Published in final edited form as:

Eur J Immunol. 2020 November ; 50(11): 1729–1745. doi:10.1002/eji.201948442.

Negative control of diacylglycerol kinase ζ -mediated inhibition of T cell receptor signaling by nuclear sequestration in mice

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Abstract

Diacylglycerol kinases (DGKs) play important roles in restraining diacylglycerol (DAG)-mediated signaling. Within the DGK family, the ζ isoform appears to be the most important isoform in T cells for controlling their development and function. DGK ζ has been demonstrated to regulate T cell maturation, activation, anergy, effector/memory differentiation, defense against microbial infection, and anti-tumor immunity. Given its critical functions, DGK ζ function should be tightly regulated to ensure proper signal transduction; however, mechanisms that control DGK ζ function are still poorly understood. We report here that DGK ζ dynamically translocates from the cytosol into the nuclei in T cells after TCR stimulation. In mice, DGK ζ mutant defective in nuclear localization displayed enhanced ability to inhibit TCR-induced DAG-mediated signaling in primary T cells, maturation of conventional $\alpha\beta$ T and iNKT cells, and activation of peripheral T cells compared with wild-type DGK ζ . Our study reveals for the first time nuclear sequestration of DGK ζ as a negative control mechanism to spatially restrain it from terminating DAG mediated signaling in T cells. Our data suggest that manipulation of DGK ζ nucleus-cytosol shuttling as a novel strategy to modulate DGK ζ activity and immune responses for treatment of autoimmune diseases and cancer.

Keywords

TCR; diacylglycerol kinases; T cell development; invariant NKT cell; Ras/MAPK

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Author contributions

DX, SZ, PC, WD, YP, JX, BL, and JW designed and performed experiments and analyzed data. XZ conceived the project, designed experiments, and was involved in data interpretation. XZ, JS, DX, and SZ wrote the manuscript.

Conflict of Interest Disclosures

The authors declare no commercial or financial conflict of interests.

INTRODUCTION

Diacylglycerol (DAG) is an important second messenger that mediates signal transduction downstream of numerous receptors. It associates with and regulates the subcellular localization and activities of multiple effector molecules. DAG-mediated signal pathways play important roles in numerous cellular processes and functions such as cell growth, differentiation, proliferation, metabolism, survival, and effector function [1, 2]. In T lymphocytes, DAG activates the RasGRP1-Ras-Erk1/2 and PKC θ -IKK-NF κ B pathways to promote T cell development and activation [3–7]. Evidence has also illustrated equal importance of tight control of DAG-mediated signaling. Dysregulation of DAG signal can cause developmental defects, abnormal cellular activation and function, and even tumorigenesis [8, 9].

One pathway involved in terminating DAG is mediated by DAG kinases (DGK) via phosphorylation and conversion to phosphatidic acid (PA) [1, 2]. PA itself is also an important second messenger that regulates the activities of multiple effector molecules such as PI3K and SHP1 [10, 11]. Thus, DGKs can function as a switch by terminating DAG signal and simultaneously initiating PA mediated signal. Several studies have suggested that such a switch could exert important roles in the immune system [8, 12, 13]. In mammals, ten DGKs encoded by different genes have been identified. Some DGKs contain splicing isoforms, adding complexity of this family of enzymes [1, 2]. DGK α , ζ , and δ are the predominant isoforms of the DGK family expressed in T cells [14–17]. While the physiological function of DGK δ in the immune system remains unclear, DGK α and ζ have been demonstrated to be important regulators in multiple immune cell lineages, particularly in T lymphocytes, by functioning synergistically or redundantly in these cells. Both DGK α and ζ , inhibit the RasGRP1-Ras-Erk1/2 pathway, the PKC θ -IKK-NF κ B pathway, PI3K-AKT and mTOR signaling [14, 18–21]. They regulate the development of conventional $\alpha\beta$ T cells, iNKT cells, mucosal associated invariant T cells, and regulatory T cells, inhibit T cell activation but promote T cell anergy, regulate activation induced T cell death, control T helper cell differentiation and effector and memory CD8 T cell responses to viral and bacterial pathogens, and inhibit CD8 T cell- and chimeric antigen receptor T cell-mediated anti-tumor responses [8, 15, 16, 19, 20, 22–36]. In T cells, B cells, and NK cells, DGK ζ appears to exert greater importance than DGK α [15, 31, 37, 38].

All DGK isoforms contain a catalytic domain and 2 – 3 cysteine rich C1-like domains. They also contain other structural features, based on which DGKs have been divided into five types. Such type-specific structural features may mediate protein-protein and protein-lipid interaction to control DGK activity, subcellular localization, and function [39–43]. Many in vitro studies with various cell line models have suggested important roles of some DGK structural features in regulating DGK function [14, 40, 41, 44]. However, in vivo importance of specific structural features of the DGK family under physiological and pathological conditions has not been illustrated thus far.

DGK ζ , belonging to type IV of the DGK family, contains several type-specific structural motifs/domains besides the catalytic and C1-like domains. A nuclear localization sequences (NLS) overlaps with a MARCKS motif and is localized between the C1 domain and the

catalytic domain; a nuclear export sequence is located within the catalytic domain; a PDZ-binding motif sits at the carboxyl terminus; and several ankyrin repeats are localized close to the C-terminus [45–47]. DGK ζ can be detected in both cytosolic and nuclear compartments [44, 48, 49]. Cytosolic location of DGK ζ allows it to quickly translate to the cytoplasmic membrane and immune synapse to assess its substrate [50]. However, the functional significance of DGK ζ nuclear localization in immune cells is still unclear.

In this report, we generated and examined multiple mouse models that allow conditional expression of wild-type (WT), kinase dead (KD) mutant, or nuclear localization mutant (NLS) DGK ζ after Cre-mediated deletion of a transcription stop cassette flanked by two LoxP sites. We demonstrated that WT DGK ζ dynamically translocated from the cytosolic compartment into the nuclei in primary T cells after TCR engagement and that mutation of its NLS prevented it from entering the nucleus. Compared with DGK ζ^{WT} , DGK ζ^{NLS} mutant displayed enhanced ability to inhibit DAG-mediated signaling, intrathymic T cell and iNKT cell development, and T cell activation. Our data reveal that nuclear localization is a mechanism to negative control of DGK ζ function by preventing its access to substrate. Additionally, we provide evidence that iNKT cell development was subjected to stricter requirement of DAG signaling than conventional $\alpha\beta$ T cells.

