PIP5-KINASE DEPENDENT PIP2 GENERATION

IMPAIRS T CELL ACTIVATION BY RIGIDIFYING THE ACTIN COTEX

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DEDICATION

To my parents and my husband for their unyielding support

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Antigen-specific activation of T cells is initiated by the interaction of T cell receptors (TCRs) with the peptide-loaded MHC complex on antigen presenting cells (APCs). A specialized junction, the immunological synapse (IS), is formed at the T cell-APC interface, where cell surface receptors, signaling molecules as well as cytoskeletal elements rearrange into a highly organized structure (Monks et al., 1998; Grakoui et al., 1999; Cemerski et al., 2006; Fooksman et al., 2009). Dynamics actin and remodeling plays a pivotal role in IS formation and T cell activation (Wülfing et al. 1998, 2003; Dustin et al., 2000; Billadeau et al., 2007; Burkhardt et al., 2008; Gomez et al., 2008). It is believed that the activities of many actin regulatory proteins are spatially and temporally regulated by phosphatidylinositol 4,5-bisphosphate (PIP2) (Sechi et al, 2000; Yin et al., 2003; Logan et al., 2006). How actin dynamics is regulated by PIP2 in T cell activation is unclear.

Generation of PIP2 at the plasma membrane can be accomplished by the three isoforms of the type I phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks): α, β, and γ

(Ishihara et al., 1996; Loijens and Anderson, 1996; Oude Weernink et al., 2004). It is proposed that different PIP5K isoforms synthesize distinct pools of PIP2 in the plasma membrane and contribute to the versatility of PIP2 function (Kanaho et al., 2007; van den Bout et al., 2009). The roles of these PIP5Ks in T cell activation have not been addressed. The experiments in this thesis are designed to investigate how regulation of PIP2 levels by changing cellular PIP5K levels affects actin cytoskeletal dynamics at the IS and T cell activation.

The major findings of this thesis are: PIP5K isoforms are differentially targeted during T cell activation by APCs. PIP5Kα and γ87 are mainly targeted to the IS, whereas β and γ90 isoforms accumulate at the uropod or distal pole region of the T cells. Overexpression of PIP5Ks caused defective actin polymerization at the periphery of the IS and rigid T cell morphology, impaired TCR proximal signaling and decreased IL-2 secretion. Knockdown of PIP5Kγ instead induced sustained peripheral actin accumulation at the IS, enhanced T cell proximal signaling and IL-2 secretion. Furthermore, we found that the activity of the actin-binding ERM proteins was impaired in PIP5K overexpressing T cells. Taken together, we propose that PIP5K dependent PIP2 production inhibits ERM inactivation triggered by TCR stimulation, which leads to increased T cell rigidity, defective actin dynamics and T cell effector function.

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ABBREVIATIONS

PIP2:	phosphatidylinositol 4,5-bisphosphate
PIP5Ks:	phosphatidylinositol 4-phosphate 5-kinases
TCRs:	T celll receptors
MHC:	major histocompatibility complex
APC:	antigen presenting cell
cSMAC:	central superamolecular activation cluster
pSMAC:	peripheral superamolecular activation cluster
dSMAC:	distal superamolecular activation cluster
IS:	immunological synapse
TIRFM:	total internal reflection fluorescence microscopy
DPC:	distal pole complex
LAT:	Linker for Activation of T cells
WASp:	Wiskott Aldrich Syndrome protein
GEF:	guanine nucleotide exchange factor
ABPs:	actin-binding proteins
BMMCs:	Bone marrow derived macrophages
ZAP70:	ζ-chain-associated protein 70 kDa
ELISA:	Enzyme-linked immunosorbent assay
MCC:	moth cytochrome C
ITAMs:	immunoreceptor tyrosine-based activation motifs
Cyto D:	Cytochlasin D
Latr. B:	Latrunculin B
PIP3:	phosphatidylinositol (3, 4, 5)-triphosphate
siRNA:	small interfering RNA
shRNA:	small or short hairpin RNA
PLCγ :	phospholipase C gamma

Chapter I Literature Reviews

1. Molecular organization of the immunological synapse and its significance in T cell activation

The T cell plays a central role in immunity to infectious diseases, autoimmune diseases, cancer and transplantation. It fulfills its function by cytokine production (helper T cells) or killing of foreign antigen-bearing cells (cytotoxic T cells). T cell activation is initiated by interaction of T cell receptors (TCRs) on a T cell with the peptide-bearing MHC molecules on an antigen-presenting cell (APC). TCR ligation causes dramatic molecular reorganizations at the interface between a T cell and an APC, where cell surface receptors, downstream signaling molecules, as well as cytoskeletal components polarize toward the T cell-APC contact site, forming the immunological synapse (IS).

1). Discovery of the immunological synapse

The IS was first described by Abraham Kupfer and collegues in 1998 (Monks et al., 1998).The researchers used fluorescently-labeled antibodies and deconvolution microscopy to visualize the molecular organization at the interface between T cell-APC conjugates fixed after 5-20 minutes of interaction. The images revealed that the T cell receptors and some signaling molecules, such as PKCϴ, were preferentially localized to the center of the T cell-APC interface and were surrounded by a ring-like structure including the adhesion molecules Talin and LFA-1. Kupfer defined the center of the interface as the central superamolecular activation clusters (cSMAC), and the peripheral ring as the peripheral superamolecular activation clusters (pSMAC). Later a distal SMAC (dSMAC) was identified outside the pSMAC, where the phosphatase CD45 was enriched (Freiberg et al., 2002) (Figure 1-1). This distinct concentric molecular pattern at the contact site between the T cell and APC intrigued scientists and led them to ask if it is functionally relevant.

2). The roles of cSMAC in T cell activation

Early studies showed that TCRs and downstream signaling molecules were concentrated in the cSMAC region, thus the cSMAC was thought to be a site of signaling potentiation relevant for productive T cell activation (Monks et al., 1998; Grakoui et al., 1999). Subsequent findings challenged this hypothesis. Using T cells transduced with GFP labeled receptors and simultaneously loaded with Ca^{2+} dye, Krummel et al (2000)

found that the initiation of calcium signaling occurs very fast (within 1min) and was coincident with very small TCR clusters at the T cell-APC interface (Krummel et al., 2000). These clusters appear to form all over the contact site and are highly dynamic. After about 5-10 minutes, these TCR clusters coalesce into one larger cluster and move toward the center of the interface, resembling the cSMAC observed by Kupfer et al (1998). Notably, Ca^{2+} signaling peaks before the central TCR clustering (Krummel et al., 2000). In addition, it was found that the characteristic cSMAC was not always formed during T cell-APC interactions. Double positive thymocytes do not form a cSMAC but instead form multi-focal TCR clusters at the interface during positive selection (Hailman, 2002). Purtic and collegues found that central TCR accumulation was not required for T cell effector function in some TCR transgenic models, but was required for IL-2 secretion in a low-affinity/avidity 5c.c7 αβ TCR transgenic cells (Purtic et al., 2005). Collectively, these data suggest that formation of the cSMAC can be affected by many factors: the nature of the T cell and the APC, the antigen quality and the stimulation type, and the tissue context in which the interaction is taking place (Friedl et al., 2005; Jacobelli et al., 2004). Thus conclusions made on the functions of cSMAC should be confined to the specific system being studied. For example, total internal reflection fluorescence microscopy (TIRFM) revealed that the TCR microclusters were formed when T cells interacted with artificial planar lipid bilayers (used as surrogate APCs) containing MHC- and ICAM-embedded proteins (Varma et al 2006). These microclusters originate at the periphery of the IS and colocalize with signaling components (phosphorylated proteins). After formation, the microclusters move toward the cSMAC region, where they lose detectable signaling activity (Varma et al 2006). Thus, the TIRFM studies support the idea that productive signaling is extinguished in the center of the synapse. A recent study by Cemerski et al provided more interesting results (Cemerski et al., 2008). The authors investigated the IS formation in $CD4^+$ T cells interacting with either planar lipid bilayer or with APCs loaded with different concentrations of antigen. They found that weak antigenic stimuli triggered sustained protein tyrosine phosphorylation predominantly at the cSMAC region (as measured by anti-phosphotyrosine and phospho-ZAP70 staining) (Cemerski et al., 2008). When strong antigenic stimuli is applied, phosphotyrosine and pZAP-70 detection at the cSMAC region was weaker, likely due to high-rate TCR

turnover (Lee et al., 2003; Varma et al., 2006). Accordingly, at higher antigenic stimuli, inhibition of TCR downregulation by treatment with chlorpromazine (an inhibitor of Clathrin-mediated endocytosis) increased phosphotyrosine staining in the cSMAC. By combining other studies, the authors propose that the cSMAC could serve as an adaptive controller of T cell activation: enhancing weak signals and attenuating strong ones (Varma et al., 2006; Lee et al., 2003). In our system, we used the *in vitro* primed, primary TCR transgenic T cells and incubated them with professional APCs to mimic the physiological situations. We imaged the spatiotemporal accumulations of 30 GFP tagged signaling molecules in T cells activated by APCs. We found that several critical TCR proximal signaling intermediates, including ZAP-70, LAT, PKCθ, Itk, PLC-γ, Rac, and Rho rapidly (within seconds) accumulated to the center of the T cell-APC interface upon tight cell couple formation, and that the formation of this 'cSMAC signaling complex' was related to efficient T cell signaling (Singleton et al., 2009).

Live cell imaging of the dynamics of the signaling molecules at the T cell-APC interface clearly demonstrates that formation of the IS is a highly dynamic and orchestrated process. Characterizing the molecular segregation at specific locations and times during IS formation under both physiological and non-physiological conditions, and in combination with downstream cellular events, will undoubtedly help us better understand how signaling is organized in the synapse and translated into specific T cell responses.

2. The role of actin dynamics in IS formation and T cell activation

It is well known that T cell activation requires efficient reorganization of the actin cytoskeleton (Burkhardt et al., 2008; Gomez et al., 2008; Billadeau et al., 2007). Upon encountering an APC bearing specific antigens, the T cell undergoes dramatic changes in its morphology and cytoskeleton. The migration is arrested, the uropod is retracted, and the leading edge is flattened and wrapped around the surface of the APC (Negulescu et al., 1996; Dustin et al., 1997). In parallel with these changes, APC binding induces rapid accumulation of F-actin beneath the cell-cell contact area, initially forming an actin-rich patch, and then the actin clears from the center of the contact, converting this patch into an actin-rich ring at the periphery of the IS (Valitutti et al., 1995; Wulfing et al., 1998b; Bunnell et al., 2001). The relationship between actin dynamics and T-cell signaling is

reciprocally interdependent and complex (Sechi et al., 2004; Gomez et al., 2008). On the one hand, the initial TCR triggering and proximal signaling are required to induce actin remodeling and the formation of stable conjugates. On the other hand, actin remodeling helps to regulate the assembly of the signaling molecules and the formation of the immunological synapse, which may facilitate sustained signaling required for full T cell activation (Burkhardt et al., 2008; Billadeau et al., 2007).

Early studies which investigated the roles of actin cytoskeleton in T cell activation were initiated by using the commonly used actin-disrupting drugs such as Latrunculin A and Cytochlasin D (CytoD) (Henney et al., 1973; Valitutti et al., 1995). The two inhibitors function by inducing net actin depolymerization by preventing new actin polymerization, while having no effect on the existing polymerized actin filaments. More specifically, Latrunculin sequesters actin monomers, whereas Cyto D binds to the fast-growing (barbed) ends of the actin filaments, preventing monomer addition (Cooper et al., 1987; Yarmola et al., 2000).Though mechanistically not fully understood, Latrunclin or CytoD treatment severely impairs IS formation at T cell-APC interface, resulting in diminished calcium signaling, deficient IL-2 production and T cell proliferation (Holsinger et al., 1998; Campi et al., 2005; Billadeau et al., 2007).

Actin dynamics is regulated by constant filamentous assembly and disassembly, which involves a variety of actin-binding proteins and lipids, in which PH domain-mediated protein-protein and protein-lipid interactions may play key roles (Logan et al, 2006; Sechi et al., 2000; Burkhardt et al., 2008). More than 60 classes of actin-binding proteins are identified so far, they function to nucleate, bundle, sever, cross link, as well as sequester actin monomers. Recent studies on individual actin-regulatory proteins have greatly advanced our understanding of how actin dynamics is regulated and functions to affect T cell activation (Figure 1-2). The followings are examples of a few well-studied ones in T cell activation.

1). WASp

WASp is the best characterized actin regulator in T cells. It is named for its causative role in Wiskott Aldrich Syndrome (WAS), a severe X-linked immunodeficiency disorder (Notarangelo et al., 2003). T cells from WAS patients or WASp-deficient mice showed defects in TCR-induced actin polymerization at IS and IL-

2 production (Zhang et al., 1999). WASp is an Arp2/3 dependent actin-nucleation-promoting factor, which catalyzes the formation of a new actin filament as a branch on a preexisting filament (Higgs et al., 2001). The primary pathway for WASp activation downstream of TCR signaling involves formation of a signaling complex including TCR, Lck, ZAP-70, LAT, SLP-76, Itk and the Rho GEF, Vav1 (Zeng et al., 2003). The molecular interaction of the complex brings active Cdc42 into proximity with WASp, together with PIP2 binding, triggers a conformational change that allows the activation of the Arp2/3 complex (Higgs et al., 2000). However, some disagreements have emerged recently on the relative importance of WASp in T cell actin regulation. T cells from WASp-knockout mice showed normal actin organization and conjugate formation in response to APC stimulation, though IL-2 production was impaired (Cannon et al., 2004). In addition, Jurkat T cells depleted of WASp by RNA interference exhibited normal actin polymerization (Nolz et al., 2006). One explanation suggested for these discrepancies is that loss of WASp function may be partially compensated by WASp homologues such as N-WASp, WAVE2 and HS1 (Burkhardt et al., 2008).

