGKα in SAP deficient cells restores the correct level of DAG and its effectors at the IS and reestablishes MTOC polarization (Ruffo et al., 2016). Accordingly, inhibition of DGKα activity had no substantial effect on the killing of target cells by activated $CD8^+$ lymphocytes whereas it enhances the weak effector function of SAP deficient lymphocytes (Chauveau et al., 2014; Ruffo et al., 2016).

These observations indicate that a signaling domain enriched in DAG is generated by the fine-tuning of localized production by PLCγ and equally localized metabolism by DGKα (Mérida et al., 2015). As evidenced in **Figure 1**, fine regulation of DGKα activity plays a central role, with a small fraction recruited and activated by PI3Kδ at the pSMAC (Chauveau et al., 2014), and the remaining enzyme activity is inhibited by SAP (Baldanzi et al., 2011a). The resulting spatial definition of DAG signaling drives T cell polarization by promoting the local recruitment of multiple PKC isoforms (Quann et al., 2011) that in turn organize molecular motors such as dynein at the IS and myosin II at the opposite cell end (Liu et al., 2013). Once established, T cell polarity is maintained by polarized vesicular trafficking toward the IS, where DGKα also plays a role. Indeed, DGKα is recruited to multivesicular bodies and to exosomes, and it promotes both the polarization of MVBs toward the IS and exosome secretion (Alonso et al., 2011).

Little is known about the function of DGKs in other types of immune synapses. In NK cells, DGKζ silencing or treatment with DGK inhibitors enhances effector functions (Prinz et al., 2014; Yang et al., 2016), but it is currently unknown if this relates to a change in the DAG gradient or to an increased efficiency of the secretory pathways.

Notably, while the role of DGKα as a DAG-driven signaling terminator has been extensively investigated, whether and how PA production by DGKs at the IS affects TCR signaling is currently unknown. However, some cues suggest this possibility as PA production by DGKα and ζ is required for T cell development (Guo et al., 2008), whereas DGKζ-generated PA promotes TLR-induced IL-12 production by negatively regulating the PI3K-AKT pathway (Liu et al., 2007). Moreover, PA is an allosteric activator of PLCγ (Jones and Carpenter, 1993), suggesting that a PA-dependent feedback mechanism can amplify the magnitude of the signal. Future studies addressing the localization and function of DGKs-generated PA in lymphocytes should provide further insights into TCR signaling.

DGKS IN DIRECTIONAL MIGRATION

The leading edge of growth factor stimulated cells is another site of intense PIPs turnover, where PLCγ is recruited to produce a local enrichment of DAG coupled to Ca^{2+} release triggered by inositol triphosphate (Piccolo et al., 2002; Mouneimne et al., 2004; Nishioka et al., 2008). We have demonstrated that upon growth factor or chemokine-mediated stimulation of epithelial cells, DGKα is activated and recruited to the plasma membrane,