RESULTS

Generation of mice carrying inducible WT and mutant DGK ζ

To investigate whether DGK ζ function is regulated by nuclear localization, we generated three lines of mice that carry either a GFP-DGK ζ^{WT} , GFP-DGK ζ^{NLS} (NLS mutant with lysines in the NLS mutated to alanine), or GFP-DGK ζ^{KD} (kinase dead with the glycine residue in the ATP binding site mutated to alanine) transgene knocked into the Rosa26 locus (Figure 1A, 1B). These transgenes contained the 104 kDa DGK ζ 1 isoform fused to the carboxyl terminus of EGFP to allow for identification of DGK ζ expressing cells and DGK ζ localization by GFP. A CAG promoter was inserted 5' to the transgene to increase its expression and a transcription STOP cassette flanked by two loxP sites was located between the promoter and the GFP-DGK ζ knockin (GFP-DGK ζ^{KI}) transgene so that transgenic GFP-DGK ζ could only be expressed after Cre-mediated deletion of the transcription STOP. Successful generation of these mice was confirmed by PCR amplification of the transgenes (Figure 1C).

After breeding these mice with the CD4Cre transgenic mice to delete the STOP cassette starting at the CD4⁺CD8⁺ double positive (DP) thymocytes, we could detect GFP-DGK ζ fusion protein in GFP-DGK ζ^{WT} -, GFP-DGK ζ^{KD} -, and GFP-DGK ζ^{NLS} -CD4Cre thymocytes with an anti-GFP antibody. Both endogenous DGK ζ 1 and ζ 2 could be detected in control total thymocyte lysates using an anti-DGK ζ antibody and the GFP-DGK ζ fusion proteins overlapped with the endogenous DGK ζ 2 (124kDa). The expression levels of these fusion proteins appeared similar in thymocytes detected by Western blot analysis (Figure 1D, 1E) and in live CD4⁺CD8⁺ double positive (DP), CD4⁺CD8⁻ single positive (SP), and CD4⁻CD8⁺ SP thymocytes based on GFP expression (Figure 1F). GFP-DGK ζ^{WT} , GFP-DGK ζ^{KD} , and GFP-DGK ζ^{NLS} fusion protein levels in thymocytes were about 3.4, 3, and 3.3 folds of the endogenous WT DGK ζ 2 in thymocytes (Figure 1D, 1E). Different from T

cells, B220⁺ B cells did not show obvious GFP expression (Figure 1G). Expression of TCR β in DN, DP, and SP thymocytes were not obviously affected by these DGK ζ transgenes (Figure 1H). Together, these results demonstrated successful generation of these conditional GFP-DGK ζ transgenic mice and that NLS and KD mutations did not obviously affect DGK ζ stability.

Inhibition of T cell maturation by DGK ζ NLS mutant

DAG-mediated signaling and its tight regulation by DGK ζ play important roles in T cell maturation in the thymus [8, 51, 52]. Among DAG effector pathways, the RasGRP1-Ras-Erk1/2 pathway mediates positive selection of DP thymocytes and maturation to CD4⁺ or CD8⁺ single positive (SP) stage [51, 52]. In GFP-DGK ζ ^{WT}-CD4Cre mice, total thymocyte numbers were similar to WT controls (Figure 2A). TCR β ⁺ thymocyte percentages and numbers were also comparable to WT control mice (Figure 2B, 2C). Similar to GFP-DGK ζ ^{WT}-CD4Cre mice, GFP-DGK ζ ^{KD}-CD4Cre mice did not display obvious differences to WT control mice in total thymic cellularity and TCR β ⁺ thymocyte numbers. Interestingly, GFP-DGK ζ ^{NLS}-CD4Cre mice contained slightly increased total thymocyte numbers with 40% increases compared to WT control mice. However, the percentages and numbers of TCR β ⁺ thymocytes were decreased, suggesting impaired T cell generation in these mice (Figure 2B, 2C).

T cells mature in the thymocytes sequentially through the CD4⁻CD8⁻ DN, CD4⁺CD8⁺ DP, to the CD4⁺ or CD8⁺ SP stages. In GFP-DGK ζ ^{WT}-CD4Cre mice and GFP-DGK ζ ^{KD}-CD4Cre mice, the percentages and numbers of DN, DP, and SP thymocytes were not obviously different from control mice, indicating no gross blockade of T cell maturation (Figure 3A, 3B, 3D). However, in GFP-DGK ζ ^{NLS}-CD4Cre mice, CD4⁺CD8⁻TCR β ⁺ and CD4⁻CD8⁺TCR β ⁺ SP mature T cells were decreased in both percentages and numbers (Figure 3A, 3C). Within DP thymocytes, the percentages and numbers of the post-selection CD69⁺TCR β ⁺ cells were decreased in GFP-DGK ζ ^{NLS}-CD4Cre mice (Figure 3E), suggesting impairment of positive-selection of developing thymocytes. The decreases of CD4⁺ SP thymocytes (69.5%) appeared more severe than the decreases of CD8⁺ SP thymocytes (32.4%), suggesting differentiation and/or positive selection of CD4⁺ T cell lineage was more sensitive to GFP-DGK ζ ^{NLS} than CD8⁺ T cell lineage. In contrast to decreases of SP thymocytes, DP thymocytes in GFP-DGK ζ ^{NLS}-CD4Cre mice were slightly increased about 39% (Figure 3D). Consistent with impaired maturation, both percentages and numbers of CD4 T cells in the spleen and lymph nodes (LNs) from GFP-DGK ζ ^{NLS}-CD4Cre mice but not from GFP-DGK ζ ^{WT}-CD4Cre mice and GFP-DGK ζ ^{KD}-CD4Cre mice were decreased compared with control mice (Figure 3F–3H). The decreases of peripheral CD8 T cells were subtler than CD4 T cells. Together, these data revealed that GFP-DGK ζ ^{NLS} but not GFP-DGK ζ ^{WT} or GFP-DGK ζ ^{KD} were capable of inhibiting T cell maturation from DP to SP stages.

Enhanced inhibition of TCR induced DAG signaling and Egr1 expression by DGK ζ NLS

Activation of RasGRP1 by DAG leads to Erk1/2 activation in T cells. Following anti-CD3 stimulation, thymocytes from GFP-DGK ζ ^{WT}-CD4Cre mice displayed decreased Erk1/2 phosphorylation (Figure 4A, 4B), suggesting that increased DGK ζ expression inhibited

activation of the RasGRP1-Ras-Erk1/2 pathway. Compared with GFP-DGK ζ^{WT} -CD4Cre thymocytes, anti-CD3-induced Erk1/2 phosphorylation in GFP-DGK ζ^{NLS} -CD4Cre thymocytes was further reduced. DAG-mediated RasGRP1-Ras-Erk1/2 functions upstream of both mTORC1 and mTORC2 activation after TCR engagement [21] and the DAG-PKC θ -CARMA1 pathway promoted mTORC1 activation [53]. DGK α and ζ deficiency led to enhanced TCR-induced mTORC1 and mTORC2 activation [21]. TCR induced S6 phosphorylation and Akt S473 phosphorylation, indicators of mTORC1 and mTORC2 activation respectively, were decreased slightly in GFP-DGK ζ^{WT} -CD4Cre thymocytes but decreased more obviously in GFP-DGK ζ^{NLS} -CD4Cre thymocytes. Together, these observations demonstrated that increased expression of DGK ζ in developing thymocytes inhibited TCR-induced Erk1/2 and mTOR activation and that GFP-DGK ζ^{NLS} mutant was a gain-of-function mutant with enhanced ability to terminate DAG signaling compared with WT DGK ζ .

