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Regulation of T Cell Receptor Signaling by Diacylglycerol Kinases and Phosphatidylinositol Transfer Proteins

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Abstract
Signals transduced through the T cell receptor (TCR) lead to T cell differentiation, proliferation, and elaboration of cytokines, all of which are required for optimal immunity. Phosphoinositide (PI) mediated signaling plays a particularly prominent role in this process. TCR signaling is amplified by the activation of phospholipase C γ1 (PLCγ1), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) to form the second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). Regulation of PI and products such as DAG are therefore essential for normal TCR signaling. DAG levels are reduced by diacylglycerol kinases (DGKs), which metabolize DAG and diminish DAG-mediated signaling. In T cells, the predominant DGK isoforms expressed are DGKα and DGKζ. Deletion of either isoform enhances DAG-mediated signaling, yet the relative importance of these enzymes is unknown. Here, we describe that DGKζ but not DGKα suppresses natural regulatory T cell development and predominantly controls Ras and AKT signaling. The differential functions of DGKα and DGKζ are not attributable to differences in expression levels or localization to the T cell-APC contact site. However, RasGRP1, a key activator of Ras signaling, associated to a greater extent with DGKζ than DGKα. In addition, DGKζ displayed greater metabolism of DAG to PA after TCR stimulation than DGKα. In silico modeling of TCR-stimulated Ras activation in DGKα- versus DGKζ-deficient T cells suggested that a greater catalytic rate for DGKζ than DGKα could lead to increased suppression of Ras-mediated signals by DGKζ. DGKζ dominant functions over DGKα, therefore, are in part due to DGKζ's greater effective enzymatic activity and association with RasGRP1. Future experiments will establish how DGK isoform function is regulated by TCR signaling. To examine how the DAG precursor PI is regulated, we performed a preliminary analysis of mice with a T cell specific deficiency of phosphatidylinositol transfer protein (PITP) α and β, which regulate PI-mediated signaling in vitro. Our initial in vivo studies suggest that deletion of PITPα and PITPβ at the double positive stage of T cell development results in loss of mature T cells in the thymus and periphery. Further experiments will establish why PITPs are required for normal T cell development.

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REGULATION OF T CELL RECEPTOR SIGNALING BY DIACYLGLYCEROL KINASES AND PHOSPHATIDYLINOSITOL TRANSFER PROTEINS

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DEDICATION

To my parents, Prakash and Jaya Joshi,

my brother, Hemant Joshi,

and my fiancée, Tripti Tewari

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ABSTRACT

REGULATION OF T CELL RECEPTOR SIGNALING BY DIACYLGLYCEROL KINASES AND PHOSPHATIDYLINOSITOL TRANSFER PROTEINS

Rohan P. Joshi

Gary A. Koretzky

Signals transduced through the T cell receptor (TCR) lead to T cell differentiation, proliferation, and elaboration of cytokines, all of which are required for optimal immunity. Phosphoinositide (PI) mediated signaling plays a particularly prominent role in this process. TCR signaling is amplified by the activation of phospholipase C γ1 (PLCγ1), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) to form the second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). Regulation of PI and products such as DAG are therefore essential for normal TCR signaling. DAG levels are reduced by diacylglycerol kinases (DGKs), which metabolize DAG and diminish DAG-mediated signaling. In T cells, the predominant DGK isoforms expressed are DGKα and DGKζ. Deletion of either isoform enhances DAG-mediated signaling, yet the relative importance of these enzymes is unknown. Here, we describe that DGKζ but not DGKα suppresses natural regulatory T cell development and predominantly controls Ras and AKT signaling. The differential functions of DGKα and DGKζ are not attributable to differences in expression levels or localization to the T cell-APC contact site. However, RasGRP1, a key activator of Ras signaling, associated to a greater extent with DGKζ than DGKα. In addition, DGKζ displayed greater metabolism of DAG to PA after TCR stimulation than DGKα. *In silico* modeling of TCR-stimulated Ras activation in DGKα- versus DGKζ-deficient T cells suggested that a greater catalytic rate for DGKζ than DGKα could lead to increased suppression of Ras-mediated signals by DGKζ. DGKζ dominant functions over DGKα, therefore, are in part due to DGKζ's greater effective enzymatic activity and association with RasGRP1. Future experiments will establish how DGK isoform function is regulated by TCR signaling. To examine how the DAG precursor PI is regulated, we performed a preliminary analysis of mice

with a T cell specific deficiency of phosphatidylinositol transfer protein (PITP) α and β, which regulate PI-mediated signaling *in vitro*. Our initial *in vivo* studies suggest that deletion of PITPα and PITPβ at the double positive stage of T cell development results in loss of mature T cells in the thymus and periphery. Further experiments will establish why PITPs are required for normal T cell development.

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CHAPTER I: INTRODUCTION

An adaptive immune system that efficiently recognizes pathogen is essential for clearance of infection. During infection, antigen-presenting cells (APCs) engulf pathogens and present pathogen-derived peptides on their cell surface. T cells, a critical arm of the adaptive immune system, recognize these peptides by engagement of cell-surface T cell receptor (TCR). Signals mediated by the TCR lead to T cell differentiation, proliferation, and elaboration of cytokines, all of which are required for optimal immunity, and elimination of infection. Receptor mediated signaling is therefore crucial for T cell function. Phosphoinositide lipid mediated signaling plays a particularly prominent role in this process. After engagement of the TCR, signaling is amplified by the activation of phospholipase C γ1 (PLCγ1), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) to form the second messengers diacylglycerol (DAG) and inositol trisphosphate (IP_3), and the activation of phosphatidylinositide 3-kinase (PI3K), which phosphorylates PIP_2 to form phosphatidylinositol-3,4,5-trisphosphate (PIP_3). DAG, IP_3, and PIP_3 each activate a diverse set of signaling cascades leading to T cell activation. An understanding of the regulation of phosphoinositides and their products is therefore essential for understanding how TCR signaling leads to T cell activation. The major focus of this thesis is on the regulation of DAG. Preliminary studies also investigated how the phosphoinositide precursor of DAG is itself regulated.

Synthesis of DAG is crucial for activation of signaling cascades including the Ras, NF-κB, and AKT pathways. DAG levels must therefore be finely tuned not only through controlled production but also by its metabolism. Diacylglycerol kinases (DGKs) are a diverse family of enzymes that phosphorylate DAG to form phosphatidic acid (PA), thereby terminating DAG signaling and initiating additional signaling events through the

synthesis of bioactive PA. The critical role of DGKs in controlling DAG in T cells has become evident over the last decade through the use of both cell line and *in vivo* models. The regulation of DGKs themselves, however, has begun to be understood only recently. This review summarizes our understanding of the role of DGKs in T cells and describes new advances in deciphering the means by which DGKs are regulated.

1.1. DGK Isotypes and their Structures

DGKs are a large family of enzymes that share a common substrate, DAG. The domain architectures of DGKs reflect this common function, as each of the ten isoforms has a catalytic domain as well as at least two C1 domains that are homologous to protein kinase C (PKC) C1 phorbol-ester/DAG binding domains.

The catalytic domains of DGKs contain two parts, a catalytic subdomain (termed DAGKc) and an accessory subdomain (termed DAGKa). The DAGKc subdomain contains a highly conserved Gly-Gly-Asp-Gly motif that serves as the ATP binding site and is necessary for enzymatic activity (Sanjuán et al., 2001; Topham et al., 1998). The role of the DAGKa subdomain has not been directly studied. However, two unrelated kinases, sphingosine kinase (SPK) and ceramide kinase (CERK), contain a domain similar to DAGKc, but do not contain a DAGKa subdomain. DAG is not a substrate for SPK and CERK, and DGKs are not specific for sphingosine or ceramide containing lipids, suggesting that the DAGKa subdomain may be required for the specificity of DGKs for DAG lipids (Raben and Wattenberg, 2008; Sugiura et al., 2002; Merida et al., 2008).

The conservation of the C1 domain across all DGK isoforms suggests that this domain is essential for DGK function. Nevertheless, the exact role of the DGK C1 domain is unclear. As DGK C1 domains are homologous to DAG/phorbol ester-binding PKC C1 domains, DGK C1 domains were presumed to mediate the binding of DGK to DAG. However, no DGK C1 domain contains the canonical phorbol-ester binding amino acid motif of the PKC C1 domain, with the exception of DGKβ and DGKγ C1 domains (Hurley and Misra, 2000). DGK C1 domains therefore may not be involved in direct DAG binding. Nevertheless, the C1 domain may have non-canonical roles in controlling DGK localization. For example, cellular exposure to phorbol myristate acetate (PMA) causes DGKγ to translocate to the plasma membrane (Shirai et al., 2000). Targeted disruption of the C1 domain of DGKζ or DGKθ prevents the translocation of these molecules to the membrane in response to G-protein coupled receptor (GPCR) activation (Santos et al., 2002; van Baal et al., 2005). The DGK C1 domain may also be involved in protein-protein interactions, as evidence suggests that DGKζ translocation may be controlled by interactions with β-arrestins, rather than interactions with DAG (Nelson et al., 2007). In addition, DGKζ directly interacts with Rac1 through its C1 domain (Yakubchyk et al., 2005). The available evidence suggests that the C1 domain may have functions unique to each isoform and could be involved in protein-protein interactions in addition to protein-lipid interactions.

1.2. DGK Subtypes

The ten DGKs are divided into five subtypes based on domains apart from the C1 and catalytic domains (Fig. 1.1). As described below, these additional domains potentially regulate DGK catalytic activity, localization, and substrate specificity.

1.2.1 Type I DGKs (DGKα, DGKβ, and DGKγ)

Type I DGKs contain an N-terminal calcium sensitive recoverin homology (RVH) domain and two EF hand motifs. The RVH domain is homologous to the N-terminus of the recoverin family of neuronal calcium receptors (Jiang et al., 2000a). *In vitro* kinase assays using purified protein suggest that deletion of the RVH domain results in loss of calcium dependent activation of DGKα; in addition, simultaneous deletion of the RVH and EF hands domains results in constitutive activation of DGKα (Jiang et al., 2000a). These data suggest that the RVH domain senses calcium, while the EF hand domain mediates suppression of kinase activity. The RVH and EF hands domains likely cooperate to control calcium dependent activation of DGKα through an intramolecular interaction with the C1 and catalytic domains (Takahashi et al., 2012). In addition to controlling enzymatic activity, binding of calcium to the EF hands may allow DGKα to translocate from the cytoplasm to the plasma membrane (Merino et al., 2007). While the RVH and EF hand domains of DGKα are important for its function, the role of these domains in DGKβ and DGKγ function has been less well studied. When expressed in isolation, the EF hands of DGKβ and DGKγ bind to calcium with a dissociation constant an order of magnitude less than that of DGKα, suggesting that calcium may not strongly regulate these isoforms (Yamada et al., 1997). However, simultaneous deletion of the

RVH and EF hands of DGKγ results in cytoplasm to plasma membrane translocation and increased filipodia-like cytoplasmic protrusions in a N1E-115 neuroblastoma cell line (Tanino et al., 2013). Thus, calcium is a key regulator of DGKα function and may regulate other Type I DGKs as well.

1.2.2 Type II DGKs (DGKδ, DGKη, and DGKκ)

Type II DGKs contain an N-terminal plekstrin homology (PH) domain that mediates interaction with lipids and proteins (Imai et al., 2005; Klauck et al., 1996; Sakane et al., 1996). The PH domain of DGKδ weakly binds to phosphatidylinositols; however, treatment of HEK293 cells with the DAG analog PMA promotes PH domain-dependent translocation of DGKδ to the plasma membrane (Park et al., 2008; Takeuchi et al., 1997; Imai et al., 2002, 2004). In addition to the PH domain, DGKδ and DGKη contain a sterile α motif (SAM) at their carboxy termini, which may be involved in oligomerization through zinc binding (Imai et al., 2002; Knight et al., 2010). DGKκ lacks a SAM domain but does contain a C-terminal motif that may bind type I PDZ domains (Imai et al., 2005). The function of this motif is unknown.

1.2.3. Type III DGKs (DGKε)

DGKε, the only member of the Type III DGK family, contains no additional structural domains other than its tandem C1 and catalytic domains. Interestingly, DGKε is the only DGK isoform known to display specificity to the acyl sidechains of the glycerol backbone of DAG (Kohyama-Koganeya et al., 1997; Rodriguez de Turco et al., 2001).

DGKε preferentially binds to DAG containing an arachidonoyl chain at middle position of the glycerol backbone (Kohyama-Koganeya et al., 1997; Rodriguez de Turco et al., 2001). This preference may be responsible for the presence of arachidonoyl containing PIP_2 in the plasma membrane (Prescott and Majerus, 1981; Shulga et al., 2011).

1.2.4. Type IV DGKs (DGKζ and DGKι)

Type IV DGKs contain myristoylated alanine-rich protein kinase C substrate (MARCKS), PDZ-binding, and ankyrin domains. The MARCKS domain is homologous to the phosphorylation-site domain (PSD) of the MARCKS protein and contains four serine residues that are possibly sites of phosphorylation by protein kinase C (PKC) α(Topham et al., 1998). In COS-7 and A172 cells, this phosphorylation event leads to nuclear translocation of DGKζ; in Jurkat T cells, serine to alanine mutation of these residues results in loss of translocation of DGKζ to the plasma membrane (Topham et al., 1998; Santos et al., 2002). The PDZ-binding and ankyrin domains are involved in protein-protein interactions. For example, the PDZ-binding domain regulates DGKζ's interaction with Sorting Nexin-27 (SNX27) and is required for SNX27 to mediate intracellular vesicle trafficking (Rincon et al., 2007). Additionally, in Purkinje neurons and muscle cells the PDZ-binding domain controls DGKζ's interaction with γ1-Syntrophin, which determines the subcellular localization of DGKζ (Hogan et al., 2001; Abramovici et al., 2003; Abramovici and Gee, 2007). Finally, the ankyrin domain regulates DGKζ's interaction with the long form of the leptin receptor in rat hypothalamus extract and may regulate leptin receptor signaling (Liu et al., 2001). The role of the MARCKS and ankyrin domains in DGKι function is unknown. However, just as has been found for DGKζ, the

PDZ-binding domain is required for the interaction of DGKι with the postsynaptic density 95 (PSD-95), a neuronal scaffolding protein (Kim et al., 2009; Yang et al., 2011).

1.2.5. Type V DGKs (DGKθ)

The only member of the Type V DGK family, DGKθ, contains an N-terminal proline-rich domain, a PH domain between its C1 and catalytic domains, and a Ras-association domain within its PH domain. The enzymatic activity of DGKθ is diminished by mutations of the PH and proline-rich domains, suggesting an essential role of these species conserved regions in DGKθ function (Houssa et al., 1997; Los et al., 2004).

1.3. Tissue Expression of DGKs

DGK expression is broad, as most tissues express multiple DGK isoforms and most DGK isoforms are expressed in multiple tissues. The major site of DGK expression is the brain, which expresses all known DGK isoforms (Shulga et al., 2011; Crotty et al., 2006). A recent review reported expressed sequence tag (EST) data available from the National Center for Biotechnology Information (NCBI) and summarized tissue expression of DGKs (Shulga et al., 2011). Interestingly, EST data suggest that DGK isoform expression is relatively narrow in several tissues: (1) DGKε is the only expressed isoform in adipose tissue; (2) DGKγ is the only isoform expressed in the pituitary gland; (3) DGKα and DGKθ are the only isoforms expressed in the bone marrow; and (4) DGKα, DGKδ, and DGKζ are the most abundant isoforms expressed in lymphocyte-rich tissue (Shulga et al., 2011). While DGKα, DGKδ, and DGKζ are all

expressed in T cells, the function of DGKδ in T cells remains unknown. As such, the role of DGKα and DGKζ in T cells is discussed in detail below.

1.4. Regulation of DGK function in T cells

1.4.1. Transcriptional Regulation of DGKs in T cells

The regulation of DGKα and DGKζ has been a focus of research over the past five years because of the key roles these molecules have in TCR signaling. Both DGKα and DGKζ are regulated at the transcriptional level. T cell lines that are induced to become unresponsive to antigen (anergic) by the administration of TCR stimulation without co-stimulation through additional receptors, such as CD28, up-regulate DGKα mRNA (Zha et al., 2006; Macián et al., 2002). In contrast, activation of murine T cells with both TCR and co-stimulation causes down-regulation of both DGKα and DGKζ mRNA transcripts (Olenchock et al., 2006; Martínez-Moreno et al., 2012). These data suggest that transcription of DGKα and DGKζ mRNA is sensitive to signals downstream of TCR and co-stimulation.

One transcription factor upregulated in response to TCR stimuli is early growth responsive gene 2 (Egr2). A binding site for Egr2 was identified within the promoter of DGKα (Zheng et al., 2012). After TCR engagement, transcription of Egr2 was found to peak before DGKα transcription increased, suggesting that Egr2 may regulate DGKα levels. It was found that Egr2 bound to the DGKα promoter and increased DGKα transcription after TCR engagement in the absence of co-stimulation. Furthermore, deletion of Egr2 resulted in decreased DGKα upregulation and anergy induction after anergy inducing conditions. In addition, forced over-expression of DGKα in Egr2

deficient T cells rescued anergy induction. These results suggest that Egr2 is a key regulator of DGKα transcription during anergy and that this regulation has important functional effects.

Recently, binding sites for members of the forkhead box O (FoxO) transcription factor family were found within the DGKα gene (Martínez-Moreno et al., 2012). In quiescent T cells, FoxO1 and FoxO3 are bound to the DGKα promoter and enhance DGKα transcription. DGKα promoter binding is lost after T cell activation, and this event is correlated with the phosphorylation of FoxO proteins downstream of TCR and AKT signaling and decreased DGKα transcription. T cells exposed to the cytokine interleukin-2 (IL-2), which is produced by activated T cells, were also found to decrease DGKα transcription in a manner dependent on AKT function. In addition, this decreased transcription correlated to decreased FoxO binding to the DGKα promoter. FoxO proteins may therefore provide a link between TCR signaling, T cell activation, and DGKα transcription. For example, in quiescent cells, FoxO mediated transcription of DGKα may be high; initial TCR signaling may lead to decreased FoxO mediated transcription and the production of IL-2, which may further serve to shut off DGKα transcription in activated T cells. Further studies using genetic tools would help determine the functional implication of FoxO regulation of DGKα on T cell activation and anergy. The factors that control DGKζ transcription have not yet been studied.

1.4.2. Post-translational Regulation of DGKs in T cells

Evidence also exists for post-translational regulation of DGKα and DGKζ. The presence of RVH and EF hand domains in DGKα suggests that cytoplasmic calcium

levels could regulate this isoform. After TCR engagement, calcium is released into the cytoplasm from the ER and extracellular environment (Smith-Garvin et al., 2009). *In vitro* data would suggest that this flux would lead to binding of calcium to the EF hands and loss of auto-inhibition (i.e. activation) of DGKα catalytic activity (Takahashi et al., 2012; Jiang et al., 2000a). Surprisingly, treatment of Jurkat T cells with an intracellular calcium chelator during TCR and co-stimulation leads to an increase rather than a decrease in DGKα catalytic activity as measured by an *ex vivo* assay of PA production (Baldanzi et al., 2011). Interestingly, intracellular calcium increases are necessary for the translocation of DGKα to the plasma membrane, where DAG is synthesized after TCR engagement (Sanjuan et al., 2003; Sanjuán et al., 2001). Thus, the *in vivo* calcium-dependent regulation of DGKα is more complex than what occurs *in vitro* with isolated protein, and calcium may serve to localize DGKα activity rather than modulate DGKα catalytic function.

Recently, the adapter protein SAP [signaling lymphocyte activation molecule (SLAM)-associated protein] was also found to regulate DGKα function during immune signaling (Baldanzi et al., 2011). Upon TCR and co-stimulation of Jurkat T cells through either CD28 or SLAM receptor engagement, DGKα is recruited to the membrane and its catalytic activity is suppressed. This suppression is dependent upon PLCγ1 activation and calcium release (Baldanzi et al., 2011). Knockdown of SAP results in a rescue of DGKα catalytic activity and reduced translocation to the plasma membrane after TCR and co-stimulation (Baldanzi et al., 2011). The investigators in this study could not find a direct protein interaction between SAP and DGKα; thus, SAP apparently directs the posttranslational regulation of DGKα through an as yet unidentified mechanism (Baldanzi et al., 2011). The Src family tyrosine kinase Lck also regulates DGKα in a T cell line (Merino et al., 2008). TCR stimulation of Jurkat T cells leads to Lck-mediated

phosphorylation of DGKα at the hinge region between its tandem C1 domains and the catalytic domain (Merino et al., 2008). Mutation of this tyrosine residue to phenylalanine abrogates DGKα translocation to the plasma membrane and suppression ERK phosphorylation (Merino et al., 2008). TCR-activated Lck phosphorylation of DGKα therefore is an important event for attenuating T cell activation.

Comparatively little is known about posttranslational modification of DGKζ in immune cells. As described above, in COS-7 and A172 cells, PKCα phosphorylates serine residues in the MARCKS domain of DGKζ and leads to its nuclear translocation (Topham et al., 1998). In Jurkat T cells, mutation of these serine residues to alanine results in loss of DGKζ translocation to the plasma membrane, but the kinase that acts on DGKζ has not been definitively identified (Santos et al., 2002; Gharbi et al., 2011). Although the MARCKS domain appears important for localization in this cell line, the importance of this domain for DGKζ regulation of downstream signaling pathways requires further study.

1.5. DGK Regulation of Immune Cell Signaling and Activation

1.5.1. An Overview of TCR and DAG Signaling in T cells

Engagement of the TCR by peptide presented by APCs starts a signaling cascade that culminates in activation of the T cell (Fig. 1.2). Early events lead to the activation of the tyrosine kinases Lck and ZAP-70, which leads to the formation of a multimolecular signaling complex involving the adapter proteins linker of activated T cells (LAT) and SH2 domain containing leukocyte protein of 76kDa (SLP-76) (Zhang et al., 1998; Motto et al., 1996; Smith-Garvin et al., 2009). PLCγ1 is recruited to and activated

by this complex and in turn hydrolyses the phospholipid PIP_2 to form membrane-diffusible DAG and cytosolic IP_3 (Imboden and Stobo, 1985). DAG recruits and activates numerous targets, of which the best characterized in T cells are RasGRP1, PKD and PKCθ, although other PKCs are targets as well (Tognon et al., 1998; Quann et al., 2011; Spitaler et al., 2006). RasGRP1 is a guanine nucleotide exchange factor that activates Ras-GTP and the MAPK cascade, along with non-DAG activated son of sevenless (SOS) (Dower et al., 2000, 1). This leads to the formation of the activator protein 1 (AP-1) transcription factor complex, which is critical for the production of interleukin-2 (IL-2) and T cell activation. Activation of PKCθ leads to the recruitment of CARMA1 and Bcl10, culminating in the activation of the NF-κB pathway, which is important for the production and secretion of pro-inflammatory cytokines (Coudronniere et al., 2000; Sun et al., 2000; Narayan et al., 2006; Wang et al., 2004; Hara et al., 2004; Jun et al., 2003). Activation of PKD promotes integrin activation through an interaction with Rap1 in T cells (Medeiros et al., 2005, 1). DGKs phosphorylate DAG to form PA, and may act as negative regulators of these pathways.

PA itself is an important signaling lipid, although its functions in T cells are not well defined. For example, in Jurkat T cells, simultaneous activation of integrin and TCR receptors leads to hydrolysis of phosphatidylcholine by phospholipase D 2 (PLD2), likely creating a pool of PA that is a source for the synthesis of DAG by phosphatidic acid phosphatase (PAP) (Mor et al., 2007). This PLD2-sourced pool of DAG is important for specific activation of k-Ras, rather than n-Ras, which is activated by TCR signaling alone (Mor et al., 2007). PA may also lead to activation of mammalian target of rapamycin (mTOR) and DGKζ specifically may influence this pathway (Foster, 2007, 2009; Fang et al., 2001; Avila-Flores, 2005). For example, in HEK293 cells, overexpression of DGKζ increased PA levels in response to mitogenic simuli. Overexpression of DGKζ also

increased phosphorylation of ribosomal S6 kinase (S6K), a molecule downstream of mTOR, in a manner dependent on mTOR's PA binding domain (Avila-Flores, 2005). These data suggest that, after engagement of the TCR, DAG and DGKs may have additional roles in regulating mTOR and other pathways downstream of PA. As discussed below and consistent with this notion, PA may have a role in the development of T cells in the thymus. Further studies are needed, however, in primary T cells to further elucidate the functions of PA in T cells.

1.5.2. DGKα in T cell Signaling and Activation

DGKs serve a crucial role in T cells by metabolizing DAG and terminating DAG activated signaling cascades. The predominant isoforms of DGK expressed in T cells are DGKα and DGKζ (Zhong et al., 2008). Studies in a transformed T cell line (Jurkat) stimulated through an ectopically expressed muscarinic type I receptor revealed a modest reduction in CD69 upregulation with overexpression of wildtype DGKα and a marked reduction of CD69 upregulation with expression of a constitutively active form of DGKα (Sanjuán et al., 2001). Overexpression of DGKα in Jurkat T cells also reduced AP-1 activation after TCR stimulation (Olenchock et al., 2006). In studies of a virally transduced primary T helper 1 cell clones, overexpression of DGKα was found to inhibit ERK phosphorylation in response to TCR and co-stimulation and translocation of RasGRP1 to the plasma membrane (Zha et al., 2006). Primary T cells deficient in DGKα also demonstrate increased ERK phosphorylation and Ras activation following TCR stimulation (Olenchock et al., 2006). This leads to increased IL-2 production and proliferation of DGKα-deficient cells during blockade of co-stimulatory signals

(Olenchock et al., 2006). Together, these data suggest that DGKα-dependent suppression of TCR signaling is crucial for the suppression of T cell activation. The effects of DGKα deficiency on primary T cell function are discussed further below.

1.5.3. DGKζ in T cell Signaling and Activation

Early experiments in which DGKζ was overexpressed in Jurkat T cells demonstrated that DGKζ suppresses Ras, ERK, and AP-1 activation as well as upregulation of the activation marker CD69 (Zhong et al., 2002). Kinase activity was found to be required for this suppression; in addition, an intact N-terminal region including the MARCKS domain was required, while the ankyrin and PDZ-binding domains were found to be dispensable (Zhong et al., 2002). Subsequent experiments in primary T cells from DGKζ-deficient mice confirmed these findings. DGKζ knockout T cells produced less PA after TCR stimulation (Zhong et al., 2003). In addition, phosphorylation of MEK and ERK was increased in DGKζ-deficient T cells. This increase correlated with increased upregulation of the activation markers CD69 and CD25 as well as increased proliferation to antigen and increased homeostatic proliferation (expansion of cell numbers when transferred into lymphopenic mice) (Zhong et al., 2003). Finally, DGKζ-deficient mice had a modest increase in responses to the pathogen lymphocytic choriomeningitis virus (LCMV), indicating that enhanced T cell signaling and activation may have functional consequences as well (discussed further below).

1.5.4. DGK Regulation of AKT-mTOR Signaling in T cells

Much evidence suggests, therefore, that DGKα and DGKζ have overlapping roles in T cell signaling, although no direct comparison of the relative contribution of DGKα and DGKζ to this regulation has been performed. Highlighting the overlapping yet complementary roles of DGKα and DGKζ, treatment of DGKζ-deficient T cells with an inhibitor of DGKα function increases IL-2 production and proliferation in response to TCR stimuli and co-stimulation blockade compared to DGKζ-deficient untreated cells (Olenchock et al., 2006). In addition, simultaneous deletion of DGKα and DGKζ in T cells [double knockout (DKO) cells] increases activation of the AKT and mTOR pathways (Gorentla et al., 2011). Increased Ras-MEK-ERK-Rsk1 signaling in DKO cells is likely responsible for increased AKT-mTOR activation (Gorentla et al., 2011). DGKα and DGKζ are therefore responsible for regulating a wide range of pathways that are important for T cell signaling, activation, proliferation, and metabolism.