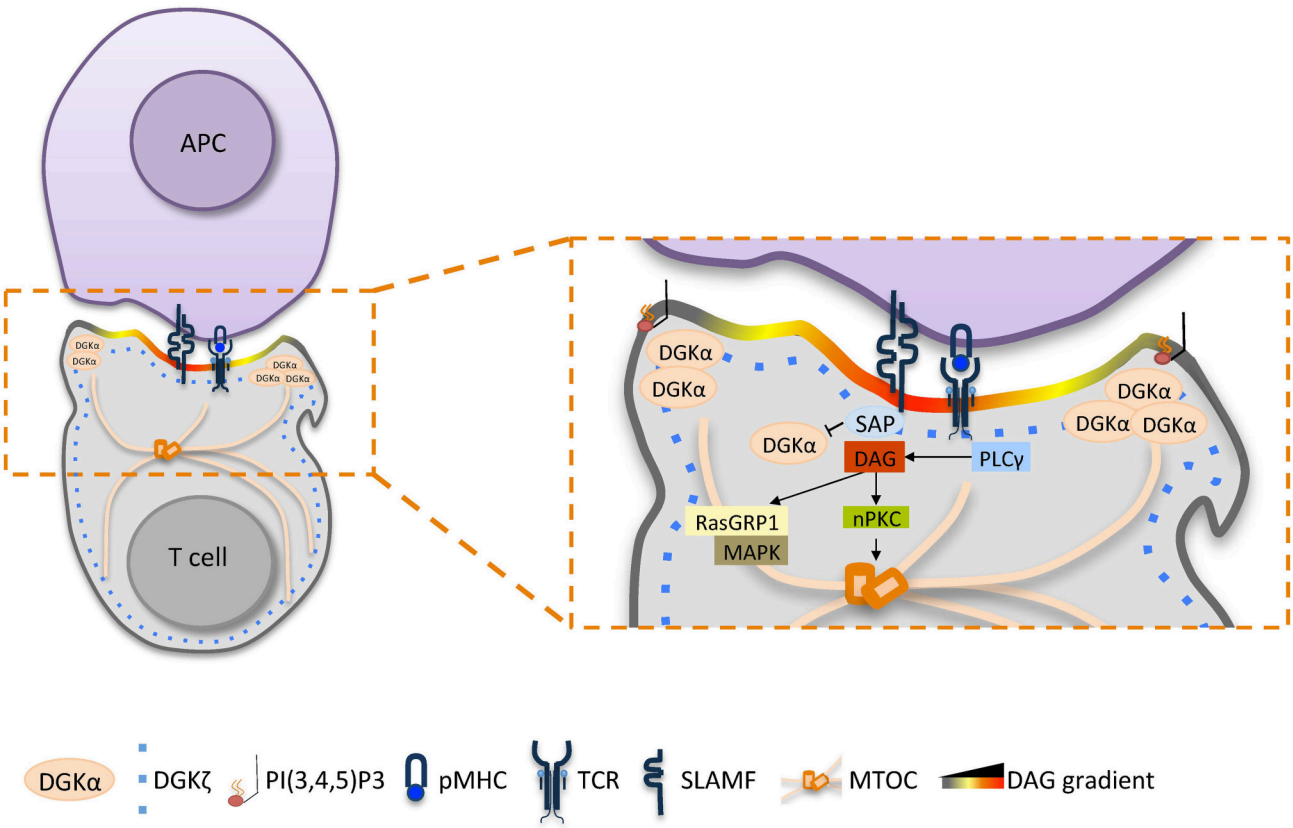


FIGURE 1 | The role of DGKα in shaping diacylglycerol signaling at the immune synapse. Upon TCR/SLAM engagement, PLCγ activation induces DAG production at the synapse and DGKα/ζ recruitment. DGKζ (blue dotted line) is responsible for the metabolism of the bulk of DAG over the entire synapse, whereas DGKα (light orange) is recruited by PI3Kδ generated $PI_{3,4,5}P3$ at the pSMAC, where DAG is constrained. Excessive DAG metabolism at the IS by DGKα is prevented by SAP-mediated inhibition. The resulting sharp DAG gradient promotes downstream MAPK signaling and the local recruitment of nPKC, which promotes MTOC reorientation.

where the PA produced by DGKα recruits PA-binding proteins such as atypical PKC ζ and ι (aPKCζ; Chianale et al., 2007, 2010; Baldanzi et al., 2008) and Rab11 family interacting protein 1 (Rab11-FIP1) (Rainero et al., 2012). DGKα activated aPKC phosphorylates RhoGDI, thus promoting the release of Rac1, actin polymerization, and elongation of invasive protrusions enriched in Integrin β_1 and metalloproteinase 9 (Chianale et al., 2007, 2010; Rainero et al., 2014). The PA produced by DGKα at the tip of invasive pseudopods is also a docking site for vesicles containing Rab11-FIP1 and Integrin $\alpha_5\beta_1$, allowing DGKα to polarize vesicular trafficking and promote directional migration (Rainero et al., 2014). Altogether, these data indicate that in epithelial cells, PA production by DGKα is essential to orchestrate the organization of the signaling machinery that promotes protrusion formation and directed cell migration (**Figure 2A**).

DGKα expression is low in mouse embryonic fibroblasts (MEFs), but other investigators have demonstrated that DGKζ plays an equivalent role in cell migration. DGKζ$^{-/-}$ MEFs have more focal adhesions at their central region due to impairment in the local recruitment of PAK1 kinase. Upon PDGF stimulation, DGKζ promotes PAK1-mediated phosphorylation of RhoGDI that releases Rac1, which drives directed cell migration (Abramovici et al., 2009). Thus, close parallelism exists between the DGKα driven aPKC recruitment in epithelial cells and the DGKζ mediated recruitment of PAK1 in MEFs, both controlling Rac1 activity and migration. Moreover, DGKζ also regulates RhoA activity in MEFs by acting as a scaffolding protein independently of PA production (Ard et al., 2012). DGKζ is also highly expressed in colon cancer cell lines and its expression correlates with enhanced cell motility due to increased Rac1 and RhoA activation (Cai et al., 2014), suggesting that DGKζ is a key regulator of Rho GTPase activity and cell migration in fibroblasts and tumor cells (**Figure 2B**).

In fibroblasts, DGKγ is also recruited to ruffles and lamellipodia where it co-localizes with Rac1 (**Figure 2C**). Surprisingly, DGKγ acts as a suppressor of growth factor-induced protrusions by recruiting and activating the β2-chimerin GAP activity (Tsushima et al., 2004; Yasuda et al., 2007). This observation clearly indicates the key contribution of DGKs to local PA accumulation that controls ruffling and lamellipodia formation (Nishioka et al., 2010), but suggests