During T cell development, the transcription factor Egr1 promotes positive selection of conventional T cells and iNKT cells downstream of the Ras/Erk1/2 pathway [54–56]. Both transcription factor ThPOK and Runx3 are critical for the development of CD4 and CD8 lineages, respectively [57–59]. Consistent with decreased Erk1/2 activation, Egr1 expression in the DGK ζ^{NLS} -CD4Cre DP, CD4⁺, and CD8⁺ thymocytes was decreased compared with WT mice. However, ThPOK and Runx3 levels in these cells were similar to WT controls (Figure 4C). These results suggest that decreased Egr1 expression may contribute to impaired positive selection of both conventional T cells and iNKT cells as will be described later and that different sensitivities of CD4 and CD8 SP thymocytes to GFP-DGK ζ^{NLS} might not be caused altered expression of ThPOK or Runx3.

DGK ζ^{NLS} displays enhanced ability to inhibit T cell activation

DAG mediated signaling pathways are important for T cell activation. CD69 upregulation is one of the early T cell activation events that is dependent on activation of the DAG-RasGRP1-Ras-Erk1/2-AP1 (activator protein 1) pathway. T cells from GFP-DGK ζ^{WT} mice showed reduced CD69 upregulation following anti-CD3 stimulation (Figure 5A, 5B). CD69 upregulation was further reduced in T cells from GFP-DGK ζ^{NLS} -CD4Cre mice compared with GFP-DGK ζ^{WT} -CD4Cre mice. In addition to CD69, CD25 is also upregulated during T cell activation. GFP-DGK ζ^{WT} was able to weakly inhibit CD25 upregulation but GFP-DGK ζ^{NLS} demonstrated stronger inhibition than GFP-DGK ζ^{WT} (Figure 5A, 5B). Using a Celltrace violet proliferation dye dilution assay, we also observed weak inhibition of TCR-induced T cell proliferation by GFP-DGK ζ^{WT} but much stronger inhibition of proliferation of both CD4 and CD8 T cells by GFP-DGK ζ^{NLS} (Figure 5C). Moreover, GFP-DGK ζ^{NLS} inhibited T cell production of IL2 after TCR stimulation (Figure 5D). Such inhibitory effects of GFP-DGK ζ^{NLS} displayed diminished magnitudes when stimulated with both anti-CD3 and anti-CD28 antibodies and were abolished, when stimulated with PMA and ionomycin (Figure 5D, Supplemental Figure S2A–2C), which was consistent with the ability of PMA to resistant to DGK activity. Different from GFP-DGK ζ^{NLS} and GFP-DGK ζ^{WT} , GFP-DGK ζ^{KD} did not obviously affected TCR induced Erk1/2 phosphorylation, CD69 or CD25 upregulation, and proliferation of T cells (Supplemental Figure S3A – 3D), indicating that the inhibitory activity of DGK ζ was dependent of its kinase activity. Together, these

observations indicate that GFP-DGK ζ ^{NLS} contained enhanced capability to inhibit T cell activation compared with GFP-DGK ζ ^{WT}.

Inhibition iNKT cell maturation by GFP-DGK ζ ^{WT} and GFP-DGK ζ ^{NLS}

RasGRP1-Ras-Erk1/2 and PKC θ -IKK-NF κ B pathways as well as mTOR signaling are important for iNKT cell maturation [33, 34, 56, 60–68]. Because DGK α and ζ inhibit activation of these signal events [6, 21, 53], we further examined the effects of DGK ζ mutants on iNKT cell development. We observed about 50% decreases of TCR β ⁺PBS57 loaded CD1d tetramer (CD1Dtet)⁺ iNKT cells in the thymus, spleen, and liver in GFP-DGK ζ ^{WT}-CD4Cre mice (Figure 6A, 6B) but not in GFP-DGK ζ ^{KD}-CD4Cre mice (Figure 6C, 6D) compared with their respectively WT controls, indicating kinase activity dependent inhibition of iNKT cell development by GFP-DGK ζ ^{WT}. The decreases of iNKT cells were much severer in GFP-DGK ζ ^{NLS}-CD4Cre mice with 92.4, 92.2, and 87.5% reduction of iNKT cells in the thymus, spleen, and liver, respectively, compared with WT controls (Figure 6E, 6F). Together, these data indicated that iNKT cell development was sensitive to increased DGK activity. The much severer iNKT cell developmental blockade in GFP-DGK ζ ^{NLS}-CD4Cre mice than in GFP-DGK ζ ^{WT}-CD4Cre further supported that DGK ζ ^{NLS} is a gain-of-function mutation.

iNKT cell development in the thymus can be defined into CD24⁺CD44⁻NK1.1⁻ stage 0, and CD24⁻CD44⁻NK1.1⁻ stage 1, CD24⁻CD44⁺NK1.1⁻ stage 2, and CD24⁻CD44⁺NK1.1⁺ stage 3 [69, 70]. We observed decreases of iNKT cells in the thymus of GFP-DGK ζ ^{NLS}-CD4Cre mice began at stage 0 and became more obvious at stages 1 – 3 (Figure 6G, 6H), suggesting that DGK ζ ^{NLS} might inhibit not only iNKT cell development at the earliest stage but also the accumulation/expansion of late stage iNKT cells.

Dynamic nucleus localization of DGK ζ in the activated-T cells during TCR stimulation

To explore the relationship between DGK ζ function and subcellular localization, we examined GFP-DGK ζ localization in peripheral T cells from GFP-DGK ζ ^{WT}-CD4Cre, GFP-DGK ζ ^{NLS}-CD4Cre, and GFP-DGK ζ ^{KD}-CD4Cre mice. In unstimulated T cells, GFP-DGK ζ ^{WT} and GFP-DGK ζ ^{KD} were detected in both the cytosol and the nucleus, with stronger fluorescence intensity in the cytosol than in the nucleus (Figure 7A, 7B). During anti-CD3 stimulation, GFP-DGK ζ ^{WT} and GFP-DGK ζ ^{KD} began to accumulate in the nucleus at 10 minutes after stimulation and such nuclear accumulation of DGK ζ appeared intensified 30' and 4 hours after stimulation and maximized after overnight stimulation, suggesting that the kinase activity did not contribute to TCR-induced DGK ζ nuclear translocation. However, GFP-DGK ζ ^{NLS} was predominantly localized in the cytoplasmic compartment with little nuclear localization in unstimulated T cells. Moreover, it did not translocate into the nucleus after anti-CD3 stimulation. To rule out the possibility that DGK ζ localization was influenced by GFP, we examined endogenous DGK ζ localization in WT T cells. We observed that endogenous DGK ζ mainly localized in the cytosol before stimulation and translocated to the nucleus after overnight TCR stimulation (Figure 7C, 7D). Thus, both endogenous DGK ζ and GFP-DGK ζ localized similarly in the cytosol in unstimulated state and translocated into the nucleus after TCR stimulation.