1.5.5. DGKζ as a Modulator of Analog-to-digital Signaling in T cells

The role of DGKζ in T cell activation has been refined recently with respect to Ras activation. T cells exhibit digital activation after TCR stimulation, such that increases in TCR ligand binding lead to increased percentages of cells with an “on” state, rather than a smooth gradient of cell activation markers (Chakraborty et al., 2009). This mechanism requires that an analog signal (number of TCR bound to ligand) be converted to a digital state (activation) through the TCR signaling machinery. The Ras pathway contributes to this conversion through the two guanine nucleotide exchange factors RasGRP1 and SOS (Roose et al., 2005). DAG allosterically activates RasGRP1; in contrast, the immediate product of RasGRP1 and SOS – Ras-GTP – allosterically

activates SOS, thereby creating a positive feedback loop (Roose et al., 2005, 2007). In other words, as Ras-GTP production by RasGRP1 reaches a certain threshold, SOS is activated and greatly enhances production of Ras-GTP. Thus, DAG-RasGRP1 units serve as an analog component, while SOS serves as an analog to digital converter (Das et al., 2009; Chakraborty et al., 2009). As DGKζ activity attenuates DAG, it is positioned to regulate the number of DAG-RasGRP1 molecules present and therefore modulate the threshold for digital Ras activation. This phenomenon was studied in $CD8^+$ T cells (Riese et al., 2011). An *in silico* model predicted that $CD8^+$ T cells deficient in DGKζ would have increased DAG-RasGRP1 functional units, leading to an increase in fraction of T cells with activated Ras signaling but no change in the amount of activated Ras in each cell. *In vitro* testing using phosphorylation of ERK as a surrogate for Ras activation confirmed this prediction. In addition, the model predicted that a lower amount of antigen-receptor engagement on DGKζ-deficient cells than wildtype cells would be necessary for the equivalent amount of Ras/ERK activation. However, *in vitro* testing of this notion found that wildtype and DGKζ-deficient $CD8^+$ T cells had similar levels of antigen-receptor engagement at 50% of the maximum ERK phosphorylation, suggesting increased complexity to how DGKζ controls RasGRP1 activity. These data therefore suggest that DGKζ activity is important for determining the threshold of T cell activation. Additional studies are needed to determine whether the presence of DGKα in T cells also affects this threshold.

1.5.6. DGK at the Immunological Synapse

The immunological synapse (IS) forms at the site where the TCR engages with antigen presented by an APC. A multimolecular signaling cluster is formed at this site, which includes PLCγ1. The localized production of DAG at the IS is important for functional TCR signaling. For example, localized DAG synthesis causes reorientation of the microtubule-organizing complex (MTOC) towards the IS, which is required for transduction of the TCR signal (Quann et al., 2009). Moreover, treatment of TCR-stimulated cells with an inhibitor of DGK activity results in diffuse DAG persistence at the IS and impaired MTOC reorientation, which is thought to be important for directional secretion of cytolytic factors and sustained TCR signaling (Quann et al., 2009, 2011). DGKs may therefore play an essential role at the IS in metabolizing DAG and restricting DAG localization to the IS. This notion was studied in Jurkat T cells (Gharbi et al., 2011). Following TCR stimulation, TCR-bound immune complexes were isolated, and both DGKα and DGKζ were present, although a GFP-tagged DGKα did not translocate to the plasma membrane as visualized by microscopy. In addition, knockdown of DGKα did not affect the *in vitro* conversion of DAG to PA by the TCR-bound complexes, while knockdown of DGKζ did. Collectively, these experiments support the notion that while both DGKα and DGKζ likely participate in the regulation of DAG at the IS, DGKζ appears to play the dominant role. It was also found that treatment of Jurkat T cells with PMA enhanced the accumulation of DGKζ in TCR-bound immune complexes, suggesting a positive feedback between DAG accumulation and DGKζ function at the IS (Gharbi et al., 2011). Although it was not formally shown, this could be due to stimulation of PKCs, as a non-phosphorylatable MARCKS domain mutant DGKζ was defective in DAG metabolism at the IS. Together, these data suggest a key role for DGKs, in particular DGKζ, in regulating signaling at the IS.

DGKs may also be involved in secretory traffic at the IS. One mechanism of target cell killing at the IS by $CD8^+$ cytotoxic T cells (CTLs) may be the engagement of Fas on target cells by Fas ligand (FasL) expressed on the surface of CTLs or by FasL secreted through exosomes (Monleón et al., 2001). Exosomes containing FasL form within multivesicular bodies (MVBs) in CTL, and may be regulated by T cell activation (Zuccato et al., 2007; Monleón et al., 2001). In a recent study, Jurkat T cells were used as a model system to study the role of DGKα in MVB exosome secretion (Alonso et al., 2011). DGKα was recruited to MVBs and appeared to have two distinct roles. Through pharmacologic inhibition, it was shown that DGKα activity negatively regulated mature MVB formation. In contrast, knockdown of DGKα resulted in increased polarization of MVBs to the IS and increased exosome secretion. These results are consistent with the notion that DGKα has a dual role in exosome secretion, with kinase activity required for suppression of MVB development but non-kinase functions required for exosome secretion. The role of DGKζ in MVB formation requires further study.

1.6. DGK Functions in T cells

1.6.1. T cell Anergy

Anergy is one mechanism by which T cells undergo peripheral tolerance. Engagement of the both the TCR as well as co-stimulatory receptors leads to normal T cell activation, whereas stimulation of T cells through the TCR alone induces anergy (Jenkins et al., 1987; Choi and Schwartz, 2007; Macián et al., 2002). Anergic cells do not proliferate or produce IL-2 in response to antigenic stimuli, thus preventing the potential activation of self-reactive cells that have escaped negative selection in the

thymus (Choi and Schwartz, 2007). Anergic T cells were found to have decreased Ras activation and translocation of RasGRP1 to the immunological synapse, although activation of nuclear factor of activated T cells (NFAT), a calcium-dependent process has been found to be intact (Fields et al., 1996; Li et al., 1996; Zha et al., 2006; Kang et al., 1992). Thus, it appears that anergy correlates with a normal TCR-induced calcium signal but defective production of DAG. As IP_3 and DAG are created in equimolar ratios by PLCγ1 cleavage of PIP_2, it was speculated that DGK metabolism of DAG could potentially alter the balance of calcium and DAG signaling, thus converting T cell activation into T cell anergy. This notion was lent further weight by microarray data in which DGKα was found to be one of the predominant transcripts upregulated in anergic T cells (Macián et al., 2002; Zha et al., 2006).

Experimental evidence from studies in which DGK levels were manipulated genetically support the role of DGKs in T cell anergy. Overexpression of DGKα in a T cell line mimics an anergic state characterized by decreased Ras activation and RasGRP1 translocation to the IS (Zha et al., 2006). Conversely, deletion of DGKα, or DGKζ, in primary cells results in cellular resistance to anergy (Olenchock et al., 2006). Engagement of cytotoxic T lymphocyte antigen 4 (CTLA4), an inhibitory receptor on the surface of T cells, in the presence of TCR stimulation normally suppresses activation and promotes anergy. However, unlike wildtype cells, stimulation of DGKα- or DGKζ-deficient T cells through the TCR in the presence of co-stimulation blockade with CTLA4-Fc resulted in proliferation and production of IL-2 (Olenchock et al., 2006). Moreover, using an *in vivo* model, DGKα-deficient mice were found to have impaired anergy induction (Olenchock et al., 2006). Together, these data demonstrate that DGKα and DGKζ both function to control T cell activation and perhaps aid peripheral tolerance.

Neither DGKα- nor DGKζ-deficient mice develop autoimmunity, which may be expected for molecules that suppress T cell activation. Autoimmune hepatitis is observed in mice that lack both isoforms, suggesting that DGKα and DGKζ have redundant functions in suppressing autoimmunity (Zhong et al., 2008). However, the DKO mice have striking defects in T cell selection and development (discussed below). Hence, although T cells deficient in both isoforms demonstrate augmented TCR activation and potentially a propensity for inducing autoimmunity, another possibility is that these T cells express an altered set of TCRs that could potentially lead to an autoimmune phenotype. Separating these possibilities is difficult in conventional DKO mice, as normal T cell development does not occur. Mice with conditional gene targeting systems would help clarify the mechanisms of autoimmunity in DGK-deleted mice, as these mice would enable normal T cell development to proceed before gene deletion of DGKα and DGKζ. These mice would also allow investigators to separate possible alterations in selection from changes in TCR signaling in mature T cells for the observed effects of DGK loss on T cell activation and anergy, described above.

1.6.2. T cell Responses to Pathogen

Effective immune responses to initial exposure to a pathogen as well as subsequent exposures are essential for survival of the host. Effector and memory T cell subsets mediate these responses, respectively. TCR signal strength modulates both effector and memory T cell differentiation and maintenance (Zehn et al., 2009; Smith-Garvin et al., 2010; Wiehagen et al., 2010; Teixeiro et al., 2009). The critical role of DGKα and DGKζ in suppressing T cell activation and the regulated expression of these molecules

during activation led to a study of how these molecules function in effector and memory responses to LCMV (Shin et al., 2012). $CD8^+$ T cells from LCMV-specific P14 transgenic TCR mice deficient in DGKα or DGKζ showed increased antigen-specific $CD8^+$ T cell expansion and increased cytokine production in response to LCMV infection. Interestingly, the enhanced effector responses to LCMV by DGKα-deficient T cells were found to be partly cell extrinsic, while the enhanced responses to LCMV by DGKζ-deficient T cells were found to be cell intrinsic; these data suggest separate roles for DGKα or DGKζ in T cell signaling. After rechallenge with LCMV, $CD8^+$ T cells deficient in DGKα or DGKζ demonstrated decreased expansion, indicating that DAG signaling may differentially control effector and memory cell formation.

The mechanism controlling this differential DGK function in effector and memory T cells is currently unclear. One intriguing possibility is that DAG signaling may control mTOR activation, which acts differently in effector and memory T cells (Araki et al., 2009; Shin et al., 2012; Pearce et al., 2009; Rao et al., 2010). Inhibition of mTOR complex 1 (mTORC1) function using rapamycin has been found to diminish $CD8^+$ effector T cell function and promote memory $CD8^+$ T cell differentiation (Rao et al., 2010; Chi, 2012). These data indicate that one function of mTORC1 is to enhance effector T cell function and diminish memory T cell formation, which mirrors the observed phenotype in mice deficient in DGKα or DGKζ. As described above, T cells lacking both DGKα and DGKζ have increased AKT and mTORC1 activation (Gorentla et al., 2011). In addition, although we have not investigated mTORC1 activation, we have found that T cells lacking DGKζ alone have increased AKT activation (unpublished observations). Taken together, the available evidence suggests that deletion of DGKs may regulate effector and memory responses by enhancing mTORC1 activity, although further studies are required.

1.6.3. T cell Anti-tumor Responses

T cell responses to tumor are often characterized by development of peripheral tolerance to tumor antigens (Staveley-O'Carroll et al., 1998). Induction of T cell anergy in particular may be one mechanism by which tumors evade the immune system (Staveley-O'Carroll et al., 1998). As deletion of DGKα or DGKζ disrupts anergy formation and promotes T cell activation, deletion of these molecules may also enhance T cell responses to tumor. Other inhibitory molecules involved in TCR signaling have been targeted successfully to increase T cell responses to tumor. For example, $CD8^+$ T cells deficient in SH2 domain-containing phosphatase 1 (SHP-1), a tyrosine phosphatase, or cbl-b, an E3 ubiquitin ligase, have enhanced responses to tumor. Recently, the role of DGKζ in tumor responses was tested using an implanted EL-4 OVA model of tumor eradication (Riese et al., 2011). Compared to tumors implanted in wildtype mice, tumors implanted into DGKζ-deficient mice were significantly smaller after three weeks. In addition, tumor antigen-specific T cells from DGKζ-deficient mice expressed higher levels of markers of activation and proliferation. To rule out the effects of DGKζ deletion in non-T cells and determine whether $CD8^+$ T cells were responsible for this effect, these experiments were also performed using adoptively transferred $CD8^+$ DGKζ-deficient T cells, with similar results.

Interestingly, DGKζ-deficient T cells did not display enhanced cytotoxicity compared to wildtype $CD8^+$ T cells. The mechanism by which DGKζ deficiency enhances tumor responses is an important question and requires further investigation. For example, DGKζ deficiency could enhance anti-tumor responses primarily by

preventing the induction of anergy during initiation of the immune response. Alternatively, DGKζ deficiency may increase anti-tumor responses by lowering the threshold for T cell activation during proliferation of effector cells. Distinguishing between these possibilities will have important implications for the timing of administration of DGKζ-targeting cancer immunotherapies. DGKζ has added promise as a therapeutic target compared to other inhibitory molecules, as unlike mice deficient in SHP-1 or cbl-b, mice lacking DGKζ do not develop overt signs of autoimmunity. Additional study is needed, however, to determine if modulation of DGKζ function is also applicable to T cell responses in non-implanted, endogenously developing tumor models.

The role of DGKα in anti-tumor responses was studied recently in human tumor infiltrating $CD8^+$ T cells (CD8-TILs) from patients with renal cell carcinoma (RCC) (Prinz et al., 2012). CD8-TILs from RCCs were defective in lytic granule exocytosis and their ability to kill target cells. While proximal signaling events were intact in response to TCR engagement, CD8-TILs exhibited decreased phosphorylation of ERK when compared to non-tumor infiltrating $CD8^+$ T cells. This impairment of lytic ability and phosphorylation of ERK correlated with an increase in DGKα expression in CD8-TILs. Treatment of CD8-TILs with an inhibitor of DGKα activity rescued killing ability of target cells, increased basal levels of phosphorylation of ERK, and increased PMA/ionomycin stimulated phosphorylation of ERK. In addition, *ex vivo* administration of IL-2 to CD8-TILs was found to decrease DGKα expression levels, rescue lytic granule exocytosis and killing ability, and increase phosphorylation of ERK in response to PMA/ionomycin treatment. These results suggest that CD8-TILs may be defective in function partially due to high DGKα levels leading to dysfunction in lytic granule exocytosis, which is consistent with the previously described role for DGKα in controlling secretory traffic at the

immunological synapse (Alonso et al., 2011). Further study is needed to elucidate the DGKα-regulated pathways in CD8-TILs that control lytic granule exocytosis.

1.6.4. T cell Adhesion

Surveillance of a host for pathogens requires that T cells constantly circulate between secondary lymphoid organs and enter and exit microvasculature. Interaction with the microvasculature requires cooperation between (1) selectins, which mediate rolling on endothelial cells, (2) chemokine receptors, which determine directionality of cellular migration and transduce signals to activate integrins on the migrating cell, and (3) integrins, which mediate the firm attachment of the cell to the underlying endothelium and allow transmigration into a tissue (Hogg et al., 2011; Sackstein, 2005). DAG-activated RasGRP2 may link chemokine receptor signaling to integrin activation by activating Rap1 (Hogg et al., 2011). Through DAG metabolism, DGKs are therefore positioned to regulate signaling from chemokine receptors to integrins, so-called "inside out" signaling. The role of DGKζ in regulating T cell adhesion was recently studied (Lee et al., 2012). Using a quantitative model of T cell integrin signaling adhesive dynamics, it was predicted that DGKζ deficiency would enhance the kinetics of integrin activation and shorten the distance to arrest of T cells under shear flow (Lee et al., 2012). To test this model, wildtype or DGKζ-deficient T cells were introduced into a flow chamber with immobilized P-selectin and intercellular adhesion molecule-1 (ICAM-1), a ligand for integrins, with or without the chemokine receptor ligand CXCL12. The time and distance to arrest of T cells of the different genotypes was then measured. DGKζ-deficient T cells had a significantly shorter time and distance before arrest than wildtype cells after

addition of CXCL12, supporting the model and indicating that DGKζ deficiency may enhance integrin activation kinetics and firm arrest of T cells. These data suggest that DGKζ regulates T cell arrest and may suppress aberrant integrin activation. Further studies are required to investigate the molecular mechanisms by which DGKζ modulates inside-out signaling and integrin activation.

1.7. DGKs and development

1.7.1. T cell Development

During T cell development in the thymus, each thymocyte expresses a TCR with a single specificity. These TCRs arise from the random rearrangement of gene segments resulting in enormous potential for diversity of sequence. The selection of thymocytes from this large number of developing cells with useful but self-tolerant TCRs is essential for a productive immune response to pathogens that does not cause autoimmunity. Signaling through the TCR of developing T cells directs the deletion of cells that cannot recognize peptide presented by self-MHC (termed positive selection) and cells that are potentially autoreactive (termed negative selection). The TCR-mediated, DAG-activated RasGRP1 is a critical molecule for the positive selection of T cells (Dower et al., 2000; Priatel et al., 2006). In addition, Ras/ERK signaling is important for both positive and negative selection of T cells (Daniels et al., 2006; Mariathasan et al., 2001; Werlen et al., 2000). Strong but transient ERK signals induce negative selection, whereas weaker but sustained ERK signals promote positive selection (Werlen et al., 2000; Daniels et al., 2006; Mariathasan et al., 2001). Recruitment of activated Ras to the plasma membrane may lead to strong selecting

signals, while recruitment to both the plasma membrane and intracellular regions may lead to weak selecting signals (Daniels et al., 2006). Together, these data suggest that alterations in DAG signaling by DGKs could alter thymocyte selection by altering strength of signal and Ras-GTP localization.

Expression of both DGKα and DGKζ is highly regulated during thymocyte development. DGKα mRNA is expressed at low levels in the earliest double negative (DN) stage of development and increases as thymocyte development progresses to the double positive (DP) and CD4 and CD8 single positive (CD4SP and CD8SP) stages (Outram et al., 2002; Wu et al., 2009; Lattin et al., 2008; Su et al., 2004). Two isoforms of DGKζ are expressed in developing thymocytes, a short form and long form that share the same domains but are distinguished by the length of the N-terminal region (Zhong et al., 2002). The long isoform is more highly expressed than the short isoform in DN thymocytes; as development continues, expression of the short isoform progressively increases while expression of the long isoform decreases (Zhong et al., 2002). Whether this switch in isoform has functional implications is unknown.

While both molecules demonstrate regulated expression during development, deficiency of DGKα or DGKζ alone does not grossly alter thymocyte selection, although a decrease in CD8SP thymocytes in mice deficient in DGKζ has been observed (Olenchock et al., 2006; Zhong et al., 2003). DGKα and DGKζ may cooperate to control selection, as simultaneous deletion of DGKα and DGKζ leads to a strong block at the DP stage of thymocyte development (Guo et al., 2008). Interestingly, upregulation of CD69, CD5, and TCRβ – events correlated with productive positive selection – are unaffected, and this defect is likely due to increased cell death once thymocytes reach the $TCR\beta^{hi}$ stage of DP development (Guo et al., 2008). Positive selection can be tested using female mice that have a transgenic TCR specific for the H-Y antigen, which is only

expressed by males. Female DKO mice crossed to the H-Y transgene have decreased CD8SP compared to wildtype, but whether this is due to a failure of positive selection or to enhanced negative selection is unclear. Mice with RasGRP1 overexpression do not have defects in positive selection, suggesting that DGK deficiency may lead to alterations in signaling through molecules other than RasGRP1 (Klinger et al., 2004; Guo et al., 2008). Although further study is needed, exogenous addition of PA to DGKα- and DGKζ-deleted fetal thymic organ cultures partially rescued development of SP thymocytes, indicating that lack of PA production during TCR signaling could lead to the observed phenotype (Guo et al., 2008). In summary, these data provide strong evidence that DGKα and DGKζ have a cooperative role in controlling the selection of developing thymocytes.

DGKs are also invoved in the development of invariant natural killer T cells (*i*NKT), a rare subset of T cells that function at the interface of innate and adaptive immunity. The PKCθ/NF-κB pathway plays a critical role in *i*NKT development at multiple stages (Bendelac et al., 2007). As DAG activates PKCs, a recent study investigated the role of DGKα and DGKζ in *i*NKT development (Shen et al., 2011). Although deficiency of DGKα and DGKζ alone does not markedly affect *i*NKT development, simultaneous deletion of both isoforms reveals a cell-intrinsic defect in *i*NKT development at the early $CD44^{lo}NK1.1^{-}$ stage. After TCR stimulation, thymocytes lacking both DGKα and DGKζ had increased ERK and IκBα phosphorylation compared to wildtype cells, suggesting that DGKs may enhance *i*NKT development through suppression of these pathways. Consistent with this notion, mice with constitutively active IKKβ had *i*NKT development that resembled that of DGKα and DGKζ double deficient mice, although there was not a correlation when all surface markers were investigated. In contrast, early *i*NKT development was unaffected in mice with

constitutively active k-Ras; instead, these mice displayed a severe block at the transition from $CD44^{hi}NK1.1^{-}$ to $CD44^{hi}NK1.1^{+}$ *i*NKT cells. These data suggest that DGKα and DGKζ may regulate the PKCθ/IKKβ/NF-κB axis during early *i*NKT development and the RasGRP1/Ras pathway during late *i*NKT development. However, further study using genetic and pharmacologic approaches is required to determine if increased PKCθ and RasGRP1 activation is causal for the observed defect in *i*NKT development with DGKα and DGKζ deletion.

1.8. Aims of this Thesis

TCR-mediated T cell activation is essential for a productive adaptive immune response. Phosphoinositide lipid based signaling, including cascades initiated by the synthesis of DAG, plays a critical role in transducing signals from the TCR. As such, regulation of these lipid molecules would be expected to play a large role in TCR signaling. Evidence obtained over the past decade has indicated that not only the synthesis of DAG and other lipids, but the metabolism of these lipids as well, is important for temporal and spatial control of signal transduction. In T cells, DGKα and DGKζ metabolize DAG to PA and terminate DAG-mediated signaling. Deletion of either isoform enhances DAG-mediating signaling, leading to resistance to anergy, augmented T cell effector responses to pathogen, and increased function of chimeric antigen receptor (CAR)-expressing T cells in cancer immunotherapy. These data suggest overlapping roles for DGKα and DGKζ in T cells. However, given these isoforms' distinct domain architectures that direct their differential regulation in enzymatic studies, isoform-specific functions may exist as well. Further investigation of isoform-selective roles in T cells is critical for developing therapies targeting DGKs because of their clear *in vivo* roles.

The first results section of this thesis is devoted to investigating dominant and selective roles for the ζ isoform of DGK in T cells. We investigate the role of DGK isoforms *in vivo* during nTreg development as well as *in vitro* in TCR signaling, which is essential for nTreg development. We describe that DGKζ and not DGKα suppresses the development of nTreg. Second, we describe a dominant role for DGKζ and not DGKα in

suppressing Ras-mediated signaling downstream of the TCR, which improves our understanding how DGKs function and how DAG-activated pathways are regulated in T cells. These findings delineate a clear separation of function for DGKα and DGKζ in T cells.

In the second results section, we investigate the mechanisms that determine these differences in function. To do so, we performed experiments investigating the expression, localization, interaction with other proteins, and effective enzymatic activity of DGKα and DGKζ. We find two novel mechanisms – binding to RasGRP1 and effective enzymatic activity – that may direct the differential function of DGKα and DGKζ. These findings are not only important for the development of therapies targeting DGKs but also are of broad interest for understanding how DGKs are regulated. In addition, these results provide clues of significance in understanding of how nTregs develop.

In the third results section, we broaden our investigation to phosphoinositides and how they themselves may be regulated. We examine the role of phosphatidylinositol transfer proteins (PITP) α and β, which may facilitate the transfer of phosphatidylinositol from the ER to the plasma membrane, in T cells using conditional genetic knockout mice. We find that while PITPα and PITPβ are grossly dispensable for normal T cell development when individually deleted, simultaneous deletion of these molecules leads a loss of mature T cells in the thymus and periphery. These findings indicate that PITPs have a critical role in T cells, and future work will investigate the mechanism of loss of T cells with PITP deletion.

Together, our results provide further evidence that regulation of phoshoinositides and their metabolic products are essential for normal T cell signaling and development. Our studies of differential functions of DGKα and DGKζ suggest that local interactions with signaling molecules and changes in enzymatic activity can direct DGK function.

Consequently, this work provides a foundation for therapies that may target DGK isoforms to enhance T cell function.

1.9. Figures

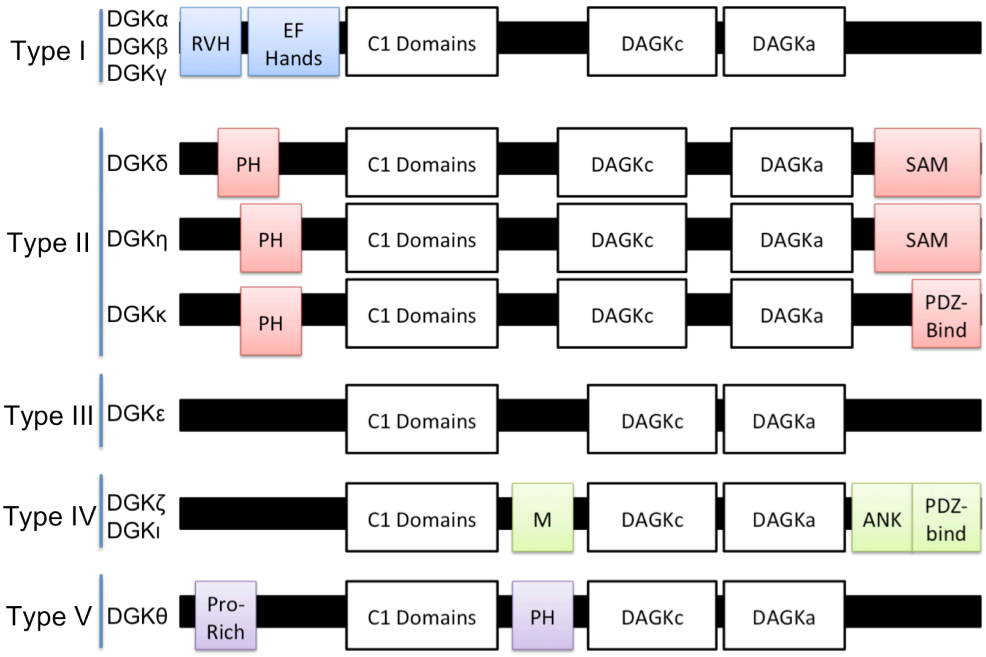


Figure 1.1: DGK isoforms are divided into five subtypes based on domains apart from the C1 and catalytic domains.

RVH: recoverin homology domain. PH: pleckstrin homology domain. SAM: sterile α motif. M: MARCKS homology domain. ANK: ankyrin repeat domain. PDZ-bind: PDZ-binding domain. Note: domain sizes are not to scale.

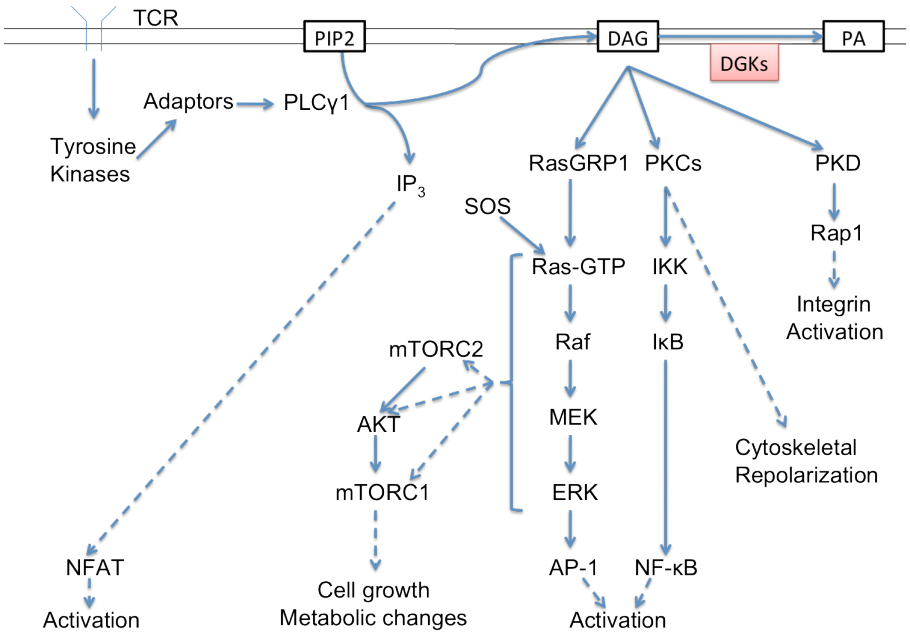


Figure 1.2: Diacylglycerol has a central role in TCR signaling.

Engagement of the TCR leads to the activation of tyrosine kinases and the formation of a multimolecular complex including adapter proteins. PLCγ1 is recruited to and activated by this complex and in turn hydrolyses the phospholipid PIP_2 to form membrane-diffusible DAG and cytosolic IP_3. DAG activates proteins including RasGRP1, PKCs, and PKD. Activation of RasGRP1 leads to Ras and AKT pathway activation. Activation of PKCs leads to the activation of NF-κB and cytoskeletal repolarization. Activation of PKD leads to integrin activation. IP_3, Ras, and PKC-θ signaling cooperate to promote T cell activation through the transcription factors NFAT, AP-1, and NF-κB, respectively. DGKs phosphorylate DAG to form PA, thereby potentially regulating a broad set of signaling pathways downstream of the TCR.