2). The ERM protein family

The ERM protein family consists of three closely-related proteins, Ezrin, Radixin and Moesin. They function by crosslinking the cortical actin cytoskeleton to the plasma membrane (Allenspach et al., 2001; Delon, et al; Bretscher et al., 2002). ERM proteins possess an N-terminal FERM domain that interacts with the cytoplasmic tails of various cell-surface receptors, including CD43, CD44, ICAM-1 and ICAM-3, anchoring them directly to the cortical F-actin through their C-terminal Actin-binding region (Charrin and alcover, 2006). ERM proteins exist in an autoinhibited conformation, via direct interaction between the FERM and C-terminal domains. Activation of ERM protein requires the binding of the FERM domain to PIP2 and the phosphorylation of a conserved Threonine in the C-terminal domain (Fievet et al., 2004; Matsui et al., 1998; Simons et al., 1998). T lymphocytes express no Radixin, traces of Ezrin and abundant Moesin. In resting T cells, a pool of active ERM (pERM) proteins is present at the cell periphery (Faure et al., 2004; Allenspach et al., 2001; Delon et al., 2001). Upon TCR engagement, ERM proteins are rapidly inactivated by dephosphorylation, through a pathway involving Vav1- regulated Rac1 (Faure et al., 2004). As a result, ERM-mediated linkage between

the plasma membrane and F-actin is severed, leading to reduced cellular rigidity, increased cell-surface protein mobility and more efficient T cell-APC conjugation. In this regard, ERM proteins are shown to affect lipid raft dynamics and TCR clustering at IS (Ilani et al. 2007, Roumier et al 2001). Within minutes following antigenic stimulation, ERM proteins are re-activated by phosphorylation, relocating to and regulating the backward movement of the proposed negative regulators such as CD43, to the distal pole region of the T cell (Allenspach et al., 2001; Ilani et al., 2007). Ezrin and Moesin exhibited some none-overlapping features. For example, Ezrin showed transient localization to the IS before its movement to the distal pole, whereas Moesin localizes to the distal pole only. Ezrin and Moesin exhibited different tyrosine phosphorylation patterns upon T cell activation. However, they were shown to function together to promote T cell activation (Shaffer et al., 2009).

3). Cofilin

Cofilin is a ubiquitously expressed 19 kDa actin-binding protein, and member of the actin-depolymerizing factor (ADF) family of proteins that plays a critical role in regulating actin dynamics (Lee et al., 2000; Eibert et al., 2004). Engagement of the TCR together with coreceptors CD2 or CD28 results in Cofilin activation and its association with the actin cytoskeleton (Lee et al., 2000). Cofilin localizes to the periphery of the IS upon T cell-APC interaction. Peptides that block Cofilin binding to actin result in impaired immunological synapse formation and severe defects in T cell cytokine production and proliferation (Eibert et al., 2004). Cofilin activity is regulated through phosphorylation and dephosphorylation at Serine-3. Phosphorylated Cofilin is unable to bind to F-actin. Thus Cofilin is inactivated by phosphorylation and activated by dephosphorylation. In unstimulated resting $CD4^+$ T cells, Cofilin exists mainly as a phosphorylated inactive form. Costimulation of TCR/CD3 and CD28 induces dephosphorylation and activation of Cofilin (Ambach et al., 2000). Active Cofilin binds to actin and induces F-actin severing and depolymerization, which is critical for enhanced actin filament turnover and rapid remodeling of the actin cytoskeleton (DesMarais et al., 2003). The pathways controlling Cofilin activity during T-cell activation are not fully understood, but recent studies have revealed some key players. The direct upstream kinases that inactivate Cofilin are the LIM kinases. It has been found that a functional

RhoA/ROCK/LIM-kinase pathway is required for actin reorganization at the effector-target interface and the subsequent cytotoxic lymphocyte mediated killing (Lou et al., 2001). The Serine/Threonine Phosphatases type 1 (PP1) and type 2A (PP2A) associate with and dephosphorylate Cofilin in activated human T lymphocytes, thereby mediating Cofilin activation (Ambach et al., 2000; Lou et al., 2001). In addition, a recent report showed that the Ras-PI3K cascade functions as a central regulator of Cofilin dephosphorylation after costimulation through CD3 and CD28 in human peripheral blood T cells (Wabnitz et al., 2006). Like several other actin-binding proteins, Cofilin contains PIP2 binding motifs and its activation can be regulated by PIP2. Binding of PIP2 prevents association of Cofilin with F-actin and inhibits actin depolymerization (van Rheenen et al., 2007; Gorbatyuk et al., 2006).

In addition to actin-binding proteins, many signaling molecules that function downstream of the TCR are also involved in reorganization of the actin cytoskeleton. TCR stimulation promotes a rapid cascade of tyrosine phosphorylation (within seconds), recruitment and activation of several signaling molecules including the TCR, Lck, ZAP-70, LAT, SLP-76, Itk and Vav1 (Figure 1-3) (Smith-Garvin et al., 2009). Defects in any of these proteins may result in disruption of actin organization at the IS. One of the most important molecules among them is LAT (Linker for Activation of T cells), a 36kDa trans-membrane adapter protein (Zhang et al., 1998). Upon tyrosine phosphorylation (Tyr 171, Tyr 191) by ZAP-70, LAT serves as a docking site for downstream signaling proteins, including Grb2, Gads, PLCγ1 and PI3K. These together form a 'LAT signalosome', acting as a bridge between TCR proximal signaling and distal effector function (Wange, 2000; Wonerow et al, 2001; Burbach et al., 2007). LAT is essential for T cell activation. T cells deficient in LAT expression exhibited defective calcium signaling, ERK phosphorylation and activation of the IL-2 promoter (Martelli et al., 2000). LAT has been shown to be required for TCR-induced spreading and actin rearrangement at the IS (Bunnell et al., 2001). The mechanisms of the regulation of actin remodeling by LAT are not well characterized. One hypothesis is that, by recruiting Gads, LAT can in turn recruit another adapter protein SLP-76, which provides docking sites for the guanine nucleotide exchange factor (GEF) Vav1, and the actin-polymerization regulating proteins WASp (Barda-Saad et al, 2005). Another possibility is

that the recruitment and activation of PLCγ by LAT will result in hydrolysis and decrease of PIP2, which can affect the activity of many actin-binding proteins.

3. Regulation of actin cytoskeleton by PIP2 and PIP5Ks.

1). Regulation of actin cytoskeleton by PIP2

It is widely believed that the activity of many actin regulatory proteins is spatially and temporally regulated by PIP2, an important lipid signaling molecule residing on the inner leaflet of the plasma membrane (Figure 1-4) (Yin and Janmey 2003; Logan et al., 2006; Sechi et al., 2000). Though only 0.5-1% of total phospholipids on the plasma membrane, PIP2 is involved in multiple cellular signaling pathways. The well known function of PIP2 is its cleavage by PLCγ to produce the two second messengers: IP3 and DAG, which in turn initiate intracellular calcium release and PKC activation, respectively. Accumulating data in recent years suggest that PIP2 itself can act as a second messenger by binding to and regulating the activity of many proteins and enzymes involved in signal transduction pathways. Among them, regulation of the actin cytoskeleton by interactions with actin-binding proteins (ABPs) plays a critical role in PIP2-mediated processes. Early evidence indicating association between PIP2 and actin come from the work by Lassing and Lindberg, who demonstrated that PIP2 directly binds to Profilin, an actin-monomer-sequestering protein, and that this binding induces the release of actin monomer from the Profilin-actin complex (Lassing and Lindberg, 1988). Subsequent biochemical data revealed more PIP2-ABP regulations, e.g., PIP2 binds to Cofilin and Gelsolin and inhibits their actin severing activity; PIP2 causes uncapping of actin filament barbed ends by binding to actin capping proteins; PIP2 can both enhance or reduce actin crosslinking activity depending on its association with α-Actinin or Filamin; PIP2 also induces the conformational change and activation of the ERM proteins, strengthening the linkage between actin filament and the plasma membrane. Structural studies showed that putative PIP2-binding sites of many ABPs are overlapping with the sites that are important for F-actin binding, and therefore in general, lipid binding would prevent actin binding (Sechi and Wehland, 2000). In support of *in vitro* studies, *in vivo* data also provide compelling evidence of intimate relationship between PIP2 and actin dynamics. Firstly, PIP2 affects the structure of actin filaments in the cortical cytoskeleton. Lowering PIP2 levels by either microinjection of PIP2 antibodies or

overexpression of the PIP2 hydrolyzing enzyme Synaptojanin in cells causes decreased membrane ruffling and disorganization of actin stress fibers (Gilmore and Burridge, 1996; Shibasaki et al., 1997). In contrast, increasing PIP2 levels by overexpression of the PIP2-synthesizing enzyme phosphatidylinositol-4-phosphate 5-kinase (PIP5K) promotes extensive actin polymerization and formation of microvilli. Secondly, PIP2 affects membrane-cytoskeleton attachment. Sequestration of PIP2 by either overexpression of the PH domain of Phospholipase C-δ that binds to PIP2, or overexpression of plasma targeted polyphosphate 5-phosphatase (Inp54) that selectively deplete 60% of membrane PIP2, induce an about three-fold decrease in cytoskeleton–membrane adhesion. At the same time, pharmacological agents that interfere with DAG and Ca^{2+} signaling pathways had little to no effect on membrane tether formation. These results strongly suggest that the observed effect is due specifically to decreased PIP2 itself rather than changes in second messenger levels (Raucher et al., 2000). Thirdly, by fluorescence and DIC imaging, the PIP2 marker, EGFP tagged PH domain of PLCδ fusion protein is found to be concentrated in highly dynamic F-actin rich regions of the plasma membrane, and that modulation of the plasma PIP2 microdomains by GMC-like proteins regulates cell cortex actin dynamics (Laux et al., 2000).

In summary, PIP2 regulation of actin dynamics is widely recognized, however the diverse actin regulators regulated by PIP2 and their different effects on actin cytoskeleton remodeling make the mechanisms of PIP2-actin regulation in a living cell as a whole under physiological or pathological conditions far from clear. Understanding how PIP2 levels are spatially and temporally regulated by a particular enzyme/protein within a cell, and how the regulations correlate with changes in actin dynamics will help define the underlying mechanisms.

2) Regulation of the actin cytoskeleton by PIP5Ks.

The major biosynthetic pathway for PIP2 production in mammalian cells is regulated by PIP5 kinases. Three PIP5K isoforms encoded from three different genes have been identified in mammalian cells: α, β, and γ. The γ isoform also consists of several splicing variants: γ87, γ90 and γ93. γ90 differs from γ87 in a 26 amino acid extension at its C-terminus. The γ93 isoform is similar to γ90, but has an extra 26 aa inserted 26 residues from the C-terminus. γ93 is the most recently discovered one and its

function is not clear (Kanaho et al., 2007; Mao and Yin, 2007). Though the three PIP5K isoforms share a highly homologous kinase core domain (80% homology), they differ greatly in their N- and C- terminus (Figure 1-5). These structural differences confer them distinct tissue distributions and subcellular localizations, as well as unique cellular functions. Here I summarize the literature investigations on their roles in cellular signaling pathways, with an emphasis on their regulation of the actin cytoskeleton. Originally the human and mouse PIP5Ks nomenclature was reversed (e.g., human α equals mouse β, and human β equals mouse α); to reduce confusion, I will use the human nomenclature in this thesis unless specified.

1) PIP5Kα

The PIP5Kα gene encodes a protein of 549 aa, with a molecular weight of ~68 kDa. Similar to PIP5Kβ, the PIP5Kα isoform has a wide tissue distribution (Loijens and Anderson 1996, Ishihara et al., 1996).

Endogenous PIPKα was detected at membrane ruffles, in the cytosol, and within the nucleus in MG-63 fibroblast cells. Upon PDGF stimulation, PIPKα translocates to membrane ruffles, which requires coordinated activation of Rac1 (Doughman, 2003). PIP5Kα was also found to localize transiently at the site of phagocytosis. Overexpression of a kinase dead mutant of PIP5Kα impairs PIP2 and F-actin accumulation at the phagocytic cup, suppressing phagocytosis (Coppolino et al., 2002). Mao et al. further demonstrated that PIP5Kα promotes particle ingestion by activating WASp to induce Arp2/3 mediated actin polymerization at the phagocytic cup (Mao et al., 2009). PIP5Kα is also shown to regulate membrane traffic. Overexpression of PIP5Kα (murine β) in 293T cells resulted in accumulation of large vesicles and disrupted endosomal trafficking (Galiano et al., 2002). Recent immunoelectron microscopy studies revealed that PIP5Kα and PIP2 are located in electron-dense structures surrounding activated FcγRIIA in U937 monocytes, suggesting the involvement of PIP5Kα in raft-based FcγRIIA signaling (Szymańska et al., 2009).

Platelets lacking PIP5Kα (murine β) exhibited impaired aggregation. Though these platelets had only a modest decrease of PIP2 in the resting state, PIP2 synthesis was significantly impaired after agonist stimulation (Wang et al., 2008a). This impaired synthesis of PIP2 resulted in a marked defect in IP3 production. Platelets lacking both

PIP5Kα and β isoform have a total loss of IP3 formation after thrombin stimulation, though these platelets still have PIP5Kγ, the most abundant PIP5K isoform in platelets. Taken together, these data suggest that in GPCR stimulated platelets, PIP5Kα and β contribute to a rapidly synthesized pool of PIP2 that allows for IP3 production (Wang et al., 2008a). Together with the results from another study, in Megakaryocytes, PIP5Kγ was shown to contribute to a distinct pool of PIP2 required for stable membrane-cytoskeleton association (Wang et al., 2008b).

2) PIP5Kβ

PIP5Kβ is a ubiquitously expressed protein, containing 540 amino acids, with an overall molecular weight ~68KDa. Endogenous PIP5Kβ was found at cytosolic vesicular structures that are around the nuclei in MG-63 human osteosarcoma fibroblasts (Doughman et al., 2003).

Overexpression of PIP5Kβ has been found to induce dramatic actin reorganization through interactions with small Rho GTPases in different cell types. However, the actin phenotypes differ greatly among cell types. For example, overexpression of wt PIP5Kβ in COS-7 cells increased the amount of F-actin, with decreased stress fibers and lamellipodia formation, but increased short actin fibers resembling "pine needles" (Shibasaki et al., 1997). Overexpression of the kinase-dead PIP5Kβ did not induce such an effect. Dominant-negative RhoA overexpression alone resulted in decreased actin fibers while it failed to do so when PIP5Kβ was overexpressed, suggesting that PIP5Kβ may function downstream of Rho-mediated actin regulation (Shibasaki, 1997). PIP5Kβ-overexpression in NIH3T3 cells did not induce the stress fibers or focal contact formation, but stimulated the activation of ERM proteins and their recruitment to plasma membranes to form microvilli, a Rho regulated actin phenotype. In addition, immunoblotting of lysates from Hela cells overexpressing PIP5Kβ revealed a ~6 fold increase of phosphorylated ERM proteins as compared to controls (Matsui, 1999). In CV1 cells, overexpression of PIP5Kβ induced actin stress fiber formation and inhibited membrane ruffling, a Rac regulated process. Inhibition of ruffling is likely due to inhibition of Gelsolin, Capping protein, and Profilin by increased PIP2 production (Yamamoto et al., 2001). Why does overexpression of one PIP5K produce so many different actin phenotypes? Small GTPases of the Rho family Rac, Rho, Cdc42 have been

shown to regulate the formation of distinct actin filament-based structures. RhoA plays a central role in induction of stress fiber formation and focal adhesions. Rac stimulates actin filament accumulation at the plasma membrane, and is responsible for membrane ruffles and lamellipodia formation. Cdc42 controls the formation of filipodia (Hall, 1998). Yamamoto et al suggest that different cellular backgrounds may favor a unique combination of actin regulators (Rho vs. Rac vs. Cdc42), and the reciprocal dominance of one signaling pathway over another can lead to the generation of distinct actin structures in different cell types (Yamamoto et al., 2001).