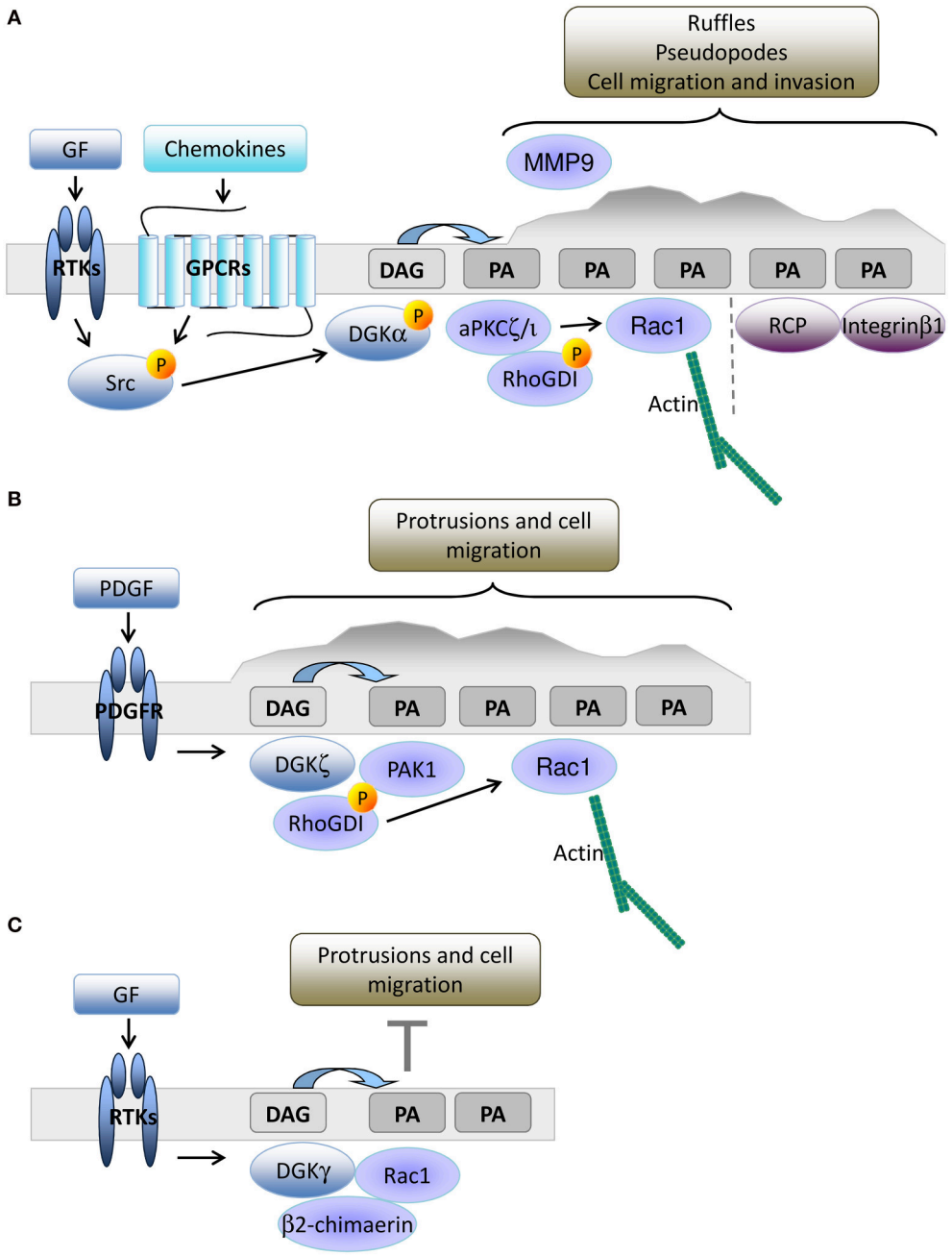


FIGURE 2 | DGKs-produced PA drives membrane protrusion and cell motility. (A) In epithelial cells, growth factors or chemokine stimulation promotes Src mediated recruitment and activation of DGKα to nascent protrusions. The resulting PA enriched domain drives protrusion elongation and matrix invasion by the local recruitment of aPKC and Rab11-FIP1. These PA binding proteins respectively control cytoskeletal remodeling through Rac1 and integrin β1 recycling. **(B)** In fibroblasts, PDGF triggers DGKζ recruitment to nascent protrusions. The resulting PA enriched domain drives focal adhesion remodeling and cell migration by recruiting and activating PAK1 and promoting Rac1 activation. **(C)** Growth factors also promote membrane recruitment and activation of DGKγ that limits Rac1 activity through β2 chimaerin.

that multiple PA pools with specific functions and interactors are involved. The relevance of protein-protein interactions in dictating the signaling outcome of DGK-produced PA is evidenced by the observation of the isoform specific complexes (i) DGKα-aPKC-RhoGDI-Rac1 (Chianale et al., 2010), (ii) DGKζ-PAK1-RhoGDI-Rac1 (Abramovici et al., 2009), and (iii) DGKγ-β2 chimaerin (Yasuda et al., 2007).

The relevance of DAG and PA in cell migration suggests that DGKs are relevant targets for the control of tumor development and metastasis (Purow, 2015). Surprisingly, both DGKα and

ζ also play a key role in cancer-cell survival, acting at either the plasma membrane, intracellular organelles, or the nucleus (Baldanzi et al., 2011b; Filigheddu et al., 2011; Dominguez et al., 2013; Kefas et al., 2013; Tanaka et al., 2013; Torres-Ayuso et al., 2014, 2015; Poli et al., 2016). However, the signaling pathways that converts PA and DAG at cell membranes in cell survival signaling are poorly understood, as few PA-regulated proteins are linked to the control of apoptosis and the cell cycle. Among these, mTOR (mammalian target of rapamycin) is notable as a target for both $PI_{3,4,5}P3$ signaling and DGKζ-produced PA (Avila-Flores et al., 2005; Chen et al., 2012; You et al., 2014).

FUTURE PROSPECTS: DO DGKS PLAY A ROLE IN THE CONTROL OF CELL POLARITY?

The illustrated data indicate the role of DGKs in the shaping of signaling domains at the plasma membrane by confining DAG signaling and contributing to the generation of PA-enriched domains. In the T cells engaged in IS, the existence of a DAG gradient is necessary and sufficient to polarize the entire cytoskeleton (Quann et al., 2009) but the relevance of DAG gradients in other polarized systems such as front/rear or apical/basal asymmetry is currently under investigation (Tsai et al., 2014).

PA gradients are even less characterized because the relevance of PA in signaling is just emerging (Jang et al., 2012). A few studies found a role of PLD generated PA in the recruitment of PA binding proteins to the apical domain of epithelial monolayers (Gloerich et al., 2012; Consonni et al., 2014), despite controversial evidences about the apical enrichment of PA (Gerl et al., 2012). Interestingly, several data link the diacylglycerol generated-PA with central players in the establishment of cell polarity: aPKC, Par3, and integrin β1 (Rainero et al., 2014). In apical/basal polarized epithelial cells, aPKC, Par3, and Par6 compose the Par complex, which is located at the apical side within the region of tight junctions, where it promotes the formation and maintenance of tight junctions and the apical domain (Horikoshi et al., 2009). Activation of aPKC is a key event in the regulation of apical/basal polarity since aPKC phosphorylates several substrates involved in polarity establishment such as Crumbs, Lgl, and GSK3β (glycogen synthase kinase-3β). Phosphorylation of Crumbs and Lgl promotes their correct intracellular localization, whereas GSK3β phosphorylation is involved in microtubule capture and stabilization, and in the maturation of cell-cell contacts (Gandalovičová et al., 2016). The link with DGKs is provided by the observation that: i) aPKC binds to and is activated by PA (Limatola et al., 1994) and ii) their localization at the invasive protrusions of cancer cells is promoted by DGKα-produced PA (Chianale et al., 2010; Rainero et al., 2014). PA might also play a role in the localization of the aPKC-Par3-Par6 complex as the *Drosophila* Par3 homolog, Bazooka also directly binds PA (Yu and Harris, 2012).

DGKα-produced PA also controls intracellular trafficking of integrin β1 through the PA binding protein Rab11-FIP1 (Lindsay and McCaffrey, 2004; Rainero et al., 2012). Interestingly, integrin β1 trafficking is essential for directional migration (Shafaq-Zadah et al., 2016), apical/basal polarity (Bryant et al., 2014), and mitotic spindle orientation (Toyoshima and Nishida, 2007). Several evidences link integrin signaling and epithelial cell polarity (Zovein et al., 2010; Myllymäki et al., 2011), suggesting an interplay between integrin trafficking and Par complex activity. We speculate that DGKs-produced PA, which regulates both the Par complex through aPKC and integrin β1 trafficking though Rab11-FIP1, contributes to the coordination of those pathways.