CHAPTER II: A DOMINANT ROLE FOR THE ZETA ISOFORM OF DGK IN T CELL RECEPTOR MEDIATED SIGNALING

2.1. Introduction

As noted above, T cell activation requires engagement of the TCR with peptide presented within MHC proteins on APCs, which leads to the production of second messengers that activate pathways critical for the normal development, activation, differentiation, and proliferation of T cells. DAG is essential for activation of diverse signaling cascades downstream of the TCR, including the Ras, NF-κB, and AKT pathways, which are integrated with other key signals to promote T cell effector function (Tognon et al., 1998; Coudronniere et al., 2000; Quann et al., 2009; Gorentla et al., 2011). Levels of DAG therefore must be finely tuned not only through its production but also its metabolism for appropriate control of a T cell response.

T cells express high levels of α and ζ isoforms of DGK in addition to the δ isoform, whose function in lymphocytes remains unknown. Deletion of DGKα or DGKζ in mice results in T cells with enhanced Ras and extracellular signal-regulated kinase (ERK) activation in response to TCR engagement (Zhong et al., 2003; Olenchock et al., 2006; Zhong et al., 2002). In addition, both molecules regulate the T cell effector response to pathogen in mice (Shin et al., 2012). These data suggest that DGKα and DGKζ have overlapping roles in T cells. Consistent with this notion, simultaneous deletion of DGKα and DGKζ in mice reveals a defect in thymocyte development at the double positive stage that is not seen in mice deficient in DGKα or DGKζ alone, suggesting a redundant function for these molecules in T cell development.

However, DGKα and DGKζ have distinct domain architectures that suggest differential regulation of these molecules, perhaps directing isoform-specific functions in addition to their redundant roles. DGKα contains a calcium-responsive EF-hands regulatory domain, while DGKζ contains myristoylated alanine-rich protein kinase C substrate (MARCKS), ankyrin and PDZ-domains (please refer to the Chapter I for a detailed description of the known functions of these domains). In Jurkat T cells, DGKζ was recently found to be the predominant regulator of DAG after TCR engagement, suggesting specific functions for this isoform (Gharbi et al., 2011). No direct investigation of the relative role of DGKα and DGKζ has been performed in primary T cells, although differences in DGKα and DGKζ function in TCR signaling have been suggested (Olenchock et al., 2006). Furthermore, whether isoform-specific functions exist *in vivo* is unknown.

Here, we show that DGKζ has dominant roles over DGKα in regulatory T cell development and TCR signaling in primary T cells. Deletion of DGKζ, but not DGKα, enhances thymic regulatory T cell development. DGKζ also demonstrates quantitatively greater control over signaling downstream of Ras after TCR engagement. Our findings suggest that DGKζ plays a selective role in suppression of Treg development and a predominant role in suppression of DAG-mediated Ras signaling.

2.2. Results

2.2.1 In vivo functions of DGKα and DGKζ

2.2.1.1 DGKζ but not DGKα suppresses nTreg development

Deletion of DGKζ results in enhanced development of natural regulatory T cells (nTregs) (*Schmidt* et al., manuscript submitted). To determine if DGKα similarly suppresses development of this lineage during thymocyte maturation, we examined the proportion of thymic FoxP3^{+} cells within the pool of CD4 single positive (CD4SP) T cells of wildtype, DGKα-deficient, and DGKζ-deficient mice (Fig. 2.1). The percent of CD4SP thymocytes was similar in each genotype (Fig. 2.2). As reported by *Schmidt* et al. (submitted), mice deficient in DGKζ have increased percentages of thymic FoxP3^{+} cells and CD25^{+}FoxP3^{-} cells, a population enriched for Treg precursors, compared to wildtype mice (Fig. 2.1 A, C-D). In contrast, mice deficient in DGKα showed no such increase in the percentage of FoxP3^{+} cells in the thymus and an intermediate frequency of CD25^{+}FoxP3^{-} cells. Splenic FoxP3^{+} cell percentages were also increased only by deficiency of DGKζ and not DGKα (Fig. 2.1 B and E). These data demonstrate that DGKζ is unique in its ability to suppress development of nTregs and has distinct functions *in vivo* from DGKα.

To understand whether DGKα and DGKζ have a dose dependent effect on Treg development, we investigated the thymi of mice with varying numbers of alleles of DGKα and DGKζ (Fig. 2.3). We applied a multilinear regression model to our data with number of DGKα and DGKζ alleles removed as predictors and percentage of Tregs as the

dependent variable and determined the significance, magnitude, and interaction of removal of DGK isoforms on nTreg percentages (R^2=0.736, p=1.75x10^-12). While deletion of DGKα alone did not significantly increase nTreg percentages (p=0.331), deletion of DGKζ alone significantly increases nTreg percentages (p=4.67x10^-12) with a magnitude of 1.89% per deleted DGKζ allele (95% confidence interval 1.49 - 2.29). These data suggest that DGKζ is unique in its ability to suppress nTreg development. We also found a significant interaction term between DGKα and DGKζ deletion (p=0.0027), such that in the absence of DGKζ alleles, deletion of DGKα alleles actually decreased nTreg percentages. This is demonstrated by the fact that DGKα$^{+/-}$DGKζ$^{-/-}$ mice have decreased nTreg percentages compared to DGKα$^{+/+}$DGKζ$^{-/-}$ mice. In comparison, DGKα$^{-/-}$DGKζ$^{+/-}$ mice have a modest increase in nTreg percentages compared to DGKα$^{-/-}$DGKζ$^{+/+}$ mice. Taken together, these data suggest that in the context of simultaneous deletion of both isoforms, the overriding effect of DGKζ deletion is to increase nTreg percentages, while the overriding effect of DGKα is to decrease nTreg percentages. DGKα deletion may decrease nTreg percentages indirectly due to alterations in CD4 single positive (CD4SP) selection, as DGKα$^{+/-}$DGKζ$^{-/-}$ mice have decreased CD4SP compared to DGKα$^{+/+}$DGKζ$^{-/-}$ mice (summary data not shown).

2.2.1.2 Examination of DGKζ re-expression on DGKζ-deficient nTreg development

As the different domain architectures of these isoforms could direct their differential suppressive function in nTreg development, we further investigated which domains of DGKζ were required for this function. We focused these experiments on the kinase and MARCKS domains of DGKζ as suppression of nTreg development could be

kinase function independent and the MARCKS domain has been shown to direct localization of DGKζ in Jurkat T cells (Ard et al., 2012; Gharbi et al., 2011). We used DGKζ with a point mutation in the catalytic domain that disrupts DGKζ catalytic activity or DGKζ with serine to alanine mutations in the MARCKS domain that prevents phosphorylation of these sites. After subcloning these constructs into a retroviral vector, we transduced DGKζ-deficient bone marrow cells, transferred these cells into lethally irradiated congenically labeled recipient mice, and waited 10 weeks for immune reconstitution. We observed a roughly equal transduction of wildtype DGKζ, kinase-dead DGKζ, and MARCKS domain mutant DGKζ into cells in these mice (data not shown). Unfortunately, however, we observed a wide variation in the ratio of FoxP3+ cells within the transduced population to non-transduced population in both kinase-dead DGKζ and MARCKS domain mutant DGKζ groups, making interpretation of this data difficult (data not shown). The wide variation could be due to effects of retroviral transduction, as mice with a greater degree of transduced cells also appeared to have the lowest ratio of FoxP3+ cells within the transduced population to non-transduced population, regardless of the vector that was used (data not shown).

2.2.1.3 $CD44^{hi}CD8^{+}$ T cells are increased in DGKζ-deficient mice compared to DGKα deficient mice

DGKζ-deficient mice have increased proportions of $CD44^{hi}CD8^{+}$ T cells but a decreased total number of $CD8^{+}$ T cells (Riese et al., 2011). We wished to determine whether DGKα-deficient mice have a similar $CD8^{+}$ T cell phenotype. In contrast to age-matched DGKζ-deficient mice, DGKα-deficient mice demonstrated a significantly smaller increase in the proportion of $CD44^{hi}$ cells within $CD8^{+}$ splenic T cells (Fig. 2.4).

Concordant with their CD44 expression phenotype, DGKα-deficient mice appeared to have an intermediate number of splenic $CD8^+$ T cells between that of wildtype and DGKζ-deficient mice. The differences in CD44 phenotype between DGKα- and DGKζ-deficient $CD8^+$ T cells therefore could be due to differences in lymphopenia within the $CD8^+$ T cell compartment. Alternatively, cell-intrinsic differences in DGK isoform function could lead to these differences. Nevertheless, these data suggest that DGKζ contributes more than DGKα to control the CD44 activation marker phenotype of $CD8^+$ T cells *in vivo*.

2.2.2. DGKζ exhibits greater control over TCR-mediated signaling than DGKα

2.2.2.1. DGKζ has greater quantitative control than DGKα over TCR-stimulated ERK phosphorylation

TCR signaling is critical for the development of nTregs and the activation of T cells. Both DGKα and DGKζ suppress DAG-mediated signaling after TCR engagement (Olenchock et al., 2006; Zhong et al., 2003; Gharbi et al., 2011), but a direct comparison of the role of DGKα and DGKζ in primary T cells has not yet been performed. We predicted that differences in the ability of DGKα and DGKζ to regulate TCR signaling might mirror the observed differences in *in vivo* function, such that DGKζ would exhibit greater control over DAG-mediated TCR signaling than DGKα. To test this possibility, we examined the phosphorylation of ERK in T cells after TCR stimulation, an assay used previously to examine modulation of DAG-mediated signaling by DGKs (Olenchock et al., 2006; Zhong et al., 2003; Riese et al., 2011; Guo et al., 2008; Zha et al., 2006; Gharbi et al., 2011). Using anti-CD3 antibody at a suboptimal concentration, we

stimulated T cells from mice with varying numbers of alleles of DGKα and DGKζ and measured ERK phosphorylation by flow cytometry. This allelic series allowed us not only to determine which isoform has greater control of ERK phosphorylation but also to quantify the relative magnitude of DGKα and DGKζ suppression of ERK phosphorylation. Deletion of DGKζ resulted in a large increase in the percentage of cells with phosphorylated ERK (phospho-ERK), while deletion of DGKα resulted in a consistently lower increase in phospho-ERK positive $CD4^+$ and $CD8^+$ T cells (Fig. 2.5 A-D).

To analyze the allelic series statistically, we applied a multiple linear regression model with the number of DGKα and DGKζ alleles removed as predictors and the natural log of the percentage of phospho-ERK positive cells as the dependent variable (Table 2.1). As mice with complete DGKα and DGKζ deletion have a severe block in thymic development (Guo et al., 2008), we eliminated these mice from our analysis. According to the regression model, deletions of DGKα and DGKζ alleles were strong and significant predictors of the percentage of phospho-ERK positive cells for both $CD4^+$ and $CD8^+$ T cells (R-squared=0.768, $p=5.74 \times 10^{-9}$ and R-squared=0.811, $p=3.98 \times 10^{-10}$, respectively). Deletion of DGKα or DGKζ significantly increased ERK phosphorylation in $CD4^+$ and $CD8^+$ T cells (Table 2.1), consistent with previous reports (Zhong et al., 2003; Riese et al., 2011; Olenchock et al., 2006). However, the magnitude of the effect of DGKζ deletion was much greater than that of DGKα deletion, with a 2.6-fold and 3.3-fold greater effect of DGKζ deletion than DGKα deletion on the natural log of phospho-ERK in $CD4^+$ and $CD8^+$ T cells, respectively. The increased ERK phosphorylation observed in DGKζ-deficient T cells was not due to the higher percentage of $CD44^{hi}CD8^+$ activated splenic T cells, as the percentage of phospho-ERK positive cells was similar between the $CD44^{hi}$ and $CD44^{lo}$ populations, regardless of genotype (Fig. 2.6). Taken together,

these results provide strong evidence that DGKζ plays a more dominant role than DGKα in suppressing signals leading to ERK phosphorylation in $CD4^+$ and $CD8^+$ T cells.

2.2.2.2. DGKζ exhibits greater control over TCR-stimulated AKT and S6 phosphorylation than DGKα

Simultaneous deletion of DGKα and DGKζ leads to increased signaling through the AKT pathway, likely due to increased Ras-MAPK-ERK signaling (Gorentla et al., 2011). We therefore tested whether AKT signaling, like ERK, was also predominantly affected by DGKζ rather than DGKα deletion. We also examined the phosphorylation of S6 ribosomal protein, an event downstream of both AKT and ERK signaling. At both 5 and 15 minutes after TCR stimulation, we observed a large increase in the percentage of cells with phosphorylated AKT and S6 in DGKζ-deficient T cells compared to wildtype T cells (Fig. 2.7 A and B, top and middle panels, C-D). In contrast and consistent with DGKα's more minor role in ERK phosphorylation, only a slight increase in percentages of phospho-AKT and phospho-S6 positive cells was observed in DGKα-deficient T cells. Of note, the phosphorylation of SH2 domain-containing leukocyte protein of 76kDa (SLP76) was similar among wildtype, DGKα-, and DGKζ-deficient T cells after TCR stimulation (Fig 2.7 A and B, bottom panels), indicating that signals proximal to DAG synthesis are unaltered with deletion of DGKs. While we observed striking increases in the proportion of phospho-AKT and phospho-S6 positive cells in DGKζ-deficient $CD8^+$ T cells, a more modest change was observed in $CD4^+$ T cells. This result may be due to differences in proximal signaling events, as less SLP76 phosphorylation was observed in $CD4^+$ compared to $CD8^+$ T cells (Fig. 2.7 A and B, bottom panels), and/or from the

increased proportion of Tregs, which are known to be less responsive to TCR stimuli than conventional $CD4^+$ T cells, in DGKζ-deficient $CD4^+$ T cells. This examination of ERK, AKT, and S6 phosphorylation demonstrates a consistently greater role for DGKζ than DGKα in suppressing DAG-mediated signaling, particularly signaling downstream of Ras, after TCR engagement. Interestingly, we observed that both DGKα- and DGKζ-deficient T cells have similar increases in the phosphorylation of IκBα (Fig. 2.8), suggesting that DGKζ's dominant suppressive role does not extend to DAG-mediated activation of PKC-θ and its downstream targets.

We wished to determine which domains of DGKζ were required for its dominant suppressive role in TCR signaling as a clue to how DGKα and DGKζ function differently. Similar to our investigation of nTreg development we focused on the kinase and MARCKS domains of DGKζ as DGKζ has been found to have a kinase-independent scaffolding role that regulates signaling and the MARCKS domain controls localization in Jurkat T cells (Ard et al., 2012; Gharbi et al., 2011). To test whether suppression of DAG-mediated TCR signaling requires functional kinase activity, we created bone marrow chimeras with DGKζ-deficient bone marrow cells expressing either retroviral vector, wildtype DGKζ, kinase-dead DGKζ, or a non-phosphorylatable MARCKS domain mutant DGKζ and evaluated ERK, AKT, and S6 phosphorylation after TCR stimulation. While wildtype DGKζ rescued suppression of ERK, AKT and S6 phosphorylation in both $CD4^+$ and $CD8^+$ T cells, kinase dead DGKζ did not (Fig. 2.9 A-D). Interestingly, cells expressing MARCKS domain mutant DGKζ displayed an intermediate phenotype and suppressed these phosphorylation events more than kinase dead but less than wildtype DGKζ. These results indicate that suppression of TCR signaling requires DGKζ's ability to phosphorylate DAG rather than a kinase-independent scaffolding function as well as an intact MARCKS domain.

2.3. Discussion

Although previous work has shown that both DGKα and DGKζ can serve as negative regulators of TCR-mediated signaling and T cell activation, a direct comparison of the roles of these two enzymes had not been previously reported. In this work, we demonstrate that DGKζ possesses functions distinct from that of DGKα both *in vivo* and *in vitro*. We found significantly enhanced development of nTregs in DGKζ-deficient mice, a phenotype that we did not observe in DGKα-deficient mice. Moreover, DGKζ and not DGKα was the predominant suppressor of Ras-mediated signals after TCR engagement.

TCR signals are critical for the development of nTregs (Jordan et al., 2001). Increased DAG signals in particular contribute to the development of nTregs, as DGKζ-deficient mice have a cell-intrinsic increase in thymic Tregs (Fig. 2.1 A and *Schmidt et al*., submitted). Notably, in contrast to DGKζ-deficient mice, DGKα-deficient mice have no increase in thymic Treg percentages. We observed a significant increase in Treg-precursor enriched $CD25^{+}FoxP3^{-}$ cells in DGKζ-deficient mice, which correlated with increased thymic Treg percentages. Surprisingly, DGKα-deficient mice also have a significant albeit lower increase in $CD25^{+}FoxP3^{-}$ cells, and this increase does not translate to an increase in thymic Treg percentages. These data suggest that DGKζ deficiency increases nTreg development not only through increased generation of Treg precursors but also through modulation of other processes during nTreg development.

When examining an allelic series of mice with different numbers of DGKα and DGKζ alleles, we found that while removal of DGKζ alleles increased the percentage of

nTregs in the absence of DGKα alleles, removal of DGKα alleles decreased the percentage of nTregs in the absence of DGKζ alleles. As DGKα$^{+/-}$DGKζ$^{-/-}$ mice have decreased CD4SP compared to DGKα$^{+/+}$DGKζ$^{-/-}$ mice, these results could be due to alterations in selection of CD4SP. Although mice deficient in DGKα or DGKζ alone display no gross defects in selection, deletion of both DGKα and DGKζ leads to a severe block at the DP to SP transition, likely due to selection defects (Guo et al., 2008). Given DGKα and DGKζ's redundant role in selection, deletion of increasing numbers of DGK alleles in our series may uncover these defects. Nevertheless, our data would suggest that these selection defects are not linked to nTreg percentages, as deletion of DGKα leads to opposite effects on nTreg percentages than deletion of DGKζ. As DGKα- and DGKζ-deficiency have similar effects on selection but disparate effects on nTreg development, DGKα and DGKζ may differentially regulate downstream pathways that are not involved in selection but do function in nTreg development. Alternatively, the signals upstream of DGKs that control nTreg development and T cell selection could be different, perhaps directing the function of both DGK isoforms during selection but only DGKζ during nTreg development. Further experiments are needed to investigate these possibilities.

We also observed that DGKα and DGKζ play different roles in suppressing Ras-mediated TCR signaling. Deletion of DGKζ had a much greater effect than deletion of DGKα on ERK, AKT, and S6 phosphorylation after TCR engagement, suggesting a dominant role for DGKζ in this process. These data are consistent with previous reports in Jurkat cells indicating that DGKζ is the main controller of PA production from DAG at the TCR, as removal of DGKζ could lead to a greater increase in DAG than removal of DGKα (Gharbi et al., 2011). In addition, our findings are also concordant with previous studies indicating that DGKζ-deficiency more profoundly increases IL-2 production than

DGKα-deficiency after TCR and co-stimulation (Olenchock et al., 2006). Our studies also suggest that phosphorylation of the MARCKS domain is important for DGKζ suppression of ERK, AKT and S6 phosphorylation. Future experiments will determine how the MARCKS domain regulates DGKζ function.

Differences in signaling phenotypes between T cells deficient in DGKα or DGKζ could provide further clues as to the molecular pathways that control nTreg development. Two possibilities are that (1) DGKα and DGKζ regulate the same pathways but with different thresholds that are required for nTreg development, and/or (2) DGKα and DGKζ regulate separate pathways that differentially control nTreg development. As signaling through ERK contributes to the development of nTregs in a dose-dependent manner (*Schmidt et al.,* submitted), DGKζ-deficient mice might display increased nTreg development compared to DGKα-deficient mice due to DGKζ's more potent role in suppression of ERK signaling, leading to an increase in percentage of developing cells with ERK signaling above a certain threshold required for nTreg development. Interestingly, both DGKα- and DGKζ-deficient T cells have similar increases in the phosphorylation of IκBα, signaling through which is also essential for nTreg development. Thus, while deletion of DGKζ increases TCR-mediated signaling through both ERK and NF-κB pathways (*Schmidt et al.*, submitted), deletion of DGKα may increase TCR-mediated signaling primarily through NF-κB. As DGKζ deficiency increases nTreg development and DGKα deficiency does not, these data suggest that activation of the NF-κB pathway alone is insufficient to increase Treg development. Rather, NF-κB and ERK signaling may need to be simultaneously engaged to increase nTreg generation.

The data presented here demonstrate that DGKα and DGKζ differentially regulate TCR signaling and nTreg development. Our studies suggest that nTreg

development is controlled by DGKζ at a step after the FoxP3^{-}CD25^{+} precursor stage. Future experiments will elucidate how and why DGKζ and not DGKα is involved in the FoxP3^{-}CD25^{+} to FoxP3^{+}CD25^{+} transition. These results also have implications for the development of DGK-specific inhibitors for therapeutic use, as DGKα inhibition would be expected to have a much different effect on nTreg development and functions requiring TCR signaling than DGKζ inhibition.

2.4. Figures

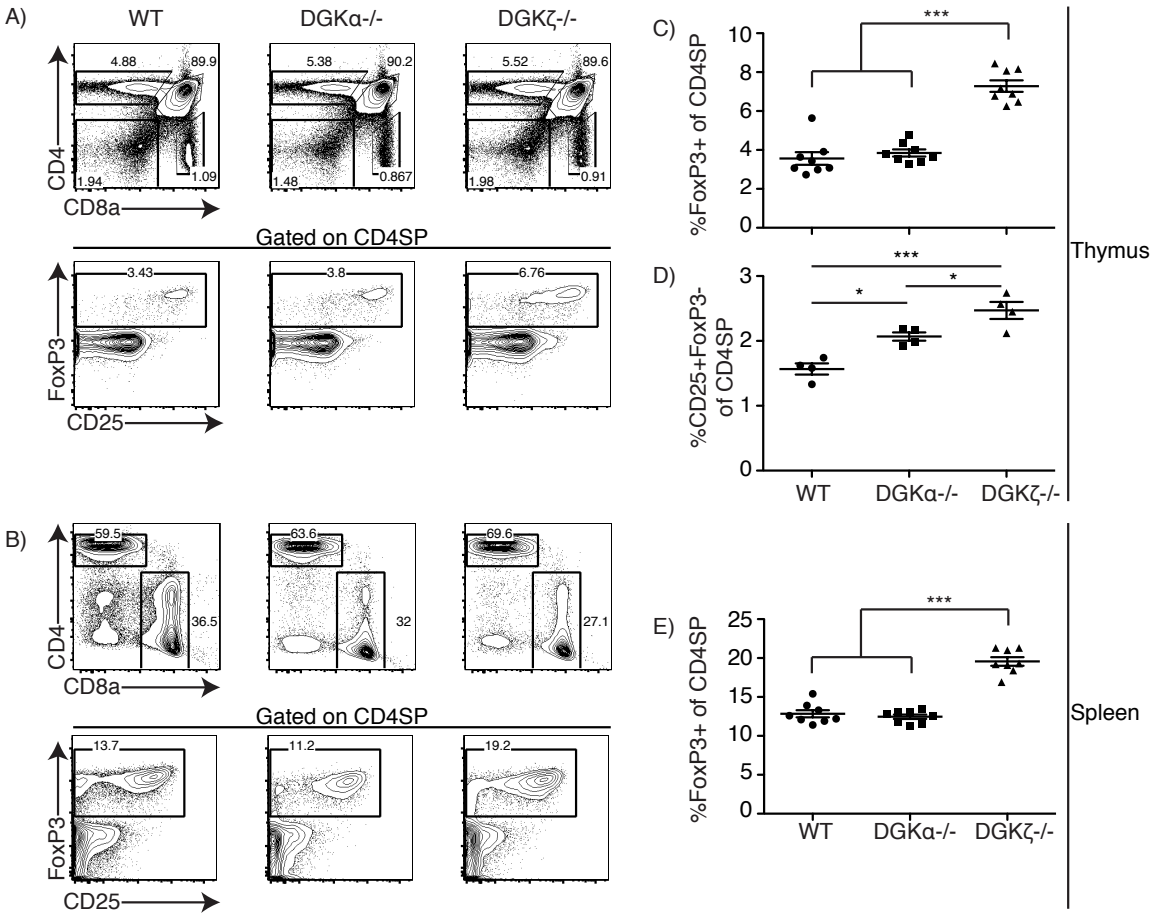


Figure 2.1: DGKα-deficient mice have no increase in Treg percentages, unlike DGKζ-deficient mice, but do have increased Treg precursor numbers.

(A) Top: Representative flow cytometric profiles of freshly isolated thymocytes gated on live singlet cells. Bottom: FoxP3$^+$ cells gated on CD4 single positive thymocytes. (B) Top: Representative flow cytometric profiles of freshly isolated splenocytes gated on live singlet cells. Bottom: FoxP3$^+$ cells gated on CD4 single positive splenocytes. (C) Summary data of percentage of FoxP3$^+$ cells among CD4SP in thymi. (D) Summary data of percentage of CD25$^+$FoxP3$^-$ cells (Treg precursors) among CD4SP in thymi. (E) Summary data of percentage of FoxP3$^+$ cells among CD4SP in spleen. N=8 from 3 independent experiments for FoxP3 expression and N=4 from 2 independent experiments for CD25 expression. (*p<0.05, **p<0.01, ***p<0.001,, one-way ANOVA with Tukey's post-test).

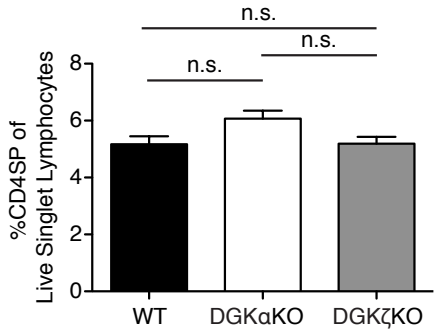
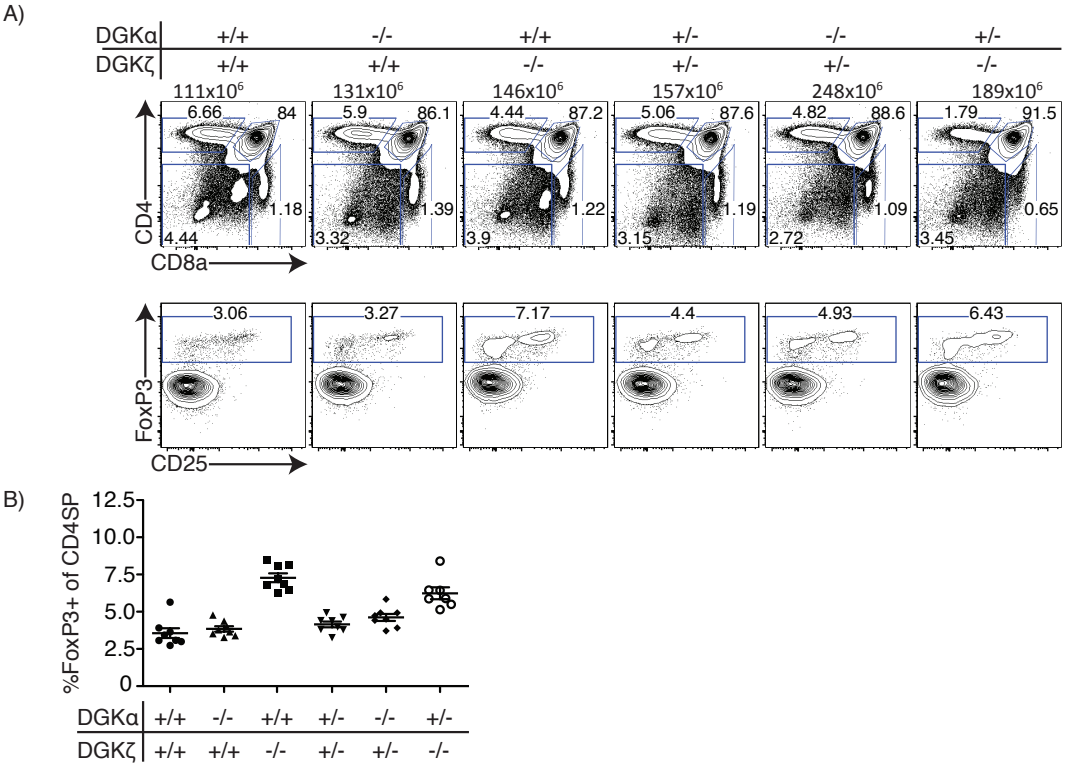


Figure 2.2: Wildtype, DGKα-deficient, and DGKζ-deficient mice have similar percentages of CD4 single positive cells.

N=8 from 3 independent experiments. One-way ANOVA with Tukey's post-test.



C)

%FoxP3+ =	p-value for term
3.39	
+ (0.197 × *DGKα Alleles Deleted*)	0.331
+ (1.89 × *DGKζ Alleles Deleted*)	5.17×10^{-12}
- (0.637 × *DGKα Alleles Deleted* × *DGKζ Alleles Deleted*)	0.0027
	overall fit
	$R^2 = 0.736$
	$p = 1.75 \times 10^{-12}$

Figure 2.3: Allelic series reveals a dose-dependent effect of loss of DGKζ but not DGKα.