Because primary T cells had very small cytosolic compartment and were difficult to distinguish cytosol and cytoplasm membrane, we established 2B4 T cell lines that expressed either GFP-DGK ζ^{WT} or GFP-DGK ζ^{NLS} to examine cytosolic and plasma membrane distribution of DGK ζ during TCR engagement. As shown in figure S4A – S4C, both DGK ζ^{WT} and DGK ζ^{NLS} were detected in the cytosol and cytoplasm membrane before TCR stimulation. After overnight anti-CD3 stimulation, DGK ζ^{WT} translocated into the nucleus or presented at the nucleus envelope. However, the DGK ζ^{NLS} mostly presented at the cytoplasm membrane.

Taken together, our results indicated that WT DGK ζ localization was dynamically regulated. It was predominantly localized in the cytosolic compartment in resting state and dynamically translocated into the nucleus after TCR stimulation and the NLS was crucial for DGK ζ nuclear localization to occur. When NLS was mutated, DGK ζ displayed increased tendency to localize in the cytosol and at the cytoplasmic membrane. Collectively, these observations together with enhanced inhibition of DAG-mediated signaling, T cell activation, and T cell development by GFP-DGK ζ^{NLS} indicated that DGK ζ nuclear translocation from the cytosol may sequester it from access to its substrates in the cytoplasmic compartment and, especially, at the cytoplasm membrane and thus functions as a negative control mechanism of DGK ζ function during T cell development and activation.

DISCUSSION

DGK isoforms are differentially expressed in various tissues to regulate diverse cellular processes. Given their important physiological functions, DGKs should be tightly regulated. Although both transcriptional and post-transcriptional mechanisms have been reported to control the expression and function of specific isoforms in multiple cell line models [17, 71, 72], mechanisms that regulate DGKs and the physiological importance of such regulation, especially in vivo settings, are still poorly understood.

In this study, we have focused on DGK ζ because its predominant roles in several immune cell lineages. Previous studies have found that DGK ζ is dynamically regulated in T cells with high expression in naïve resting T cells, anergic T cells, and tumor infiltration T cells but downregulated expression during T cell activation [15, 16, 72]. High levels of DGK ζ in naïve T cells might be important to prevent DAG accumulation in these cells so that tonic TCR signal would not trigger spontaneous T cell activation; while downregulation of DGK ζ expression during T cell activation may allow full activation of these cells. Such dynamic expression of DGK ζ is in part controlled by miR34a that directly targets DGK ζ [72]. Elevated DGK ζ in anergic and tumor-infiltrating T cells may contribute to their impaired anti-tumor immunity [23, 73]. To explore additional mechanisms that regulate DGK ζ function, we have generated three lines of mice with conditional expression of GFP-DGK ζ^{WT} , GFP-DGK ζ^{KD} , and GFP-DGK ζ^{NLS} in developing thymocytes and peripheral T cells. We have demonstrated that expression of GFP-DGK ζ^{WT} but not GFP-DGK ζ^{KD} weakly inhibits TCR-induced Erk1/2 activation, CD69 and CD25 upregulation, and proliferation of T cells and causes moderate reduction of iNKT cells but not conventional $\alpha\beta$ T cells. These data not only extend previous studies in cell line models that overexpression of DGK ζ inhibits TCR signaling and upregulation of activation markers to

primary cells but also reveals important consequences when DGK ζ is overexpressed in vivo and revealed that iNKT cell development is more sensitive to altered DGK ζ activity than $\alpha\beta$ T cells. More importantly, we show that GFP-DGK ζ^{NLS} displays much stronger effects on T cells than GFP-DGK ζ^{WT} in inhibiting DAG-mediated signaling, T cell activation, and iNKT cell development. Moreover, GFP-DGK ζ^{NLS} but not GFP-DGK ζ^{WT} is able to impede conventional $\alpha\beta$ T cell maturation. Based on these observations, we conclude that DGK ζ^{NLS} is a gain-of-function mutant in developing thymocytes and peripheral T cells with enhanced ability to suppress DAG-mediated signaling.

Subcellular localization of DGK ζ varies dramatically among different cell types. It has been shown to localize predominantly in the nucleus, in the cytosolic compartment, or distribute in both nucleus and cytosol dependent on different cell types and tissues examined [41, 44, 47, 74–77]. Our data indicate that DGK ζ function is dynamically regulated in T cells via shuttling between the cytosol and nucleus and that nuclear localization of DGK ζ serves as a negative control of DGK ζ function by preventing it from accessing its substrate in the cytoplasmic membrane. In primary T cells, GFP-DGK ζ^{WT} as well as endogenous DGK ζ preferentially localizes in the cytosolic compartment with a small portion of the protein residing in the nucleus in unstimulated primary T cells. TCR stimulation induces rapid DGK ζ translocation and accumulation in the nucleus accompanying concordant depletion in the cytosol. An event critical for TCR-induced T cell activation is the activation of PLC γ 1 and subsequent production of DAG. Newly generated DAG localizes in the cytoplasmic membrane, particularly in the immune synapse [78]. Nuclear translocation of DGK ζ during T cell activation may prevent it from accessing the newly generated DAG to ensure DAG-mediated activation of downstream signal cascades for T cell activation. Failure of nuclear translocation of the DGK ζ^{NLS} mutant allows for its persistence in the cytosol, leading to accelerated termination of DAG generated after TCR engagement.

It is interesting to note that DGK ζ is mainly localized in the nucleus in several other cell types such as neurons and myoblasts. Nuclear localization of DGK ζ in these cells is important for its stability as cytosolic DGK ζ is subjected to ubiquitination and proteasomal degradation and for its ability to negative control of cell cycle [44, 48, 49, 71, 79, 80]. Different from these cells, both endogenous DGK ζ and GFP-DGK ζ are primarily localized in the cytosol in primary T cells in the quiescent state but translocate into the nucleus during T cell activation. In addition, we do not observe decreased expression of GFP-DGK ζ^{NLS} compared with GFP-DGK ζ^{WT} . Such differences between T cells and neurons as well as several other cell types suggest that T cells utilize different mechanisms to control DGK ζ localization and stability.