Starting from the observation of the central role of DGKs in establishing lymphocyte polarity and in directional migration, we propose that this family of enzymes may play a widespread role in the establishment of membrane domains that dictates cell polarization. The neuronal system is a very promising field to explore, where several DGK isoforms are expressed (Ishisaka and Hara, 2014) and control both neurite growth and branching (Shirai et al., 2010) and synapse stability (Kim et al., 2009; Shirai et al., 2010).

This review aims to prompt further studies investigating the link between DGKs activity, membrane asymmetry, and cell fate.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

AG is supported by grants from Telethon (GGP13254), AIRC (Associazione Italiana per la Ricerca sul Cancro) (IG13524) and PRIN (2015 4CWJH4). GB is supported by the TIPSO grant from Regione Piemonte (PAR FSC 2007–2013 Asse I—Innovazione e transizione produttiva—Linea di azione 3: "Competitività industria e artigianato" linea d—Bando regionale sullemalattie Autoimmuni e Allergiche). Salary of VM and VB is provided by Telethon and TIPSO to AG and GB respectively.

REFERENCES

Abramovici, H., Mojtabaie, P., Parks, R. J., Zhong, X. P., Koretzky, G. A., Topham, M. K., et al. (2009). Diacylglycerol kinase zeta regulates actin cytoskeleton reorganization through dissociation of Rac1 from RhoGDI. *Mol. Biol. Cell* 20, 2049–2059. doi: 10.1091/mbc.E07-12-1248

Almena, M., and Mérida, I. (2011). Shaping up the membrane: diacylglycerol coordinates spatial orientation of signaling. *Trends Biochem. Sci.* 36, 593–603. doi: 10.1016/j.tibs.2011.06.005

Alonso, R., Mazzeo, C., Rodriguez, M. C., Marsh, M., Fraile-Ramos, A., Calvo, V., et al. (2011). Diacylglycerol kinase α regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of Fas ligand-containing exosomes in T lymphocytes. *Cell Death Differ.* 18, 1161–1173. doi: 10.1038/cdd.2010.184

Ard, R., Mulatz, K., Abramovici, H., Maillet, J. C., Fottinger, A., Foley, T., et al. (2012). Diacylglycerol kinase ζ regulates RhoA activation via a kinase-independent scaffolding mechanism. *Mol. Biol. Cell* 23, 4008–4019. doi: 10.1091/mbc.E12-01-0026

Avila-Flores, A., Santos, T., Rincón, E., and Mérida, I. (2005). Modulation of the mammalian target of rapamycin pathway by diacylglycerol kinase-produced phosphatidic acid. *J. Biol. Chem.* 280, 10091–10099. doi: 10.1074/jbc.M412296200

Baldanzi, G., Cutrupi, S., Chianale, F., Gnocchi, V., Rainero, E., Porporato, P., et al. (2008). Diacylglycerol kinase-alpha phosphorylation by Src on Y335 is required for activation, membrane recruitment and Hgf-induced cell motility. *Oncogene* 27, 942–956. doi: 10.1038/sj.onc.1210717

Baldanzi, G., Pietronave, S., Locarno, D., Merlin, S., Porporato, P., Chianale, F., et al. (2011b). Diacylglycerol kinases are essential for hepatocyte growth factor-dependent proliferation and motility of Kaposi's sarcoma cells. *Cancer Sci.* 102, 1329–1336. doi: 10.1111/j.1349-7006.2011.01953.x

Baldanzi, G., Pighini, A., Bettio, V., Rainero, E., Traini, S., Chianale, F., et al. (2011a). SAP-mediated inhibition of diacylglycerol kinase α regulates TCR-induced diacylglycerol signaling. *J. Immunol.* 187, 5941–5951. doi: 10.4049/jimmunol.1002476

Brindley, D. N., Pilquil, C., Sariahmetoglu, M., and Reue, K. (2009). Phosphatidate degradation: phosphatidate phosphatases (lipins) and lipid phosphate phosphatases. *Biochim. Biophys. Acta* 1791, 956–961. doi: 10.1016/j.bbalip.2009.02.007

Bryant, D. M., Roignot, J., Datta, A., Overeem, A. W., Kim, M., Yu, W., et al. (2014). A molecular switch for the orientation of epithelial cell polarization. *Dev. Cell* 31, 171–187. doi: 10.1016/j.devcel.2014.08.027

Cai, K., Mulatz, K., Ard, R., Nguyen, T., and Gee, S. H. (2014). Increased diacylglycerol kinase ζ expression in human metastatic colon cancer cells augments Rho GTPase activity and contributes to enhanced invasion. *BMC Cancer* 14:208. doi: 10.1186/1471-2407-14-208

Cannons, J. L., Qi, H., Lu, K. T., Dutta, M., Gomez-Rodriguez, J., Cheng, J., et al. (2010b). Optimal germinal center responses require a multistage T cell:B cell adhesion process involving integrins, SLAM-associated protein, and CD84. *Immunity* 32, 253–265. doi: 10.1016/j.immuni.2010.01.010

Cannons, J. L., Wu, J. Z., Gomez-Rodriguez, J., Zhang, J., Dong, B., Liu, Y., et al. (2010a). Biochemical and genetic evidence for a SAP-PKC-θ interaction contributing to IL-4 regulation. *J. Immunol.* 185, 2819–2827. doi: 10.4049/jimmunol.0902182

Cannons, J. L., Yu, L. J., Hill, B., Mijares, L. A., Dombroski, D., Nichols, K. E., et al. (2004). SAP regulates T(H)2 differentiation and PKC-θ-mediated activation of NF-κB1. *Immunity* 21, 693–706. doi: 10.1016/j.immuni.2004.09.012

Carquin, M., D'Auria, L., Pollet, H., Bongarzone, E. R., and Tyteca, D. (2016). Recent progress on lipid lateral heterogeneity in plasma membranes: from rafts to submicrometric domains. *Prog. Lipid Res.* 62, 1–24. doi: 10.1016/j.plipres.2015.12.004