(A) Top: Representative flow cytometric profiles of freshly isolated thymocytes gated on live singlet cells. Bottom: FoxP3$^+$ cells gated on CD4 single positive thymocytes. (B) Summary data of percentage of FoxP3$^+$ cells among CD4SP in thymus. (C) A multilinear regression was performed using the number of DGKα and number of DGKζ alleles removed as predictors and the natural log of the percentage of FoxP3+ cells as the dependent variable. Data were fit according to the model z = ax + by + C. The magnitude of coefficients and significance of the independent variables are shown. N=8 from 3 independent experiments for FoxP3 expression. (*p<0.05, **p<0.01, ***p<0.001,, one-way ANOVA with Tukey's post-test).

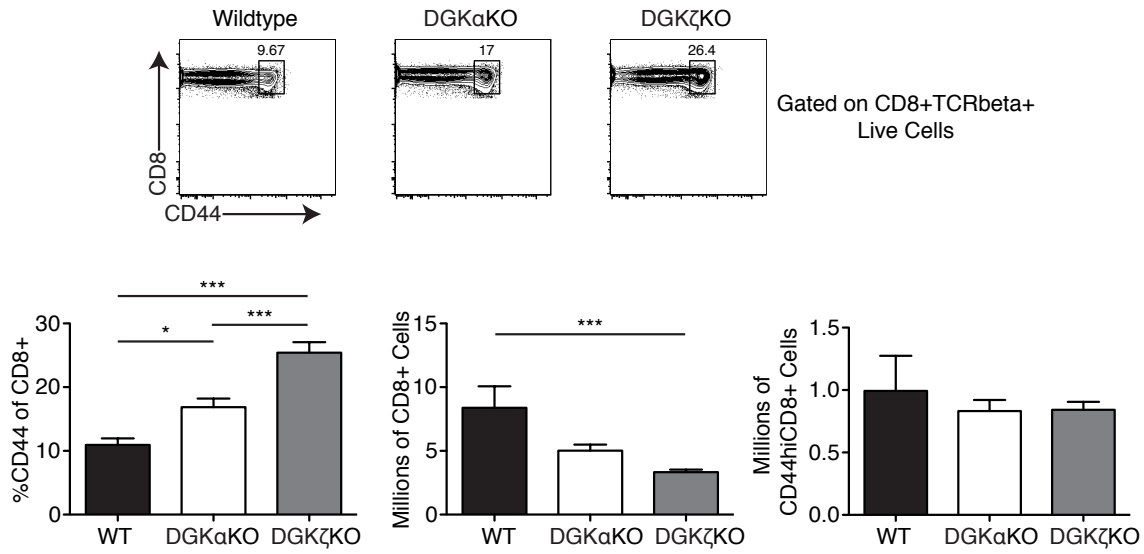


Figure 2.4: DGKζ-deficient CD8+ T cells have increased CD44 expression compared to DGKα-deficient CD8+ T cells.

Freshly isolated splenocytes were stained with antibodies to TCRβ, CD8 and CD44 and examined by flow cytometry. Representative flow plots shown gated on live CD8+TCRβ+ splenocytes. Summary data of the percent of CD44hi cells among CD8+ T cells, number of CD8+ T cells, and number of CD44hiCD8+ are shown. N=5-6 from 3 independent experiments. (*p<0.05, ***p<0.001, one-way ANOVA with Tukey's post-test)

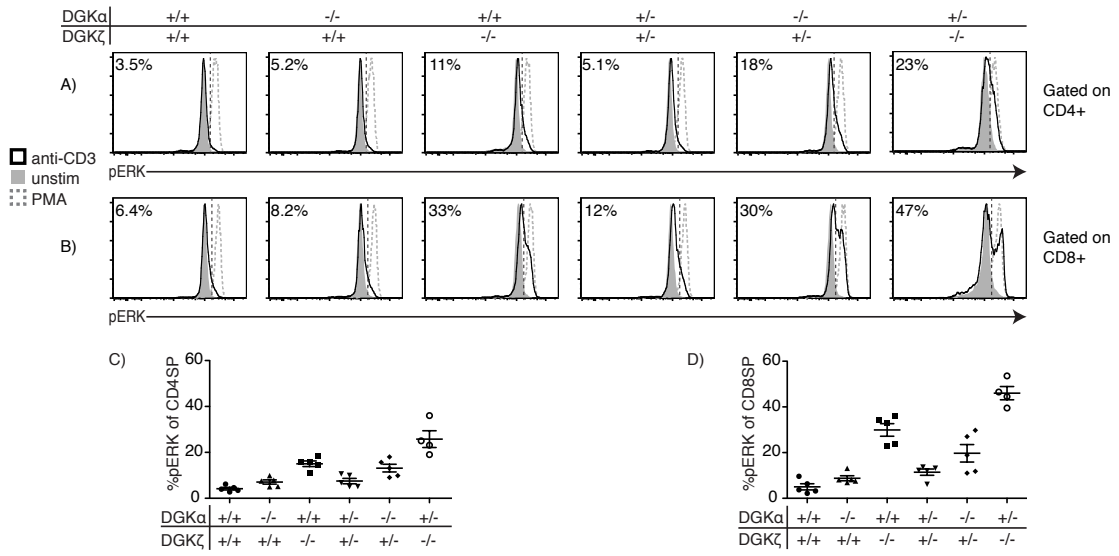


Figure 2.5: DGKζ has a quantitatively greater role in suppression of ERK phosphorylation than DGKα.

Splenic cells were freshly isolated, rested for two hours in serum-free media, and stimulated with anti-CD3 antibody for 15 minutes. Representative flow cytometric plots of ERK phosphorylation shown gated on (A) CD4 single positive splenocytes or (B) CD8 single positive splenocytes. Genotypes are indicated at the top and percentages indicate the percent of phospho-ERK positive cells at 15 minutes. The phospho-ERK positive gate, indicated by the dotted line, was defined based on maximum stimulation using PMA. Summary data of phospho-ERK positive cells is shown for CD4 cells (C) and CD8 cells (D). For statistical analysis, see Table 1. PMA: phorbol myristate acetate. N=4-5 for each genotype from 2 independent experiments.

Table 2.1: Multiple linear regression of number of DGKα and DGKζ alleles deleted predicting percent of ERK phosphorylation.

Dependent Variable	Independent Variable	Coefficient ± SE	P Value
Log %pERK^{+} of CD4 cells	# DGKα Alleles Deleted	0.270 ± 0.075	0.00130
	# DGKζ Alleles Deleted	0.705 ± 0.077	1.23×10^{-9}
	Constant	1.31 ± 0.13	2.70×10^{-10}
Log %pERK^{+} of CD8 cells	# DGKα Alleles Deleted	0.287 ± 0.089	0.00330
	# DGKζ Alleles Deleted	0.958 ± 0.091	6.99×10^{-11}
	Constant	1.44 ± 0.16	1.26×10^{-9}

A multilinear regression was performed using the number of DGKα and number of DGKζ alleles removed as predictors and the natural log of the percentage of phospho-ERK (pERK) positive cells as the dependent variable. Data were fit according to the model z = ax + by + C. The magnitude of coefficients and significance of the independent variables are shown.

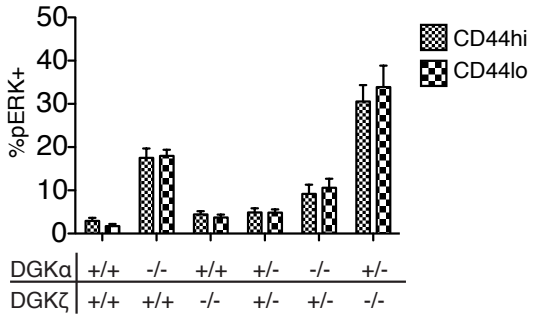


Figure 2.6: ERK phosphorylation after TCR stimulation is independent of level of CD44 expression on CD8+ T cells.

Splenic cells were freshly isolated, rested for two hours in serum-free media, stimulated with anti-CD3 antibody 15 minutes, and analyzed by flow cytometry using antibodies to phospho-ERK, CD8, CD4, and CD44. Percent phospho-ERK positive cells of CD44lo or CD44hi CD8+ cells is shown. N=4-5 from 2 independent experiments.

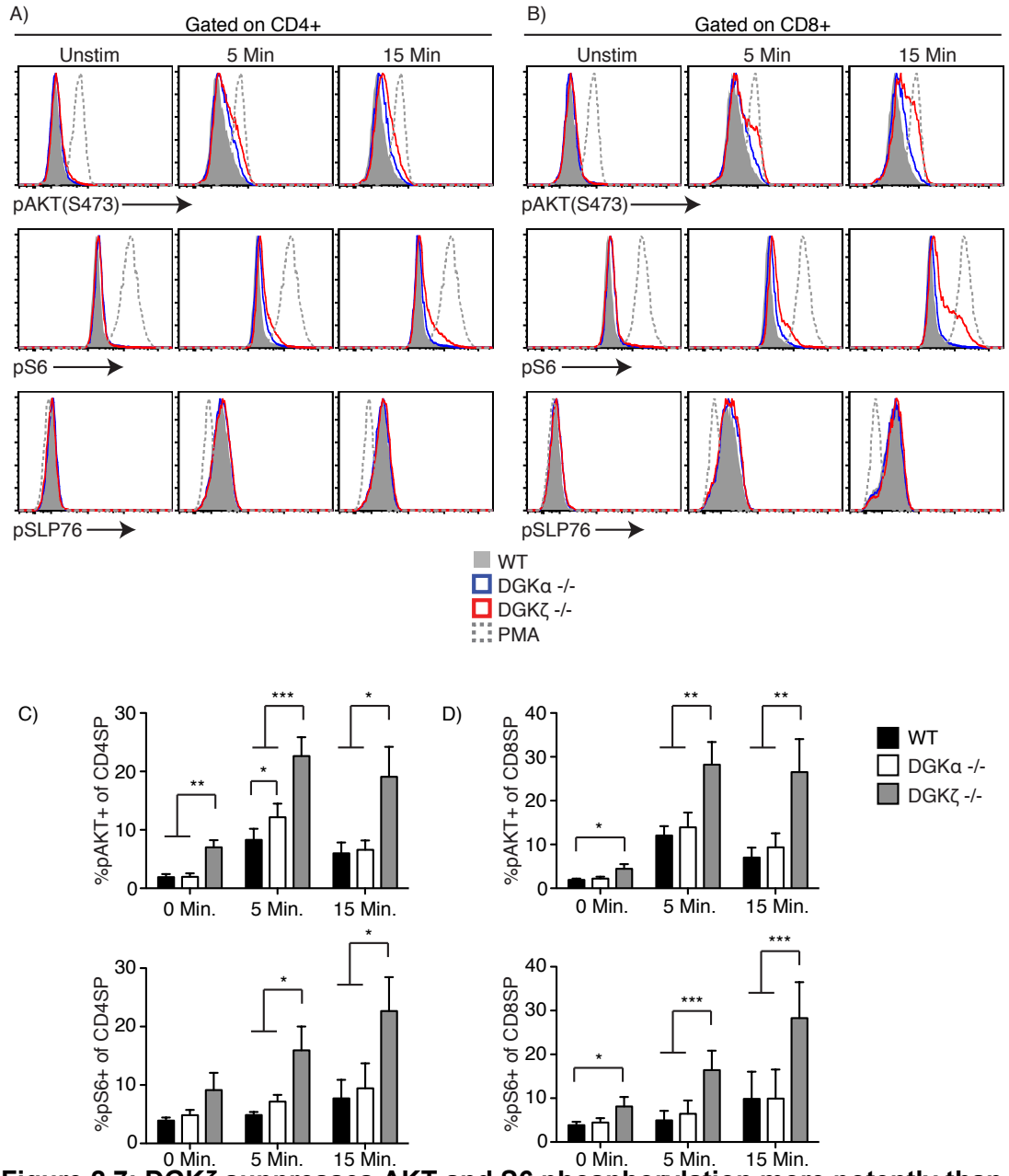


Figure 2.7: DGKζ suppresses AKT and S6 phosphorylation more potently than DGKα.

Splenic cells were freshly isolated, rested for two hours in serum-free media, and stimulated with anti-CD3 antibody for 5 or 15 minutes. Representative flow cytometric plots of SLP76, AKT(S473), and S6 phosphorylation gated on (A) CD4 single positive live splenocytes or (B) CD8 single positive live splenocytes. (C-D) Summary data of phospho-AKT and phospho-S6 positive cells gated on CD4SP and CD8SP, respectively. Phospho-positive gates were defined based on maximum stimulation using PMA. (*p<0.05, **p<0.01, ***p<0.001, Repeated-Measures ANOVA with Tukey's post-test, 4 independent experiments).

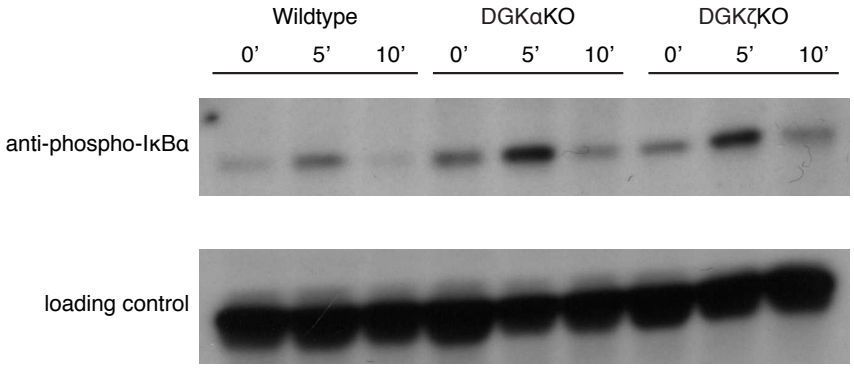


Figure 2.8: Phosphorylation of IκBα is increased similarly in DGKα- and DGKζ-deficient T cells.

Purified CD4+CD25-CD45RBhi T cells were stimulated by crosslinking using biotinylated antibodies to CD3, CD4, and CD28 and analyzed by western blot at the indicated timepoints. Representative of 2 experiments. Data acquired and analyzed by Amanda Schmidt.

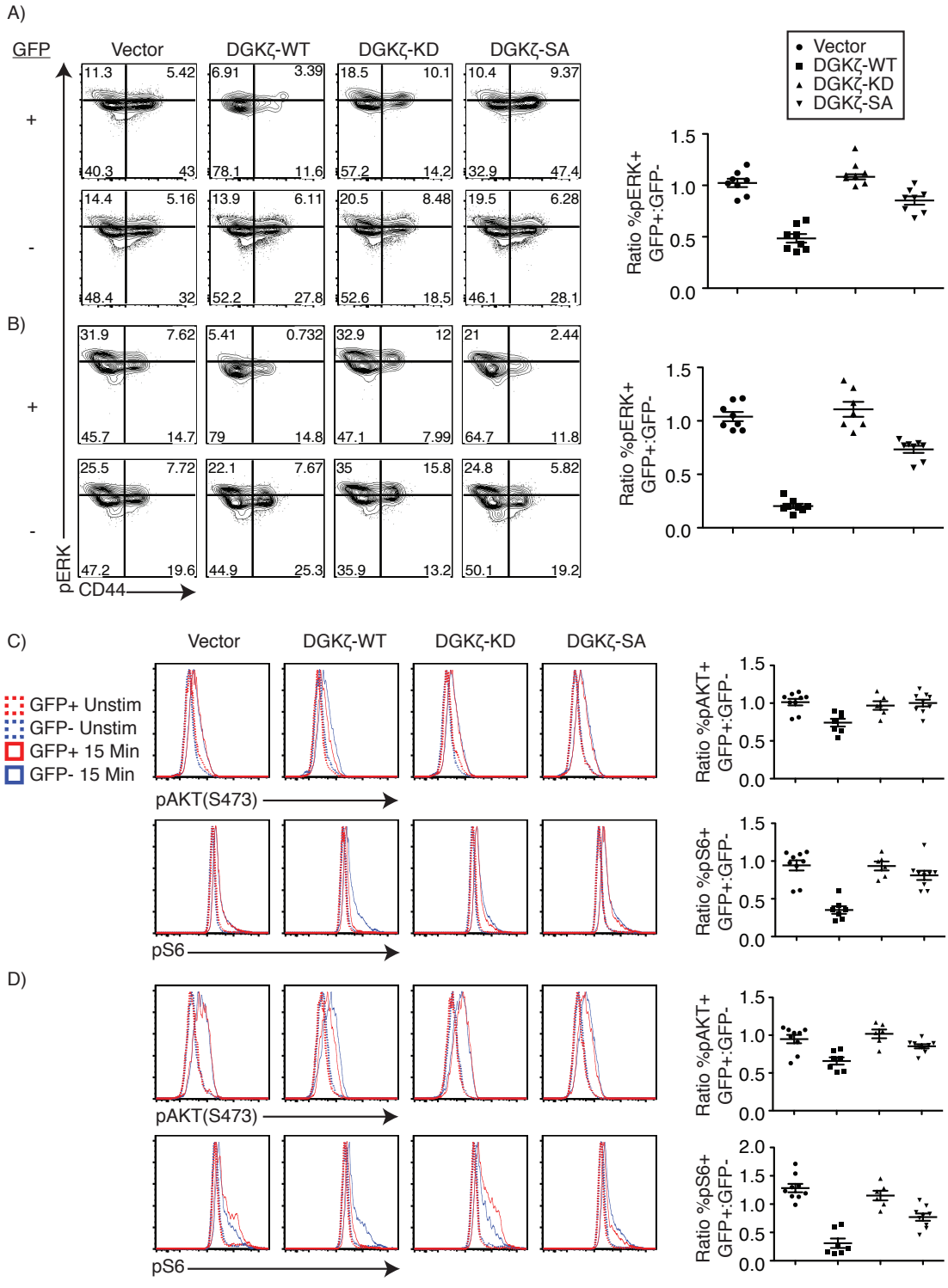


Figure 2.9: Kinase activity is required for DGKζ suppression of signaling downstream of Ras.

$CD45.2^{+}DGK\zeta^{-/-}$ bone marrow was transduced with empty virus (vector) or virus encoding wildtype DGKζ (DGKζ-WT), non-phosphorylatable MARCKS domain mutant DGKζ (DGKζ-SA) or kinase-dead DGKζ (DGKζ-KD) and transferred into $CD45.1^{+}$ irradiated host mice. After reconstitution, splenocytes were isolated and stimulated for 15 minutes using an anti-CD3 antibody. Representative flow cytometric plots of ERK, AKT, and S6 phosphorylation gated on (A and C) $CD45.2^{+}CD4^{+}$ or (B and D) $CD45.2^{+}CD8^{+}$ live cells and then GFP^{+} or GFP^{-} cells as indicated. Right: The ratio of the percent of phospho-ERK, phospho-AKT, and phospho-S6 positive cells of GFP^{+} to GFP^{-} cells in (A and C) $CD45.2^{+}CD4^{+}$ or (B and D) $CD45.2^{+}CD8^{+}$ live cells. Ratio=1 and ratio<1 corresponds to similar or less $phospho^{+}$ cells in transduced to non-transduced cells, respectively. Phospho-positive gates were defined based on maximum stimulation using PMA. Differences between all groups were significant with $p<0.01$.

2.5. Materials and Methods

Mice

Mice deficient in DGKα or DGKζ were described previously and backcrossed 7 times to C57Bl/6 (Olenchock et al., 2006; Zhong et al., 2003). Mice with varying number of alleles of DGKα and DGKζ were generated by crossing mice deficient in DGKα or DGKζ. DGKα−/−OT-II and DGKζ−/−OT-II mice were generated by crossing C57Bl/6 OT-II to DGKα−/− or DGKζ−/− mice, respectively. All experiments were performed using age-matched mice. Animal maintenance and experimentation were performed in accordance with the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Flow cytometric analysis of ERK, AKT, and S6 phosphorylation

Spleens were isolated, red blood cells were removed using ACK lysis buffer (155mM ammonium chloride, 10mM potassium bicarbonate, 1mM EDTA), and splenocytes were rested for two hours in serum-free media. For experiments examining ERK phosphorylation in an allelic series, rested splenocytes were stimulated for 15 minutes with anti-CD3 (500A2) in RPMI. For experiments examining AKT and S6 phosphorylation in wildtype, DGKα-deficient, DGKζ-deficient cells, or splenocytes isolated from bone marrow chimeras, rested splenocytes were stimulated for 5 or 15 minutes with anti-CD3 (500A2) in the presence of Live/Dead Aqua in PBS. Similar results were obtained when cells were stimulated in RPMI. Stimulation was stopped and cells were fixed by adding 3mL of 1X BD Phosflow Lyse/Fix buffer and incubating cells for 10-15 minutes. Cells were washed with FACS buffer and surface stained with anti-CD4-PerCPCy5.5, anti-CD8a-PECy7, and CD44-AF700. For cells from bone marrow chimeras, cells were stained with anti-CD45.2-Pacific Blue, anti-CD4-PerCPCy5.5, and

anti-CD8a-PECy7. Cells were then washed, permeablized in BD Perm/Wash buffer for 30 minutes, and stained with rabbit anti-phospho-ERK (Cat# 9101S, Cell Signaling) 1:100, and/or anti-phospho-AKT(S473)-PE (BD Pharmingen) 1:5 and rabbit anti-phospho-S6 (Cell Signaling) 1:100. Finally, cells were washed and stained with anti-rabbit-PE or anti-rabbit-AF647 antibodies. Flow cytometry was performed using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Flow cytometric analysis of thymi and spleen

Thymi and spleens from wildtype, DGKα-deficient, or DGKζ-deficient mice were freshly isolated and surface stained using antibodies to TCRβ, CD4, CD8, CD44 and CD25. Cells were then fixed using FoxP3 fixation/permeabilization (eBiosciences) and stained using anti-FoxP3 (eBiosciences) in the presence of FoxP3 staining buffer (eBiosciences). Flow cytometry was performed using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Retroviral transduction of bone marrow and creation of bone marrow chimeras

C57Bl/6 8-12-week-old DGKζ-deficient mice were injected intraperitoneally with 5mg of 5-fluorouracil. Four days later, cells from femurs, tibias, and hip bones were isolated by mortar and pestle (Lo Celso and Scadden, 2007). Cells were resuspended at 2-5×106 cells/mL in stimulation media (Iscove's Modified Dulbecco's Media supplemented with 15% fetal bovine serum, 10ng/mL IL-3, 10ng/mL IL-6, and 50ng/mL SCF) and incubated overnight. The following day, cells were harvested in resuspended at 2-5×106/mL in fresh stimulation media plated in 3mL per well of a 6 well plate. Cells were infected by addition of 1mL of retrovirus containing supernatant and polybrene at a final concentration of 4mg/mL, centrifugation at 1300g for 2 hours, and incubation overnight. The next day, cells were infected again according to the same protocol. Four hours after spinfection, cells were injected retroorbitally into CD45.1 congenically

marked 950 rads irradiated recipient mice. Mice were maintained on sterile water supplemented with trimethoprim/sulfamethoxazole for 2-3 weeks.

Constructs and Cloning

DGKα (kind gift from Anthony DeFranco) or DGKζ (Thermo Scientific, cat# MMM1013-9200165) were subcloned into pEGFP-C1 (Clontech) or MIGR1 for transduction of bone marrow. eGFP-DGKα or eGFP-DGKζ were subcloned into pK1 retroviral vector (kind gift from Warren Pear) to create pK1-eGFP-DGKα or pK1-eGFP-DGKζ for transduction of primary murine T cells. Wildtype DGKζ or kinase-dead DGKζ (kind gift from Isabel Merida) were subcloned into MIGR1 for transduction of bone marrow.

CHAPTER III: MECHANISMS CONTROLLING DGK ISOFORM FUNCTION

3.1. Introduction

Modulation of DGKs has been shown to regulate T cell responses to cancer and the cytolytic ability of chimeric antigen receptor (CAR) transduced T cells (Riese et al., 2011, 2013). An understanding of isoform specific functions of DGKs in T cells is therefore important for the development of new therapies. In the previous chapter we demonstrated that DGKζ and not DGKα has a dominant role in nTreg development and TCR signaling. However, the mechanisms that dictate differences in DGK isoform regulation of TCR signaling, which we explore here, are not well understood.

3.1.1 DGKζ and DGKα structure

The domain architectures of DGKα and DGKζ may provide clues to how the functions of these molecules are regulated post-translationally. DGKα and DGKζ share two domains, a C1 domain and a catalytic domain. While the C1 domain is classically involved in DAG binding, in DGKs its primary role appears to be in protein-protein interactions (Zhong et al., 2008; Joshi and Koretzky, 2013). The catalytic domain is comprised of a subdomain with a true ATP binding site for catalysis, and an accessory domain that cannot bind ATP but may be involved in the enzymatic conversion of DAG to PA. The catalytic subdomains of DGKα and DGKζ have 66% sequence identity, while the accessory subdomains have 45% sequence identity. These data suggest that DGKα and DGKζ may have different enzymatic activities or may act on DAGs with varying acyl

chain lengths. However, neither DGKα nor DGKζ display specificity toward acyl side chain composition (Shulga et al., 2011).

The remaining domains of DGKα and DGKζ are distinct (please refer to the Chapter I for a detailed description of the known functions of these domains). DGKα contains an EF hand domain that binds Ca^{2+} and regulates its translocation to the plasma membrane (Sanjuán et al., 2001; Sanjuan et al., 2003). DGKζ has three unique domains: a myristoylated alanine-rich C-kinase substrate (MARCKS) domain, a PDZ domain, and an Ankyrin repeat domain. Akin to the EF hands of DGKα, the unique MARCKS, PDZ, and Ankyrin domains of DGKζ may regulate localization of DGK activity.

3.1.2 Localization of DGKs

Localization of DGK activity is likely important for function. Functional TCR signaling requires the localized production of DAG at the site of the immunological synapse (IS). For example, localized DAG synthesis after TCR stimulation is sufficient to cause reorientation of the microtubule-organizing complex (MTOC) to the IS, which is required for normal TCR signaling (Quann et al., 2009). Moreover, treatment of TCR stimulated cells with an inhibitor of DGK activity results in diffuse DAG persistence at the IS and impaired re-orientation of the MTOC (Quann et al., 2011, 2009). As such, DGKs likely play an essential role at the IS in metabolizing DAG and restricting DAG localization to the IS. Translocation of DGKs to the IS therefore could be important for function. In addition, interaction with molecules at the IS could similarly play a role in regulation DGK function. For example, although DGKζ and DGKι have the same domain architectures, DGKζ has been found to regulate Ras signaling, while DGKι regulates Rap1 signaling. This correlates with DGKζ's ability to associate with

RasGRP1, which regulates Ras activation, and DGKι's ability to associate with RasGRP3, which mediates Rap1 activation. In addition to localization and protein interactions, another mechanism that could dictate DGK function in T cells is the relative protein expression of one isoform versus the other. We predicted, given its dominant role in TCR signaling, that DGKζ would be expressed more highly than DGKα in the T cell populations we have studied. In this chapter of this thesis we compare and contrast subcellular localization, protein expression levels, and association with key effectors of T cell activation of DGKα versus DGKζ.

3.2. Results

3.2.1. Expression levels of DGKα and DGKζ do not explain differences in function

We reasoned that one potential explanation for the dominant impact of DGKζ on TCR signaling could be due to predominant expression of DGKζ in the cell compared to the alpha isoform. Thus, if expression of DGKα is low compared to DGKζ, genetic deletion of DGKα would result in removal of fewer total numbers of DGK molecules and possibly more subtle effects on DAG-mediated TCR signaling than deletion of DGKζ. To investigate this possibility, we examined protein expression of DGKα and DGKζ in T cells (Fig. 3.1). As we lacked the necessary reagents to create a standard curve using known quantities of pure DGKα and DGKζ, we performed a western blot using lysate from 293T cells expressing either eGFP-DGKα or eGFP-DGKζ fusion protein and created a standard curve relating GFP intensity to DGKα or DGKζ intensity by densitometry. We examined wildtype purified T cells using antibodies to DGKα or DGKζ and calculated a GFP intensity equivalent, allowing us to relate expression levels of DGKα and DGKζ. To our surprise, DGKα was expressed at ~3-fold higher levels (range of 1.4 to 6.1) than DGKζ in T cells (Fig. 3.1).