The first genetic evidence for a physiological role of PIP5Kβ *in vivo* comes from the research by Sasaki et al., who generated the PIP5Kα-deficient (mouse-PIP5Kα represents human PIP5Kβ) mice. These mice are viable, fertile and have no observable histological abnormalities despite the wide tissue distributions of mouse PIP5Kα (Sasaki et al., 2005). Bone marrow derived mast cells (BMMCs) from PIP5Kα-/- mice showed normal expression of the other two PIP5Ks, PIP5Kβ and PIP5Kγ. However mouse-PIP5Kα-/- BMMCs exhibited enhanced degranulation, intracellular calcium signaling, cytokine production and hyperphosphorylation of several signaling proteins in response to FCεRI stimulation. Moreover, anaphylactic responses are enhanced in mice deficient for mouse-PIP5Kα. BMMCs lacking mouse-PIP5Kα showed statistically significant decrease of PIP2 content, while surprisingly, increased production of IP3 and PIP3. In contrast, F-actin content in mouse PIP5Kα-/α- BMMCs was consistently decreased to 70-80% of the wt level, suggesting an involvement of actin in causing the mouse-PIP5Kα-/α- phenotype. In support of this, WT BMMCs pretreated with Latrunculin showed enhanced degranulation in response to FCεRI stimulation. However, Latrunculin treatment did not enhance cytokine production or phosphorylation of signaling molecules, nor did it lead to elevated intracellular calcium, suggesting factors other than the actin cytoskeleton are involved. Finally, the authors found that the presence of FCεRIγ in the lipid raft fraction was significantly enhanced in mouse-PIP5Kα-/α- BMMCs even before aggregation. Collectively, these authors suggest that mouse PIP5Kα acts as a negative regulator of FCεRI-mediated anaphylactic responses, which functions by controlling both the actin cytoskeleton and the dynamics of FCεRI signaling (Sasaki et al., 2005).

PIP5Kβ was also shown to regulate membrane trafficking. siRNA specific knockdown of PIP5Kβ, but not of PIP5Kα or γ, affected the pool of PIP2 involved in constitutive receptor-mediated endocytosis in CV-1 and Hela cells. Drugs that disrupt the actin cytoskeleton did antagonize increased endocytosis observed in cells overexpressing PIP5Kβ (Padrón et al 2003).

A recent study revealed another function of PIP5Kβ in neutrophil polarity. PIP5Kβ was found to localize to the uropod of polarized neutrophil-differentiated HL60 cells. The localization was independent of its kinase activity, but required the 83 C-terminal amino acids, which interacts with EBP50, and indirectly with ERM proteins and RhoGDI. The polarized localization of PIP5Kβ depends on G12/G13 signaling but not RhoA signaling. PIP5Kβ-WT overexpression did not increase total PIP2 level. The GFP-PH-PLCδ1 accumulated to both the leading edge and the uropod of polarized mock and PIP5Kβ-WT overexpressing cells. In addition, knockdown of PIP5Kβ by RNAi prevented the neutrophil polarity and chemotaxis (Lacalle et al., 2007).

3) PIP5Kγ

Two groups independently made the PIP5Kγ knockout mice. In one study, the PIP5Kγ-null mice died soon after birth. There was a 40% reduction in PIP2 levels in the brain of PIP5Kγ-/- animals, and several defects found in neuronal synaptic transmission. In the other study, the PIP5Kγ-/-mice died prenatally at embryonic day 11.5 of development. Loss of PIP5Kγ caused widespread developmental and cellular defects, including cardiovascular and neurological development (Di Paolo et al., 2004; Wang et al., 2007).

There are at least five PIPKγ splice variants have been identified in mammals, γ87 (635aa), γ90 (661aa), γ93 (687aa), PIPKIgamma_i4 (700aa) and PIPKIgamma_i5 (707aa) (Schill et al., 2009). The last three γ splice variants are newly discovered ones and their functions are not characterized yet. I will focus on γ87 and γ90 isoforms in this thesis. γ87 and γ90 migrate at 87 and 90 kDa on a SDS-PAGE gel, respectively. They differ only in the C-terminal 26 amino acid extension in γ90, whereas they showed distinct cellular localization and functions. PIP5Kγ87 is more abundantly expressed than PIP5Kγ90 in most cells, whereas PIP5Kγ90 is mostly enriched in the brain and focal adhesions (Wenk et al., 2001; Di Paolo et al., 2002; Wang et al., 2004). Literature studies

on γ87-specific functions are limited, likely due to its mild effect on cellular function when overexpressed or knocked down. In one study, γ87 was shown to be involved in Phospholipase D2 stimulated Integrin-mediated adhesion in HeLa cells (Powner et al., 2005). In another study, γ87 was found to be the major supplier to the PIP2 pool that mediates GPCR-mediated Ca^{2+} signaling (Wang et al., 2004). It is shown that although RNAi mediated knockdown of both γ87 and γ90 decrease total cellular PIP2 by less than 15%, it blocks histamine-induced IP3 production by ~70%, with diminished Ca^{2+} signaling. In contrast, knockdown of γ90 alone, or of α or β had minimal effect. In addition, both PIP5Kγ87 and PIP5Kβ RNAi decreased plasma membrane PIP2 significantly, as detected by two independent methods, single cell fluorescence imaging using overexpressed GFP-PLCδ-PH and anti-PIP2 staining. However, only the former suppressed GPCR-mediated IP_3/Ca^{2+} signaling, suggesting that the plasma membrane PIP2 pools generated by γ87 and β may be functionally compartmentalized (Wang et al., 2004). In support of this 'compartmentalized PIP2' hypothesis, one study demonstrated that the PIP5Kβ and γ regulate distinct stages of Ca2+ signaling in mast cells (Vasudevan et al., 2009). PIP5Kγ contributes to the pool of PIP2 that is hydrolyzed by PLC to mediate Ca^{2+} release from ER stores in BMMCs, whereas PIP2 generated by PIP5Kβ negatively regulates SOCE (store-operated calcium entry) on the plasma membrane, possibly by negatively regulating the gating of the Orai1 Ca^{2+} channel or other channels. The authors propose that different lipid raft localization of the two PIP5K isoforms may be responsible for their distinct effects (Vasudevan et al., 2009). A more recent study showed that though both PIP5K γ and α were recruited to the phagocytic cup during FcγR mediated phagocytosis, they regulate different phases of the process (Mao et al., 2009). PIP5K γ-/- bone marrow derived macrophages showed a dramatic increase (more than 50%) of polymerized actin and were defective in FcγR microclustering and particle attachment. Delivery of PIP2 or low dose Latrunculin B treatment restored particle attachement to PIP5K γ-/- BMMs. In contrast, PIP5Kα-/- BMMs exhibited impaired actin polymerization at the phagocytic cup and were defective in particle ingestion. In addition, γ87 was transiently tyrosine phosphorylated (activated) by Syk during phagocytosis, while PIP5K α was not (Mao et al., 2009). Mechanistically, the authors demonstrated that lack of PIP5Kγ altered RhoA/Rac1 balance, favoring the dominant activation of RhoA,

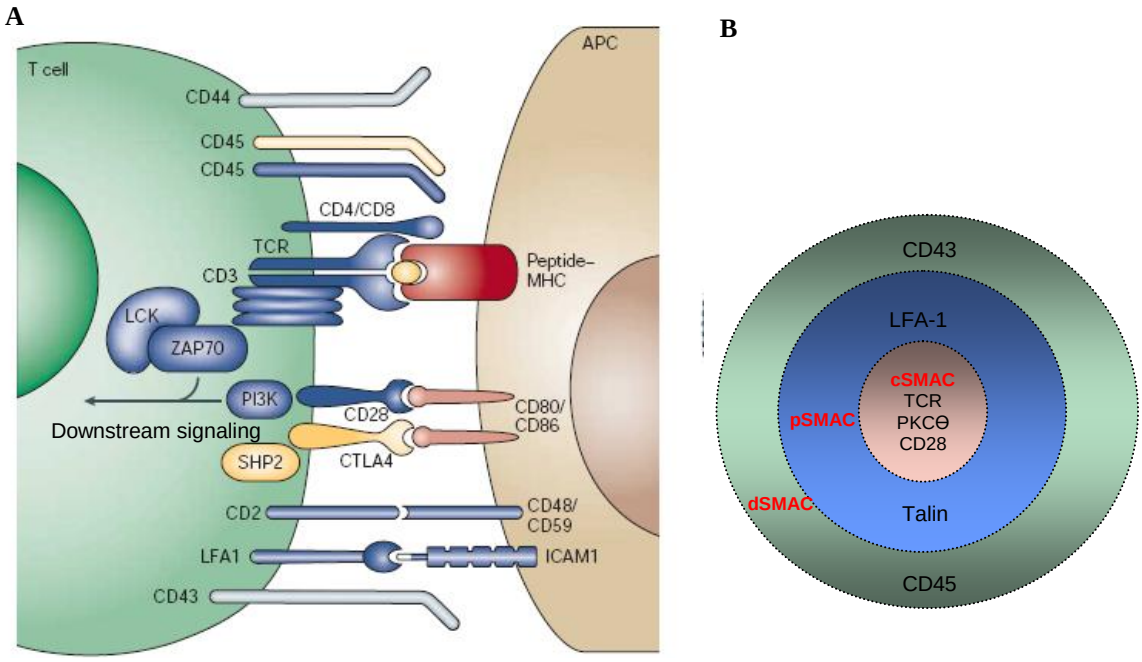
which results in enhanced actin polymerization and defective receptor clustering and particle attachment. On the other hand, lack of PIP5Kα reduced PIP2 production at the nascent phagocytic cup, preventing activation of WASp to induce *de novo* Arp2/3 mediated actin nucleation, therefore, particle ingestion was inhibited. As the two PIP5K isoforms showed different access to Syk, it is proposed that γ87 may be preferentially recruited to the raft microdomains (Mao et al., 2009).

The PIP5Kγ90 isoform is predominantly localized to the neuronal synapse and focal adhesions. It is implicated in the regulation of synaptic transmission, membrane trafficking, actin dynamics and focal adhesion formation (Wenk et al., 2001; Di Paolo et al., 2002). PIP2 plays a critical role in Clathrin-regulated endocytosis. Accumulating data suggest that γ90 is involved in this PIP2 regulated process. It is found that the C terminal tail of γ90 directly interacts with the β2 subunit of the AP-2 protein of the Clathrin-coated complex (Nakano-Kobayashi et al., 2007). This interaction allows for preferential targeting of the kinase to the site of Clathrin coat assembly and the production of PIP2 locally to regulate endocytosis. The γ90-AP2 interaction was also found to regulate E-Cadherin trafficking and adhesion junction assembly, implicating a potential role for PIP5K γ90 in epithelial tumor progression (Ling et al., 2007). Talin interacts with the same C terminal 26 aa of γ90 as AP-2, through its FERM domain. Binding of talin targets γ90 to focal adhesions and activates it to produce PIP2 locally, regulating the focal adhesion dynamics (Di Paolo et al., 2002).

Lokuta et al recently found that γ90 was enriched in the uropod during chemotaxis of primary neutrophils and dHL-60 cells. Overexpression of a kinase-dead γ90 compromised uropod formation and the rear retraction in dHL-60 cells, revealing a previous unknown function of γ90 as a novel uropod component. This finding is reminiscent of the similar findings for the β isoform in controlling neutrophil polarity. Why these two PIP5K isoforms, with no similarity in their N or C terminus, preferentially regulate uropod function is of interest to know.

In summary, PIP2 is a highly versatile molecule regulating various signal transduction pathways and cellular events. One of the plausible explanation for its multifunction is that PIP2 is produced at different times and locations by different PIP5K isoforms in response to different environmental stimulations. Further characterization and

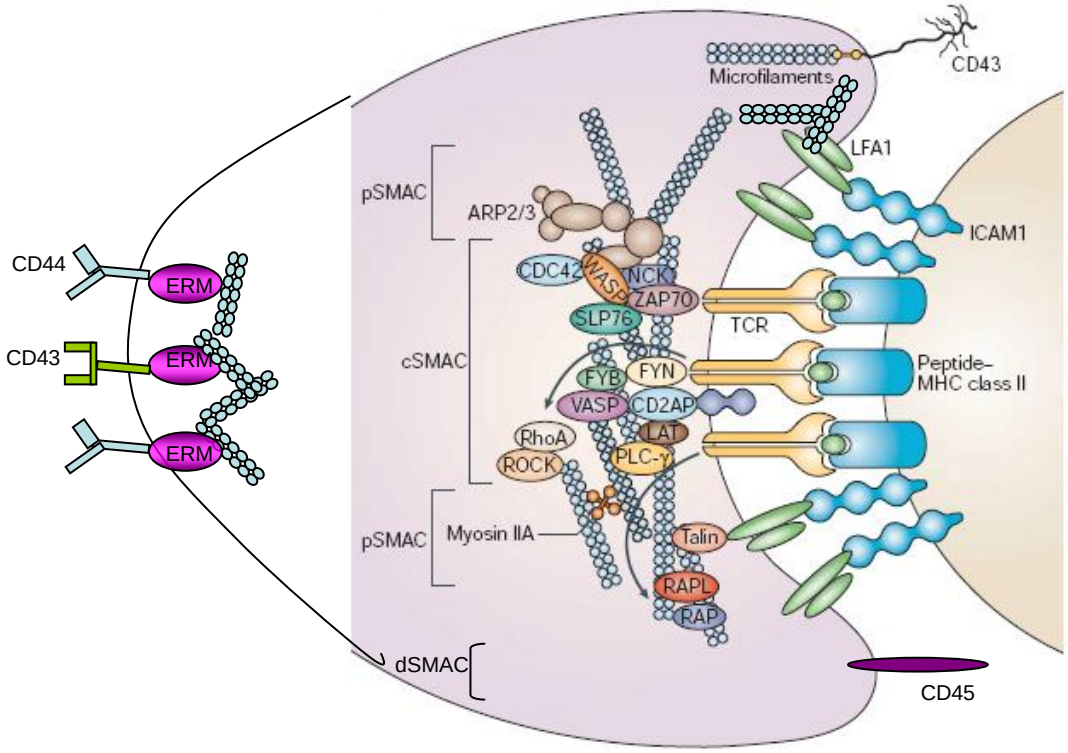
comparison of PIP2 regulation by individual PIP5K isoform in different cellular contexts remains to be done to elucidate the complex signaling pathways that PIP2 is involved in.