Chauveau, A., A., Le Floc'h, Bantilan, N. S., Koretzky, G. A., and Huse, M. (2014). Diacylglycerol kinase α establishes T cell polarity by shaping diacylglycerol accumulation at the immunological synapse. *Sci. Signal.* 7, ra82. doi: 10.1126/scisignal.2005287

Chen, G., Chen, S. M., Wang, X., Ding, X. F., Ding, J., and Meng, L. H. (2012). Inhibition of chemokine (CXC motif) ligand 12/chemokine (CXC motif) receptor 4 axis (CXCL12/CXCR4)-mediated cell migration by targeting mammalian target of rapamycin (mTOR) pathway in human gastric carcinoma cells. *J. Biol. Chem.* 287, 12132–12141. doi: 10.1074/jbc.M111.302299

Chianale, F., Cutrupi, S., Rainero, E., Baldanzi, G., Porporato, P. E., Traini, S., et al. (2007). Diacylglycerol kinase-alpha mediates hepatocyte growth factor-induced epithelial cell scatter by regulating Rac activation and membrane ruffling. *Mol. Biol. Cell* 18, 4859–4871. doi: 10.1091/mbc.E07-02-0177

Chianale, F., Rainero, E., Cianflone, C., Bettio, V., Pighini, A., Porporato, P. E., et al. (2010). Diacylglycerol kinase alpha mediates HGF-induced Rac activation and membrane ruffling by regulating atypical PKC and RhoGDI. *Proc. Natl. Acad. Sci. U.S.A.* 107, 4182–4187. doi: 10.1073/pnas.0908326107

Consonni, S. V., Brouwer, P. M., van Slobbe, E. S., and Bos, J. L. (2014). The PDZ domain of the guanine nucleotide exchange factor PDZGEF directs binding to phosphatidic acid during brush border formation. *PLoS ONE* 9:e98253. doi: 10.1371/journal.pone.0098253

de Saint Basile, G., Ménasché, G., and Fischer, A. (2010). Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. *Nat. Rev. Immunol.* 10, 568–579. doi: 10.1038/nri2803

Di Paolo, G., and De Camilli, P. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* 443, 651–657. doi: 10.1038/nature05185

Dominguez, C. L., Floyd, D. H., Xiao, A., Mullins, G. R., Kefas, B. A., Xin, W., et al. (2013). Diacylglycerol kinase α is a critical signaling node and novel therapeutic target in glioblastoma and other cancers. *Cancer Discov.* 3, 782–797. doi: 10.1158/2159-8290.CD-12-0215

Dustin, M. L. (2014). The immunological synapse. *Cancer Immunol. Res.* 2, 1023–1033. doi: 10.1158/2326-6066.CIR-14-0161

Dustin, M. L., Chakraborty, A. K., and Shaw, A. S. (2010). Understanding the structure and function of the immunological synapse. *Cold Spring Harb. Perspect. Biol.* 2:a002311. doi: 10.1101/cshperspect.a002311

Ferraz-Nogueira, J. P., Díez-Guerra, F. J., and Llopis, J. (2014). Visualization of phosphatidic acid fluctuations in the plasma membrane of living cells. *PLoS ONE* 9:e102526. doi: 10.1371/journal.pone.0102526

Filigheddu, N., Sampietro, S., Chianale, F., Porporato, P. E., Gaggianesi, M., Gregnanin, I., et al. (2011). Diacylglycerol kinase α mediates 17-β-estradiol-induced proliferation, motility, and anchorage-independent growth of Hec-1A endometrial cancer cell line through the G protein-coupled estrogen receptor GPR30. *Cell. Signal.* 23, 1988–1996. doi: 10.1016/j.cellsig.2011.07.009

Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R. A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* 109, 611–623. doi: 10.1016/S0092-8674(02)00755-9

Gandalovičová, A., Vomastek, T., Rosel, D., and Brábek, J. (2016). Cell polarity signaling in the plasticity of cancer cell invasiveness. *Oncotarget* 7, 25022–25049. doi: 10.18632/oncotarget.7214

Gassama-Diagne, A., Yu, W., ter Beest, M., Martin-Belmonte, F., Kierbel, A., Engel, J., et al. (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nat. Cell Biol.* 8, 963–970. doi: 10.1038/ncb1461

Gerl, M. J., Sampaio, J. L., Urban, S., Kalvodova, L., Verbavatz, J. M., Binnington, B., et al. (2012). Quantitative analysis of the lipidomes of the influenza virus envelope and MDCK cell apical membrane. *J. Cell Biol.* 196, 213–221. doi: 10.1083/jcb.201108175

Gharbi, S. I., Rincón, E., Avila-Flores, A., Torres-Ayuso, P., Almena, M., Cobos, M. A., et al. (2011). Diacylglycerol kinase ζ controls diacylglycerol metabolism at the immunological synapse. *Mol. Biol. Cell* 22, 4406–4414. doi: 10.1091/mbc.E11-03-0247

Gloerich, M., ten Klooster, J. P., Vliem, M. J., Koorman, T., Zwartkruis, F. J., Clevers, H., et al. (2012). Rap2A links intestinal cell polarity to brush border formation. *Nat. Cell Biol.* 14, 793–801. doi: 10.1038/ncb2537

Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., et al. (1999). The immunological synapse: a molecular machine controlling T cell activation. *Science* 285, 221–227. doi: 10.1126/science.285.5425.221

Guo, R., Wan, C. K., Carpenter, J. H., Mousallem, T., Boustany, R. M., Kuan, C. T., et al. (2008). Synergistic control of T cell development and tumor suppression by diacylglycerol kinase alpha and zeta. *Proc. Natl. Acad. Sci. U.S.A.* 105, 11909–11914. doi: 10.1073/pnas.0711856105

Horikoshi, Y., Suzuki, A., Yamanaka, T., Sasaki, K., Mizuno, K., Sawada, H., et al. (2009). Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. *J. Cell Sci.* 122(Pt 10), 1595–1606. doi: 10.1242/jcs.043174

Ishisaka, M., and Hara, H. (2014). The roles of diacylglycerol kinases in the central nervous system: review of genetic studies in mice. *J. Pharmacol. Sci.* 124, 336–343. doi: 10.1254/jphs.13R07CR

Jang, J. H., Lee, C. S., Hwang, D., and Ryu, S. H. (2012). Understanding of the roles of phospholipase D and phosphatidic acid through their binding partners. *Prog. Lipid Res.* 51, 71–81. doi: 10.1016/j.plipres.2011.12.003

Jones, G. A., and Carpenter, G. (1993). The regulation of phospholipase C-γ1 by phosphatidic acid. Assessment of kinetic parameters. *J. Biol. Chem.* 268, 20845–20850.