The incongruity between DGKα's higher expression levels and its weaker role in suppression of TCR-mediated signaling downstream of Ras suggests that DGKα cannot function redundantly for DGKζ after TCR engagement. To further test this notion, we created bone marrow chimeras with DGKζ-deficient bone marrow cells over-expressing DGKα or re-expressing DGKζ using a retroviral vector. After immune reconstitution, we examined ERK, AKT, and S6 phosphorylation after TCR stimulation. Even though

DGKα was overexpressed 2-3 fold higher than endogenous levels in transduced cells (Fig. 3.2 A), we observed no change in ERK, AKT, and S6 phosphorylation with DGKα overexpression in either $CD4^+$ or $CD8^+$ T cells (Fig. 3.2 B and C). In contrast, re-expression of DGKζ significantly suppressed ERK, AKT and S6 phosphorylation after TCR engagement. Taken together, these results demonstrate that the relative expression levels of DGKα and DGKζ do not explain their differences in function and that DGKζ has a non-redundant and dominant role in suppression of Ras pathway-mediated TCR signaling.

3.2.2. DGKζ and DGKα localize similarly to the T cell-APC contact site

After TCR stimulation, DAG is synthesized locally at the IS (Quann et al., 2009), and translocation of DGK molecules to this T cell-APC contact site could be one mechanism of regulation of DGK function. We investigated this possibility in primary T cells. We expressed either eGFP-DGKα or eGFP-DGKζ fusion proteins in OT-II transgenic TCR, DGKζ-deficient $CD4^+$ T cells and incubated these cells with ovalbumin-peptide pulsed B cells to allow conjugates to form. In unconjugated T cells, both DGKα and DGKζ were diffusely localized throughout the cell (Fig. 3.3). After formation of the IS, both DGKα and DGKζ translocated to the proximal and distal poles of the cell (Fig. 3.4 A). Using unbiased analysis with an automated script, we quantified the amount of translocation by determining the average GFP intensity in thirds (proximal to the IS, middle of the cell, and distal to the IS) compared to the average GFP intensity of the whole cell. Both DGKα and DGKζ equally and significantly localized to the T cell-APC contact site at 5, 15, and 30 minutes after conjugate formation (Fig. 3.4 B, Fig. 3.5 for 15

and 30 minutes, and Fig. 3.4 C). These results suggest that, as a percentage of total DGKα and DGKζ in the cell, DGKα and DGKζ grossly localize to similar degrees to the T cell-APC contact site after initiation of TCR signaling.

3.2.3. RasGRP1 associates with DGKζ at higher levels than with DGKα

Our experimental data suggest that the proportion of total DGKα molecules that localize to the T cell-APC contact site is similar to the proportion of total molecules that localize to the contact site. However, our data also demonstrate that DGKζ is expressed at lower levels than DGKα, suggesting that the total number of DGKζ molecules that localize to the contact site after TCR engagement is lower than the total number of DGKα molecules. The dichotomy between having a fewer number of DGKζ molecules near the contact site but a greater role in TCR signaling could be explained by differential binding to RasGRP1, which activates the Ras pathway. The association of DGKs with downstream DAG-activated molecules may allow DGKs to regulate these molecules (Gharbi et al., 2011; Regier et al., 2005), and in HEK-293 cells, DGKζ co-immunoprecipitates with RasGRP1 (Topham and Prescott, 2001). One possibility that could explain differences in function, therefore, is that DGKα does not associate with RasGRP1 as DGKζ does. To test this notion, we expressed RasGRP1 in 293T cells, eGFP-DGKα transduced 293T cells, and eGFP-DGKζ transduced 293T cells and then co-immunoprecipitated complexes using an anti-GFP antibody (Fig. 3.6 A). We consistently observed greater co-immunoprecipitation of RasGRP1 with eGFP-DGKζ than eGFP-DGKα (mean 2.94-fold greater RasGRP1 association, range 1.28 - 5.61), suggesting that RasGRP1 may more strongly associate with DGKζ than DGKα.

Unexpectedly, eGFP-DGKα and eGFP-DGKζ were detected at similar levels when co-immunoprecipitated using anti-RasGRP1. This finding could be due to the additional immunoprecipitation of lower molecular weight RasGRP1 species that do not complex with DGKα and DGKζ, thereby limiting the detection of differences between RasGRP1-DGKα and RasGRP1-DGKζ associations. Overall, these results suggest that differences in DGKα and DGKζ binding to RasGRP1 may correlate with DGKζ's predominant functions in T cells.

3.2.4. DGKζ is more effective at generating PA than DGKα after TCR stimulation

We speculated that differences in ability to metabolize DAG between DGKα and DGKζ could also explain why a fewer number of DGKζ molecules at the T cell-APC contact site would have more of an effect on TCR signaling than a larger number of DGKα molecules. As DGKs may undergo posttranslational modifications after TCR stimulation, *in vitro* measurement of enzymatic activity is difficult. We therefore investigated DGKα and DGKζ's effective enzymatic activity, defined by the *in vivo* production of PA after TCR engagement. We predicted the following: PA would increase after stimulation in wildtype T cells; PA would increase slightly less than wildtype in DGKα-deficient T cells due to remaining highly functional DGKζ, even though a large total number of DGK molecules have been removed; and PA would barely increase in DGKζ-deficient T cells due to the remaining less functional DGKα, even though a small total number of DGK molecules have been removed. Consistent with our hypothesis, PA levels increased in wildtype cells, slightly less in DGKα-deficient cells, and hardly at all in DGKζ-deficient cells (Fig. 3.7 A). These data demonstrate that DGKζ plays a more

critical role in regulating PA production following TCR engagement than DGKα and suggest that DGKζ has greater effective enzymatic activity following TCR stimulation than DGKα in intact cells.

3.2.5. In silico modeling suggests differences in kinase activity and binding affinity to RasGRP1 lead to differences in function

To further explore the effects of effective enzymatic activity and binding affinity to RasGRP1 on T cell signaling, we used a previously developed mathematical model of Ras activation in T cells (Table 3.1 and 3.2) . Past experimental data suggest that this model correctly predicts the qualitative features of Ras activation kinetics following TCR stimulation of various signaling strengths in both wildtype and DGKζ-deficient T cells (Das et al., 2009; Riese et al., 2011). We stimulated wildtype, DGKα-deficient, and DGKζ-deficient splenocytes with increasing concentrations of anti-CD3 antibody *in vitro* and examined ERK phosphorylation as a readout of Ras activation. At all ranges of anti-CD3 antibody concentrations, we observed greater ERK phosphorylation in DGKζ-deficient T cells compared to DGKα-deficient T cells (Fig. 3.7 B). We then performed *in silico* modeling to examine whether differences in binding to RasGRP1 and/or effective enzymatic activity could lead to our observed experimental results. Effective enzymatic activity integrates concentrations of DAG and DGKs and kinase activity into a single parameter. As we lack tools to measure exact *in vivo* concentrations of DAG and DGKs after TCR stimulation, we entered our best approximation of these individual parameters into the model and varied catalytic rate (k_{cat}). Based on our experimental data, we

assumed a 3-fold larger number of DGKα molecules than DGKζ molecules in the system and equal availability of DAG to DGKα and DGKζ.

As expected, with equal catalytic rates and RasGRP1 binding affinities, *in silico* DGKα-deficient T cells have greater Ras activation at all ranges of TCR stimulus strength than DGKζ-deficient T cells (Fig. 3.7 C, top left). With equal catalytic rates but a 3-fold greater RasGRP1 binding affinity for DGKζ than DGKα, DGKα-deficient T cells still exhibit greater Ras activation than DGKζ-deficient T cells, suggesting that differences in binding affinity to RasGRP1 alone may not lead to our observed experimental results (Fig. 3.7 C, bottom left). Consistent with our experimental data, with a greater catalytic rate for DGKζ than DGKα but equal RasGRP1 binding affinity, *in silico* DGKζ-deficient T cells would have greater Ras activation than DGKα-deficient T cells, suggesting that differences in DGK catalytic activity are critical for differences in function (Fig. 7 C, top right). While a greater binding affinity of RasGRP1 to DGKζ than DGKα alone does not lead to differences in Ras activation (Fig 3.7 C top and bottom left), in concert with an increased catalytic rate for DGKζ, an increased RasGRP1 binding affinity appears to enhance differences in Ras activation between DGKα- and DGKζ-deficient T cells (Fig. 3.7 C, bottom right). Taken together, these data suggest that with a fewer number of DGKζ than DGKα molecules, DGKζ's greater effective enzymatic activity and binding to RasGRP1 contribute to its predominant role in TCR-driven Ras-mediated signaling.

3.3. Conclusions

It is intriguing that deletion of DGKζ has a greater impact on T cell function and TCR signaling than deletion of DGKα, despite higher expression levels of DGKα than DGKζ in T cells. We wondered whether subcellular localization of DGKα and DGKζ within the T cell accounts for the differences, as DGKζ but not DGKα localizes to the T cell-APC contact site in Jurkat T cells (Gharbi et al., 2011). However, we found that in primary murine T cells, both DGKα and DGKζ display sustained localization to the contact site, at least to 30 minutes after stimulation. The observed discrepancy could be due to differences between Jurkat T cells and primary T cells. For example, in Jurkat T cells, DGKζ diffusely localizes to the plasma membrane of the cell (Gharbi et al., 2011). In contrast, our data demonstrate that DGKζ primarily localizes to the proximal and distal poles of the cell (Fig 3.4 B). We also found that, as a percentage of GFP-tagged molecules expressed in T cells, DGKα and DGKζ grossly localize to similar degrees to the contact site after initiation of TCR signaling. Because the endogenous expression of DGKα is higher than DGKζ, the total number of DGKα molecules near the contact site is probably greater than the total number of DGKζ molecules. These data strongly suggest that isoform-specific differences in function are not due to differences in gross localization. However, differences in localization on a smaller scale cannot be excluded. For example, greater localization of DGKζ than DGKα to regions where RasGRP1 is present after TCR engagement by an APC could dictate DGK function. As observed by total internal reflection fluorescence microscopy, interference with the function of DGKs leads to disruption of DAG accumulation at the IS, suggesting that DGKα and/or DGKζ

may localize specifically to the IS (Quann et al., 2009). More refined imaging using these techniques could help determine if DGKα and DGKζ localize to different regions of the IS and specifically to the site of RasGRP1 localization.

Although our localization data do not explain DGKζ's dominant role in TCR signaling, association with RasGRP1 may be one mechanism controlling the differential functions of DGKα and DGKζ. Studies of DGK isoforms have suggested that specific interaction with target proteins is important for regulating the DAG-mediated activation of these molecules. For example, while DGKζ negatively regulates Ras signaling, its structurally related isoform, DGKι, negatively regulates Rap1 signaling (Regier et al., 2005). This difference in function correlated with DGKζ's ability to associate with RasGRP1, which regulates Ras activation, and DGKι's ability to associate with RasGRP3, which mediates Rap1 activation. In a similar fashion, an increased ability of DGKζ to bind to RasGRP1 could contribute to its greater function in T cells. This notion is supported by our data demonstrating 3-fold greater association of RasGRP1 with DGKζ than DGKα and *in silico* modeling data that suggest differences in binding affinity to RasGRP1 enhance the effects of differences in catalytic activity (Fig. 3.7 C top and bottom right). The ability of DGKs to associate with DAG-activated molecules could also potentially explain DGKα and DGKζ's overlapping role in NF-κB signaling but not RasGRP1 signaling. To this end, it would be interesting to examine potential interactions of DGKα and DGKζ with PKC-θ, which appears to be a shared target of these DGK isoforms in T cells.

In addition to DGKζ's stronger association with RasGRP1, we uncovered effective enzymatic activity as another potential mechanism that correlates with DGKζ's dominant role in attenuating TCR-mediated signaling downstream of Ras. Direct measurement of the physiologically relevant relative catalytic activity of the two isoforms

is difficult because both DGKα and DGKζ undergo posttranslational modifications that regulate their activity in ways that cannot be mimicked rigorously *in vitro*. For example, the concentration of cytosolic free calcium has been shown to influence DGKα function (Baldanzi et al., 2011), and measurement of the local concentration of calcium around DGKα molecules after TCR stimulation is difficult. Phosphorylation of the MARCKS domain of DGKζ may also regulate its catalytic activity (Luo et al., 2003), and measurement of phosphorylation of this specific site after TCR engagement is similarly complicated. We therefore investigated the production of PA after TCR stimulation *in vivo*. We observed a reproducible increase in whole-cell PA production in wildtype and DGKα-deficient T cells after TCR stimulation, suggesting that loss of DGKα enzyme has little impact on metabolism of DAG to PA. In contrast, the TCR-stimulated increase in PA was largely abrogated in cells deficient in DGKζ, suggesting that DGKζ rather than DGKα is the predominant regulator of PA after TCR stimulation. These data may explain why DGKζ has greater control over TCR signaling than DGKα, even though the total number of DGKα molecules near the T cell-APC contact site are probably more than that of DGKζ.

Given that DGKα molecules are likely more abundant than DGKζ molecules in the T cell and at the T cell-APC contact site, mathematical modeling of Ras activation suggests that DGKζ must have a greater effective catalytic activity than DGKα to account for the increased Ras activation observed in DGKζ-deficient T cells. A remaining question is why DGKζ function is greater than that of DGKα after TCR stimulation. For example, is this difference observed at baseline or does TCR-mediated signaling modulate DGK isoform activity? In terms of regulation, TCR and co-stimulatory signals have been found to inhibit DGKα activity in a manner dependent on signaling lymphocyte activation molecule-associated protein (SAP) as well as calcium release and

PLCγ1 activation (Baldanzi et al., 2011). However, much less is known about regulation of DGKζ kinase activity in T cells. Serine-to-aspartic acid phospho-mimetic mutations in the MARCKS domain lead to decreased catalytic activity *in vitro*, suggesting that MARCKS domain phosphorylation by protein kinase Cs (PKCs) may suppress its function (Luo et al., 2003). Whether the same regulation is true *in vivo* requires further study. Thus, investigation of TCR-mediated regulation of DGK activity could help decipher why DGKα and DGKζ display different enzymatic function *in vivo*.

Our data demonstrate that re-expression of DGKζ but not overexpression of DGKα in DGKζ-deficient T cells rescues suppression of TCR signaling, suggesting that DGKα cannot function redundantly for DGKζ in suppression of these pathways. At the same time, however, TCR stimulation of T cells deficient in DGKα does lead to modest increases in ERK, AKT, and S6 phosphorylation, suggesting that DGKα has some impact on these pathways. One possibility that would reconcile these results is that two different pools of DAG mediate Ras pathway activation, with a DGKζ-regulated pool potently stimulating RasGRP1 activity and a DGKα-regulated pool weakly stimulating RasGRP1 activity. DAG species are not homogenous, as acyl side chains of varying lengths and saturation may be connected to the glycerol backbone, and specific DAGs may maximally regulate downstream targets, such as PKCs, although this notion is controversial (Sánchez-Piñera et al., 1999; Hinderliter et al., 1997). Development of approaches to differentiate DAG pools *in vivo* will be required to rigorously test this notion in primary T cells.

While our data reveal functions in which DGKζ has a dominant role, the co-expression of DGKα in T cells begs the question of whether DGKα has dominant roles in other processes. One possibility is in reorientation of the microtubule organizing complex (MTOC) during formation of the IS. DAG synthesis at the IS has been found to

closely precede and be sufficient for reorientation of the MTOC (Quann et al., 2009). Further investigation in DGKα- and DGKζ-deficient T cells and higher resolution microscopic analysis of DGKα and DGKζ subcellular localization at the IS could help decipher the relative roles of these two isoforms in MTOC reorientation.

Our data presented in Chapter 2 demonstrate that unlike DGKα, DGKζ has an important role in nTreg cell development and a predominant function in TCR-mediated signaling downstream of Ras. The experiments presented in this chapter suggest that multiple mechanisms may explain these disparities, including differences in binding to downstream DAG-activated molecules and in effective enzymatic function. As DGKs have been identified as potential targets in cancer immunotherapy (Riese et al., 2013), defining these clear functions for DGKζ in T cells will help with the development and therapeutic use of isoform-specific inhibitors of DGKs.

3.4. Figures

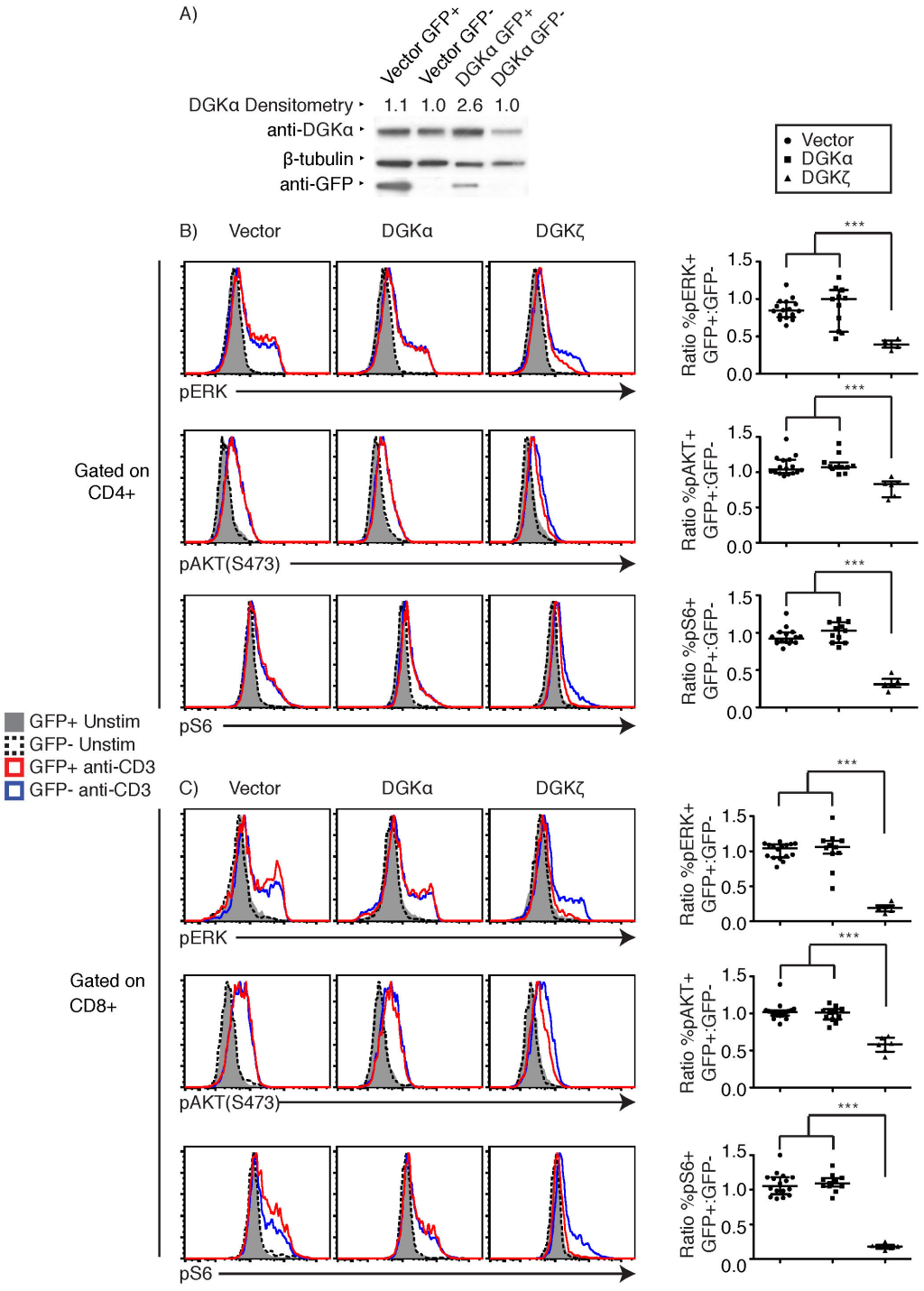


Figure 3.1: DGKα is not redundant for DGKζ in suppression of ERK, AKT and S6 phosphorylation.

$CD45.2^{+}DGK\zeta^{-/-}$ bone marrow was transduced with empty virus (vector) or virus encoding DGKα, or DGKζ and transferred into $CD45.1^{+}$ irradiated host mice. (A) Splenic T cells isolated from vector or DGKα transduced $DGK\zeta^{-/-}$ bone marrow chimeras were sorted for GFP^{+} and GFP^{-} cells, lysed for western blotting, and probed with anti-DGKα antibody to determine over-expression of DGKα. (B-C) Splenocytes were stimulated for 15 minutes using an anti-CD3 antibody. Left: Representative flow cytometric plots of ERK, AKT, and S6 phosphorylation gated on (B) $CD45.2^{+}CD4^{+}$ or (C) $CD45.2^{+}CD8^{+}$ live cells and then GFP^{+} or GFP^{-} cells as indicated. Right: The ratio of the percent of phospho-ERK, phospho-AKT, and phospho-S6 positive cells of GFP^{+} to GFP^{-} cells in (B) $CD45.2^{+}CD4^{+}$ or (C) $CD45.2^{+}CD8^{+}$ live cells. Ratio=1 and ratio<1 corresponds to similar or less phospho-positive cells in transduced compared to non-transduced cells, respectively. Phospho-positive gates were defined based on maximum stimulation using PMA. (***$p<0.001$, one-way ANOVA on log-transformed data with Tukey's post-test)

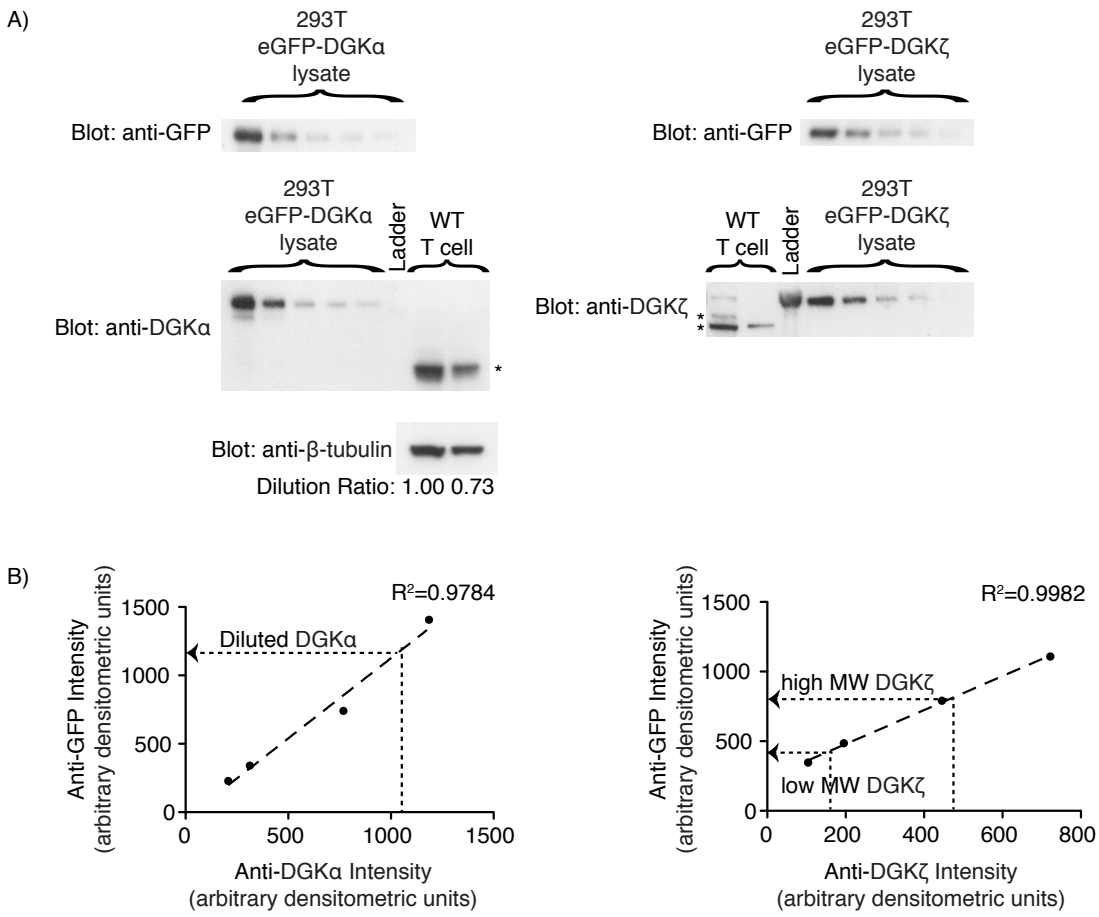


Figure 3.2: DGKα is higher than DGKζ expression in T cells.

Serial dilutions of lysate from 293T cells expressing eGFP-DGKα or eGFP-DGKζ were subjected to Western blot alongside undiluted or diluted wildtype purified T cell lysate. (A) Top left: Serial dilutions of eGFP-DGKα expressing 293T lysate blotted with anti-GFP antibody. Middle left: Serial dilutions of eGFP-DGKα expressing 293T lysate and undiluted or diluted wildtype T cell lysate blotted with anti-DGKα antibody. Bottom left: dilution ratio of wildtype T cell lysate was determined using b-tubulin as a loading control. Top right: Serial dilutions of eGFP-DGKζ expressing 293T lysate blotted with anti-GFP antibody. Bottom right: Serial dilutions of eGFP-DGKζ expressing 293T lysate and undiluted or diluted wildtype T cell lysate blotted with anti-DGKζ antibody. * denotes DGKα and the two isoforms of DGKζ. (B) Analysis of western blot data by densitometry for (left) DGKα or (right) DGKζ. Dots represent data of GFP intensity and DGK intensity. The dashed line represents a linear regression of DGK intensity versus GFP intensity. The dotted line indicates wildtype T cell intensity for DGKα or the two forms of DGKζ and the calculated equivalent GFP value. In the experiment shown, DGKα expression levels were found to be 1.44 times higher than DGKζ expression levels. Data are representative of 3 experiments. In an average of 3 experiments, DGKα expression was found to be 3-fold higher than DGKζ expression by densitometry. MW = molecular weight.

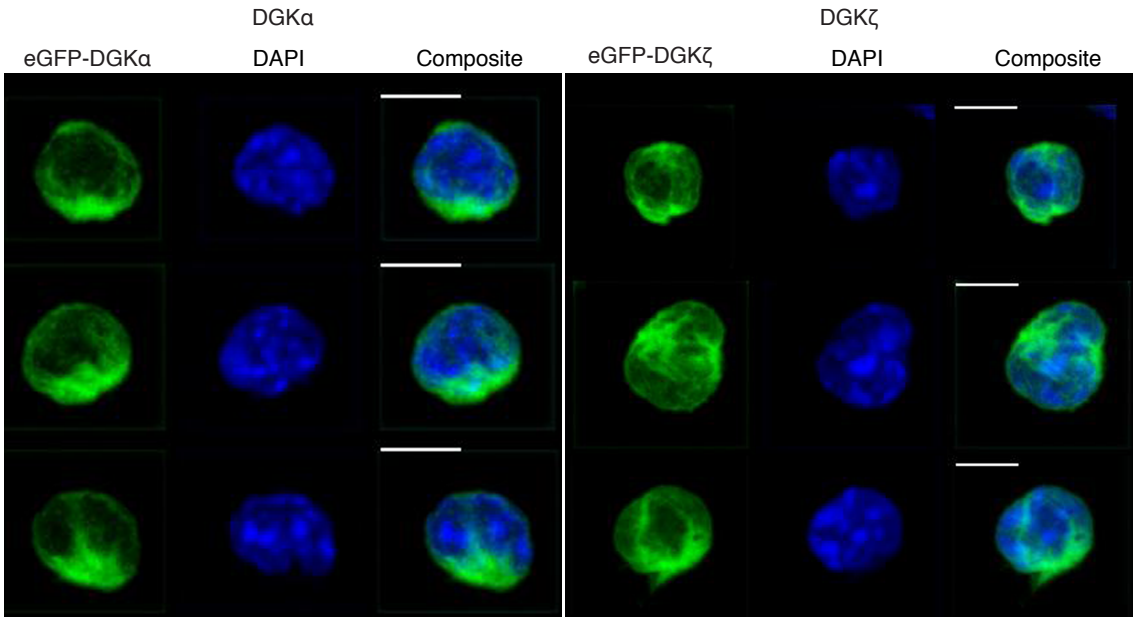


Figure 3.3: DGKα and DGKζ diffusely localize in unconjugated T cells.

eGFP-DGKα or eGFP-DGKζ transduced OT-II DGKζ−/− T cells were fixed and stained with DAPI. Representative confocal microscopy images 5 minutes after initiation of conjugation are shown. GFP is shown in green and DAPI in blue. White bar indicates 5 microns.