Immunological synapse

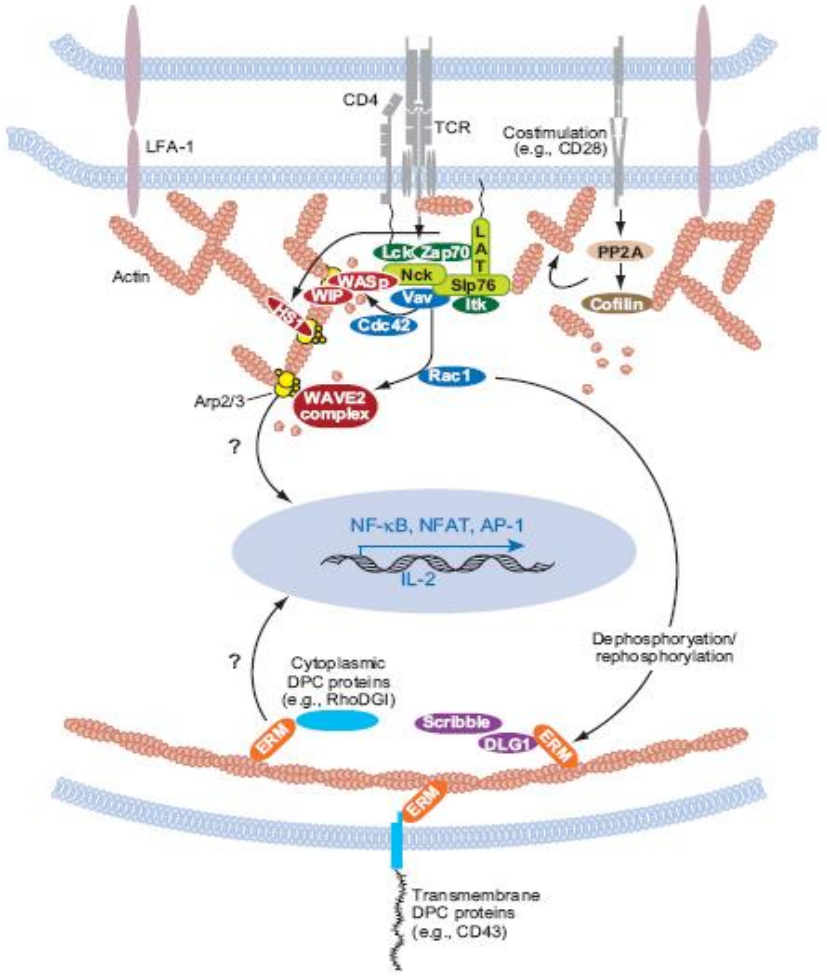
Modified from Davis et al, Nature Reviews Immunology, 2003

Figure 1-1. Overview of a mature immunological synapse. A.The side view of a mature immunological synapse formed between a T cell and an antigen presenting cell. A selection of the receptors and signaling molecules that are involved in T-cell activation is shown. B. The En face view of the synapse (T cell side) with the characteristic concentric ring structure, including the central supramolecular activation cluster (cSMAC) (pink), the peripheral supramolecular activation cluster (pSMAC, blue) and the distal supramolecular activation cluster that is outside the pSMAC (dSMAC, green)



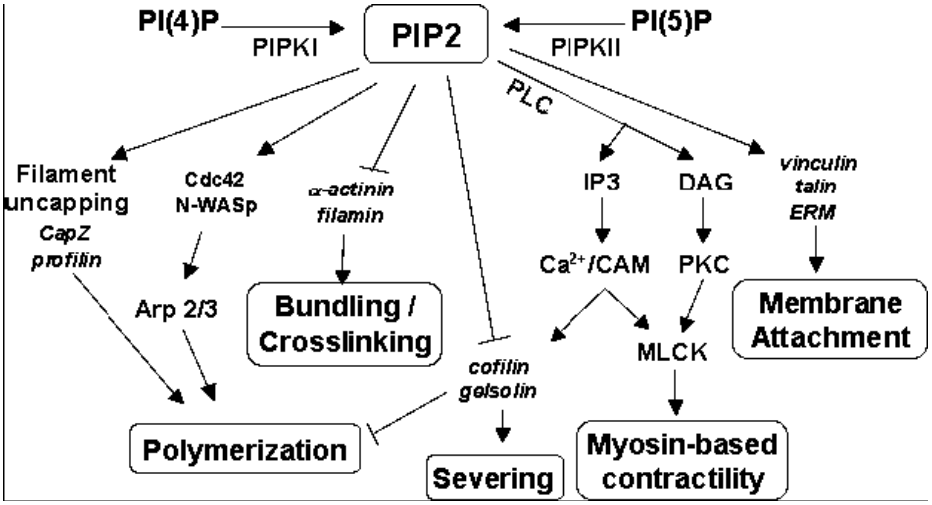
Modified from Sánchez-Madrid et al., Nature Reviews Immunology, 2004

Figure 1-2 Regulation of immunological synapse formation by TCR-mediated actin reorganization. TCR triggering induces recruitment and phosphorylation of a variety of signaling proteins, such as Fyn, Lck, ZAP70 (ζ-chain-associated protein 70 kDa), the scaffolding proteins LAT and SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa), as well as actin regulatory protiens WASP (Wiskott-Aldrich syndrome protein). WASP together with small Rho GTPases Cdc42 regulate Arp2/3 mediated actin nucleation and polymerization. Costimulation of TCR leads to activation of cofilin, which depolymerize and sever actin filaments, creating new barbed ends for filament growth. TCR ligation also leads to rapid inactivation of the ERM proteins, resulting in disanchorage of the cortical actin cytoskeleton from the plasma membrane and promoting the molecular rearrangement at immunological synapse. The reactivation of ERM proteins shortly after is believed to direct the movement of negative regulators such as CD43 to the distal pole of the T cells.



From Burkhardt et al., Annul Review of Immunology, 2008

Figure 1-3. Simplified model of signaling pathways linking TCR signaling to actin remodeling. Encounter of an APC induces a signaling cascade comprised of tyrosine kinases (*dark green*), adaptor proteins (*light green*), and immediate upstream actin-regulatory proteins (*dark blue*). The latter transduce signals to several nucleation-promoting factors (*red*) that direct the polymerization of branched actin filaments at sites of TCR engagement. Engagement of costimulatory molecules leads to activation of proteins that sever actin filaments (*dark brown*), creating new barbed ends as substrates for filament growth. Signals emanating from the TCR also lead to transient dephosphorylation and rephosphorylation of ERM proteins (*orange*), which direct the localization of transmembrane and cytoplasmic proteins (*light blue*) to cortical actin filaments at the distal pole complex (DPC). Also localized to the DPC are proteins containing PDZ (PSD-95/Discs large/ZO-1) domains (*purple*), which form a network at this site. Question marks indicate the unknown mechanisms by which actin remodeling leads to IL-2 promoter activation and other changes in gene expression (Burkhardt et al., 2008)



From Logan and Mandato, Biol Cell, 2006

Figure 1-4. Regulation of actin remodeling by PIP2. PIP2 plays a key role in re-structuring the actin cytoskeleton in several ways. (i) PIP2 promotes actin polymerization by either activating N-WASp/Arp2/3-mediated actin branching, or impairing the activity of actin-severing proteins, suchas Gelsolin and Cofilin, or uncapping actin filaments for the addition on new actin monomers. (ii) PIP2-mediated actin polymerization is counteracted by its hydrolysis to IP3. IP3-mediated activation of Ca2+/CaM promotes actin severing by inhibiting Gelsolin or Cofilin function. (iii) PIP2 also modulates the function of several actin cross-linking and regulatory proteins which are critical for the assembly of stress fibres, focal adhesions and membrane attachment (Logan and Mandato. 2006)

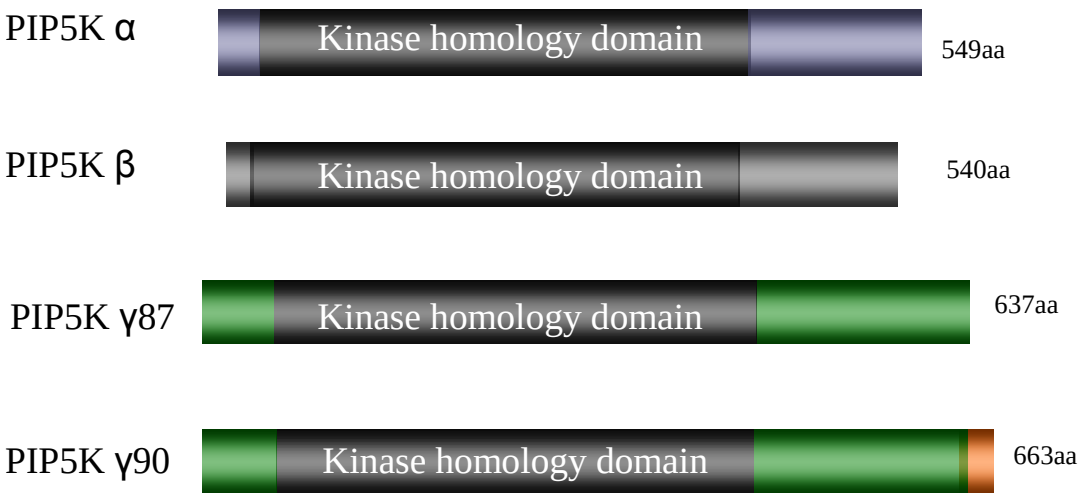


Figure 1-5. Domain structures of human PIP5K isoforms. The three PIP5K isoforms α, β, and γ share a highly homologous kinase domain (80% homology). There is little conservation at their N and C terminal domains. The two γ isoforms differ only in the 26 amino acids at the C terminus of γ90.

Chapter II Materials and Methods

Cells, reagents and antibodies

In vitro primed, primary T cells from 5c.c7 TCR transgenic mice (Seder et al., 1992) were used in all experiments. The use of these mice has been reviewed and approved by the UT Southwestern Institutional Animal Care and Use Committee. As APCs, CH27 (H-2^a, I-E^k) B cell line was used. Agonist moth cytochrome C peptide (MCC88-103, ANERADLIAYLQADK) was used as a specific antigen for T cell activation. The ecotropic virus packaging line Phoenix E cells was a gift from G. Nolan, Stanford University. Latrunculin B was purchased from Invitrogen. Alexa Fluor 594 phalloidin was purchased from Molecular Probes. The following antibodies were used: Rabbit polyclonal antibodies against mouse phospho-LAT Y191, phospho-PLC-γ Y783, phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) (Cell Signaling, Danvers, MA); HRP conjugated anti-GAPDH anti-mouse Abs (Abcam Inc., Cambridge, MA). Rabbit polyclonal anti-panPIP5Kγ antibodies and mouse monoclonal anti-actin antibodies were kindly provided by the laboratory of Dr. Helen Yin (University of Texas Southwestern Medical center, Dallas, TX).

Retroviral transduction

For virus production, the Phoenix E cells at a confluency of approximately 75% were transfected with the retroviral plasmids encoding different EGFP-tagged signaling molecules using calcium phosphate precipitation methods. Medium was changed the next day and the cells were cultured for additional 48 h to allow for maximal viral production and immediately used after collection.

At 24 h before infection (day 0), lymphocytes were isolated from lymph nodes of the 5c.c7 αβ TCR transgenic mice and stimulated in 24-well plates in the presence of 3 μM MCC peptide at a density of 4×10^6 cells per well and per mL of complete medium (RPMI with L-glutamine (Mediatech, Inc) containing 10% FCS (Hyclone),100 U/ml penicillin/streptomycin (Hyclone), 50 μM β-mercaptoethanol (Sigma-Aldrich). On day 1, viral supernatants were collected and added to the overnight primed 5c.c7 lymphocytes at a concentration of 2ml per well per 4 million cells. The mixture was then added with 10 μg/ml protamine and centrifuged in 24-plates at 2,500r.p.m at 32 °C for 2h. After the spinduction, the cells were incubated at 37°C. On day 2, viral supernatants were replaced

with 2ml of fresh complete medium supplemented with 100U/ml IL-2. On day 5, GFP positive cells were sorted by FACS such that only those cells that are one log away from the negative cells and within a fivefold range of low abundance of GFP, which matched the high end of microscope sensitivity, were collected. The sorted cells were maintained in IL-2 medium and used for imaging within the next 48 h. The CH27 B lymphoma cell line was maintained in complete RPMI 1640 medium. To stimulate 5c.c7 T cells, CH27 cells were preincubated with 10uM MCC peptide for at least 4h (< 24h) before use.

Retroviral constructs

The retroviral expression vectors used were derived from the Moloney murine leukemia virus and have full-length Moloney long-terminal repeats, an extended Ψ sequence and an internal ribosomal entry site (IRES). The cDNAs encoding GFP tagged PIP5Kα, β, γ87, γ87-D316K or γ90 were obtained from the Yin laboratory at UTSW and subcloned into the retroviral vector. To make the dual expression constructs, cDNA encoding GFP-PH-PLCδ1, GFP-actin or GFP-TCRzeta were first cloned into the retroviral vector after the IRES site, and then individual cDNA encoding PIP5K isoform was inserted in front of the IRES site.