Joshi, R. P., Schmidt, A. M., Das, J., Pytel, D., Riese, M. J., Lester, M., et al. (2013). The ζ isoform of diacylglycerol kinase plays a predominant role in regulatory T cell development and TCR-mediated ras signaling. *Sci. Signal.* 6, ra102. doi: 10.1126/scisignal.2004373

Kadamur, G., and Ross, E. M. (2013). Mammalian phospholipase C. *Annu. Rev. Physiol.* 75, 127–154. doi: 10.1146/annurev-physiol-030212-183750

Kefas, B., Floyd, D. H., Comeau, L., Frisbee, A., Dominguez, C., Dipierro, C. G., et al. (2013). A miR-297/hypoxia/DGK-α axis regulating glioblastoma survival. *Neuro Oncol.* 15, 1652–1663. doi: 10.1093/neuonc/not118

Kim, K., Yang, J., Zhong, X. P., Kim, M. H., Kim, Y. S., Lee, H. W., et al. (2009). Synaptic removal of diacylglycerol by DGKzeta and PSD-95 regulates dendritic spine maintenance. *EMBO J.* 28, 1170–1179. doi: 10.1038/emboj.2009.44

Kim, S., Kedan, A., Marom, M., Gavert, N., Keinan, O., Selitrennik, M., et al. (2013). The phosphatidylinositol-transfer protein Nir2 binds phosphatidic acid and positively regulates phosphoinositide signalling. *EMBO Rep.* 14, 891–899. doi: 10.1038/embor.2013.113

Kobayashi, N., Hozumi, Y., Ito, T., Hosoya, T., Kondo, H., and Goto, K. (2007). Differential subcellular targeting and activity-dependent subcellular localization of diacylglycerol kinase isozymes in transfected cells. *Eur. J. Cell Biol.* 86, 433–444. doi: 10.1016/j.ejcb.2007.05.002

Kurooka, T., Yamamoto, Y., Takai, Y., and Sakisaka, T. (2011). Dual regulation of RA-RhoGAP activity by phosphatidic acid and Rap1 during neurite outgrowth. *J. Biol. Chem.* 286, 6832–6843. doi: 10.1074/jbc.M110.183772

Lacalle, R. A., Gómez-Moutón, C., Barber, D. F., Jiménez-Baranda, S., Mira, E., C., et al. (2004). PTEN regulates motility but not directionality during leukocyte chemotaxis. *J. Cell Sci.* 117(Pt 25), 6207–6215. doi: 10.1242/jcs.01545

Lemmon, M. A. (2008). Membrane recognition by phospholipid-binding domains. *Nat. Rev. Mol. Cell Biol.* 9, 99–111. doi: 10.1038/nrm2328

Leslie, N. R., Batty, I. H., Maccario, H., Davidson, L., and Downes, C. P. (2008). Understanding PTEN regulation: PIP2, polarity and protein stability. *Oncogene* 27, 5464–5476. doi: 10.1038/onc.2008.243

Limatola, C., Schaap, D., Moolenaar, W. H., and van Blitterswijk, W. J. (1994). Phosphatidic acid activation of protein kinase C-zeta overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. *Biochem. J.* 304 (Pt 3), 1001–1008. doi: 10.1042/bj3041001

Lindsay, A. J., and McCaffrey, M. W. (2004). The C2 domains of the class I Rab11 family of interacting proteins target recycling vesicles to the plasma membrane. *J. Cell Sci.* 117(Pt 19), 4365–4375. doi: 10.1242/jcs.01280

Liu, C. H., Machado, F. S., Guo, R., Nichols, K. E., Burks, A. W., Aliberti, J. C., et al. (2007). Diacylglycerol kinase zeta regulates microbial recognition and host resistance to Toxoplasma gondii. *J. Exp. Med.* 204, 781–792. doi: 10.1084/jem.20061856

Liu, X., Kapoor, T. M., Chen, J. K., and Huse, M. (2013). Diacylglycerol promotes centrosome polarization in T cells via reciprocal localization of dynein and myosin II. *Proc. Natl. Acad. Sci. U.S.A.* 110, 11976–11981. doi: 10.1073/pnas.1306180110

Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V., et al. (2007). PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell* 128, 383–397. doi: 10.1016/j.cell.2006.11.051

Mérida, I., Andrada, E., Gharbi, S. I., and Ávila-Flores, A. (2015). Redundant and specialized roles for diacylglycerol kinases α and ζ in the control of T cell functions. *Sci. Signal.* 8, re6. doi: 10.1126/scisignal.aaa0974

Mérida, I., Avila-Flores, A., and Merino, E. (2008). Diacylglycerol kinases: at the hub of cell signalling. *Biochem. J.* 409, 1–18. doi: 10.1042/BJ20071040

Merino, E., Avila-Flores, A., Shirai, Y., Moraga, I., Saito, N., and Mérida, I. (2008). Lck-dependent tyrosine phosphorylation of diacylglycerol kinase alpha regulates its membrane association in T cells. *J. Immunol.* 180, 5805–5815. doi: 10.4049/jimmunol.180.9.5805

Mittelbrunn, M., Molina, A., Escribese, M. M., Yá-ez, M. Ó. M., Escudero, E., Yáñez-Mó, M., et al. (2004). VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11058–11063. doi: 10.1073/pnas.0307927101

Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* 395, 82–86. doi: 10.1038/25764