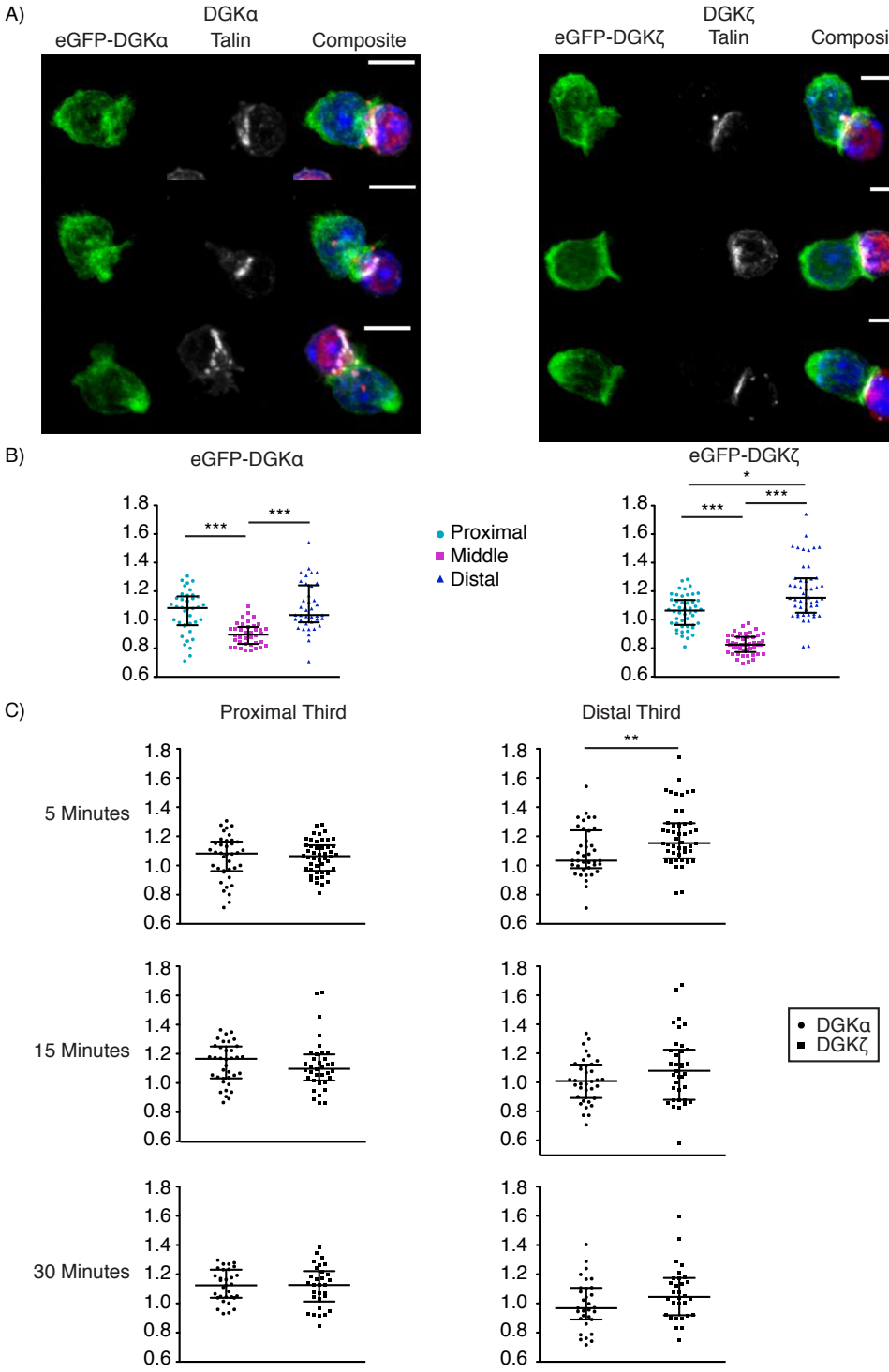


Figure 3.4: DGKα and DGKζ localize in similar degrees to the T cell-APC contact site.

(A) eGFP-DGKα or eGFP-DGKζ transduced OT-II DGKζ$^{-/-}$ T cells were conjugated with OVA-peptide pulsed B cells, fixed, and probed with an anti-talin antibody to mark the IS. Representative confocal microscopy images 5 minutes after initiation of conjugation are shown. GFP is shown in green, talin in white, DAPI in blue, and the CMTMR labeled B cell in magenta. White bar indicates 5 microns. (B) Ratio of average GFP intensity in thirds of the cell (proximal to the IS, the middle of the cell, or distal to the IS) to the average GFP intensity of the whole cell. Data shown 5 minutes after initiation of conjugation in eGFP-DGKα or eGFP-DGKζ transduced cells. (*p<0.05, ***p<0.001, Kruskal-Wallis with Dunn's post-test) (C) Comparison of eGFP-DGKα or eGFP-DGKζ accumulation ratio at the T cell-APC contact site or distal pole of the cell at 5, 15, and 30 minutes. Only significant differences are shown. N>=30 for all sets of images. (**p<0.01 Mann-Whitney test)

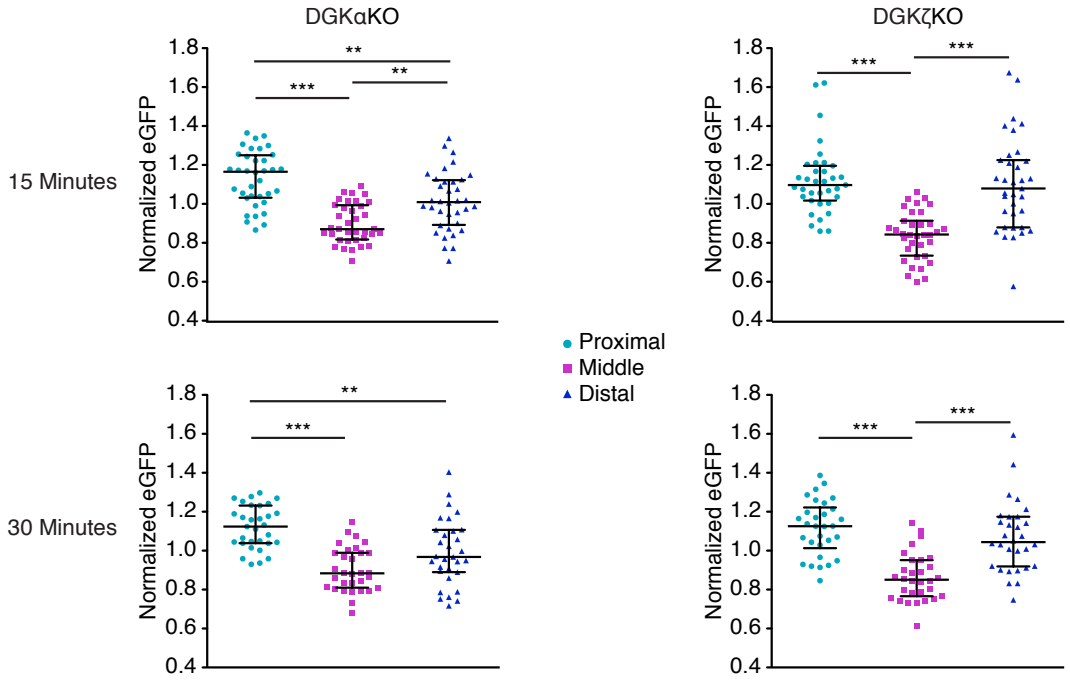


Figure 3.5: DGKα and DGKζ localize to the T cell-APC contact site.

Ratio of average GFP intensity in thirds of the cell (proximal to the IS, the middle of the cell, or distal to the IS) to the average GFP intensity of the whole cell. (**p<0.01, ***p<0.001, Kruskal-Wallis with Dunn's post-test)

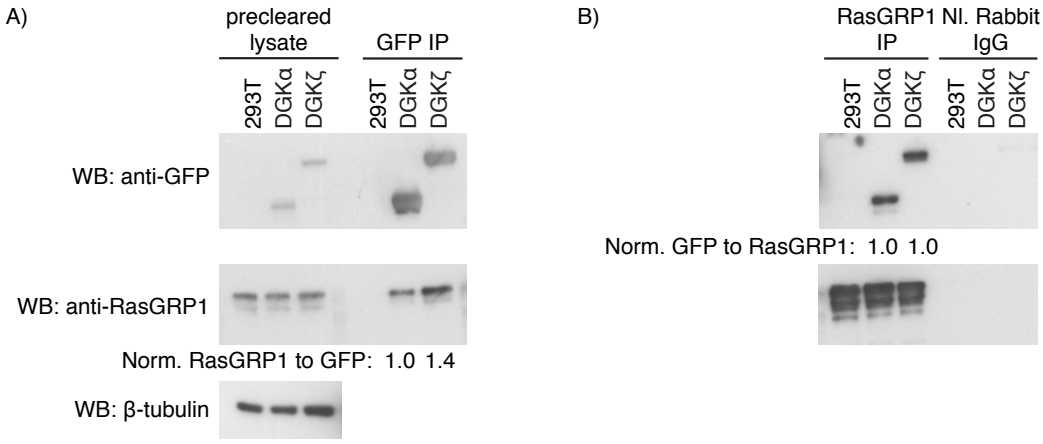


Figure 3.6: RasGRP1 associates in greater amounts with DGKζ than DGKα.

293T, eGFP-DGKα transduced 293T, and eGFP-DGKζ transduced 293T were transfected with a RasGRP1-expressing vector. Lysate was divided into thirds. (A) Immunoprecipitation was performed using anti-GFP and blotted using anti-GFP or anti-RasGRP1 antibodies. (B) Immunoprecipitation was performed using anti-RasGRP1 or normal rabbit IgG and blotted using anti-GFP or anti-RasGRP1 antibodies. Quantification of bands performed by densitometry. Representative of 4 experiments.

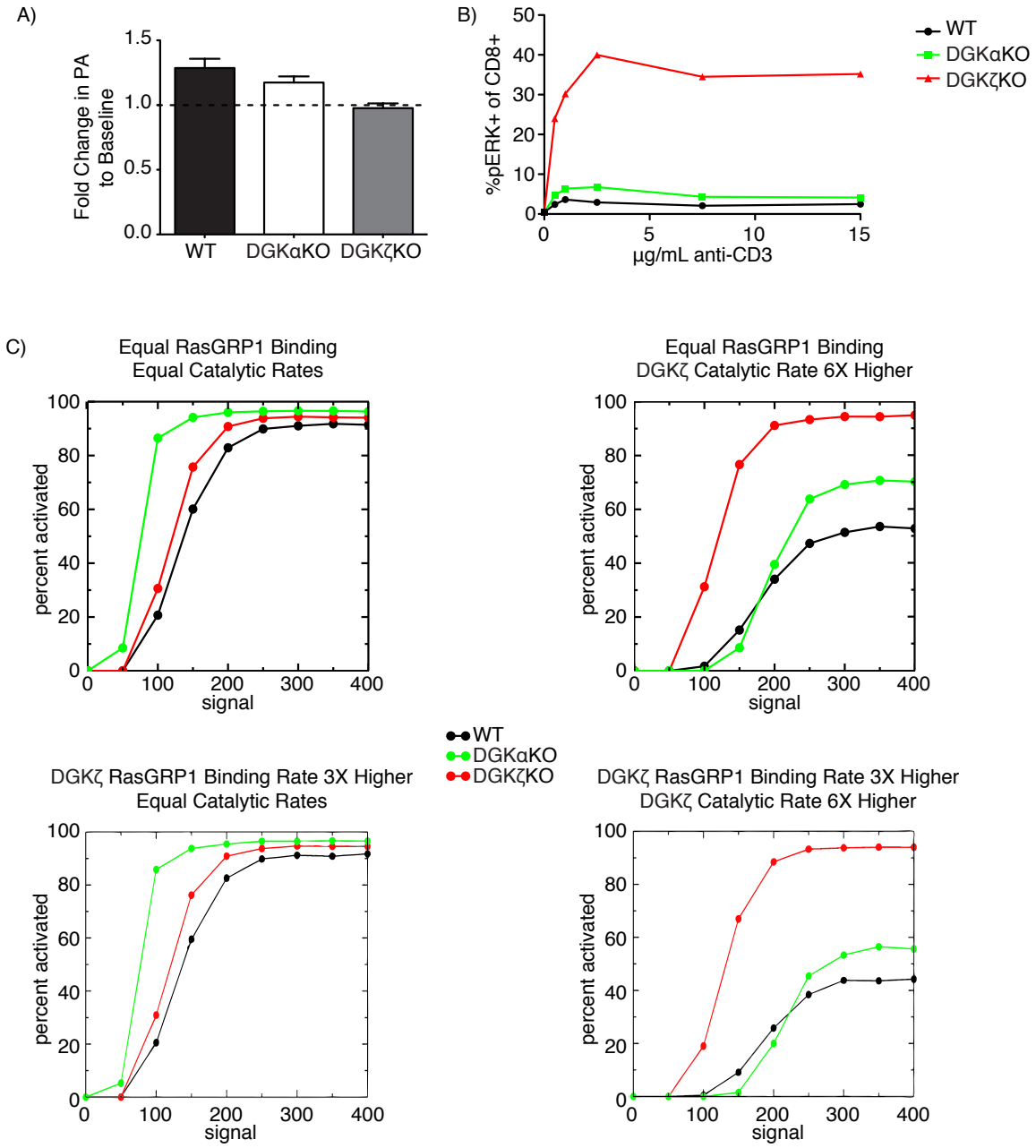


Figure 3.7: DGKζ has greater effective enzymatic activity than DGKα in T cells.

(A) T cells were isolated from wildtype, DGKα$^{-/-}$, or DGKζ$^{-/-}$ mice and stimulated for 7.5 minutes with an anti-CD3 antibody. PA concentration in unstimulated and stimulated cells was determined and data is expressed as the fold change in PA in stimulated cells to unstimulated cells. Error bars represent triplicate measurements of PA. Representative of 2 independent experiments. (B) Splenic cells were freshly isolated,

rested for two hours in serum-free media, and stimulated with increasing concentrations anti-CD3 antibody for 15 minutes. ERK phosphorylation was determined by flow cytometry and the percent of phospho-ERK positive cells among $CD8^+$ cells is shown for indicated concentrations of anti-CD3 antibody. Representative of 2 experiments. (C) Shows variation of the fraction of the cells that produced RasGTP concentrations larger than one third of the total Ras concentration as the signal concentration is increased. The signal represents the TCR-LAT signalosome created upon antigen stimulation. The description of the *in silico* model, the simulation method, and the parameters used in the model are given in the materials and methods section and the supplementary material. The concentration of DGKα, as found in experiments, is assumed to be 3 times larger than that of DGKζ. The results shown here are from the simulations done on 10,000 *in silico* "cells". Ras activation at 15 mins for the wildtype, $DGK\zeta^{-/-}$, and $DGK\alpha^{-/-}$ cells (top left) when the catalytic rates and RasGRP1 binding rate of DGKζ and DGKα are the same, (bottom left) when the RasGRP1 binding rate of DGKζ is 3-fold larger than that of DGKα and catalytic rates of DGKζ and DGKα are the same, (top right) when the catalytic rate of DGKζ is 6-fold larger than that of DGKα and RasGRP1 binding rates equal, or (bottom right) when the RasGRP1 binding rate of DGKζ is 3-fold larger than that of DGKα and the catalytic rate of DGKζ is 6-fold larger than that of DGKα. Professor Jayajit Das performed the *in silico* experiments, with intellectual input from Rohan Joshi and Professor Edward Behrens.

Table 3.1: Reactions and the kinetic rates used in the *in silico* model

Reactions	Binding rate (k_{on}) $(mM)^{-1}s^{-1}$	Unbinding rate (k_{off}) s^{-1}	Dissociation constant (K_D) mM	Catalytic Rate (k_{cat}) s^{-1}
S^a + SOS $\rightleftarrows$ S-SOS	5.0	0.1	0.02	NA
S-SOS_c+R_D $\rightleftarrows$ S-SOS_c-R_D $\rightarrow$ S-SOS_c + R_T	0.27	4.0	14.8	0.0005
S-SOS_a+R_D $\rightleftarrows$ S-SOS_a-R_D	0.122	3.0	24.6	
S-SOS_a+R_T $\rightleftarrows$ S-SOS_a-R_T	0.11	0.4	3.6	
S-(SOS_a-R_D)$_c$+R_D $\rightleftarrows$ S-(SOS_a-R_D)$_c$-R_D $\rightarrow$ S-(SOS_a-R_D)$_c$ + R_T	0.068	1.0	14.7	0.003
S-(SOS_a-R_T)$_c$+R_D $\rightleftarrows$ S-(SOS_a-R_T)$_c$-R_D $\rightarrow$ S-(SOS_a-R_T)$_c$ + R_T	0.053	0.1	1.89	0.038
S+PLCγ1 $\rightleftarrows$ S-PLCγ1	0.0425 *	0.01 *	0.23 *	
S-PLCγ1+PIP_2 $\rightarrow$ S-PLCγ1+DAG				$8x10^{-7}$ *
DAG+RasGRP1 $\rightleftarrows$ DAG-RasGRP1	0.05	0.1	2.0	
DAG-RasGRP1+R_D $\rightleftarrows$ DAG-RasGRP1-R_D $\rightarrow$ R_T+ DAG-RasGRP1	0.32	1.0	3.1	0.01
GAP+ R_T $\rightleftarrows$ GAP+ R_T $\rightarrow$ GAP+ R_D	1.25	0.2	0.16	0.1
DAG+DGKα $\rightleftarrows$ DAG-DGKα $\rightarrow$ PA+DGKα	0.05 *	0.01 *	0.2 *	0.01 *
RasGRP1-DAG+DGKα $\rightleftarrows$ RasGRP1-DAG-DGKα $\rightarrow$ PA+RasGRP1+DGKα	0.05 *	0.01 *	0.2	0.01 *
DAG+DGKζ $\rightleftarrows$ DAG-DGKζ $\rightarrow$ PA+DGKζ	0.05 *	0.01 *	0.2 *	Varied from 0.01 to 0.06

				*
RasGRP1-DAG+DGKζ ⇄ RasGRP1-DAG-DGKζ → PA+RasGRP1+DGKζ	Varied from 0.05 to 0.15 *	0.01 *	Varied from 0.2 to 0.066 *	Varied from 0.01 to 0.06 *

[a]S denotes the species 'signal', which represents the TCR-LAT signalosome in the model. *Parameters were estimated to reproduce the time scales (~15 mins) for robust Ras activation in the *in silico* models consistent with Erk activation as observed in experiments. The binding and unbinding rate, dissociation constant, and catalytic rates of the reactions used in the *in silico* model are shown. Parameter values in Table S1 are taken from *Riese et al., Das et al*., and *Stephens et al.*

Table 3.2: Concentrations of proteins and lipids used in the *in silico* model

Molecular species	Concentration
S[a]	0 – 125 molecules/$(mm)^2$
SOS	1000 molecules/$(mm)^3$
RasGRP1	1500 molecules/$(mm)^3$
PLCγ1	62 molecules/$(mm)^2$ *
PIP_2[b]	4250 molecules/$(mm)^2$
GAP	125 molecules/$(mm)^3$
DGKα	2250 molecules/$(mm)^3$ *
DGKζ	750 molecules/$(mm)^3$ *

[a]S denotes the species 'signal', which represents the TCR-LAT signalosome in the model.

[b]Phosphatidylinositol 4,5-bisphosphate. * = Parameters were estimated to reproduce the time scales (~15 mins) for robust Ras activation in the *in silico* models consistent with Erk activation as observed in experiments. The concentration of molecular species included in the *in silico* model is summarized. Parameter values in Table S2 are taken from *Riese et al., Das et al*., and *Stephens et al.* Additional parameters that were estimated for the specific model in Fig. S4 are indicted with a * symbol.

3.5. Materials and Methods

Mice

See Section 2.5 for a description of mice used in these experiments.

Constructs and Cloning

DGKα (kind gift from Anthony DeFranco) or DGKζ (Thermo Scientific, cat# MMM1013-9200165) were subcloned into pEGFP-C1 (Clontech) or MIGR1 for transduction of bone marrow. eGFP-DGKα or eGFP-DGKζ were subcloned into pK1 retroviral vector (kind gift from Warren Pear) to create pK1-eGFP-DGKα or pK1-eGFP-DGKζ for transduction of primary murine T cells.

Cellular phosphatidic acid assay

T cells from wildtype, DGKα$^{-/-}$, or DGKζ$^{-/-}$ mice were isolated using magnetic selection with CD90.2 microbeads (Miltenyi), rested for 2 hours, and left unstimulated or stimulated for 7.5 minutes with an anti-CD3 (500A2) antibody in RPMI. Cells were washed once with PBS and immediately lysed using sonication. Protein concentration was determined by bicinchoninic assay (BCA, Thermo Scientific). Total cellular phosphatidic acid content was determined according to manufacture protocol using the total phosphatidic acid kit (Cayman Chemical, cat #700240) and normalized to protein concentration as described previously (Bobrovnikova-Marjon et al., 2012).

In silico modeling

We use a continuous time Monte Carlo method or the Gillespie method (Gillespie, 1977) to solve the Master equation associated with signaling network described in Fig. S4 and Tables S1-S2. The simulation method includes copy number fluctuations of signaling molecules, also known as the intrinsic noise fluctuations (Swain et al., 2002), that occur due to random nature of stochastic biochemical reactions. We

also include cell-to-cell variations of the total protein and lipid abundances due to extrinsic noise fluctuations (Swain et al., 2002). The extrinsic noise fluctuations are implemented in the following way. In each individual cell, the total concentrations of protein or lipid were chosen from uniform distributions with the average values shown in Table 2. The upper and lower bounds of a uniform distribution for a specific signaling species was chosen by decreasing and increasing the corresponding average value by a factor of 0.0175 (Volfson et al., 2006). The signaling reactions were simulated in a spatially homogeneous simulation box of size, V = (area (4 mm2) × height (0.02 mm)) with two compartments representing the plasma membrane and the adjoining cytosolic region (26). This particular choice of the simulation box size ensures that the system is well-mixed. The results shown are from the simulations done on 10,000 in silico "cells". Further details regarding the simulation can be found in references 20 and 26. The simulations are carried out by using the software package Stochastic Simulation Compiler (SSC) (Lis et al., 2009). The codes for the simulations are available at the link, http://planetx.nationwidechildrens.org/~jayajit/.

Immunoprecipitation

293T cells, 293T cells transduced with eGFP-DGKα, and 293T cells transduced with eGFP-DGKζ were grown on 10cm culture dishes in DMEM supplemented with 10% fetal bovine serum and antibiotics. These cell lines were transfected with pEF6-huRasGRP1-myc/his WT (kind gift from Dr. Jeroen Roose) using the calcium phosphate method. Two days later, cells were washed with PBS and lysed in 0.1% Tween-20, 150mM NaCl, and 25mM Tris-HCl pH 7.4 supplemented with phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Roche Applied Sciences) for 30 minutes. Lysate was centrifuged and supernatant was pre-cleared by incubation with protein A-sepharose beads for 1 hour at 4°C with in a rotating stand. Pre-cleared lysate

was divided into three samples receiving either 1uL/mL lysate anti-GFP antibody (Abcam, cat# Ab290), 10μL/mL lysate anti-RasGRP1 (H120, Santa Cruz Biotech, cat# sc-28581), or 5μL/mL lysate normal rabbit IgG (Santa Cruz Biotech, cat# sc-2027). Protein A-sepharose beads were added and samples were incubated overnight at 4°C on a rotating stand. The following day, samples were washed 3 times with lysis buffer and 3 times with lysis buffer containing 500mM NaCl and denatured in SDS sample loading buffer.

Western blotting for DGKα expression

Splenocytes were isolated from bone marrow chimeras, and T cells were purified using magnetic selection with CD90.2 microbeads (Miltenyi). GFP+ and GFP− cells were sorted using a FACS Aria (BD Biosciences) and lysed in 1% Non-idet P-40 supplemented with protease inhibitors. Lysates were subjected to western blotting using anti-DGKα (Santa Cruz, cat# sc-271644), anti-GFP (Clontech, cat #632375), or anti-b-tubulin (Cell Signaling, cat #2146S).

Transduction of primary murine T cells

CD4+ T cells from OT-II; DGKζ$^{-/-}$ mice were isolated using CD4+ T cell Isolation Kit (Miltenyi) and stimulated from 24-30 hours on 1mg/mL anti-CD3 (2C11) and 5μg/ml anti-CD28 coated 24-well plates in TCM (Iscove's Modified Dulbecco's Media supplemented with 10% fetal bovine serum and antibiotics) supplemented with 50U/mL recombinant human IL-2 (rhIL-2) at a concentration of 2-3 × 106 cells/mL. On the same day, 293T cells were transfected using calcium phosphate method with pK1-eGFP-DGKα or pK1-eGFP-DGKζ along with ecotropic packaging vectors pCGP and pHIT123 (kind gift from Warren Pear). The next day, 1mL virus containing supernatant from 293T cells was bound to wells of a 24-well plate that was coated with retronectin (Takara Bio., Inc.) according to the manufacturers protocols by spinning at 1200g for 2 hours at 30°C.

CD4+ T cell blasts were harvested and resuspended in TCM supplemented with 80U/mL rhIL-2 at a concentration of 2-3 × 106 cells/mL. The virus-coated wells were washed once with PBS, T cells were aliquoted, and a spinfection was performed using centrifugation at 1200g for 2 hours at 30°C. The following day, cells were harvested and replated on a non-retronectin coated plate. Transduction efficiencies of greater than 30% were routinely achieved.

Conjugation of T cells and antigen presenting cells

B cells were selected from wildtype mice using magnetic selection using CD19 microbeads (Miltenyi). B cells were washed with serum-free RPMI and stained with CMTMR (5-(and-6)-(((4-Chloromethyl)Benzoyl)Amino)Tetramethylrhodamine, Life Technologies) at a final concentration of 1μM. B cells were then washed with TCM and divided into two samples. One sample received OVA peptide (323-339) (Genscript) at a final concentration of 5μg/mL and samples were incubated for 4 hours at 37°C. B cells and transduced T cells were harvested, washed once with serum-free RPMI, and resuspended at 6.66 × 106 and 13.33 × 106 cells/mL serum-free RPMI, respectively. 75mL of B cells were added to 75μL T cells in a FACS tube, spun at 1000rpm for 2 minutes, and incubated for 5-30 minutes in a 37°C water bath. After conjugation, 50μL of cells were coated onto poly-L-lysine (Sigma) coated microscope cover slips and incubated at 37°C for 10 minutes in a humidity chamber. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 0.01% saponin, 0.25% fish skin gelatin in PBS. Cells were stained with anti-Talin (Sigma, Cat #T3287) followed by anti-mouse Alexa Fluor 647 (Life Technologies) and anti-GFP-Alexa Fluor 488 (Life Technologies) to enhance GFP signal. Cells were mounted onto slides in Prolong Gold Antifade Reagent with DAPI (Life Technologies). Conjugates were selected by observation of a GFP expressing cell next to a CMTMR expressing

cell. Images were acquired at room temperature using Volocity (Improvision) software and a spinning-disk confocal (UltraView ERS 6; PerkinElmer) microscope (Axiovert200; Carl Zeiss) equipped with an ORCA-ER camera (Hamamatsu Photonics) and a 63× oil-immersion plan-achromatic objective with a 1.4 N.A.

Image Processing and Analysis

Images were processed in Fiji (Schindelin et al., 2012), using the "subtract-background" function and adjusting the dynamic range based on background intensity. Conjugates in which Talin accumulated at the IS were analyzed using a custom automated MATLAB script. Briefly, GFP images were flattened and subjected to a threshold based on background noise intensity. The user was then asked to draw a line overlaying the Talin image in order to rotate the image such that the conjugate is oriented vertically. After selecting the top and bottom of the cell, the GFP image is divided into thirds along the y-axis and the average GFP intensity in each third as well as the average GFP intensity of the whole cell are calculated. As a control analysis, cells were divided into thirds along the x-axis to determine if the presence of the nucleus affects this analysis. GFP was not found to be excluded from the x-axis middle third of the cell using this analysis regardless of molecule transduced. In addition, analysis using this method on GFP-alone transduced cells demonstrated no particular GFP localization to any third of the cell.

Endogenous DGK protein expression

eGFP-DGKα transduced 293T or eGFP-DGKζ transduced 293T were lysed and serial dilutions were created. T cells from wildtype mice were magnetically purified using CD90.2 microbeads (Miltenyi), lysed, and left undiluted or diluted two-fold. eGFP-DGKα, eGFP-DGKζ, and wildtype T cell lysate were then subjected to western blotting using antibodies to GFP and DGKα or DGKζ. Densitometry was used to create a standard

curve relate GFP staining intensity to DGKα or DGKζ staining intensity and a linear regression was performed. The DGKα or DGKζ staining intensity of wildtype T cell was then used to calculate a GFP staining equivalent to examine the relative amount of DGKα or DGKζ protein.

Flow cytometric analysis of ERK, AKT, and S6 phosphorylation

See methods section of Chapter 2.