To make the PIP5Kγ-shRNA-EGFP construct, 4 different shRNA duplexes against the PIP5Kγ gene were first generated by the online shRNA designer (Dharmocon) and synthesized by integrated DNA technologies. The 4 synthesized shRNA duplexes were then ligated into the retroviral vector behind a CMV promoter followed by a GFP selective marker. To test the knockdown efficiency of PIP5Kγ by each γ-shRNA duplex, the 4 shRNAs were individually transfected into NIH3T3 cells. 48 or 96h later, the cells were lysed and the PIP5Kγ protein level was detected by the anti- PIP5Kγ antibody. We found that the 3331 γ-shRNA gave the best knockdown results and it consistently led to more than 70% knockdown of the endogenous PIP5Kγ protein. The 3331 γ-shRNA was then used in all knockdown experiments. The shRNA sequence used were: forward, 5'-TGAGCGCTCCCAGCACTTGGAAAGTTAATAGTGAAGCCACAGATGTATTAACTTTCCAAGTGCTGGGAT -3', and reverse, 5'- GTGGCTTCACTATTAACTTTCCAAGTGCTGGGAGGGCAATCCCAGCACTTGGAAAGTTAATACATCT-3'

Live cell imaging and image analysis

The imaging system is based on a Zeiss Axiovert S100TV microscope equipped with a 175-W Xenon source (Sutter Instruments, Novato, CA). The temperature was controlled at 37 °C by air heating. The T cell-APC interactions were imaged in imaging buffer (PBS with 10% FBS, 1mM CaCl2 and 0.5mM MgCl2) with a 40 x Fluar /NA = 1.3 oil objective.

To hold optimal numbers of T cells and APCs for imaging, either a 384-well or a 1536-well glass-bottom plate was used, depending on the numbers of sorted GFP+ T cells. For imaging with a 384 well plate, 50 µl of imaging buffer were added first to each well, then ~5µl of ~20,000 B cells were added slowly and allowed to settle onto the bottom of the well before the addition of 5 µl of ~15,000 T cells. To image with 1536-well plate, 5µl of 1000-2500 T cells were added first to each well, followed by slow addition of 1 µl of ~6000 B cells. Immediately upon the T or B cells settling at the bottom of the well, the microscopic images were acquired. For each of the 15-min experiments, every 20 seconds1 DIC and 21 GFP images spaced 1µm in z-stacks covering the entire cell were collected. For analysis, three-dimensional reconstructions were made and analyzed with the Metamorph software package.

Cell couple formation was determined in DIC images. For every T cell in contact with an APC for ≥ 6 frames (i.e. > 90 sec) it was determined, whether a tight cell couple formed or not. For each condition, more than 50 cell couples were analyzed. The time point zero of a tight cell couple formation was defined as either the first time point with a maximally spread T cell/APC interface, or 40 s after the first T cell-APC contact, whichever occurred first (Singleton et al, 2009).

Accumulation patterns of signaling molecules at the interface were determined with the Metamorph software package. Data used for this analysis were a maximum projection for the identification of the x and y coordinates of an area of accumulation and maximum-type three-dimensional reconstructions for the determination of the z coordinates. Fluorescence intensities were displayed as a pseudo-color image such that the minimal GFP fluorescence is black, maximal is red, and intermediate shown as increasing from blue through green to yellow. A region of accumulation of a given protein was defined as a region with an average

fluorescence intensity of at least > 135% of the background cellular fluorescence and scaled that only the red color was counted as accumulation. Twelve time points were analyzed for the accumulation in each cell couple, from -40 sec to 2 min in 20 sec intervals, and then at 3 min, 5 min, and 7 min. The following interface accumulation patterns were recorded: central, diffuse, peripheral, asymmetric, lamella, any interface and distal, as defined in reference (Singleton et al, 2009.Figure 3-2). Distal accumulation was scored separately from the interface accumulation, as a T cell could display both interface and distal accumulation at the same time point. For accumulations of each protein, more than 25 cell couples were analyzed. The percentage of cell couples with a particular accumulation was then calculated at each time point and plotted as a function of time. Standard errors were calculated.

The statistical significance of differences between conditions that were analyzed as a percentage of cell couples with a particular phenotype was determined using a two-sample proportion *z* test

Quantitative PCR

Total RNA was extracted from DO11.10 T cell blasts and reverse transcribed to generate cDNA for PCR in an ABI Prism 7000 sequence detection system (Applied Biosystems). The mRNA level of PIP5K was determined by comparing its mean threshold cycle to that of beta2-microglobulin. Primers used were: beta2-microglobulin forward, 5'-GCTATCCAGAAAACCCCTCAA-3', and reverse, 5'-CATGTCTCGATCC CAGTAGACGGT-3'; PIP5K-α forward, 5'-AGGAGATCGTATCCTCCATC-3', and reverse, 5'-AATGATGGAGTGCTGGGTAC-3'; PIP5K-β forward, 5'-AGAAGTTGG AGCACT CTTGG-3', and reverse, 5'-GAGAAGGCTTCAAGGGAATC-3'; PIP5K-γpan forward, 5'-TGCAGCCTCTGTGGA AATAG-3', and reverse, 5'-ATAGAAGTTAAAAA TAGATGTCTG-3'; and PIP5K-γ90 forward, 5'-CTCTGTGGAAATAGACGCTG-3', and reverse, 5'- TATAGTGAAGCGG GGAGTAC-3'.

Tat fusion PH-PLCδ

The cDNA encoding tat-PH-PLCδ was cloned into a pET15b plasmid (Novagen/EMS, La Jolla, CA). For protein preparation, the plasmids were expressed in BL21(DE3).pLysS E. coli at room temperature. The Bacteria were lysed under native conditions by sonication and tat-PH-PLCδ was purified over a Nickel-NTA column by

Imidazole gradient elution under native condition according to the instructions of the manufacturer (Qiagen, Valencia, CA). Finally, a buffer exchange to PBS was performed. The tat-PH-PLCδ purity and concentration was determined by SDS-PAGE gel and a Spectrometer. For protein transduction, different concentrations of tat-PH- PLCδ were loaded into wt or transduced 5c.c7 T cells for 30 min at 37 °C by simple incubation in standard cell culture medium. Then APCs were added and the images were taken.

Enzyme-linked immunosorbent assay (ELISA)

The OptEIA™ ELISA Kits were purchased from BD Biosciences. Due to limited numbers of transduced PIP5K-GFP+ T cells, the cells were cultured in 384 well plates to ensure maximal contacts between T cells and APCs and to reduce well to well variation. This way, IL-2 produced from as few as 2,000 APC activated T cells can be detected. To reduce variation between samples analyzed with small numbers of T cells, the same numbers of transduced GFP+ and non-transduced GFP- T cells from the same culture were sorted by flow cytometry. The assays were done in triplicate for each condition. The T cell: APC ratio is set from 1:2 to1:5 depending on the numbers of sorted GFP+ T cells. For PIP5Kγ87, PIP5Kγ90 and γ-shRNA overexperession, ~10,000 T cells (either GFP+ or GFP-) and 20,000 antigen pulsed-APCs were added to each well to a final volume of 60 ul in IL-2 free complete medium. For PIP5Kβ overexpression, ~5000 T cells and 25,000 APCs were added each well. The T cell-APC mixtures were then briefly centrifuged and incubated at 37°C. After either 4 h or16 h of incubation, supernatants were removed and either directly assayed for IL-2 production or frozen at -20°C for later detection. The ELISA protocol was slightly modified from the standard protocol included in the kits. Briefly, 96-well ELISA plates were coated with capture antibodies and incubated either at 4°C overnight or at 37°C for 2 h. After three washes, the plates were blocked with assay diluents (PBS with10%FBS) for 1 h, RT. After another three washes, the supernatants (1:5 dilution in assay diluents) or IL-2 standards were added to the plates and incubated at RT for 2 h. The following standard concentrations of IL-2 were used to generate the standard curve (pg/ml): 3200, 1600,800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.1 and 0. After sample incubation, the plates were washed 5 times and incubated with the detection antibodies for 1 h, RT. The last washes were done 7 times, with 1 min soak each time. Then the plates were developed with TMB substrate solutions and incubated in

the dark for 3-10 min for color development. Stop solution (1M H_2SO_4) was added to the plates and the plates were read at 450 nm within 30 min with λ correction at 570 nm. For detecting IL-2 secretion in Latrunculin B treated cells, different concentrations of Latr. B were first added to each well of T cells and incubated for 1h at 37°C, then antigen pulsed APCs were added. After 16 h, supernatants were collected for IL-2 ELISA.

Western Blot Analysis

The same numbers of retrovirally transduced PIP5Kγ (or γshRNA)-GFP+ and non-transduced GFP-T cells were sorted by flow cytometry. For anti-phosphoLAT, anti-phosphoPLCγ immunoblots, $1x10^5$ 5c.c7 T cells were stimulated with equal numbers of 10μM MCC pulsed CH27 cells. For anti-phosphoERM immunoblot, $1x10^5$ 5c.c7 T cells were stimulated with 10μg/ml soluble anti-CD3/CD28 antibodies plus secondary crosslinking antibodies (anti-American and Syrian Hamster IgG (G192-1) mAb, anti-American and Syrian Hamster IgG1(G94-56)mAb) (BD Pharmingen). For APC stimulation, T cells were briefly centrifuged together with APCs at 800 rpm for ~20s and allowed to interact in imaging buffer for various periods of time at 37°C. For CD3/28 activation, T cells were incubated in imaging buffer with 10 μg/ml antibody mixtures at 37°C for various times. After the incubation, the cells were briefly centrifuged and the cell pellets were then resuspended in 20μl 1x cold lysis buffer (20 mM Tris [pH7.6], 30 mM NaCl, 2 mM EDTA, pH7.6, 1%Triton-100, 1 μM Pepstatin A, 2 mM NaF/Naorthovandate, 1 mM PMSF and 10% phosphatase inhibitor cocktail 1[Sigma]) for 30 min and then centrifuged for 15 min at 14,000 rpm at 4°C. Pellets were discarded and the supernatants were resuspended in 4 μl 6xSDS-sample buffer and boiled for 5 min at 100°C. The samples were run in a 0.75 mm thick, 10% SDS-PAGE gel. After migration, the proteins were transferred onto a PVDF membrane (Millipore, Bedford) at constant voltage (80v) for 3h at 4°C. The membrane was blocked in TBS-T with 5% milk for 1h at room temperature (RT) and then incubated with primary antibody (1:1000 dilution in TBS-T with 5%BSA) with gentle agitation overnight at 4°C. After washing with TBS-T, the membrane was incubated with anti-rabbit-HRP secondary antibody (1:3000 dilution in TBS-T with 5% milk) at RT for 1h and then the phospho-proteins were visualized using the ECL detection reagent. To control for sample loading, the membrane was

stripped according to manufacturer's protocol (Pierce) and reprobed with a HRP conjugated-GADPH mouse monoclonal antibody.

Densitometry quantification was performed by scanning and analyzing X-ray films using Metamorph software. Density values for each blot were first standardized by dividing the corresponding internal GAPDH band and then normalized by giving the arbitrary value of 1 to the integrated band intensity corresponding to non-transduced T cells.

Staining for F-actin (phalliodin)

The experimental procedures were slightly modified from the protocol included with the product (Molecular probes). Briefly, 5c.c7 T cells transduced with different PIP5K isoforms or PIP5Kγ-shRNA, were either fixed directly, or stimulated with 10μg/ml anti-CD3/CD28 antibodies for 2min first, and then fixed with 4% paraformaldehyde in PBS for 10 min. After washing, the cells were permeabilized with 0.1% Triton X-100 for 3-5min and stained for 30 min with Alexa Fluor-594-phalloidin (1:50). The intracellular polymerized actin was then analyzed with a FACSCalibur flow cytometer and FlowJo software.

Chapter III Spatial and temporal regulation of actin and TCR dynamics at immunological synapse by PIP5Ks during early T cell activation

Introduction

Antigen specific T lymphocyte activation is triggered by binding of T cell antigen receptors on the surface of a T cell with foreign-peptide bearing major histocompatibity complex (MHC) presented on an antigen presenting cell (APC). T cell-APC engagement initiates not only a rapid tyrosine phosphorylation cascade proximal to the TCR but also dramatic changes in the molecular organization at the contact site between a T cell and an APC, forming a specialized cell-cell junction called the immunological synapse (IS). The classical 'mature immunological synapse' contains a cSMAC at the center of the IS, where TCR and downstream signaling molecules such as PKCϴ are concentrated, and a pSMAC that surrounds the cSMAC, where integrin proteins such as LFA-1 are enriched. A dSMAC where the phosphatase CD45 accumulates encircles the pSMAC. Though the exact function of this highly organized concentric structure is controversial, the immunological synapse is believed to be critical for T cell signal integration and directed secretion (Cemerski et al., 2006; Fooksman et al., 2009; Friedl et al., 2005).

The actin cytoskeleton plays a key role in immunological synapse formation and T cell activation. Within seconds of TCR engagement by an APC, actin rapidly polymerizes at the T cell-APC interface, forming actin-rich lamellipodia at the periphery of the IS (Tskvitaria-Fuller et al., 2003). This dynamic accumulation of actin is proposed to have at least two functions. First, the actin flow may help to reorganize the signaling molecules to allow for IS formation; Second, the actin-rich lamellipodium may serve as a structural support that stabilizes the interactions between the T cell and the APC (Billadeau et al., 2007; Burkhardt et al., 2008). Pretreatment of T cells with drugs that prevent actin polymerization such as Latrunculin or CytoD, abolishes IS formation, calcium influx and IL-2 production. Interference with the function of several actin regulators, such as WAVE, Vav1, Cofilin, HS1 or ERM proteins, causes defective actin reorganization at IS and T cell-APC interaction; subsequently, T cell activation is impaired. Together these data indicate that reorganization of the actin cytoskeleton at the

IS plays a pivotal role in regulating T cell activation (Gomez et al., 2008; Burkhardt et al., 2008).

PIP2 is believed to be a key actin regulator in cells. It regulates the activity of many actin regulatory proteins by binding to their lipid binding domains, such as PH and FERM domains, and either activate or inactivate their functions. For example, together with GTP-Cdc42, PIP2 activates WASp, which in turn stimulates the *de novo* actin nucleation mediated by Arp2/3 complex (Higgs et al., 2000). WASp deficient T cells have been shown to have defective T cell signaling transduction leading to IL-2 production, though its importance for controling actin polymerization at IS is controversial (Zhang et al., 1999; Cannon and Burkhardt, 2004; Nolz et al., 2006). Binding to PIP2 inhibits the activity of Profilin and Cofilin, regulating the dynamic turnover and restructuring of the actin cytoskeleton (Lassing and Lindberg 1985; van Rheenen et al., 2007; Sechi et al., 2000). ERM proteins act as general crosslinkers between the cortical actin cytoskeleton and the plasma membrane. The crosslinking function of ERM proteins is thought to be activated by binding of the FERM domain to PIP2 and phosphorylation at a C-terminal conserved Threonine site (Bretscher et al., 2002; Charrin et al., 2006; Shaffer et al., 2009). A recent study demonstrates that PLC-mediated hydrolysis of PIP2 alone inactivates and releases ERM proteins from lymphocyte membrane, revealing a key role of membrane PIP2 in regulating ERM function (Hao et al., 2009).