Mor, A., Campi, G., Du, G., Zheng, Y., Foster, D. A., Dustin, M. L., et al. (2007). The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. *Nat. Cell Biol.* 9, 713–719. doi: 10.1038/ncb1592

Mouneimne, G., Soon, L., DesMarais, V., Sidani, M., Song, X., Yip, S. C., et al. (2004). Phospholipase C and cofilin are required for carcinoma cell directionality in response to EGF stimulation. *J. Cell Biol.* 166, 697–708. doi: 10.1083/jcb.200405156

Myllymäki, S. M., Teräväinen, T. P., and Manninen, A. (2011). Two distinct integrin-mediated mechanisms contribute to apical lumen formation in epithelial cells. *PLoS ONE* 6:e19453. doi: 10.1371/journal.pone.0019453

Nishioka, T., Aoki, K., Hikake, K., Yoshizaki, H., Kiyokawa, E., and Matsuda, M. (2008). Rapid turnover rate of phosphoinositides at the front of migrating MDCK cells. *Mol. Biol. Cell* 19, 4213–4223. doi: 10.1091/mbc.E08-03-0315

Nishioka, T., Frohman, M. A., Matsuda, M., and Kiyokawa, E. (2010). Heterogeneity of phosphatidic acid levels and distribution at the plasma membrane in living cells as visualized by a Föster resonance energy transfer (FRET) biosensor. *J. Biol. Chem.* 285, 35979–35987. doi: 10.1074/jbc.M110.153007

Olenchock, B. A., Guo, R., Carpenter, J. H., Jordan, M., Topham, M. K., Koretzky, G. A., et al. (2006). Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nat. Immunol.* 7, 1174–1181. doi: 10.1038/ni1400

Piccolo, E., Innominato, P. F., Mariggio, M. A., Maffucci, T., Iacobelli, S., and Falasca, M. (2002). The mechanism involved in the regulation of phospholipase C γ1 activity in cell migration. *Oncogene* 21, 6520–6529. doi: 10.1038/sj.onc.1205821

Poli, A., Fiume, R., Baldanzi, G., Capello, D., Ratti, S., Gesi, M., et al. (2016). Nuclear localization of diacylglycerol kinase Alpha in K562 cells is involved in cell cycle progression. *J. Cell. Physiol.* doi: 10.1002/jcp.25642. [Epub ahead of print].

Prinz, P. U., Mendler, A. N., Brech, D., Masouris, I., Oberneder, R., and Noessner, E. (2014). NK-cell dysfunction in human renal carcinoma reveals diacylglycerol kinase as key regulator and target for therapeutic intervention. *Int. J. Cancer* 135, 1832–1841. doi: 10.1002/ijc.28837

Purow, B. (2015). Molecular pathways: targeting diacylglycerol kinase alpha in cancer. *Clin. Cancer Res.* 21, 5008–5012. doi: 10.1158/1078-0432.CCR-15-0413

Quann, E. J., Liu, X., Altan-Bonnet, G., and Huse, M. (2011). A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. *Nat. Immunol.* 12, 647–654. doi: 10.1038/ni.2033

Quann, E. J., Merino, E., Furuta, T., and Huse, M. (2009). Localized diacylglycerol drives the polarization of the microtubule-organizing center in T cells. *Nat. Immunol.* 10, 627–635. doi: 10.1038/ni.1734

Rainero, E., Caswell, P. T., Muller, P. A., Grindlay, J., McCaffrey, M. W., Zhang, Q., et al. (2012). Diacylglycerol kinase α controls RCP-dependent integrin trafficking to promote invasive migration. *J. Cell Biol.* 196, 277–295. doi: 10.1083/jcb.201109112

Rainero, E., Cianflone, C., Porporato, P. E., Chianale, F., Malacarne, V., Bettio, V., et al. (2014). The diacylglycerol kinase α/atypical PKC/β1 integrin pathway in SDF-1α mammary carcinoma invasiveness. *PLoS ONE* 9:e97144. doi: 10.1371/journal.pone.0097144

Ruffo, E., Malacarne, V., Larsen, S. E., Das, R., Patrussi, L., Wülfing, C., et al. (2016). Inhibition of diacylglycerol kinase α restores restimulation-induced cell death and reduces immunopathology in XLP-1. *Sci. Transl. Med.* 8, 321ra7. doi: 10.1126/scitranslmed.aad1565

Sakane, F., Imai, S., Kai, M., Yasuda, S., and Kanoh, H. (2007). Diacylglycerol kinases: why so many of them? *Biochim. Biophys. Acta* 1771, 793–806. doi: 10.1016/j.bbalip.2007.04.006

Sánchez-Madrid, F., and Serrador, J. M. (2009). Bringing up the rear: defining the roles of the uropod. *Nat. Rev. Mol. Cell Biol.* 10, 353–359. doi: 10.1038/nrm2680

Sanematsu, F., Nishikimi, A., Watanabe, M., Hongu, T., Tanaka, Y., Kanaho, Y., et al. (2013). Phosphatidic acid-dependent recruitment and function of the Rac activator DOCK1 during dorsal ruffle formation. *J. Biol. Chem.* 288, 8092–8100. doi: 10.1074/jbc.M112.410423

Sanjuán, M. A., Jones, D. R., Izquierdo, M., and Mérida, I. (2001). Role of diacylglycerol kinase alpha in the attenuation of receptor signaling. *J. Cell Biol.* 153, 207–220. doi: 10.1083/jcb.153.1.207

Serrador, J. M., Nieto, M., and Sánchez-Madrid, F. (1999). Cytoskeletal rearrangement during migration and activation of T lymphocytes. *Trends Cell Biol.* 9, 228–233. doi: 10.1016/S0962-8924(99)01553-6

Shafaq-Zadah, M., Gomes-Santos, C. S., Bardin, S., Maiuri, P., Maurin, M., Iranzo, J., et al. (2016). Persistent cell migration and adhesion rely on retrograde transport of β(1) integrin. *Nat. Cell Biol.* 18, 54–64. doi: 10.1038/ncb3287