Activation of the RasGRP1-Ras-Erk1/2 pathway is critical for positive selection of DP thymocytes to develop into SP mature T cells as well as iNKT cells [34, 51, 56]. Decreases of CD69⁺TCR β ⁺ post-selection DP thymocytes and SP mature T cells by GFP-DGK ζ^{NLS} suggests that positive selection is impaired. These data indicate that certain levels of DAG are needed in DP thymocytes for successful execution of positive selection and DGK ζ^{NLS} may deplete the DAG pool necessitated for activation of the RasGRP1-Ras-Erk1/2 pathway for positive selection. Of note, although GFP-DGK ζ^{WT} weakly inhibits DAG-mediated signaling and T cell activation, it does not cause obvious reduction of SP thymocytes, which

could be resulted from alterations of T cell repertoire to compensate for reduced DAG levels. Different from conventional $\alpha\beta$ T cells, iNKT cells express an invariant TCR-V α 14 chain and limited TCRV β chains. Their semi-fixed TCR repertoire may limit their ability to accommodate slightly weakened DAG signaling caused by GFP-DGK ζ^{WT} , leading to their hypersensitivity to altered DAG signaling during development.

Although we consider the impact of DGK ζ^{NLS} is mainly caused by enhanced terminating DAG due to its failure to shuttle to the nucleus, our data do not rule out that DGK ζ^{NLS} may act through other mechanisms. DGK ζ^{NLS} may overproduce PA in the cytoplasmic membrane, which may itself have important biological consequences. It has also been reported that cytoplasmic DGK ζ reduces p53-mediated cytotoxicity against doxorubicin-induced DNA damage by facilitating cytoplasmic anchoring and degradation of p53 through an ubiquitin-proteasome system [41]. DGK ζ also interacts with the adaptor molecules γ 1-syntrophin, which links ion channels and signaling proteins to dystrophin and its homologues, and such interaction is important for γ 1-syntrophin translocation to the nucleus and for anchoring DGK ζ to the cytoplasm membrane [75, 81]. Accumulation of DGK ζ^{NLS} in the cytosol during T cell activation could potentially affect these molecules and exert other functions besides regulating DAG signaling. Additionally, DGK ζ may perform important functions inside the nucleus. The nucleus contains different pools of DAG and nuclear DGK ζ has been shown to reduce nuclear DAG concentration in HEK293 cells [44]. In the nucleus of skeletal muscle, nuclear DGK ζ interacts with the class O of forkhead box transcription factor (FoxO) to mitigate FoxO-mediated ubiquitin-proteasome system dependent proteolysis to prevent muscle atrophy, which is independent on its kinase activity but dependent on its nuclear localization [77]. Whether nuclear DGK ζ performs similar functions in T cells remains to be determined. Because the endogenous DGK ζ , which co-expresses with GFP-DGK ζ^{NLS} , could still translocate to the nucleus, it is unlikely that nuclear DGK activity would be defective in GFP-DGK ζ^{NLS} expressing T cells to cause the phenotypes we have observed.

An important question remains to be addressed is how DGK ζ shuttling between the cytosol and nucleus is regulated. In HEK293 cells, DGK ζ nuclear localization is regulated by PKC-mediated phosphorylation in its MARCKS motif [44]. Because the MARCKS motif and NLS overlap within DGK ζ , phosphorylation of the serine residues within MARCKS motif may control DGK ζ cytosol/nucleus shuttling directly or via binding to yet to be identified proteins. Additionally, the K to A mutations in DGK ζ^{NLS} could also influence MARCKS motif phosphorylation, which needs to be determined in the future. It has also been reported that nucleosome assembly protein 1-like 1 (NAP1L1) and NAP1L4 shuttle between the nucleus and the cytoplasm in HEK293 cells and they interact with DGK ζ to prohibit its nuclear import via blocking its association with import carrier proteins, Qip1 (importin α 3) and NPI1 (importin α 5) [82]. Future studies should determine whether these mechanisms are involved regulating DGK ζ subcellular localization in T cells.

In summary, our data demonstrate that DGK ζ nuclear localization serves as a mechanism to negatively control DGK ζ function during T cell development and activation. We propose that manipulating cytosolic and nuclear DGK ζ levels as a novel strategy to enhance or inhibit T cell activation for cancer immunotherapy or for treatment of autoimmune diseases.

To achieve this, future studies should illustrate mechanisms that control DGK ζ nuclear localization.

MATERIALS AND METHODS

Mice

Cd4Cre mice were acquired from Charles River Laboratory. Coding sequences for GFP-DGK ζ^{WT} , GFP-DGK ζ^{KD} , and GFP-DGK ζ^{NLS} fusion proteins were generated by attaching WT and mutant DGK ζ 1 coding sequence to the carboxyl terminus of EGFP and subsequently cloned into the Asc I site of the *Rosa26-hCD2* targeting vector using standard molecular cloning approaches. DGK ζ^{KD} contains a G355D mutation in the ATP binding site within the catalytic domain. DGK ζ^{NLS} contains L₂₅₅KASKKKKRASFKRKSSKKG₂₇₄ to LAASAAAARASFAAASSAAG in its NLS that overlaps with the MARCKS motif. Linearized targeting vectors were transfected into the C57BL/6N ES cells. Successfully targeted clones, identified by PCR screen, were used to generate chimeric founder mice. Founders were bred with *Cd4Cre* (*B6.Cg-Tg(CD4-cre)1Cwi N9*) mice [83] to induce GFP-DGK ζ expression in T cells. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Duke University.

Antibodies, tetramers, and flow cytometry

Fluorochrome-conjugated TCR- β (clone H57–597), NK1.1 (clone PK136), CD62L (clone MEL-14), CD44 (clone IM7), CD24 (clone M1/69), CD3e (clone 145–2C11), CD69 (clone H1.2F3), CD25 (clone 3C7), CD4 (clone GK1.5), CD8a (clone 53–6.7), ThPOK (clone 11H11A14), and CD73 (clone TY/11.8), Ultra-LEAFTM purified anti-mouse CD3 antibody (clone 145–2C11), and anti-mouse CD28 antibody (clone 37.51) were purchased from Biolegend. Fluorochrome-conjugated Egr1 (clone 44D5) antibody was purchased from Cell Signaling Technology. PE- or APC-conjugated PBS57 loaded CD1d tetramer was kindly provided by the NIH tetramer facility. Cell death was identified using the Live/Dead Fixable Violet Dead Cell Stain (ThermoFisher) or 7-AAD. Rabbit anti-DGK ζ monoclonal antibody (ab239080) was purchased from Abcam. FITC-labeled goat anti-rabbit IgG (31635) and Alexa 488 conjugated anti-GFP antibody (A21311) was purchased from ThermoFisher. Anti-phospho-Erk1/2 T202/Y204 (D13.14.4E), phospho-S6 S235/236 (2F9), and phospho-Akt S473 (193H12) were from Cell Signaling Technology. Anti- β -actin was from Sigma-Aldrich. Single cell suspensions of the thymus, spleen, lymph nodes (LNs), and liver mononuclear cells (MNCs) in complete IMDM media (IMDM-10, 10% FBS, penicillin/streptomycin, 50 μ M 2-mercaptoethanol) were prepared as previous described [34]. Cells were stained with fluorescence conjugated antibodies and tetramers in 2% (vol/vol) FBS in PBS at 4°C for 30 min. Data were collected using BD FACSCanto II and BD LSRFortessa X-20 flow cytometer and analyzed with Flowjo. Flow cytometry analyses were adhered to the ‘Guidelines for the use of flow cytometry and cell sorting in immunological studies’ [84]. Gating strategies are shown in supplemental Figure S1.