The major cellular pathway for PIP2 production is regulated by PIP5Ks. Three PIP5K isoforms: α, β and γ have been identified in mammalian cells. PIP5Kγ also contains several splicing isoforms such as γ87, γ90 and γ93. PIP5K isoforms have a common and highly homologous kinase domain which catalyzes the phosphorylation of PI4P on its D-5 position to produce PIP4,5P2. The N and C termini of PIP5K isoforms show little homology. Different PIP5K isoforms were shown to localize in distinct cellular compartments and regulate different signaling pathways. For example, PIP5Kα has been found in membrane ruffles in MG-63 fibroblasts and within the nucleus of the fibroblasts, and is implicated in Rac1 mediated actin reorganization and pre-mRNA processing, respectively (Doughman et al., 2003; Boronenkov et al, 1998). PIP5Kβ is

found in perinuclear region in MG-63 fibroblasts and is implicated in membrane trafficking (Doughman et al., 2003). PIP5Kβ is also shown to regulate actin remodeling and participates in Rho mediated microvilli formation by activating and recruiting ERM proteins onto the microvilli of NIH3T3 cells (Matsui et al., 1999). PIP5Kγ87 is reported to specifically mediate a GPCR regulated Ca2+ signaling pathway in HeLa cells (Wang et al., 2004). PIP5Kγ90 is enriched in the neuronal synapse and focal adhesions, where it regulates synaptic transmission and focal adhesion dynamics (Di Paolo et al., 2004; Di Paolo et al., 2002). Both PIP5Kβ and γ90 have been shown to localize to the uropod of polarized neutrophils, regulating neutrophil polarity or signaling at the back (Lacalle et al., 2007; Lokuta et al., 2007). Taken together, these data suggest that the versatility of PIP2 function may be, or at least partially, attributed to its spatial and temporal production by different PIP5K isoforms.

Which PIP5K isoforms exist in T cells? Wher do they localize and how do they contribute to local PIP2 production, actin reorganization as well as TCR clustering in T cell activation by APCs? In the present study, we aim to address these questions. To visualize the spatial and temporal distributions of these signaling molecules in T cell activation, we used enhanced green fluorescent protein (EGFP) fusion proteins that either directly label the wildtype proteins (e.g., PIP5K isoforms), or label a widely used specific binding motif for the molecule (PH domain of PLCδ1 for PIP2), or label part of a receptor complex that should yield similar distributions as the receptor itself (zeta chain of the TCRs). These various EGFP fusion constructs were then transduced into the primary, in vitro primed 5c.c7 αβTCR transgenic T cells, and their *in vivo* dynamics were acquired by live cell imaging immediately upon the addition of the antigen-pulsed APCs.

Results

1. Expression and localization of PIP5K isoforms in resting T cells

First, we sought to examine the endogenous expression and relative abundance of each PIP5K isoform in primary mouse T cells. Real-time PCR results showed that primary mouse T cell blasts express all three PIP5K isoforms, including γ (γ87 and γ90),α and β (Figure 3-1). γ87 was the most abundant isoform: about 5 fold of γ90 and

3.6 fold of α. The β isoform was the least abundant, barely detectable in primary T cells. Next, we wanted to know where each PIP5K isoform localizes in primary T cells. EGFP-tagged full length enzymes were retrovirally tranduced into 5c.c7 T cells and their distributions were observed by fluorescence microscopy. T cells maintained in culture medium showed either rounded or polarized phenotype. For rounded quiescent T cells, all exogenously expressed PIP5K isoforms demonstrated a preferential localization to the plasma membrane, with little intracellular distribution (data not shown). Interestingly, in polarized T cells with a lamellapodium in the front and a uropod at the back, the γ90 and β isoforms showed uropod accumulation, which was found in 91% (32 out of 35) and 98% (42 out of 43) of polarized γ90 and β overexpressing T cells respectively. These observations are reminiscent of two recently published studies on the chemotactic neutorphils. In one study, PIP5Kβ was found at the uropod of polarized HL-60 cells and was shown to regulate neutrophil polarity and directional movement (Lacalle et al., 2007). In the other study, PIP5K γ661 (γ90) was identified as a novel uropod component that regulates the backness signal during neutrophil chemotaxis (Lokuta et al., 2007). These results together suggest that the uropod localization of β and γ90 seen in polarized T cells might have similar functions.

2. Differential polarization of PIP5K isoforms in APC activated T cells

We next looked at the localizations of each PIP5K isoform in T cells interacting with antigen-pulsed APCs. Briefly, primary 5c.c7 T cells transduced with individual EGFP-PIP5K isoform were mixed with MCC peptide-pulsed CH27 B cell APCs, both DIC and GFP time-lapse images were simutaneously acquired to record the temporal localizations of each GFP tagged enzyme in T cells upon making contacts with APCs. The images were acquired every 20 s for 15 min. This time range coincides with the peak of biochemically detectable signaling (Singleton et al., 2009). After the images were taken, GFP 3D reconstructions were made. Accumulation of each protein at different time points in every T cell-APC couple was analyzed by combining the DIC image and the corresponding GFP 3D reconstruction data. For each protein localization, at least 25 T cell-APC couples were analyzed. Seven accumulation patterns were recorded for every GFP tagged signaling protein, including interface accumulation, which consists of five different accumulations at the T cell-APC contact site: peripheral, asymmetric, diffuse,

central and lamella. The distal accumulation was recorded separately (detailed definition of each accumulation pattern can be found in Figure 3-2). The percentage of a particular accumulation pattern in the total analyzed T cells coupling with APCs was calculated and plotted as a function of time (Figure 3-3B). Time point 0 of tight cell couple formation was defined as either the first time point with a maximally spread T cell/APC interface, or 40 s after the first T cell-APC contact, whichever occurred first (Singleton et al., 2009). Immediately upon T cells forming tight cell couples with APCs, the PIP5Kγ87 and α rapidly accumulated to the T cell-APC interface. As shown in Figure 3-3&3-4, 76±7% and 86±6% of T cells exhibited interface γ87 and α accumulation, respectively, at time point 40 sec. At 7 min after tight cell couple formation, the percentage of cells with interface accumulation decreased to 13±9% for γ87 and 44±12% for α (Figure 3-4B, 3-3B). As the IS is classified as cSMAC and pSMAC according to the distinct localization of specific signaling molecules, we further looked at the five different interface accumulation patterns. We found no particular spatial preference for cSMAC or pSMAC for PIP5Kγ87 and PIP5Kα. Though both isoforms exhibited interface accumulation upon T cell activation, their accumulation patterns were not identical. PIP5Kα accumulated a little faster and stayed longer at IS than did PIP5Kγ87. In addition, there was almost no α accumulation at the distal pole of the T cells, whereas 26±7% of T cells at time point 0 showed uropod/distal pole accumulation of γ87(Figure 3-3B, 3-4B). To test if the kinase activity is required for the faithful targeting of PIP5K, we transfected a kinase deficient variant of PIP5K γ87 (D316K) into the T cells, and analyzed its accumulation patterns in response to APC activation (Figure 3-4C). Though there were slight variations in the accumulation patterns between the wt and kinase-negative γ87, no statistical significant difference was found at any time point analyzed upon cell couple formation, which is consistent with previous results showing that the kinase domain is dispensable for PIP5K localization (Lokuta et al., 2007). In contrast to the interface accumulation of γ87 and α, the γ90 and β isoforms accumulated specifically to the uropod/distal pole region in the majority of T cells forming cell couples with APCs (84±6% and 88±5% at 1min, respectively), and this posterior enrichment was maintained even after 7 min of tight cell couple formation, 64±10% and 45±15% of T cells with distal accumulation of β and γ90, respectively (Figure 3-5B, 3-6B). Taken together, our results demonstrate that different

PIP5K isoforms target to different poles of the T cells activated by APCs and they likely contribute to different pools of PIP2 production.

3. Imaging PH-PLCδ (PIP2) dynamics in antigen stimulated T cells

The dynamics of PIP2 in cells is balanced by its production and consumption, which is controlled by local activity of inositol lipid kinases, lipase and phosphatases. PIP5Ks is the major PIP2 producers in mammalian cells. Hydrolysis of PIP2 by PLCγ is a widely known mechanism for PIP2 depletion. In addition, manipulated targeting of phoshpatase has been shown to lower cellular PIP2 levels (Varnai et al., 2006; Hao et al., 2009). Though it is long believed that upon TCR triggering, PIP2 is enriched in lipid-raft regions and rapidly hydrolyzed to IP3 and DAG, when and where PIP2 is changed during early T cell activation is not well defined. To monitor PIP2 kinetics in live cells, we used the PIP2-specific fluorescent reporter, PH domain of PLCδ1 fused to EGFP, to visualize PIP2 accumulation in primary 5c.c7 T cells upon interacting with APCs. In quiescent T cells, the GFP-PH-PLCδ1 was located both in the plasma membrane and the cytosol, with a more pronounced enrichment on the cell membrane. Immediately upon T cell-APC coupling, the PH domain rapidly accumulated to the two cell contact area, which was found in 83±5% of T cells at time point 0 (Figure 3-7B). This accumulation of PH-PLCδ1 is short lived. After 3 min of stable cell-cell contact, only 23±6% of T cells showed interface PH accumulation, and after 5 min, less than 5% of T cells had interface PH-PLCδ1 accumulation (Figure 3-7B, interface). When specific patterns of interface accumulation of the PH domain were analyzed, we found that most of these markers are diffusely distributed throughout the IS, extending far beneath the membrane region of the T cells. We call this deep diffuse accumulation pattern, ‘Lamella’, which is characteristic of PH- PLCδ1 accumulation (Figure 3-2, 3-7A). In addition to the interface PH accumulation, 32±6% of T cells showed enrichment of PH- PLCδ1 at the opposite side of the IS right upon T cells conjugating with APCs (Figure 3-7B, distal). This rear accumulation of PH- PLCδ1 dissipated quickly, with less than 2% of cells showing distal accumulation at 3 min of stable cell-cell contact. When comparing the PIP2 spatio-temporal patterns with those of PIP5Ks, none of the isoforms were identical to PIP2. However, we found close correlations between the overall PIP5K accumulations and PIP2. First, they showed similar localizations: both PIP2 and PIP5Ks are enriched in the

plasma membrane of resting T cells and they all target to the two poles of activated T cells. Second, they showed similar initial targeting kinetics: within 1 min upon T cell-APC couple formation, PIP5Kγ87 and α rapidly accumulated to the T cell-APC interface, a time frame when PH-PLCδ1 is also found at the interface, suggesting the two PIP5K isoforms may contribute to the interface PIP2 production in early T cell activation. The difference is that PH-PLCδ1 interface accumulation was relatively transient. At 5 min, only 4% of T cells had interface PH-PLCδ1 accumulation, whereas there were 27±11% of T cells with interface PIP5Kγ87 accumulation and 73±8% of T cells with interface PIP5K α accumulation (Figure3-3B, 3-4B, Figure 3-7B). The later phase discrepancy in accumulation of the two PIP5K isoforms and PH-PLC δ1 can be explained by either rapid consumption of PIP2 (e.g., hydrolysis by activated PLC γ), or increased IP3 production, as IP3 was shown to bind with 10 fold higher affinity to PH-PLCδ in vitro. The similar kinetics observed for PIP5Kγ87 and PIP2, together with γ87 being the most abundant isoform in T cells indicate that γ87 may be the major PIP2 producer at the IS. In addition, the uropod/distal accumulation of PH-PLCδ1 found in ~30% of activated T cells suggest that the distal pole PIP2 is produced by PIP5K γ90 and/or β isoform (Figure 3-5B&3-6B, distal). A simplified cartoon was drawn to summarize the relative accumulations of PIP5K isoforms, PIP2, actin and TCR upon T cell-APC coupling (Figure 3-8).

4. The dynamics of PH-PLCδ1 in T cells overexpressing PIP5Ks

To further understand how PIP2 dynamics is regulated by differentially targeted PIP5K isoforms during T cell activation, we co-expressed the PIP5Kγ87, γ90 or β isoforms together with the EGFP tagged PH-PLCδ1 in 5c.c7 T cells by using the IRES-containing retroviral vector. Overexpression of the major PIP5K isoform γ87, which targeted to the interface of the T cell and APC, led to sustained interface PH accumulation as expected. At 5min after tight couple formation, 24±9% of PIP5Kγ87 overexpressing T cells had interface PH-PLCδ1 accumulation compared to only 4% of control T cells ($p=0.002$). At 7 min after cell couple formation, when there was no PH-PLCδ1 found in the interface in control T cells, still 18±8% of PIP5K γ87 overexpressing T cells exhibited interface PH accumulation ($p<0.001$). However, a decrease of PH binding was observed at earlier time points. For example, only 48±9% of PIP5K γ87

overexpressing T cells showed interface PH-PLCδ1 accumulation as compared to 83±5% of controls at time point 0 (p=0.001) (Figure 3-9). One possibility for this decrease is that overexpression of γ87 may, by an unknown mechanism, cause increased PIP2 consumption, thus, less interface PIP2 for PH- PLCδ1 to bind. It might be that γ87 preferentially localizes to a membrane compartment that has access to abundant activated PLCγ (or a phosphatase), which rapidly catalyzes PIP2. Several studies with this seemingly paradoxical observations supported this 'compartmentalized PIP5K/PIP2' hypothesis (Mao et al, 2009; Wang et al., 2004; Vasudevan et al., 2009; Chen et al., 2009). Overexpression of γ90 also caused sustained interface PH accumulation. At 3 min, 5 min and 7 min after tight cell couple formation, the interface PH-PLCδ1 accumulation was found in 50±10% v.s. 23±6% (p=0.008), 58±11% v.s.4% (p<0.001) and 13% v.s. 0% (p=0.001) of PIP5K γ90 overexpressing v.s. wt T cells, respectively (Figure 3-9). This is likely due to a later increase of interface γ90 accumulation in activated T cells (Figure 3-5B), though this isoform was mainly enriched in the uropod/distal pole region of the T cells. Interestingly, though PIP5K β is the least abundant PIP5K isoform in T cells, its overexpression dramatically changed the PH-PLCδ1 accumulation in activated T cells. Only 22±6% T cells overexpressing β exhibited interface PH accumulation upon tight cell couple formation at time 0, as compared to that found in 83±5% of wt T cells (p<0.001). Instead the majority of T cells showed sustained PH accumulation at the uropod/distal pole region, which lasted for more than 7 min upon APC stimulation (Figure 3-9). In contrast, after 3 min of tight cell couple formation, almost no rear accumulation of PH-PLCδ1 was found in wt T cells.