CHAPTER IV: THE ROLE OF PHOSPHATIDYLINOSITOL TRANSFER PROTEINS IN T CELLS

4.1. Introduction

Phosphatidyl inositols (PIs), a subset of phospholipids, play a crucial role in receptor-mediated signaling in all mammalian cells. PIs are a substrate for the production of key signaling intermediates including diacylglycerol (DAG), inositol triphosphate (IP_3), and phosphatidylinositol triphosphate (PIP_3), which activate pathways including PKCs, Ras, calcium, and Akt. In T cells, these PI-derived signals are integrated with other signals leading to T cell activation, differentiation, proliferation, and elaboration of cytokines. Consequently, the regulation of PI abundance in the plasma membrane, hydrolysis of its products, and the metabolism of PI products each potentially represent a critical checkpoint during initiation of an immune response. In Chapters II and III of this thesis we have investigated the metabolism of DAG and its functional effects on T cell development and signaling. Here, we turn our attention to the regulation of plasma membrane PI, from which PIP_2, DAG, IP_3, and PIP_3 are all derived, as this presents a possible point of regulation which would critically affect many signaling pathways.

4.1.1. PI Transport

PIs are synthesized only in the endoplasmic reticulum (ER) and traffic to the plasma membrane before they can serve as substrates for signal transduction. These hydrophobic molecules therefore must transverse the hydrophilic cytosol of the cell

(Prinz, 2010). While vesicular transport from the ER to the plasma membrane is one mechanism of transfer of phospholipids, when vesicular transport is blocked using pharmacological means phospholipids are still efficiently shuttled to the plasma membrane (Kaplan and Simoni, 1985). These data suggested the presence of molecules that can shuttle PIs from the ER to the plasma membrane. Phosphatidylinositol transfer proteins (PITPs) were found to have this ability to transfer PIs *in vitro* between [^{3}H] PI labeled donor microsomes and a liposome acceptor compartment (Helmkamp et al., 1974). Thus, PITPs could potentially serve as PI transport molecules in intact cells and facilitate TCR signaling.

4.1.2. The PITP family

PITPs are divided into two classes based on sequence homology. Class I PITPs, PITPα and PITPβ, are cytosolic proteins that contain a single PI binding domain. Both PITPα and PITPβ are conserved across mammalian species as well as unicellular and multicellular eukaryotes (Cockcroft and Carvou, 2007). In mice, the genes for these molecules are located on separate chromosomes and code for small proteins of 270-271 amino acids that have a sequence similarity of greater than 90%. PITPβ is expressed as two splice variants that differ by a single amino acid at the C-terminus. In spite of their high degree of sequence similarity, PITPα and PITPβ localize differently in cells. While both splice variants of PITPβ localize to the Golgi, PITPα localizes primarily to the cytosol and plasma membrane (De Vries et al., 1996; Morgan et al., 2006).

In contrast to class I molecules, class II PITPs contain PI binding domains that are homologous to phospholipid binding domains of RdgB. Class IIB contains RdgBβ, a

single domain molecule like class I PITPs. In contrast, class IIA molecules are multi-domain proteins with additional domains that simultaneously target the molecule to two membrane compartments of the cell (Cockcroft and Carvou, 2007). While it is easy to envision how class IIA molecules transfer lipids between two targeted compartments, the mechanism of specificity is less clear for class IIB and PITPα and PITPβ.

As all five members of the PITP family can bind to phospholipids, overlap in function between these molecules likely exists. However, the functional importance of PITPα and PITPβ alone as molecules is highlighted by the phenotypes of mice deficient in these isoforms. *Vibrator* mice harbor a natural mutation resulting in an 80% reduction in PITPα, leading to neurodegeneration and death (Hamilton et al., 1997). In addition, mice completely deficient in PITPα die perinatally, while deletion of PITPβ causes embryonic lethality (Alb et al., 2002, 2003). Because of the clear phenotypes of these mice, PITPα and PITPβ are the focus of this chapter.

4.1.3. Class I PITP structure

PITPα and PITPβ display a high degree of specificity for PIs, although they do bind and transfer phosphatidylcholine (PC) to a lesser degree (Helmkamp et al., 1974). The structures of PITPα and PITPβ revealed that both PI and PC bind to these molecules with their polar head group buried deep within the core of the PITP molecule. The overall structure of PITPα bound to PI or PC is highly similar, suggesting that the lipid species bound does not significantly alter the function of PITPs (Yoder et al., 2001; Tilley et al., 2004). However, four specific residues of PITPα make hydrogen bond contacts with the inositol ring of PI, likely explaining the specificity of PITPs for PIs.

Interestingly, mutation of these four residues abrogates PI binding, but leaves PC binding intact (Tilley et al., 2004). These mutant PITPs could be useful in demonstrating which functions of PITPs are dependent on its ability to bind to PI rather than PC. Mutational analysis also demonstrated that a two-tryptophan motif present in both PITPα and PITPβ facilitates binding to lipid membranes and is required for the PI lipid transfer ability of PITPα *in vitro*. Mutation of this WW motif likely prevents membrane docking of PITP molecules and presents another potential tool for studying how PITPs function within intact cells.

4.1.4. Biochemical functions of PITPs

A connection between PITP function and signaling in cells was found through the use of permeabilized cells. Using this method, cells were depleted of their cytosolic contents including PITPα and PLCγ and selectively reconstituted with PITPα and/or PLCγ in the presence or absence of epidermal growth factor (EGF) (Kauffmann-Zeh et al., 1995). Restoration of stimulated PLCγ activity and PI-4 kinase dependent phosphorylation was only observed in cells reconstituted with PITPα and PLCγ, not with PLCγ or PITP alone. These data suggest that PLC activity requires the presence of PITPs and, interestingly, that the phosphorylation of PI requires the stimulated activity of PI-4 kinase activity and the presence of PITPs. Furthermore, PITPα was found to complex with PLCγ in a manner dependent on EGF, suggesting that PITPα might have an active role in facilitating these signaling events. Similar findings were observed with PITPβ (Cunningham et al., 1996). In addition, a similar requirement for PITPs for signaling was observed for PLCβ and PLCδ1 (Allen et al., 1997; Thomas et al., 1993).

It is unknown whether *in vitro* transfer ability reflects actual transfer ability in cells. Indeed, unequivocal evidence of *in vivo* PI transfer of PITPs has been difficult to obtain due to lack of methods to track lipid movement. The strongest evidence for PITP transfer of PI *in vivo* was obtained by examining fluorescence resonance energy transfer (FRET) of GFP-tagged PITPα and BODIPY-tagged PI and in live cells (Larijani et al., 2003). FRET efficiency was significantly increased at the plasma membrane upon administration of EGF and diminished by inhibition of PLCγ, suggesting that PITPs do associate with PIs during receptor-mediated signaling *in vivo*. These data are consistent with a PI transfer role for PITPs in intact cells. However, it remains possible that PITPs' main function is to act as a cofactor for signaling by facilitating access to PI rather than by transferring PI between compartments.

4.1.5. In vivo PITP functions

While the exact *in vivo* biochemical role of PITP is unclear, the phenotypes of mice lacking PITPα or PITPβ are striking. *Vibrator* mice and mice deficient in PITPα die shortly after birth due to spinocerebellar degeneration, intestinal and hepatic steatosis, and hypoglycemia (Alb et al., 2003). This degenerative death correlated with derangements in the smooth ER of neurons, enterocytes, and hepatocytes, suggesting that PITPα may function *in vivo* at the ER; however, other cellular pathologies could also lead to this phenotype. Surprisingly, examination of PLC signaling in fibroblasts from *vibrator* mice revealed no differences in PI metabolism after stimulation from wildtype cells. These data could be explained by redundancy of PITPβ.

In contrast to PITPα, deletion of PITPβ leads to early embryonic lethality, preventing study of this isoform in mature cells. However, studies in other organisms suggest that PITP molecules may be involved in basic cellular processes in addition to their signaling function. In *Drosophila*, the single class I PITP present, giotto, was found to be required for both meiotic and mitotic cytokinesis (Gatt and Glover, 2006; Giansanti et al., 2006). In flatworms, PITPs were found to have a critical role in stem cell viability and regeneration of the organism (Reddien et al., 2005; Cockcroft and Carvou, 2007). Taken together, these data suggest that PITPs are involved in a broad range of cellular processes, including signaling, lipid homeostasis, and cell cycling.

4.1.6. Conclusion

The role of PITPs in T cells is completely unknown. Signaling mediated by PI is critical in T cells, as signaling through the TCR, and PLCγ1 in particular, dictates the fate of T cells during positive and negative selection, T cell activation, and T cell tolerance (Fu et al., 2010). Thus, PITPs could play a role in each of these processes in T cells. In addition, given the tools available to immunologists, such as bone marrow chimeras and gene reconstitution using retroviral transfers, T cells provide a useful platform to study the cell biology of PITPs. Here, we use mice with T cell specific deletion of PITPα and/or PITPβ to examine the function of these molecules in T cells.

4.2. Results

4.2.1. PITPα and PITPβ expression in T cells and role in Jurkat T cell growth

We first wished to determine whether PITPα and PITPβ were expressed in T cells. We found that both PITPα and PITPβ were expressed in splenic and lymph node T cells, at roughly equivalent levels to non-T cells from these organs (Fig. 4.1). Because PITPα localizes to the plasma membrane and PITPβ does not, we chose to begin our investigation with a focus on the function of PITPα in T cells. As an initial approach, we studied Jurkat T cells in which we have knocked down PITPα. Jurkat T cells express PITPα and after transduction of a vector containing shRNA to PITPα levels of protein were greatly reduced compared to Jurkat T cells transduced with vector alone. After knockdown of PITPα, we noted that Jurkat T cell growth was impaired in two independently created Jurkat T cell lines (Fig. 4.2 A and B). As shRNAs can have off target effects, we repeated this experiment using a set of 5 shRNA sequences that target different areas of PITPα mRNA and a non-targeting shRNA sequence as a control. We again found that cell growth was reduced in cells with knockdown of PITPα (Fig. 4.2 C and D). Cell growth tracked with expression levels of PITPα with the exception of one shRNA. These data suggested that PITPα may play a role in T cells.

4.2.2. Phenotype of mice with a T cell specific deletion of PITPα or PITPβ

Based on these preliminary data, we proceeded with generation and characterization of CD4-Cre^{+}PITPα$^{loxP/loxP}$ mice, in which Cre is turned on at the double positive stage of T cell development in the thymus. As expected, T cells from CD4-

Cre^{+}PITPα$^{loxP/loxP}$ mice were deficient in PITPα, suggesting that the gene is specifically deleted in the T cell compartment (Fig. 4.3 A). As PI-mediated TCR signals are essential for positive and negative selection, we hypothesized that defects in mice with a T cell specific deletion of PITPα would manifest as aberrant T cell development. Surprisingly, we found that T cell development was grossly intact in CD4-Cre^{+}PITPα$^{loxP/loxP}$ mice, with similar percentages of double positive, CD4SP, and CD8SP (Fig. 4.3 B). A closer look at CD69^{hi}TCRbeta^{hi} cells undergoing positive selection also revealed no gross defects. In the spleen, we found similar percentages of T cells, ratio of CD4^{+} to CD8^{+} T cells, and CD44^{hi} CD4^{+} and CD8^{+} T cells to control (Fig. 4.3 C and D). These data indicate no gross defects in PITPα-deficient T cells. We next performed experiments using a competitive bone marrow chimera system to reveal if PITPα deficient T cells are less fit than wildtype cells. We mixed bone marrow from CD4-Cre^{+}PITPα$^{loxP/loxP}$ or CD4-Cre^{-}PITPα$^{loxP/loxP}$ mice with congenically marked wildtype competitor bone marrow and transferred these cells into lethally irradiated congenically marked recipient mice. After immune reconstituion, we found that deletion of PITPα from T cells did not markedly affect the gross phenotype of thymocytes and splenocytes (data not shown). Thus, these preliminary results suggest that PITPα is dispensable for grossly normal T cell development.

As PITPβ could function redundantly for PITPα, we also examined T cells that were deficient in PITPβ using CMV-Cre^{+}PITPβ$^{loxP/loxP}$ mice, in which PITPβ deletion should occur in all cellular compartments. After confirming that PITPβ levels were reduced in T cells (data not shown), we examined the thymus and spleen of this mouse. Similar to our findings with PITPα, we found that thymic T cell development and the phenotype of peripheral T cells were grossly normal in PITPβ-deficient mice (Fig. 4.4). These data suggest that PITPβ is also dispensable for normal T cell development.

4.2.3. Phenotype of mice with a T cell specific of both PITPα and PITPβ

To determine if PITPα and PITPβ have redundant functions in T cells, we bred mice with a targeted deletion of both PITPα and PITPβ in T cells (CD4-Cre^{+}PITPα$^{loxP/loxP}$PITPβ$^{loxP/loxP}$ mice). With deletion of both Type I PITP isoforms, we observed a marked reduction in T cell percentages in the spleen and lymph node (Fig. 4.5 A and B). We also noted an increase in CD44^{hi} cells within the remaining CD4^{+} and CD8^{+} T cells. The remaining splenic T cells in CD4-Cre^{+}PITPα$^{loxP/loxP}$PITPβ$^{loxP/loxP}$ mice expressed both PITPα and PITPβ at levels comparable to mice not expressing Cre, indicating that these cells have escaped Cre-mediated deletion (Fig. 4.6 A).

To determine if these decreases in T cells in the periphery were due to a defect in T cell development, we examined thymi of mice in which both PITPα and PITPβ were deleted. Surprisingly, we found that DP, CD4SP, and CD8SP percentages were grossly normal (Fig. 4.5 C). In addition, we observed no decrease in percentage of TCRβ^{hi}CD69^{hi} cells, which are thought to be productively signaling cells undergoing positive selection. We observed a decrease in PITPα and PITPβ expression in DP cells compared to control DP cells, indicating that PITPα and PITPβ were indeed deleted in these cells (Fig. 4.6 B). Because of the discordance between the phenotype of peripheral T cells and thymic DP cells, we more closely examined the SP population. Interestingly, we found a striking decrease in TCRβ^{+}HSA^{lo} cells, indicating a defect in the development of mature single positive cells (Fig. 4.5 C). These preliminary data suggest that the presence of PITPα and PITPβ allows the development of mature T cells. Given PITPs potential role in signaling, we were surprised by the lack of a defect at the DP to SP transition. To determine if DP cells deficient in both PITPα and PITPβ are less fit than wildtype cells, we created mixed bone marrow chimeras. We again found no defect

at the DP to SP transition, suggesting that PITPs are unnecessary for this process when deleted at the DP stage (data not shown). We also observed a defect at the immature SP to mature SP transition in CD4-Cre^{+}PITPα$^{loxP/loxP}$PITPβ$^{loxP/loxP}$ cells but not wildtype competitor cells (data not shown), indicating that this defect is cell-intrinsic.

4.3. Conclusions

While the biochemical function of PITPs has been studied extensively *in vitro*, the role of PITPs in live cells is less understood. The PITP conditional gene knockout mice described here provides a useful tool for elucidation of *in vivo* PITP function. PI-derived signaling is essential for T cell development, and given the *in vitro* role of PITPs we predicted that deficiency of PITPs would impair T cell development. Our preliminary experiments suggest that PITPα and PITPβ are dispensable for grossly normal T cell development when deleted individually. Surprisingly, our initial results suggest that simultaneous deletion of PITPα and PITPβ at the DP stage in T cells does not result in impairment of positive selection or differentiation into single positive cells, events that depend on TCR signaling. Rather, simultaneous deletion of PITPs leads to the impairment of development of mature single positive cells. This defect is reflected in peripheral organs, in which T cell percentages are greatly decreased and the remaining T cells have escaped Cre-mediated deletion of PITPs.

In vitro studies have suggested that PITPα localizes to the plasma membrane and acts as a cofactor for PLC-mediated signaling (Kauffmann-Zeh et al., 1995). Given that mice deficient in PLCγ1 have severe defects in T cell development, we expected that deletion of PITPα would similarly affect T cell development (Fu et al., 2010). However, CD4-Cre mediated deletion of PITPα did not markedly affect T cell development or the phenotype of T cells in the spleen or lymph node, suggesting that PITPα is not required for these processes. When competed with wildtype cells, we still did not observe gross defects in T cells in PITPα-deficient T cells, suggesting that PITPα-deficient T cells are not less fit than wildtype T cells. These preliminary data

suggest that if PITPα-deficiency causes any defects in TCR signaling, they are minor enough to not affect T cell development.

In contrast to PITPα, PITPβ localizes primarily to the Golgi apparatus of the cell and has been speculated to coordinate PI metabolism with vesicle trafficking (Morgan et al., 2006; Cockcroft and Carvou, 2007). We investigated the role of PITPβ using mice with CMV-Cre mediated deletion. Under the CMV promoter, PITPβ should be deleted in all tissues. Although deletion of germline PITPβ results in early embryonic defects, our conditionally deleted PITPβ mice surprisingly displayed no gross developmental defects. These data suggest that the CMV-Cre expression has incomplete penetrance. Nevertheless, T cells in these mice expressed lower levels of PITPβ, allowing their study. Like PITPα-deficient mice, we found that T cell development and phenotype was grossly normal in PITPβ-deficient mice. These initial data suggest that like PITPα, PITPβ is not required in T cells for grossly normal T cell development. *In vitro* studies have suggested that PLC signaling can be rescued in permeabilized mammalian cells by not only the addition of PITPα, but also PITPβ and Sec14p, evolutionarily unrelated yeast PI transfer protein (Cunningham et al., 1996). PITPβ, PITPα, and other PI transfer proteins such as class II PITPs could therefore be functionally redundant in T cells.

Mice with a T cell specific deletion of both PITPα and PITPβ displayed grossly normal T cell development, but a striking defect in T cell percentages in the periphery. These preliminary data suggest that PITPα and PITPβ have a redundant role in T cells. The defect in T cell percentages in the periphery correlated with a decrease in percentage of mature $TCR\beta^{+}HSA^{lo}$ single positive T cells, and we speculate that single positive cells deficient in PITPs cannot mature properly. The phenotype of these double knockout cells were also unchanged by competition with wildtype T cells in mixed bone marrow chimeras, suggesting that dual deficient DP and immature SP cells are not less

fit than wildtype cells. However, it is formally possible that PI depletion in the plasma membrane due to PITP deletion takes sufficient enough time to allow the DP to SP transition to occur. Further investigation is necessary.

While the preliminary data described here suggest a critical function for PITPs in T cells, the exact role of these molecules requires much further study. Future experiments will examine whether the incomplete block at the $TCR\beta^{+}HSA^{hi}$ to $TCR\beta^{+}HSA^{lo}$ transition in the thymus is due to cell death. Experiments using *in situ* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining to examine cell death and retroviral transduction of Bcl-xL to rescue cell death would be useful in this regard. Given the severity of phenotype of germline deletion of PITPα and PITPβ, we speculate that these proteins may function redundantly in fundamental cellular processes that keep a cell alive. This homeostatic function could overshadow any function of these molecules in cell signaling. For this reason, we wish to determine whether this defect in thymocytes would also occur in other cell populations in which both PITPs are deleted. Floxed mice bred to a tamoxifen inducible Cre will allow us to examine the effect of global deletion of PITPα and PITPβ in different cell populations. Of particular interest is whether deletion of PITPα and PITPβ causes smooth ER pathology leading to cell death, as similar defects were found in germline PITPα deficient mice in neurons, hepatocytes, and enterocytes (Alb et al., 2003). In summary, the preliminary data presented here suggest that PITPα and PITPβ have a role in T cells that may or may not be related to TCR signaling. Further experiments will help uncover the exact function of these molecules *in vivo*, which appears to be much more complex than their *in vitro* function.

4.4. Figures

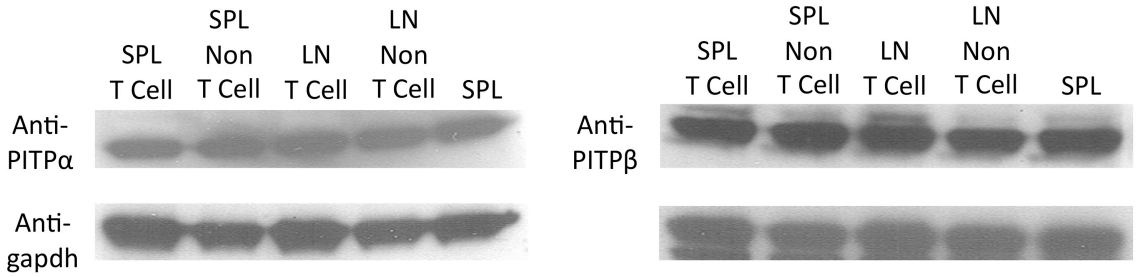


Figure 4.1: PITPα and PITPβ are expressed in T cells.

T cells and were isolated by magnetic selection from spleen and lymph nodes of wildtype mice. Flow-through was collected to isolate non-T cells. A western blot was then performed using the indicated antibodies. N=1.

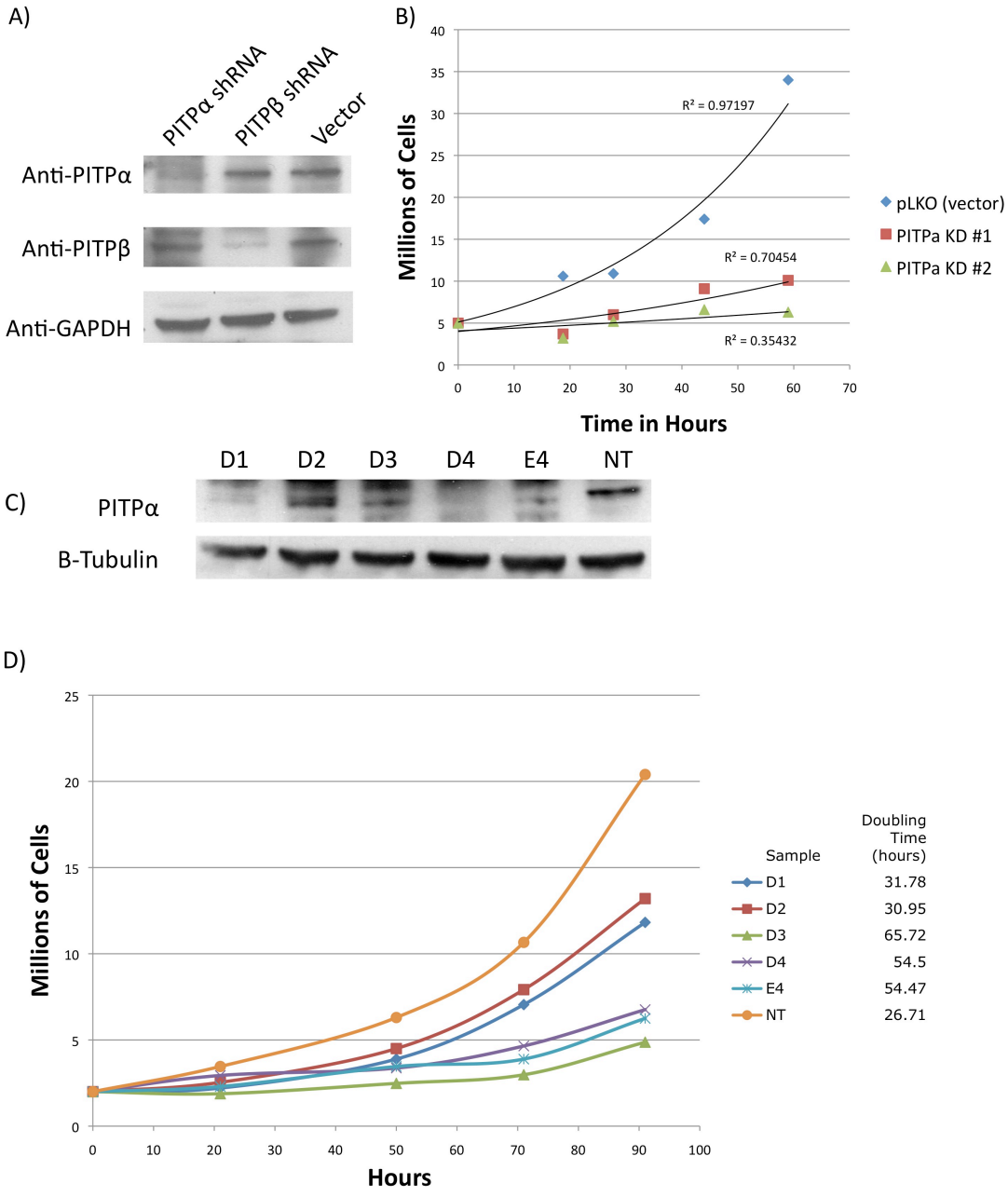


Figure 4.2: PITPα knockdown results in reduced growth of Jurkat T cells.

A) Western blot of PITPα and PITPβ expression 3 days after transduction with empty vector or shRNA to PITPα. B) Growth of indicated Jurkat T cells lines re-plated at equal concentrations at time 0. C) Western blot of PITPα and PITPβ expression 3 days after transduction with non-targeting control shRNA or 5 unique shRNAs to PITPα. D) Growth of indicated Jurkat T cells lines re-plated at equal concentrations at time 0. N=1.

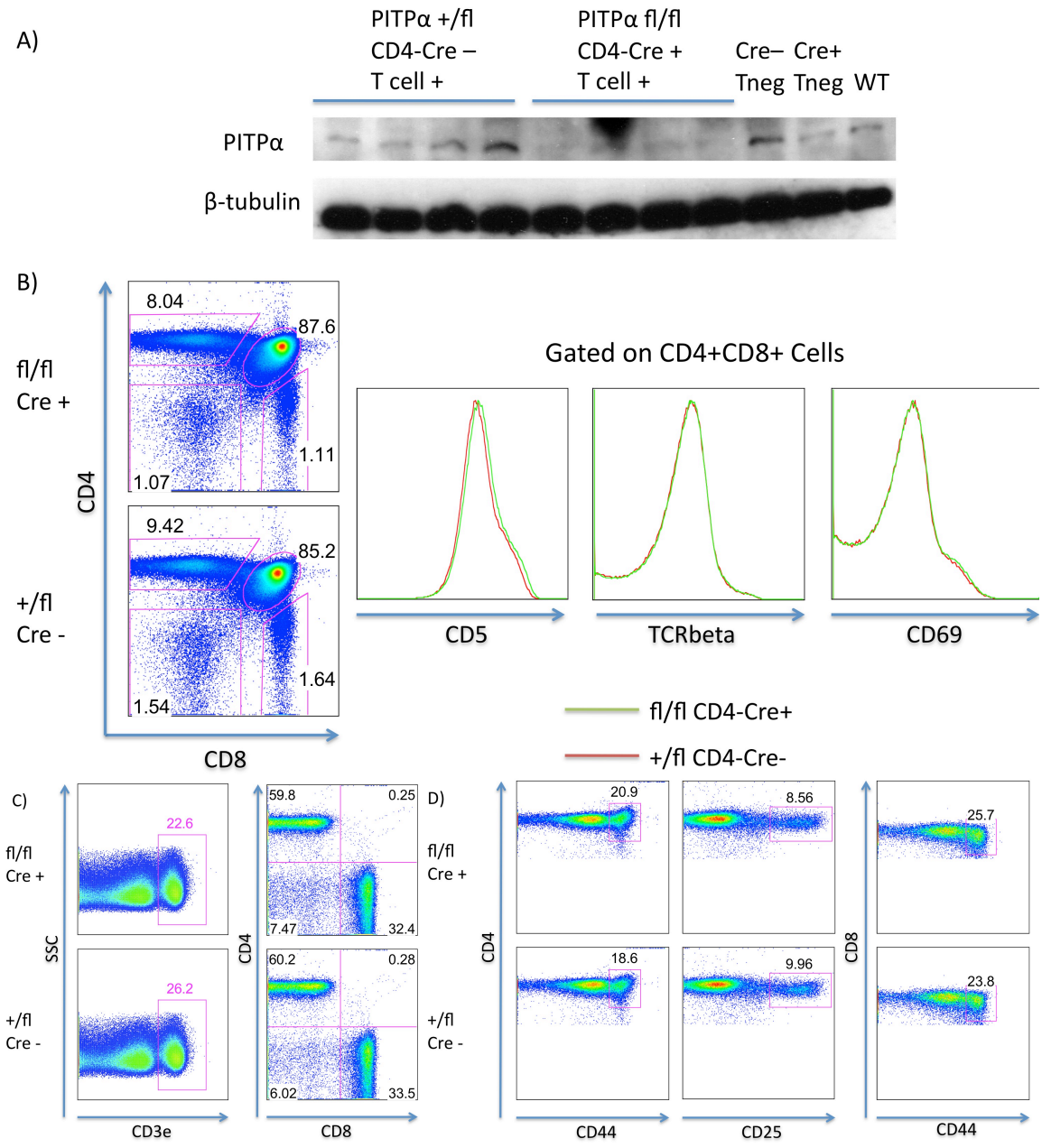


Figure 4.3: T cell specific deletion of PITPα does not grossly alter T cell development or peripheral T cell phenotype.