Establishing GFP-DGK ζ^{WT} and GFP-DGK ζ^{NLS} T cell lines.

Retrovirus-mediated gene transfer was used to express GFP-DGK ζ^{WT} and GFP-DGK ζ^{NLS} proteins in the 2B4 cell line. GFP-DGK ζ^{WT} and GFP-DGK ζ^{NLS} coding sequences were

cloned in a retroviral vector, Migr1. Retroviral vectors were transfected into the Phoenix-Eco packaging cell line using the calcium phosphate method. Viral supernatants were collected 48 hours after transfection. For infection, 2B4 cells were mixed with viral supernatants and 5 µg/ml of polybrene (Sigma-Aldrich). Cells were then centrifuged at 2500g for 90 minutes at room temperature. The culture medium was changed with fresh RPMI 1640 (containing 10% FBS) 7 and 24 hours after infection. GFP⁺ cells were sorted using SONY SH800 sorter 48 hours after infection.

Immunoblotting

Thymocytes were rested in Dulbecco's PBS containing calcium and magnesium at 37°C for 30 minutes, followed by stimulation with 5µg/mL of anti-CD3e (500A2) or PMA (10 ng/ml) plus ionomycin (1 µg/ml) for the indicated times. After stimulation, cells were lysed in RIPA lysis buffer with freshly added protease and phosphatase inhibitors and were then subjected to SDS/PAGE and Western blot analysis with the indicated antibodies.

T cell stimulation in vitro

Splenocytes or LN cells were either labeled or unlabeled with CellTrace Violet (CTV) cell proliferation kit (C34571, ThermoFisher) or eFluor 670 cell proliferation dye (65-0840-85, ThermoFisher) following the manufacturer's protocols. Cells were plated in a 96-well U bottom plate (0.5×10^6), left unstimulated or stimulated with anti-CD3e (145-2C11) at 37 °C. Unlabeled cells were stained for CD4, CD8, CD69, CD25, and 7AAD or live/dead dye for analysis of activation marker expression after overnight stimulation. CTV- or eFluorTM 670-labeled cells were stained for CD4 and CD8 to assess proliferation seventy-two hours after stimulation.

ELISA

LN T cells from WT and GFP-DGK ζ ^{NLS} mice were stimulated in the indicated conditions for 48 hours. IL-2 levels in the supernatants were measured using ELISA following the manufacture's instruction (Biolegend, 431001).

Immunofluorescence and confocal microscopy

LN T cells in IMDM-10 from GFP-DGK ζ ^{WT}-Cd4Cre and GFP-DGK ζ ^{NLS}-Cd4Cre mice were stimulated with an anti-CD3e antibody (145-2C11, 1µg/ml) in a U-bottom 96-well plate for 10 minutes, 30 minutes, 4 hours, or 22 hours. 2B4 cells that overexpressed GFP-DGK ζ ^{WT} or GFP-DGK ζ ^{NLS} were stimulated with plate bound anti-CD3e antibody (145-2C11, 1µg/ml) for 22 hours. Cells were immediately subjected to fixation using cold methanol, blocked in 5% bovine serum albumin with 0.2% Triton X-100/PBS (30 min, on ice), then stained with Alexa 488 conjugated anti-GFP antibody (1:100 in PBS with 0.2% Triton X-100 and 2% FBS) overnight at 4°C. The nucleus was stained with DAPI (Biolegend). After staining, cells were washed in PBS, followed by cytospin and mounting. Images were captured using a Leica SP5 confocal microscope. GFP-DGK ζ ^{WT}, GFP-DGK ζ ^{KD}, and GFP-DGK ζ ^{NLS} intensity in the cytosol, cytoplasmic membrane, and nucleus was quantified using the ImageJ software. Mean fluorescence intensity (MFI) of GFP in the nuclear and cytoplasmic regions were measured and subtracted from the

background. The localization index of GFP-DGK ζ fusion proteins was calculated as the ratio of nuclear/cytoplasmic MFI in individual cells [85]. For endogenous DGK ζ localization, WT LN CD4⁺ T cells purified using CD4 microbeads (130-117-043, Miltenyi Biotec) were stimulated with plate bound anti-CD3 ϵ antibodies (145-2C11, 1 μ g/ml) in a flat-bottom 96-well plate for 22 hours. After fixation, blockage, and permeabilization, cells were stained with a rabbit anti-DGK ζ primary antibody (ab239080) at 4°C overnight, cells were washed with 0.2% Triton X-100/PBS buffer twice, and then subjected to staining with FITC-labeled a goat anti-rabbit IgG secondary antibody at 4°C for 4 hours. Stained cells were washed in PBS, followed by cytospin and mounting and microscopic visualization.

Statistical analysis

Data were presented as mean $\pm$ SEM and analyzed for statistical differences using the Prism 6.01/GraphPad software. Scatter plots in each figure were pooled from multiple experiments. Data distribution was analyzed with Shapiro-Wilk test (if $n \geq 3$) using SPSS Statistics 23 software, Kolmogorov-Smirnov test (if $n \geq 5$) and D'Agostino & Pearson omnibus normality test (if $n \geq 8$) using Prism 6.01/GraphPad software. Data with normal distribution were compared using two-tailed Student *t*-test or Two-Way ANOVA test. Other data were compared using a Mann-Whitney non-parametric test. *P*-values less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Cheryl Bock, Meilang Flowers, and Julie Kent in the Transgenic and Knockout Mice facility at Duke Cancer Institute for generating DGK ζ knocking-in mice, Dr. Matthew Topham for DGK ζ plasmids, Dr. Jörn Coers for the Rosa26-hCD2 targeting vector, Dr. Warren Pear for the Migr1 vector, and the Flow Cytometry Core Facility and the Light Microscopy Core Facility at Duke Cancer Institute for the service. This study is supported in part by NIH R01AI079088 and R56AG060984 (XZ).