In addition to the accumulation patterns of a molecule at the IS, the amount of the molecules recruited to IS is also an important parameter for the function of a molecule. We call the latter 'interface enrichment'. The following equation is used to calculate PH-PLCδ-GFP interface enrichment:

Interface enrichment=I interface/I cellular background.

We found that PIP5Kγ87 overexpressing T cells exhibited increased interface PH-PLCδ1 accumulation as compared to wt T cells upon activation (at time point 100 and 120 sec, p<0.05, Figure 3-9D). Overexpressing of the other PIP5K isoforms did not cause significant changes in interface PH-PLCδ1 accumulation.

5. Coexpression of PIP5Kγ-shRNA with PH-PLCδ1-GFP caused cell death

As PIP5Kγ87 is the most abundant PIP5K isoform in T cells, and it accumulated to the T cell-APC interface where the majority of PIP2 was produced, we decided to focus our study mainly on the role of PIP5Kγ in T cell activation. We constructed a short hairpin shRNA against the common coding region shared by both the short and the long isoforms of PIP5Kγ and subcloned it into a retroviral vector containing an EGFP selective marker. T cells transduced with PIP5Kγ-shRNA-EGFP showed ~90% knockdown of the γ isoform by western blotting using a specific anti-PIP5Kγpan antibody (Figure 3-10D). No observable changes in cell shape and viability were found in the PIP5Kγ-shRNA transduced 5c.c7 T cells cultured in IL-2 medium. To see if knockdown of PIP5Kγ has any effect on PIP2 dynamics in T cells, the GFP was replaced with PH-PLCδ-GFP in the PIP5Kγ-shRNA-GFP construct. The rationale is that T cells tranduced with this construct will express both PIP5Kγ-shRNA and PH-PLCδ-GFP, thus the dynamics of PH-PLC δ accumulation would be imaged in PIP5Kγ knockdown T cells. Unexpectedly, coexpression of the PIP5Kγ-shRNA with PH-PLC δ-GFP was highly toxic to the Phenix packaging cell line used to produce virus for T cell transduction, such that after 1-2 days of transfection of the retroviral producing cell line, all cells floated and were found dead. This phenomenon was repeatedly found. Coexpression of γ-shRNA with actin-GFP did not cause cell death in either Phenix or T cells. It is possible that additional PH binding to free PIP2 further decreases cellular PIP2 levels in PIP5Kγ knockdown cells, and that the low PIP2 level is lethal.

6. Overxpression of PIP5K isoforms affected actin reorganization in APC activated T cells

The actin cytoskeleton plays a key role in the formation of immunological synapse and T cell activation. PIP2 binds to a variety of actin regulators and has been shown to regulate actin remodeling in various signaling pathways. It is thus of interest to know how changes in local PIP2 production by PIP5K isoforms will affect actin dynamics in activated T cells. To do this, cDNAs encoding EGFP tagged actin and individual PIP5K isoforms were subcloned together into the IRES containing retroviral vector, and transduced into 5c.c7 T cells. Immediately upon wt T cell-APC couple formation, the actin-GFP rapidly accumulated at the interface of 98±2% of T cells at time point 0

(Figure3-10A). The interface actin accumulation dissolved slowly, with 57±8% of the T cells exhibited interface accumulation at 7min after stable cell couple formation. Though the overall interface actin accumulation in T cells was not significantly changed upon PIP5Ks overexpression, individual accumulation patterns were affected. There was a significant decrease of actin-rich lamellapodium formation at the periphery of the IS in PIP5K overexpressing T cells as compared to wt controls. As shown in Figure3-10A, right upon tight cell couple formation (time 0), there was a ~30% decrease of the peripheral actin accumulation in γ90 overexpressing T cells compared to wt T cells (38±6% vs 68±6%, p=0.005). At the same time, an increase in diffuse actin accumulation (35±6% v.s 16±5%, p=0.01) was observed in these T cells. Similar defects in actin reorganization were also found in β and γ87 overexpressing T cells, though the phenotype was less pronounced (Figure 3-10A). We also examined actin interface enrichment in the PIP5K overexpressing cells, no significant changes were found as compared to wt T cells (3-10C). We noted that although overexpression of different PIP5K isoforms affected PH-PLCδ accumulation differently in T cells upon activation, it caused similar defects in actin reorganization at the IS. It is probable that in addition to generating isoform specific pools of PIP2, different PIP5K isoforms may provide a common pool of PIP2 in the T cell membrane to regulate the actin cytoskeleton.

7. Knockdown of PIP5Kγ caused sustained actin accumulation at IS

To see if down-regulation of PIP5K has any effect on actin dynamics in T cell activation, we made a retroviral construct that co-expresses the γ-shRNA (knockdown of the major PIP5K isoform) with actin-EGFP and tranduced it into 5c.c7 T cells. Interestingly, knockdown of PIP5Kγ caused sustained interface actin accumulation. At 7 min after cell couple formation, more than 91±5% of PIP5Kγ knockdown T cells exhibited interface actin accumulation as compared to 57±8% of control T cells ($p<0.001$)(Figure 3-10A). Pattern analysis revealed that this sustained interface actin accummulation was primarily a result of increased peripheral actin accumulation at the IS. At 3 min after tight cell couple formation, 52±8% vs 13±5% of PIP5Kγ knockdown v.s control T cells displayed peripheral actin accumulation ($p<0.001$), whereas the diffuse actin accumulation was comparable in PIP5Kγ KD and WT cells. These results were, at first glance, unexpected, as it is generally believed that increased PIP2 promotes actin

polymerization, so decreased PIP2 should favor actin depolymerization. However, this might be too simple to understand the complex mechanisms that cells respond to disturbed PIP2 homeostasis. It was found that, although PIP5Kγ-/- bone-marrow derived macrophages did not show significant changes in cellular PIP2 level (γ is the major isoforms in these cells), they had a 50% increase in F-actin concentration compared to wt cells (Mao et al, 2009). Mechanistically, Mao et al demonstrated that lack of PIP5Kγ altered RhoA/Rac1 balance, favoring the dominant activation of RhoA, which results in enhanced actin polymerization. In addition, decreases in membrane PIP2 by PIP5Kγ knockdown may exert an effect on the activity of an actin regulator(s) in T cells that may influence subsequent actin remodeling upon T cell activation. For example, ERM proteins, which function to crosslink the actin cytoskeleton to the plasma membrane, are phosphorylated and active in resting T cells. Activation of ERM proteins requires PIP2 binding. Upon TCR triggering, ERM proteins are rapidly inactivated, leading to relaxation of the cortical actin cytoskeleton from the T cell plasma membrane. As a consequence, T cell fluidity is increased and efficient T cell-APC coupling occurs (Faure et al., 2004). PIP5Kγ-knockdown induced PIP2 reduction may favor the inactivation of the ERM protein, decreasing cellular rigidity and promoting efficient actin remodeling. In contrast, overexpression of PIP5K should have the opposite effect by activation of the ERM proteins, resulting in increased cellular rigidity and impaired actin remodeling. This hypothesis was supported by later experiments that showed PIP5K overexpression inhibited ERM inactivation upon TCR triggering (see details in the next Chapter).

8. PIP5K overexpressing T cells exhibited increased cellular rigidity upon interacting with antigen pulsed APCs

In addition to a defect in actin remodeling in PIP5K overexpressing T cells, we found that these T cells displayed increased cellular rigidity when interacting with APCs. First, we observed a lack of lamellipodium formation in 20-40% PIP5K overexpressing T cells when they initiated interactions with APCs (Figure 3-11A-B). The lamellipodium is a cytoskeletal actin projection on the motile front edge of the cell, in which the actin meshwork aids in the retrograde flow of particles throughout. The absence of the lamellelipodia formation in PIP5K overexpressing T cells further suggest a defect of the actin cytoskeleton in these cells. In addition, we found that PIP5K overexpressing T cell

exhibited a relatively narrow interface when coupling with an APC. As shown in Figure 3-11C, the interface diameters at time point 0 were significantly decreased in T cells overexpressing γ87, γ90 and β, while not in T cells with γ knockdown, as compared to wt T cells. Moreover, the decrease in interface diameter was highly correlated with the absence of lamellipodium formation in these cells (Figure 3-11D), indicating an overall increase in cellular rigidity in these PIP5K overexpressing T cells.

9. Overexpression of PIP5Ks impaired TCR accumulation upon APC activation

As interface actin dynamics is critical for IS formation, we asked if changes in actin reorganization upon PIP5K overexpression can affect the dynamics of a key component of the cSMAC, TCRs. To do this, we made the PIP5K-IRES-TCRzeta-GFP construct and transduced it into T cells. We found that T cells overexpressing PIP5K isoforms exhibited significant decrease or delay in TCR centralization compared to wt cells (Figure 3-12). For example, at 40 sec after cell couple formation, when 52±8% of the wt T cells exhibited TCR central accumulation, only 28±9%, 21±5% and 14±6% of PIP5Kβ, γ87 and γ90 overexpressing T cells displayed the same phenotype ($p=0.03$, $p=0.006$ and $p<0.001$ respectively). The delayed TCR centralization is more pronounced in PIP5Kβ and γ90 overexpressing T cells. For example, the wt T cells showed peak TCR centralization at 3min after tight cell couple formation, whereas γ90 or β overexpressing T cells exhibited peak TCR centralization at 5min after tight cell couple formation (3-12B). Though γ87 overexpression did not cause delayed TCR centralization, it caused less TCR centralization at early time points: e.g., at time 0, 7±3% of γ87 overexpressing T cells showed TCR central accumulation compared to 24±6% of wt T cells ($p=0.007$) (Figure 3-12). PIP5Kγ overexpression also resulted in a delayed release of TCRzeta from the distal region of the activated T cells: e.g., at 100 sec after tight cell couple formation, when none of the wt T cells exhibited any distal accumulation of TCRzeta, there were still 28±6% and 47±8% of PIP5Kγ87 and γ90 overexpressing T cells had this phenotype ($p<0.001$, $p<0.001$) (Figure 3-12B). The exact role of TCR centralization in T cell activation is still not clear, some suggest it may be a way of strengthening TCR signaling, some argue it is a way of terminating or modulating TCR signaling. No matter what the real mechanism is, it is agreed that intact actin dynamics plays a crucial role in regulating TCR dynamics (Tskvitaria-Fuller et al., 2003; Kaizuka et al., 2007). Together, our data

suggest that defective actin reorganization in response to PIP5K overexpression may be responsible for the delayed TCR centralization, likely due to the inability to clear the actin from the cSMAC where the TCR is supposed to accumulate (increased actin diffuse accumulation in PIP5K overexpressing T cells Figure3-10C).

10. Knockdown of PIP5Kγ caused decreased TCR interface accumulation

To look at TCR dynamics in T cells with PIP5K down-regulation, PIP5KγshRNA-TCRzeta-GFP construct was cloned and transduced into 5c.c7 T cells. We observed a slight but not significant decrease of TCR central accumulation in PIP5Kγ knockdown cells as compared to wt controls. However, these PIP5Kγ knockdown T cells exhibited a significant decrease in total interface TCR accumulation at later time points: e.g., 50±9% of PIP5Kγ knockdown T cells v.s 93±5% of wt T cells at 7min after cell couple formation exhibited interface TCR accumulation (p=0.001). One possibility for this phenotype is that in PIP5Kγ KD cells, the sustained actin accumulation at the periphery of IS may be static and can not provide the dynamic 'actin flow' for efficient TCR central accumulation. Another possibility is that TCR interface accumulation is not only affected by actin dynamics, but also by PIP2 regulated membrane trafficking. The combined action may cause the observed phenotype. Together, TCR central accumulation can be affected by many factors, it may not be a easily accessible marker for evaluation of T cell activation.

11. Effects of overexpression of a kinase deficient mutant of PIP5Kγ87 on PH-PLCδ, actin and TCRzeta accumulation in T cells upon APC activation

To further investigate if the observed effect of PIP5Ks on regulating actin and TCRzeta reorganization at IS was due to its production of PIP2, we transfected into 5c.c7 T cells a kinase-negative mutant of γ87 in which Asp316, which has a role in general base catalysis, was changed into a lysine residue (γ87-D316K) (Giudici et al., 2004; Mao and Yin, 2007). We then looked at the effects of γ87-D316K overexpression on the dynamics of PH-PLCδ, actin and TCRzeta in APC activated T cells. Surprisingly, overexpression of the γ87-D316K mutant led to the displacement of membrane PH-PLCδ into the cytosol in the majority of T cells (27 out of 32), and severely affected PH-PLCδ accumulation upon T cell activation (Figure 3-9A). In addition, overexpression of the γ87-D316K caused increased actin diffuse accumulation in activated T cells: e.g, at 7min

upon tight cell couple formation, 38±8% of γ87-D316K overexpressing T cells showed diffuse actin accumulation at IS compared to 14±6% of wt T cells (p=0.004) (Figure3-10B). In addition, γ87-D316K overexpressing T cells exhibited significant decrease in TCR central clustering and interface accumulation as well as a dramatic increase in TCR distal accumulation. For example, at time point 40 sec, 43±6% and 15±5% of γ87-D316K overexpressing T cells as compared to 82±6% and 52±8% of wt T cells exhibited interface and central TCR accumulation, respectively ($p<0.001$, $p=0.005$). In contrast, 82±5% of γ87-D316K overexpressing T cells showed distal TCR accumulation compared to 34±6% of wt T cells ($p<0.001$) (Figure 3-12B). The cause of the severe defects in TCR accumulation in these γ87-D316K overexpressing T cells was not clear. It has been shown that transfection of the kinase inactive PIP5Kγ (D316K) in neurons greatly reduced the length and complexity of the neuronal processes, accompanied by an extensive loss of neurons (Giudici et al., 2004). It has also been reported that most of the kinase dead variants of PIP5Ks still have partial kinase activity which makes the their transfection phenotype difficult to interpret (Yamamoto et al., 2001; Coppolino et al., 2002; Giudici et al., 2004). In summary, the PIP5Kγ87-D316K mutant is not an ideal control for probing PIP5K function.