A) T cells from CD4-Cre^{+}PITPα$^{loxP/loxP}$ or CD4-Cre^{-}PITPα$^{+/loxP}$ mice were isolated by magnetic selection and subjected to western blotting. Flow through was collected to isolate non T cells ("Tneg"). Shown are T cells from 4 mice of each genotype. B) Thymi and C) spleen from CD4-Cre^{+}PITPα$^{loxP/loxP}$ or CD4-Cre^{-}PITPα$^{+/loxP}$ mice were isolated and analyzed by flow cytometry. Representative of 2 mice.

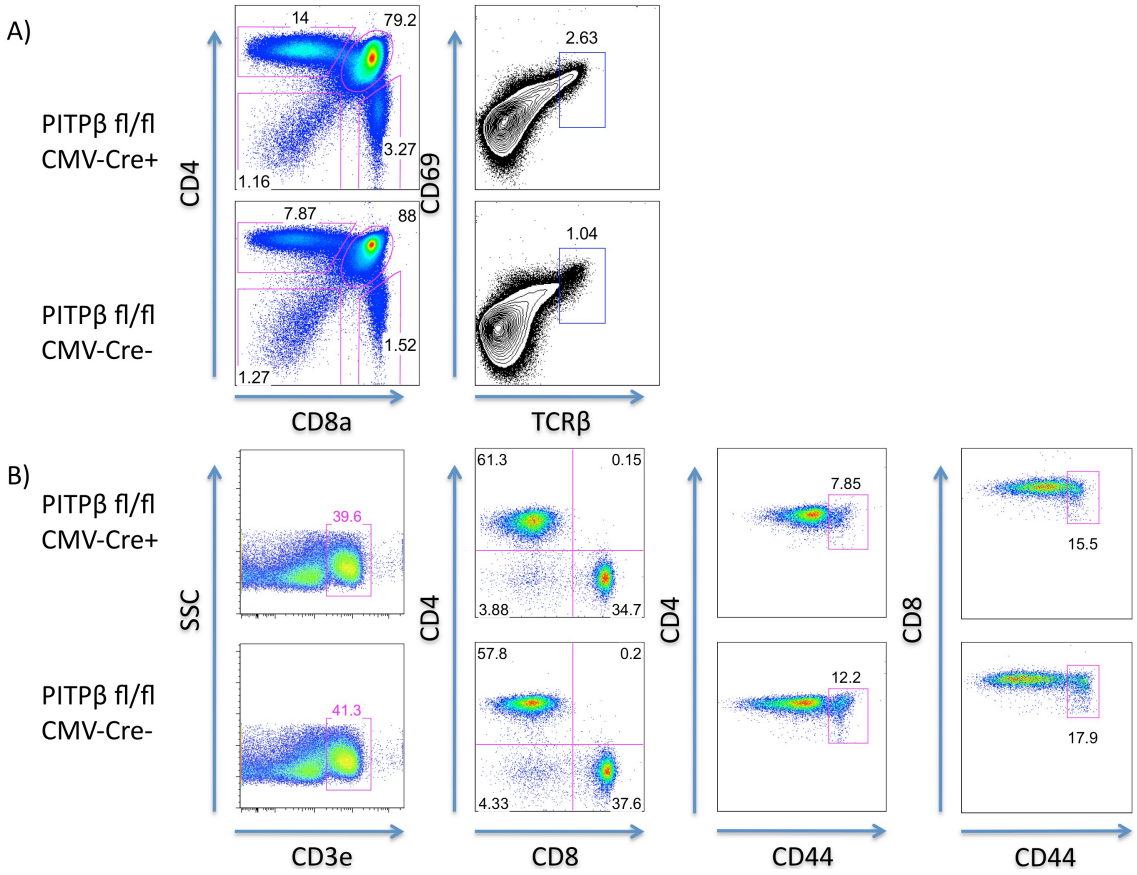


Figure 4.4: T cell specific deletion of PITPβ does not grossly alter T cell development or peripheral T cell phenotype.

A) Thymi from mice of the indicated genotype were isolated and analyzed by flow cytometry. Left: Gated on live singlets. Right: Gated on DP cells. B) Spleen from mice of the indicated genotype were isolated and analyzed by flow cytometry. From left to right: Gated on live singlets, gated on $CD3e^+$, gated on $CD3e^+CD4^+$, or gated on $CD3e^+CD8^+$ cells. Representative of 2 mice.

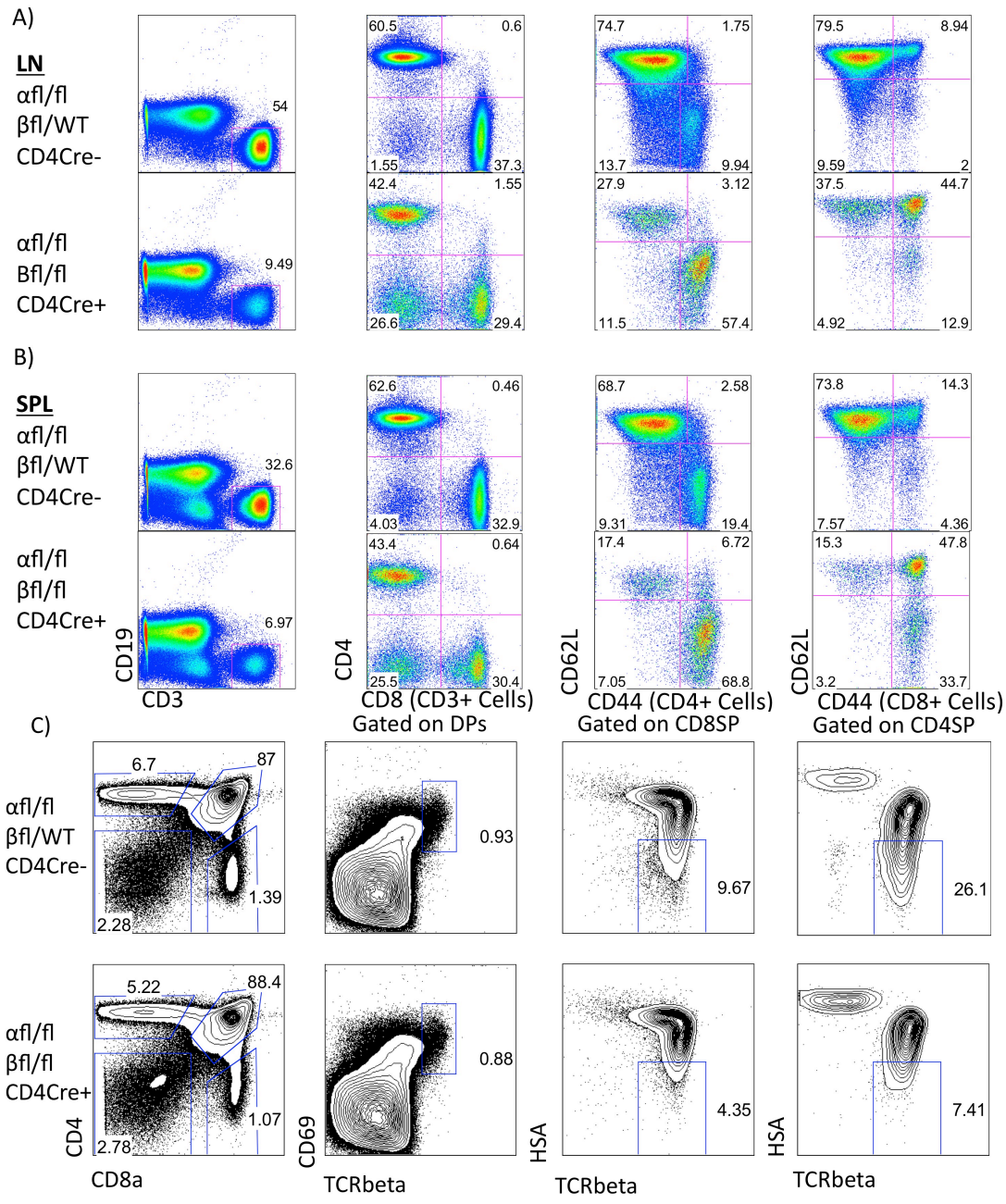


Figure 4.5: T cell specific deletion of PITPα and PITPβ results in loss of peripheral T cells and impaired development of mature SP thymic T cells.

A) Lymph nodes and B) spleen were isolated from mice of the indicated genotype and analyzed by flow cytometry. Cells gated on live singlets or as indicated in parentheses. C) Thymi from mice of the indicated genotype and analyzed by flow cytometry. Representative of 2 mice.

Figure 4.6: T cells from mice with T cell specific deletion of PITPα and PITPβ are PITP replete in the periphery and PITP deficient in the thymus.

A) Splenic T and non-T cells from mice of the indicated genotype were separated using magnetic selection with a CD90.2 antibody, and subjected to western blotting with anti-PITPα, anti-PITPβ, anti-ZAP70 as a control for T cell isolation, and anti-β tubulin as a loading control. B) DP and non-DP cells were isolated from thymi of mice of the indicated genotype using magnetic selection with antibodies to CD4 and CD8. Western blotting was performed for PITPα and PITPβ expression as in A. Numbers indicate densitometric measurements. N=1.

4.5. Materials and Methods

Mice

PITPα$^{loxP/loxP}$ and CMV-Cre;PITPβ$^{loxP/loxP}$ mice were a kind gift from the laboratory of Charles Abrams and were on a C57Bl/6 background. PITPα$^{loxP/loxP}$ were crossed to CD4-Cre mice to generate CD4-Cre;PITPα$^{loxP/loxP}$. All experiments were performed using age-matched mice. Animal maintenance and experimentation were performed in accordance with the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Western blotting for PITP expression

Splenocytes or thymocytes were isolated and T cells were purified using magnetic selection with CD90.2 microbeads or combined CD4 and CD8 microbeads (Miltenyi), respectively. Cells were lysed in 1% Non-idet P-40 supplemented with protease inhibitors. Lysates were subjected to western blotting using anti-PITPα (Santa Cruz, cat# sc-13569) 1:500, anti-PITPβ (ProteinTech Group, cat #13110-1-AP) 1:1200, and/or anti-β-tubulin and anti-ZAP70 (Cell Signaling).

Jurkat T cell growth

Cells were transduced with shRNAs in puromycin resistance vectors to PITPα (Open Biosystems) and allowed to rest for 2 days. On day 2, cells were treated with puromycin. On day 5, cells were harvested and 5x10^6 cells were replated in puromycin-containing media, after which cell growth was monitored for several days.

Flow cytometric analysis of thymi and spleen

Thymi and spleens from wildtype, DGKα-deficient, or DGKζ-deficient mice were freshly isolated and surface stained using antibodies to CD3e, TCRβ, CD4, CD8, CD44,

CD62L, HSA and/or CD25. Flow cytometry was performed using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Mixed bone marrow chimeras

Bone marrow was harvested from CD45.2^{+}Thy1.2^{+} sample mice and CD25.2^{+}Thy1.1^{+} wildtype mice, mixed at a 50:50 ratio, and transferred into CD45.1^{+}Thy1.2^{+} lethally irradiated recipient mice. Tissues were harvested after immune reconstitution at 10 weeks.

CHAPTER V: DISCUSSION AND FUTURE DIRECTIONS

In this thesis, we first investigated differential functions for DGKα and DGKζ. We found a selective role for DGKζ in suppression of nTreg development and a dominant role for DGKζ in TCR stimulated Ras-mediated signaling. These results revealed a previously unappreciated divergence in the roles of the two most highly expressed DGK isoforms in T cells. We next turned to the mechanisms that may control differential DGK isoform functions and performed experiments examining expression levels, localization, interaction with other proteins, and effective catalytic activity. We found that while DGKζ has dominant functions in TCR signaling, DGKα protein is more highly expressed in T cells and cannot function redundantly for DGKζ. While experiments in Jurkat T cell lines have found differential localization of DGKα and DGKζ, our experiments in primary T cells suggested similar gross localization of these molecules after TCR stimulation. We described two major mechanisms that correlate with DGKζ's dominant function in T cells. First, DGKζ associates in greater amounts with the Ras-activator RasGRP1. Second, DGKζ demonstrates greater effective enzymatic activity than DGKα. *In silico* modeling suggests that greater catalytic function for DGKζ would explain its greater role, and that greater RasGRP1 binding would serve to magnify differences observed due to catalytic function. We then examined a broader question of how phosphatidylinositol, the ultimate precursor of DAG in T cells, is itself regulated by examining PITPs. Our preliminary results reveal that simultaneous deletion of PITPα and PITPβ at the DP stage of T cell development results in a loss of peripheral and mature SP thymic T cells. These findings have important implications for our understanding of DAG and PI function.

5.1. DAG metabolism is critical for normal nTreg development

The signals that direct nTreg development in the thymus have begun to be elucidated. High-affinity TCR interactions in the thymus are thought to direct signals that activate NF-κB transcription factor and lead to transcription of FoxP3 (Jordan et al., 2001; Long et al., 2009; Ruan et al., 2009; Hsieh, 2009). However, the signaling molecules that link the TCR with NF-κB activation during nTreg lineage choice are less well understood. In T cells, DAG-mediated activation of PKCθ causes the formation of a multimolecular complex that activates the NF-κB (Coudronniere et al., 2000; Sun et al., 2000). We observed that deletion of DGKζ increases nTreg development, consistent with the notion that higher DAG levels increases NF-κB signaling to direct nTreg differentiation. However, DAG may also act through pathways other than NF-κB to increase nTreg development. Further investigation of other DAG-activated pathways, including the Ras and PKD, could lead to the discovery of novel mediators of nTreg development. In support of this notion, the AP-1 transcription factor, whose formation is thought to be Ras pathway dependent, has been shown to bind to the FoxP3 promoter (Wu et al., 2006). A manuscript in submission by *Schmidt et al.* suggests that ERK signals, which are downstream of Ras, help direct the development of nTreg. In addition, the catalytic function of PKD2 is required for optimal CD4+ T cell production of IL-2, a critical cytokine for nTreg development (Matthews et al., 2010). Examination of nTreg development in mice with deficiencies in the Ras and PKD pathways could be productive.

One of our striking findings was that though deficiency of either DGKα or DGKζ leads to an increase in FoxP3-CD25+ precursors, DGKα deletion does not lead to increased nTreg development while DGKζ deficiency does. These data suggest that

deletion of DGKζ does not simply increase nTreg development by enhancing CD25 upregulation. Rather, we speculate that DGKζ deficiency leads either to epigenetic changes after TCR stimulation that enhance accessibility of the FoxP3 promoter and/or increase responsiveness to IL-2R mediated signals. As phosphorylation of IκBα appeared to be similar between DGKα- and DGKζ- deficient T cells, these results also suggest that DAG-mediated signals, possibly through Ras or PKD2, differentially regulate the ability to respond to IL-2R signaling. Further experiments examining IL-2R signaling in DGKα- and DGKζ-deficient thymocytes would help in this regard.

5.2. Regulation of DGK metabolism of DAG in T cells

Data described in this thesis and from other labs demonstrate that DGK regulation of DAG is critical for normal TCR signaling. How DGKs themselves are regulated, however, is less understood in primary T cells. Our results highlight the importance of regulation of DGK function. We found that even though DGKα protein is expressed at three-fold higher levels than DGKζ, DGKζ is the isoform that primarily regulates Ras-mediated TCR signals. Moreover, overexpression of DGKα in DGKζ-deficient T cells was not sufficient to rescue suppression of TCR signaling. DAG metabolism by DGKζ must therefore differ from DAG metabolism by DGKα, and how this occurs is an interesting question.

Experiments in Jurkat T cells have focused on the localization of DGKα and DGKζ. In these experiments, as we describe here for primary T cells, DGKζ was found to be the dominant regulator of TCR signaling (Gharbi et al., 2011). This phenotype correlated with DGKζ's ability and DGKα's inability to localize to the IS. In contrast, we found that DGKα and DGKζ grossly localized to similar degrees to the IS in primary T

cells, suggesting that gross localization is not a major regulator of DGKα and DGKζ function during TCR signaling. However, regulation of function through localization on a smaller scale is not ruled out. One particularly useful system to study localization at the scale of the IS is total internal reflection fluorescence (TIRF) microscopy in combination with photo-activated MHC-peptide coated coverslips (Quann et al., 2009). After a pulse of ultraviolet light, T cells undergo polarization, allowing high-resolution temporal and spatial visualization of the IS. Experiments studying the localization of GFP-tagged DGKα and DGKζ molecules in conjunction with DAG-visualization probes such as fluorescent protein tagged PKC C1 domains would be interesting. These experiments could reveal if DGKα and DGKζ associate with different pools of DAG and/or regions of the IS.

Our data suggest that RasGRP1 associates to a greater extent with DGKζ than DGKα, and this could augment differences in function due to differences in catalytic activity. Thus, localization of DGKs to regions of the IS where RasGRP1 is present could be a major regulator of DGK function in T cells. Interactions of DGKα and DGKζ with RasGRP1 can be studied *in vivo* after TCR engagement using two complementary techniques. The first is tagging DGKs and RasGRP1 with two different fluorescent proteins, and observing whether these molecules colocalize after TCR stimulation using TIRF microscopy. While this technique is suggestive of an interaction between DGK and RasGRP1 molecules, it does not definitively indicate an association. A more rigorous approach would be to use fluorescence resonance energy transfer (FRET) microscopy. In this system, a donor fluorescent molecule, such as cyan fluorescent protein (CFP), excites an acceptor molecule, such as yellow fluorescent protein (YFP), only when in close proximity, on the order of 1-10nm. Thus, CFP tagged DGK molecules and YFP

tagged RasGRP1 molecules could be used to examine *in vivo* association of these molecules.

What signals direct DGK translocation to the T cell-APC contact site? Our experiments suggest that signals transduced after conjugation of T cells with APCs are necessary for the gross localization of DGKζ, but the exact signals that direct this translocation are unknown. PKC-mediated phosphorylation of the MARCKS domain of DGKζ is thought to be important for its translocation to the IS in Jurkat cells, but our preliminary experiments in primary T cells suggest that MARCKS domain phosphorylation is not required for gross translocation (data not shown). The localization pattern of DGKα and DGKζ in T cells resembles that of Ezrin/Radixin/Moesin (ERM) proteins, which anchor receptors to the actin cytoskeleton (Martinelli et al., 2013). DGKζ contains a PDZ-binding domain that has been demonstrated to mediate interaction of other proteins with ERM proteins (Mery et al., 2002; Mañes et al., 2010). Experiments testing the role of ERM proteins in DGKζ localization as well as the function of its PDZ-binding domain could prove fruitful for understanding DGKζ localization.

We are also interested in understanding which TCR dependent signals are required for DGKζ association with RasGRP1 in primary T cells. While we observed association of DGKζ with RasGRP1 in 293T cells, we found only a minimal interaction in Jurkat T cells in the absence of TCR stimulation (data not shown). Investigation of signals that promote DGK translocation may also reveal how DGKζ comes to associate with RasGRP1 in primary T cells.

5.3. DGK catalytic activity interfaces with analog-to-digital signaling

T cells exhibit digital activation after TCR stimulation, converting TCR ligand binding (the analog signal) to an “on” or “off” state (a digital signal) for a given cell (Chakraborty et al., 2009). This effect has been specifically shown for the activation of Ras after TCR stimulation, with DAG-activated RasGRP1 activation as an analog component and allosteric activation of SOS as an analog-to-digital converter (Das et al., 2009). DAG levels are modulated by DGKs, which are therefore poised to modulate the threshold for TCR-mediated activation of Ras signaling by increasing the number of DAG-RasGRP1 functional units. For example, DGKζ has been found to decrease the threshold for T cell activation using both experimental data and *in silico* modeling of Ras activation (Riese et al., 2011).

This analog-to-digital signaling phenomenon has important implications for the function of DGKs. Changes in DGK catalytic activity could lead to dramatic differences in the percentage of Ras-activated cells, for example. Our data suggest that this is the case for DGKα and DGKζ, with DGKζ’s greater catalytic activity leading to a much higher fraction of cells with activated Ras when DGKζ is deleted. By extension, we speculate that normal physiologic processes that modulate DGK activity could also have major effects on Ras and T cell activation. *In vitro* and *in vivo* studies have suggested that the activity of DGKα and DGKζ can be modulated through post-translational modification. For example, calcium may augment DGKα catalytic activity, although whether this occurs *in vivo* is less clear (Jiang et al., 2000a; Takahashi et al., 2012). Furthermore, TCR mediated SAP activation may modulate DGKα catalytic activity (Baldanzi et al., 2011). Phosphorylation of the MARCKS domain may diminish DGKζ catalytic activity (Luo et al., 2003). An examination of the effects of these phenomena on the threshold

for Ras activation would be interesting – while localization of DGKs has been recognized to be a major form of regulation of function for these enzymes (Shulga et al., 2011), catalytic activity has been less studied with respect to regulation of function.

5.4. Dominant functions of DGKα

While we have uncovered a dominant role for DGKζ in nTreg development and Ras-mediated signaling, the question of why DGKα is present in T cells remains. While mice deficient in DGKα demonstrate impaired induction of T cell anergy, mice deficient in DGKζ also show features of impaired anergy induction, such as unsuppressed production of IL-2, and often to a more severe extent (Olenchock et al., 2006). In addition, DGKα and DGKζ transcription appears to be coordinately regulated, with levels of both isoforms decreasing after T cell activation (Olenchock et al., 2006; Martínez-Moreno et al., 2012). Some clues of selective roles for DGKα exist. For example, treatment of cells with DGK-II (R59949), an inhibitor that primarily interferes with the function of type I DGKs such as DGKα rather than DGKζ (Jiang et al., 2000b), results in impaired MTOC reorientation after TCR stimulation (Quann et al., 2009). As DAG synthesis at the IS has been found to closely precede and be sufficient for reorientation of the MTOC, DGKα rather than DGKζ may play the major role in this process. Should a selective role for DGKα be found, an examination of why DGKα and not DGKζ controls the process would be interesting. Experiments analogous to those performed here – investigation of expression levels, localization, protein interactions, and enzymatic activity – would be useful. In addition, uncovering a dominant role for DGKα would also enable more thorough investigation of the domains that control DGK function. DGKα and

DGKζ domains could be swapped, with clear-cut readouts of whether function is also swapped.

5.5. DGKs, PITPs, and PI signaling

Phosphoinositols and their products are distinctive in some ways from other signaling intermediates, such as phosphorylated proteins. First, PIs and products including PIP_2, PIP_3, and DAG are spatially restricted to membranes, limiting their signaling effects to a very specific part of the cell. Second, PIs are heterogeneous lipid species that contain acyl chains of varying length, adding additional complexity and specificity to their signal transduction role. Third, PIs and their products are easily manipulated by chemical reactions to form molecules with highly distinct functional roles, as in the case of DAG and IP3. Fourth, because PIs and their products can serve both as docking sites and activators of proteins, strict temporal control over their presence is necessary. Finally, PIs provide a small reservoir (about 4-5% of the plasma membrane) for signaling precursors and are consumed during the signal transduction process. This necessitates regeneration of the supply of PIs for further signal transduction.

The phenotype of DGK isoform-deficient cells described here and in other labs demonstrates the temporal and spatial features of the PI product DAG as well as the importance of heterogeneity of DAG species (Joshi and Koretzky, 2013; Shulga et al., 2010). How the PI reservoir is regenerated is still an open question, as PI is synthesized in the ER and must be transported to other membranes. In this thesis, we have begun to explore this question through examination of PITPs, which bind to PIs and may transport lipids. Our preliminary data suggest that simultaneous deletion of PITPα and PITPβ leads to cellular defects in maturing thymocytes. While experiments to

investigate the exact mechanism of this defect are ongoing, we speculate that these maturing cells are dying, possibly due to smooth ER pathology that has been described in germline PITPα-deficient mice (Alb et al., 2003). Investigation of these cells using electron microscopy and other techniques could help in this regard. Nevertheless, the smooth ER phenotype of PITPα-deficient mice is intriguing as the ER is the site of synthesis of PIs. Should we obtain similar findings in T cells, these data could suggest that PIs are not simply innocuous species involved in signaling and vesicular transport. Rather, PIs may be toxic if they accumulate in certain parts of the cell. By extension, we speculate that PI mediated signaling could be important for consumption of PIs and prevention of PI buildup. Lipid compartment fractionation, lipid extraction, and quantification could be useful for our future experiments involving PITPs to determine if PIs accumulate in the ER.

5.6. Therapeutic manipulation of DGKs for disease

The two most highly expressed DGKs in T cells, DGKα and DGKζ, are clearly involved in regulating many processes: T cell selection, *i*NKT development, T cell activation, T cell anergy, T cell responses to pathogen, and T cell anti-tumor responses. In a general sense, the attenuation and termination of TCR-mediated DAG signaling by DGKα and DGKζ is common to all these processes. As DAG has numerous downstream targets, one would predict that potential therapies to modulate DGK function would be most useful in areas where broad manipulation of TCR signaling is desirable, such as in suppressing autoimmunity or enhancing immune responses to cancer or pathogen. However, one hurdle in targeting DGKα and DGKζ to modulate T cell function is that these isoforms are expressed endogenously in numerous tissues. In

fact, based on EST data, DGKα and DGKζ are the most commonly tissue-expressed DGK isoforms of all ten DGKs (Shulga et al., 2011). In other tissues, DGKα and DGKζ regulate diverse processes, such as neurite outgrowth, leptin signaling, cardiac remodeling, and cancer cell migration and invasion (Merida et al., 2008; Shulga et al., 2011). DGKα- and DGKζ-modulating therapies may therefore be most useful in settings in which T cells can be specifically targeted, such as approaches using adoptive and autologous T cell transfer immunotherapies.

Targeting of DGKs could be useful for patients with HIV, which left untreated leads to chronic viremia, decreased CD4+ T cell counts, and impairment of the immune response to opportunistic pathogens. In the setting of HIV infection, cytotoxic T cells develop an exhausted state, characterized by decreased cytokine production and responses to antigen (Yi et al., 2010). While the vast majority of patients (termed "progressors") are unable to mount effective immune responses to HIV, a small cohort of patients (termed "elite controllers" or long term non-progressors (LTNP)) have productive cytolytic T cell responses to HIV infected CD4+ T cells (Migueles et al., 2000, 2008). Interestingly, pre-treatment of CD8+ T cells with the PMA and ionomycin has been shown to rescue the cytolytic ability of progressors' CD8+ T cells to nearly the same level of that of LTNP (Migueles et al., 2008). While increased NFAT translocation due the effects of ionomycin correlate with this rescue, the function of PMA in this phenomenon is unknown. As impairing the function of DGKs increases DAG levels, a study of the role of DGKs in cytolytic responses to HIV-infected cells would be interesting. If these studies are successful, autologous transfer of DGK-inhibitor treated T cells could be developed into new therapies for chronic infection.

Recently, autologous T cell transfer of chimeric antigen receptor (CAR)-transduced T cells was successfully used to treat patients with chronic lymphoid

leukemia (CLL) (Kalos et al., 2011; Porter et al., 2011). CARs consist of a high affinity antigen-binding extracellular domain, a transmembrane domain, and an intracellular signaling domain (Curran et al., 2012). The generation of productive signaling from CARs allows appropriate cellular activation, cytotoxicity, and persistence to specific tumor antigens, and how to induce productive signaling is a topic of much research (Curran et al., 2012). Although the signal transduction pathways that are activated upon CAR encounter with antigen are not well understood, deletion of DGKα and/or DGKζ has been shown to enhance cytokine and cytolytic responses to mesothelioma in CAR-transduced murine CD8+ T cells (Riese et al., 2013). Interestingly, DGKα-deficient and DGKζ-deficient CAR-transduced T cells displayed similar cytolysis of mesothelioma cells. These data stand in contrast to the dominant function of DGKζ described here and elsewhere (Olenchock et al., 2006; Gharbi et al., 2011). Similar experiments examining the cytolytic ability of DGKα and DGKζ in un-manipulated CD8+ may be useful. If results from these experiments reflect the dominant role of DGKζ described here, a difference in signaling machinery between CAR and the TCR would be suggested. For example, CAR signal transduction may have greater reliance on DAG-mediated signaling for its effects than TCR signaling. In this way, future experiments examining signal transduction of CAR versus the TCR may dovetail with investigation of DGKs in augmenting CAR function.

5.7. Conclusions

In this thesis, we define disparate functions of DGKα and DGKζ in nTreg development and TCR signaling and find that DGKζ functions dominantly due to its

greater effective enzymatic function and greater association with RasGRP1. We also describe preliminary data suggesting a role for PITPα and PITPβ in T cells. These findings have implications for the understanding of how DGKs, DAG, and TCR signaling are regulated, and how PIs may be involved in cellular processes. Although further investigation is needed, the future is bright for translating our understanding of DGK function to helping patients.

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