Abbreviation list:

DAG	Diacylglycerol
PA	phosphatidic acid
DGK	Diacylglycerol kinase
DP	double positive
SP	signal positive
pLN	peripheral lymph node
BM	bone marrow
$\alpha\beta$T cell	conventional $\alpha\beta$ T cells
MNCs	mononuclear cells

CTV	CellTrace Violet
DKO	DGK α and ζ double deficient
MARCKS	myristoylated alanine-rich C-kinase substrate
NLS	nuclear localization sequences
EGFP	enhanced green fluorescent protein
iNKT	invariant NKT
mTORC	mammalian target of rapamycin complex
PI3K	Phosphoinositide 3-kinases
RasGRP1	RAS guanyl nucleotide-releasing protein1
Erk1/2	extracellular signal-regulated protein kinases 1 and 2
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
IKK	I κ B kinase
PKC	Protein kinase C
TCR	T-cell receptor
CARMA1	Caspase recruitment domain-containing membrane-associated guanylate kinase protein-1
PLCγ1	Phospholipase C, gamma 1
FBS	fetal bovine serum
DAPI	4',6-diamidino-2-phenylindole
MFI	Mean fluorescence intensity
Ctrl	control
ATP	adenosine triphosphate
PCR	polymerase chain reaction

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Figure 1. Generation of WT and mutant DGK ζ transgenic mice.

A. Schematic structure of DGK ζ . **B.** GFP-DGK ζ knock-in mice. Rosa: Rosa26 locus; P: CAG promoter; STOP, transcription stop cassette. **C.** Genotyping of GFP-DGK ζ^{KI} mice by PCR. **D.** Detection of DGK ζ by western blot with an anti-DGK ζ antibody and anti-GFP antibody. Endogenous DGK ζ_1 and ζ_2 are indicated with horizontal lines. Red arrow indicates GFP-DGK ζ function proteins, which overlap with the endogenous DGK ζ_2 . Loading control is detected with an anti-TCR β antibody. **E.** Quantification the GFP-DGK ζ fusion protein levels relative to endogenous DGK ζ_2 in WT thymocytes using Image J. **F.** Detection of GFP expression in thymocytes. **G.** Detection of GFP expression in B220⁺ B cells. **H.** TCR β expression in different thymocyte subsets. Data in F and H were measured by flow cytometry. Each histogram represents 100% of the indicated cell population. Data

shown are representative of or pooled from three experiments and error bars represent mean $\pm$ SEM, N = 3 mice for WT (Ctrl), GFP-DGK ζ^{WT} -CD4Cre, GFP-DGK ζ^{NLS} -CD4Cre, and GFP-DGK ζ^{KD} -CD4Cre mice in D – H.

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Figure 2. Impaired T cell generation by DGK ζ NLS.

Thymocytes from 6 – 10 weeks old GFP-DGK ζ^{WT} -CD4Cre, GFP-DGK ζ^{KD} -CD4Cre, GFP-DGK ζ^{NLS} -CD4Cre, and control mice were examined. **A.** Total thymocyte numbers. **B.** Representative flow cytometry plots showing TCR β staining in thymocytes. **C.** Percentages and numbers of TCR β^+ thymocytes. Data shown are representative of (B) or pooled (A, C) from at least eight experiments and are shown as mean $\pm$ SEM. Each circle or square represents one mouse of the indicated genotypes. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$ determined by Student t -test. (N=11 for both DGK ζ^{WT} -CD4Cre and control mice, N=13 for both DGK ζ^{NLS} -CD4Cre and control mice, N=9 for both DGK ζ^{KD} -CD4Cre and control mice)

Figure 3. Inhibition of T cell maturation by DGK ζ NLS.

Thymocytes (A – D) and splenocyte (E – G) from 6 – 10 weeks old GFP-DGK ζ^{WT} -CD4Cre, GFP-DGK ζ^{KD} -CD4Cre, GFP-DGK ζ^{NLS} -CD4Cre, and control mice were examined by flow cytometry. **A.** Representative flow cytometry plots showing CD4 and CD8 staining of thymocytes. **B.** Percentages and numbers of indicated thymocyte populations in GFP-DGK ζ^{WT} -CD4Cre and control mice. **C.** Percentages and numbers of indicated thymocyte populations in GFP-DGK ζ^{NLS} -CD4Cre and control mice. **D.** Percentages and numbers of indicated thymocyte populations in GFP-DGK ζ^{KD} -CD4Cre and control mice. Data shown are representative of (A) and pooled from (B–D) at least seven experiments (N=9 in both B and C, N=7 in D) and error bars represent mean $\pm$ SEM. **E.** Representative flow cytometry plots showing CD69 and TCR β staining of DP thymocytes. Scatter plots

show percentages and numbers of CD69⁺TCRβ⁺ cells in DP thymocytes. Data shown are representative of (plot) or pooled from five experiments, N=5 for both WT and DGKζ^{NLS}-CD4Cre mice and error bars represent mean ± SEM. **F.** Percentages and numbers of CD4⁺ and CD8⁺ splenic and LN T cells in GFP-DGKζ^{WT}-CD4Cre and control mice. Data shown are pooled from at least five experiments, N=9 for both WT and GFP-DGKζ^{WT}-CD4Cre mice in spleen, and N=5 for both WT and GFP-DGKζ^{WT}-CD4Cre mice in pLN. **G.** Percentages and numbers of CD4⁺ and CD8⁺ splenic and LN T cells in GFP-DGKζ^{NLS}-CD4Cre and control mice. Data shown are pooled from at least eight experiments. N=10 for both WT and GFP-DGKζ^{NLS}-CD4Cre mice in spleen, and N=8 for both WT and GFP-DGKζ^{NLS}-CD4Cre mice in pLN. **H.** Percentages and numbers of CD4⁺ and CD8⁺ splenic and LN T cells in GFP-DGKζ^{KD}-CD4Cre and control mice. Data shown are pooled from at least six experiments. N=9 for both WT and GFP-DGKζ^{KD}-CD4Cre mice in spleen, and N=6 for both WT and GFP-DGKζ^{KD}-CD4Cre mice in pLN. Each circle or square represents one mouse of the indicated genotypes. (F–H) error bars represent mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, determined by Student *t*-test (Fig B, E, F, G, and H) or by Mann-Whitney test (Fig C and D).

Figure 4. Enhanced inhibition of DAG-mediated signaling and downregulation of Egr1 expression by DGK ζ^{NLS} .