Shi, S. H., Jan, L. Y., and Jan, Y. N. (2003). Hippocampal neuronal polarity specified by spatially localized mPar3/mPar6 and PI 3-kinase activity. *Cell* 112, 63–75. doi: 10.1016/S0092-8674(02)01249-7

Shirai, Y., Kouzuki, T., Kakefuda, K., Moriguchi, S., Oyagi, A., Horie, K., et al. (2010). Essential role of neuron-enriched diacylglycerol kinase (DGK), DGKbeta in neurite spine formation, contributing to cognitive function. *PLoS ONE* 5:e11602. doi: 10.1371/journal.pone.0011602

Spira, F., Mueller, N. S., Beck, G., von Olshausen, P., Beig, J., and Wedlich-Söldner, R. (2012). Patchwork organization of the yeast plasma membrane into numerous coexisting domains. *Nat. Cell Biol.* 14, 640–648. doi: 10.1038/ncb2487

Spitaler, M., Emslie, E., Wood, C. D., and Cantrell, D. (2006). Diacylglycerol and protein kinase D localization during T lymphocyte activation. *Immunity* 24, 535–546. doi: 10.1016/j.immuni.2006.02.013

Tanaka, T., Okada, M., Hozumi, Y., Tachibana, K., Kitanaka, C., Hamamoto, Y., et al. (2013). Cytoplasmic localization of DGKζ exerts a protective effect against p53-mediated cytotoxicity. *J. Cell Sci.* 126(Pt 13), 2785–2797. doi: 10.1242/jcs.118711

Torres-Ayuso, P., Daza-Martín, M., Martín-Pérez, J., Ávila-Flores, A., and Mérida, I. (2014). Diacylglycerol kinase α promotes 3D cancer cell growth and limits drug sensitivity through functional interaction with Src. *Oncotarget* 5, 9710–9726. doi: 10.18632/oncotarget.2344

Torres-Ayuso, P., Tello-Lafoz, M., Mérida, I., and Ávila-Flores, A. (2015). Diacylglycerol kinase-ζ regulates mTORC1 and lipogenic metabolism in cancer cells through SREBP-1. *Oncogenesis* 4, e164. doi: 10.1038/oncsis.2015.22

Toyoshima, F., and Nishida, E. (2007). Integrin-mediated adhesion orients the spindle parallel to the substratum in an EB1- and myosin X-dependent manner. *EMBO J.* 26, 1487–1498. doi: 10.1038/sj.emboj.7601599

Tsai, F. C., Seki, A., Yang, H. W., Hayer, A., Carrasco, S., Malmersjö, S., et al. (2014). A polarized Ca2+, diacylglycerol and STIM1 signalling system regulates directed cell migration. *Nat. Cell Biol.* 16, 133–144. doi: 10.1038/ncb2906

Tsushima, S., Kai, M., Yamada, K., Imai, S., Houkin, K., Kanoh, H., et al. (2004). Diacylglycerol kinase gamma serves as an upstream suppressor of Rac1 and lamellipodium formation. *J. Biol. Chem.* 279, 28603–28613. doi: 10.1074/jbc.M314031200

Van Horn, W. D., and Sanders, C. R. (2012). Prokaryotic diacylglycerol kinase and undecaprenol kinase. *Annu. Rev. Biophys.* 41, 81–101. doi: 10.1146/annurev-biophys-050511-102330

Yang, E., Singh, B. K., Paustian, A. M., and Kambayashi, T. (2016). Diacylglycerol kinase ζ is a target to enhance NK cell function. *J. Immunol.* 197, 934–941. doi: 10.4049/jimmunol.1600581

Yasuda, S., Kai, M., Imai, S., Kanoh, H., and Sakane, F. (2007). Diacylglycerol kinase gamma interacts with and activates beta2-chimaerin, a Rac-specific GAP, in response to epidermal growth factor. *FEBS Lett.* 581, 551–557. doi: 10.1016/j.febslet.2007.01.022

You, J. S., Lincoln, H. C., Kim, C. R., Frey, J. W., Goodman, C. A., Zhong, X. P., et al. (2014). The role of diacylglycerol kinase ζ and phosphatidic acid in the mechanical activation of mammalian target of rapamycin (mTOR) signaling and skeletal muscle hypertrophy. *J. Biol. Chem.* 289, 1551–1563. doi: 10.1074/jbc.M113.531392

Yu, C. G., and Harris, T. J. (2012). Interactions between the PDZ domains of Bazooka (Par-3) and phosphatidic acid: *in vitro* characterization and role in epithelial development. *Mol. Biol. Cell* 23, 3743–3753. doi: 10.1091/mbc.E12-03-0196

Zhao, F., Cannons, J. L., Dutta, M., Griffiths, G. M., and Schwartzberg, P. L. (2012). Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytolysis. *Immunity* 36, 1003–1016. doi: 10.1016/j.immuni.2012.05.017

Zhong, X. P., Hainey, E. A., Olenchock, B. A., Jordan, M. S., Maltzman, J. S., Nichols, K. E., et al. (2003). Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nat. Immunol.* 4, 882–890. doi: 10.1038/ni958

Zovein, A. C., Luque, A., Turlo, K. A., Hofmann, J. J., Yee, K. M., Becker, M. S., et al. (2010). Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. *Dev. Cell* 18, 39–51. doi: 10.1016/j.devcel.2009.12.006

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read, for greatest visibility

COLLABORATIVE PEER-REVIEW
Designed to be rigorous – yet also collaborative, fair and constructive

FAST PUBLICATION
Average 85 days from submission to publication (across all journals)

COPYRIGHT TO AUTHORS
No limit to article distribution and re-use

TRANSPARENT
Editors and reviewers acknowledged by name on published articles

SUPPORT
By our Swiss-based editorial team

IMPACT METRICS
Advanced metrics track your article's impact

GLOBAL SPREAD
5'100'000+ monthly article views and downloads

LOOP RESEARCH NETWORK
Our network increases readership for your article

Frontiers
EPFL Innovation Park, Building I • 1015 Lausanne • Switzerland
Tel +41 21 510 17 00 • Fax +41 21 510 17 01 • info@frontiersin.org
www.frontiersin.org

Find us on