A, B. Thymocytes from WT, GFP-DGK ζ^{WT} -CD4Cre, and GFP-DGK ζ^{NLS} -CD4Cre mice were left unstimulated or stimulated with an anti-CD3 antibody (500A2) at 37°C for 5 or 15 minutes. Cell lysates were subjected to immunoblotting analyses using the indicated antibodies. β-actin was detected as loading controls (A). Quantification of relative intensity of western blot bands (B). Data shown are representative of (A) or pooled from (B) three experiments (N=3 for WT, GFP-DGK ζ^{WT} -CD4Cre, and GFP-DGK ζ^{NLS} -CD4Cre mice). Error bars represent mean ± SEM. **C.** Representative histograms showing Egr1, ThPOK, and Runx3 expression in DP, CD4SP, and CD8SP thymocytes from WT and GFP-DGK ζ^{NLS} -CD4Cre mice. Each histogram represents 100% of the indicated cell population. Data shown are representative of four experiments. N = 4 mice for Ctrl and GFP-DGK ζ^{NLS} -CD4Cre

mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, determined by Two-way ANOVA test.

Figure 5. Enhanced inhibition of T cell activation by DGK $\zeta^{\Delta\text{NLS}}$.

A. Representative flow cytometry of overlaid histograms of CD69 and CD25 expression in WT, DGK ζ^{WT} , and DGK $\zeta^{\Delta\text{NLS}}$ CD4 and CD8 T cells after overnight incubation of splenocytes in the presence or absence of an anti-CD3 antibody (0.1 $\mu\text{g}/\text{ml}$). Each histogram represents 100% of the indicated cell population. **B.** Relative MFI of CD69 and CD25 in the indicated T cells. N = 3 for Ctrl, DGK ζ^{WT} -CD4Cre and GFP-DGK $\zeta^{\Delta\text{NLS}}$ -CD4Cre mice. Data shown are representative of (A) or pooled from (B) three experiments, N = 3 for Ctrl, GFP-DGK ζ^{WT} -CD4Cre, and GFP-DGK $\zeta^{\Delta\text{NLS}}$ -CD4Cre mice. Error bars represent mean $\pm$ SEM. **C.** Representative flow cytometry overlaid histograms showing WT, DGK ζ^{WT} , and DGK $\zeta^{\Delta\text{NLS}}$ CD4 and CD8 T cell proliferation after in vitro stimulation for 72 hours with indicated concentrations of anti-CD3 antibodies. Data shown are representative of three

experiments, N = 3 for Ctrl, GFP-DGK ζ^{WT} -CD4Cre, and GFP-DGK ζ^{NLS} -CD4Cre mice. Each histogram represents 100% of the indicated cell population. **D.** ELISA for IL-2 concentrations in the supernatant from LN T cells with indicated-treatments for 48 hours. Data are pooled from three experiments. N = 3 for both WT and GFP-DGK ζ^{NLS} -CD4Cre mice. Error bars represent mean $\pm$ SEM. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001, determined by Two-way ANOVA test (Fig B) or by Student *t*-test (Fig D).

Figure 6. Effects of DGK ζ^{WT} and DGK ζ^{NLS} on iNKT cell development.

Thymocytes, splenocytes, and liver mononuclear cells from 6 – 10 weeks old GFP-DGK ζ^{WT} -CD4Cre, GFP-DGK ζ^{KD} -CD4Cre, GFP-DGK ζ^{NLS} -CD4Cre, and control mice were examined. **A, C, E.** Representative flow cytometry plots showing TCR β and PBS57-loaded CD1d tetramer (CD1dTet) staining in live gated cells from thymocytes, splenocytes, and liver mononuclear cells from GFP-DGK ζ^{WT} -CD4Cre and control mice (**A**), GFP-DGK ζ^{KD} -CD4Cre and control mice (**C**), GFP-DGK ζ^{NLS} -CD4Cre and control mice (**E**). **B, D, F.** Percentages and numbers of iNKT cells in the indicated organs and mice. Data shown are representative of (**A**) and pooled from (**B**) at least five experiments and are shown as mean $\pm$ SEM. For the experiment of iNKT cell percentages and numbers in thymus, N=8 for both Ctrl and DGK ζ^{WT} -CD4Cre mice. For the experiment of iNKT percentages and

numbers in both spleen and liver, N=5 for both Ctrl and DGK ζ ^{WT}-CD4Cre mice. Data shown are representative of six (C) or ten (E) and pooled from six (D) or ten (F) experiments and are shown as mean $\pm$ SEM. Each experiment included one ctrl and one test mice. **G.** Representative flow cytometry plots showing CD24 and CD44 expression in live gated Lin (CD11b, CD11c, B220, Ter119, F4/80)⁻ iNKT cells in the thymus (top panels) and CD44 and NK1.1 expression in CD24⁻ iNKT cells (bottom panels). **H.** Percentages and numbers of indicated iNKT cell stages. CD24⁺CD44⁻ stage 0 iNKT cells were calculated after exclusion of NK1.1⁺ cells. Data shown in G are representative of at least three experiments. Data shown in H are calculated from one experiment with three pairs of test and control mice and are shown as mean $\pm$ SEM. N = 3 for both Ctrl and GFP-DGK ζ ^{NLS}-CD4Cre mice. Each circle or square represents one mouse of the indicated genotypes. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001 determined by Student *t*-test.

Figure 7. TCR-induced nuclear localization of DGK ζ .

A. LN T cells from GFP-DGK ζ^{WT} -CD4Cre, GFP-DGK ζ^{NLS} -CD4Cre, and GFP-DGK ζ^{KD} -CD4Cre mice were stimulated with or without anti-CD3 (145–2C11) for the indicated times, followed in fixation/permeabilization, staining with an Alexa 488 conjugated anti-GFP antibody, and confocal microscopy. Objective magnification: 63 $\times$; Zoom: 6 $\times$; Scale bar: 8 μ M. Representative images are shown. **B.** Quantification of anti-GFP intensity in the cytosol and nucleus. Each dot shows nuclear/cytoplasm ratio of MFI of GFP in a T cell. **C.** WT LN CD4 $^{+}$ T cells were unstimulated or stimulated with a plate-bound anti-CD3 antibody (145–2C11) for 22 hours, followed sequential by fixation/permeabilization, staining with a rabbit anti-DGK ζ antibody and then a FITC conjugated goat anti-rabbit IgG antibody, and visualization by confocal microscopy. Objective

magnification: 63×; Zoom: 6×; Scale bar: 8 μM. Representative images are shown. **D.** Quantification of FITC intensity in the cytosol and nucleus. Each dot shows nuclear/cytoplasm ratio of MFI of FITC in a T cell. Figure A and B are representative of or pooled from four experiments for GFP-DGKζ^{WT}-CD4Cre and GFP-DGKζ^{NLS}-CD4Cre mice and three experiments for GFP-DGKζ^{KD}-CD4Cre mice. N = 1 for each genotype in each experiment. Figure C and D are representative of or pooled from three experiments. One WT mouse was used for each experiment. Error bars represent mean ± SEM. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001, determined by Mann-Whitney test (Fig B) or by the Student *t* test (Fig D).