12. Transduction of a tat-PH-PLCδ fusion protein into T cells affected actin reorganization at IS

Addition of a 10aa peptide from the HIV Tat protein confers membrane permeability to peptides or proteins. Protein transduction by tat-tagged protein into cells allows dose response and careful titration of the reagents to manipulate protein activity *in vivo* (Tskvitaria-Fuller et al., 2007). As another way to interfere with PIP2 function, we made a tat-fusion PH-PLCδ protein, which can be transduced into cells, binds to PIP2 and thus interferes with PIP2 function. We first tested if transduction of the Tat-PH-PLCδ domain can affect the PH-PLCδ-GFP accumulation in APC activated T cells. We found that treatment of 5c.c7 T cells with 5μM tat-PH-PLCδ caused modest but significant changes in PH-PLCδ-GFP interface accumulation (Figure 3-9). Concentrations higher than 5μM of the tat-PH-PLCδ protein are not stable in solution and tend to precipitate, so we could not use concentrations higher than 5μM for the tat-PH-PLCδ transduction experiments. However, 5μM tat-PH-PLCδ transduced T cells exhibited significantly

increased actin lamella (deep diffuse) accumulation at the IS: e.g., at time point 80 sec, 17±7% of tat-PH-PLCδ transduced T cells exhibited actin lamella accumulation compared to 0% of wt T cells ($p<0.001$). Neither the actin interface enrichment nor the interface diameter was significantly affected by 5μM tat-PH-PLCδ treatment (Figure 3-10C, 3-11C).

Discussion

Although PIP2 is a minor component of the plasma membrane, it is an essential regulator of many cellular processes. For instance, PIP2 is not only the precursor of three important second messengers: IP3, DAG and PIP3, but also functions as a second messenger itself though binding to and regulation of the localization and activity of many signaling proteins. Many PIP2 effectors contain lipid binding domains such as PH domains and FERM domains, which mediate their interactions with the phosphorylated inositol group on PIP2. Examples of these effectors include the components of endocytic machinery such as AP2 (adapter protein complex 2) and numerous Clathrin-associated sorting proteins (CLASPs), that possess the PIP2 binding domains which facilitate their recruitment and assembly onto the sites of potential endocytosis (Padrón et al., 2003). Other important PIP2 effectors include actin regulatory proteins such as Profilin, Cofilin, ERM proteins and WASp, whose interactions with PIP2 regulate the dynamics of the actin cytoskeleton (Yin and Janmey, 2003).

How can a single molecule regulate so many different cellular processes? One explanation for the diversity of PIP2 function is that PIP2 is spatially and temporally generated by different PIP5Ks. In support of this hypothesis, different PIP5K isoforms (α, β, γ87 and γ90) are found to express differently in various tissues, localize to distinct subcellular compartments and regulate non-redundant signaling pathways in response to various stimulus (Doughman et al., 2003; Ling et al., 2002; Di Paolo et al., 2004; Coppolino et al., 2002; Sasaki et al., 2005). Despite the wealth of study on investigating the roles of PIP5Ks in many cell types, such as macrophages, platelets, mast cells, fibroblasts and neural cells, little is known about their involvement in T cell signal transduction and activation.

In initial attempts to probe for PIP5K functions in T cell activation, in this study, we looked at the endogenous expression of PIP5K isoforms in primary mouse T cells,

their dynamic localization in early T cell activation by APCs, and compared their accumulation patterns with that of a PIP2 marker, the PH domain of PLCδ1. We also modulated the cellular PIP5K levels by either overexpression or knockdown of individual isoforms, to see how these changes would affect local PIP2 dynamics, as well as actin reorganization in T cell activation. In addition, we looked at TCR centralization as a general marker for the IS formation, in activated T cells with PIP5K overexpression or knockdown. Several findings are made in this study. 1. At the mRNA level, γ87 is the most abundant PIP5K isoform in primary mouse T cells, followed by α, γ90, and β. 2. When exogenously expressed, PIP5K isoforms are mostly enriched in the plasma membrane of the resting T cells. Immediately upon T cell activation by antigen presenting cells, PIP5Kγ87 and α isoforms preferentially accumulated to the T cell-APC interface, where the majority of PH-PLCδ is found concentrated, suggesting that these two isoforms are responsible for T cell interface PIP2 production upon APC activation. In contrast, PIP5Kβ and γ90 isoforms mainly targeted to the uropod/distal pole region of the APC activated T cells, where a small percentage of PH-PLCδ was enriched, suggesting that these two isoforms are responsible for PIP2 production at the distal region of the activated T cells. Together, the dynamic localizations of PIP5K isoforms, though not identical, correlate well with that of the PIP2 marker, PH-PLCδ1 in T cell activation. It is easy to understand the interface PIP2 accumulation in activated T cells as it is where TCR signaling is initiated and transduced in the cells. Interface PIP2 is required for generation of important signaling intermediates such as IP3, DAG and PIP3, which are indispensible for downstream signaling leading to T cell effector functions. What then are the functions of distally located PIP2/PIP5Ks in the activated T cells? This raises a fundamental question of what the function of the distal pole complex (DPC) in T cell activation is. The DPC is an actin rich protein complex distal to TCR triggered signaling. As some negative regulators of TCR signaling such as CD43 were found in the DPC, the DPC is thought to facilitate T cell activation by sequestering inhibitory proteins away from the IS (Cullinan et al., 2002; Allenspach et al., 2001). The ERM proteins are central organizers of the DPC, which function to link the plasma membrane receptors to the actin cytoskeleton. Overexpression of an ERM dominant negative mutant completely inhibited the distal movement of CD43 and other DPC proteins induced by TCR engagement; as a

consequence, cytokine production was diminished (Delon et al., 2001; Allenspach et al., 2001; Shaffer et al., 2009). Based on these previous findings, our results suggest that PIP5Kβ and γ90 may be new components of the DPC in T cells. It would be interesting to see if their localization is also regulated by ERM proteins. The PIP2 produced at the DPC might function to regulate the activity of the ERM proteins, as it is well documented that ERM activation requires PIP2 binding and phosphoylation on an activating Threonine. The DPC also contains several polarity proteins such as PKCζ, DLG1 and Scribble, which play important roles in regulating T cell polarity (Charrin et al., 2006). Two recent reports independently showed that PIP5K β and γ90 localized to the uropod of polarized neutrophils in response to chemokine stimulation, which match nicely to our findings of the two isoforms concentrating in the uropod/distal pole region of antigen stimulated T cells. The uropod and distal pole complexes are functionally similar structures. Upon APC stimulation, the T cell uropod retracts and becomes the distal pole region of the T cell. In one study, PIP2 synthesized by γ90 was shown to be important for regulating uropod retraction during chemotaxis (Lokuta et al., 2007). In a second study, the PIP5Kβ isoform was demonstrated to interact with an ERM binding protein EBP50, which enabled its interaction with ERM and RhoGDI. In addition, siRNA mediated knockdown of PIP5Kβ inhibited the neutrophil polarity and directional movement (Lacalle et al., 2007). Samelson et al., found that STIM1 and Orai1, the two important components of the CRAC channel in T cells, accumulated both at the immunological synapse and the distal pole of the activated T cells. They speculate that the caps structure formed at the DPC may provide a secondary site of calcium entry (Samelson et al., 2009). If this is the case, it would be interesting to see if PIP2 produced at the DPC would contribute to the second site of calcium signaling. 3. We found that overexpression of different PIP5K isoforms caused different PH-PLCδ accumulation patterns, however, they do not match very well with that of the enzyme when expressed alone. This is not so surprising since PIP2 dynamics in cells are finely balanced by its production and consumption. It is known that total cellular PIP2 levels are quite stable and do not undergo dramatic changes even after a stimulation (McLaughlin et al., 2002; Hinchlife et al., 2000). It is possible that the cells rapidly trigger a feedback mechanism, by increasing either the expression level or the activity of a reverse-functioning enzyme, to

counterbalance the changes in PIP2 concentration. The initial decrease in interface PH-PLCδ accumulation found in APC activated γ87-overexpressing T cells may be subject to this type of regulation. γ87 may localize to a membrane subcompartment which contains abundant enzymes for which PIP2 is a substrate such as PLCγ, PI3K or phosphoinositide phosphatases, whereas the other PIP5K isoforms do not. It is interesting to see that overexpression of the least abundant PIP5K isoform β, which localizes to the distal pole of the activated T cells, caused diminished PH-PLCδ interface accumulation but sustained distal pole accumulation. However overexpression of the other distal pole located PIP5K isoform γ90 did not lead to such a phenotype; instead a sustained accumulation of interface PH-PLCδ was observed. A later increase in interface γ90 accumulation may help explain the sustained interface PH accumulation. It is also probable that the β isoform is the major isoform contributing to distal PIP2 production during early T cell activation. 4. Overexpression of any of the three PIP5K isoforms, γ87, γ90 and β, significantly decreased actin-rich lamellapodium formation at the periphery of the IS, though to different degrees, with γ87 the mildest, and γ90 the strongest. Meanwhile actin was concentrated diffusely all over the interface, with the total actin interface accumulation comparable to that observed in wt cells. These changes in actin remodeling at the IS may have important biological consequences on T cell activation. Peripheral actin polymerization is believed to be critical for proper segregation of signaling complexes at IS and for stable T cell-APC interaction (Bunnel et al., 2001; Dustin and Cooper, 2000; Tskvitaria-Fuller et al., 2003). In the studies of IS formation between T cells and the lipid planar bilayers embedded with TCR ligands and coreceptors (act as surrogates for APCs), the TCR microclusters were found to form continuously at the periphery of the IS and are transported to the center (Kaizuka et al.,2007). Fluorescence speckle microscopy revealed that the dynamic actin network centripetally transports TCR and adhesion microdomains and that differential actin interactions may be involved in partitioning adhesion and TCR proteins into pSMAC and cSMAC zones, respectively. Application of Latrunculin to the T cells before stimulation abolished microcluster formation and TCR signaling (Kaizuka et al., 2007). In our study, how does overexpression of PIP5Ks result in decreased actin peripheral accumulation while diffuse actin patterning is increased at the IS? One possibility is that PIP5K mediated PIP2

production causes an imbalance in the activities of actin regulators so that net actin polymerization is decreased in the periphery and increased in the center of IS. Clearly the regulation of F-actin polymerization and depolymerization at the immunological synapse is a complex and coordinated process. So far the exact form of the F-actin that is generated (branched versus linear actin) and the actin-severing and actin-nucleating proteins that are involved in its generation are still relatively ill-defined. Further identification and characterization of individual actin regulatory protein that affect actin dynamics at the IS, and analyzing them at a system level should provide insights into the underlying mechanism. Another possibility for the observed defect in actin remodeling might be due to the inability to disconnect the cortical actin cytoskeleton from the plasma membrane during TCR ligation. A well known group of actin regulators that are regulated by PIP2 and function to crosslink cortical actin cytoskeleton to the plasma membrane is the ERM protein family. ERM proteins are rapidly inactivated in response to TCR trigering (Delon et al., 2001; Faure et al., 2004). The ERM inactivation decreases the T cell rigidity and releases the interactions between cortical actin filament and the plasma membrane normally mediated by active phosphorylated ERM proteins, and allows for efficient T cell-APC couple formation. ERM protein activation is regulated by PIP2 binding and phosphorylation (Faure et al., 2004; Shaffer et al., 2009). It is possible that PIP5K regulated PIP2 production prevented ERM inactivation upon TCR triggering so that the F-actin can not be efficiently cleared from the center of the T cell-APC interface, resulting in increased diffuse actin accumulation and decreased peripheral actin accumulation at IS. The increased cellular rigidity found in PIP5K overexpressing T cells further suggest a possibility of increased ERM protein activity. 5. TCR centralization is a characteristic feature of the IS formation. It is also an actin-dependent process. We found that PIP5K-overexpressing T cells showed significantly delayed TCR centralization compared to wt cells, more strikingly in γ90 and β overexpressing cells. These results correlate well with the PIP5K-overexression actin phenotype, suggesting that impaired peripheral actin polymerization may not provide sufficient ‘actin flow’ to drive TCR central accumulation. Interestingly, T cells with PIP5Kγ knockdown also exhibited decreased TCR central accumulation. One possibility is that peripheral actin filaments in these cells are ‘static’, and are unable to generate the dynamic centripedal actin flow to

drive TCR central clustering. This kind of defect in actin remodeling has been observed in Coronin-1A deficient T cells. Coronin-1A is a hematopoietic specific actin- and Arp2/3 complex binding protein. Coronin1A-/-T cells displayed excessive accumulation yet reduced dynamics of F-actin at the IS following TCR trigerring, correlating with basal activation of the kinases sAPK/JNK1/2 and deficient TCR-induced Ca^{2+} signaling (Mugnier et al., 2008). Another possibility that activated T cells with PIP5Kγ KD showed less TCR central clustering may be that PIP2 mediated membrane trafficking pathways are defective.

In summary, we show that different PIP5K isoforms are differentially targeted to the two poles of T cells upon APC stimulation. Overexpression of different PIP5K isoforms in T cells led to different PH-PLCδ accumulation patterns, suggesting that each isoform may contribute to different pools of PIP2 in the T cell plasma membrane upon activation. T cells overexpressing different PIP5K isoforms demonstrated similar defects in actin reorganization at the IS and increased cellular rigidity, suggesting that PIP5K isoforms are also functionally redundant, possibly by affecting the same actin regulator(s) in T cells. Further experiments are needed to investigate how changes in PIP5K levels affect TCR triggered downstream signaling and effector functions as well as to identify the possible actin regulator(s) involved in the proposed pathway.

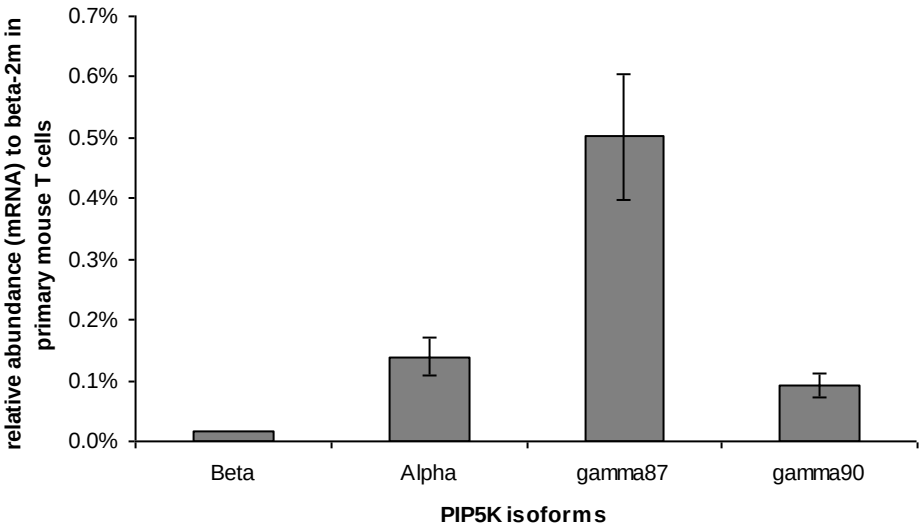
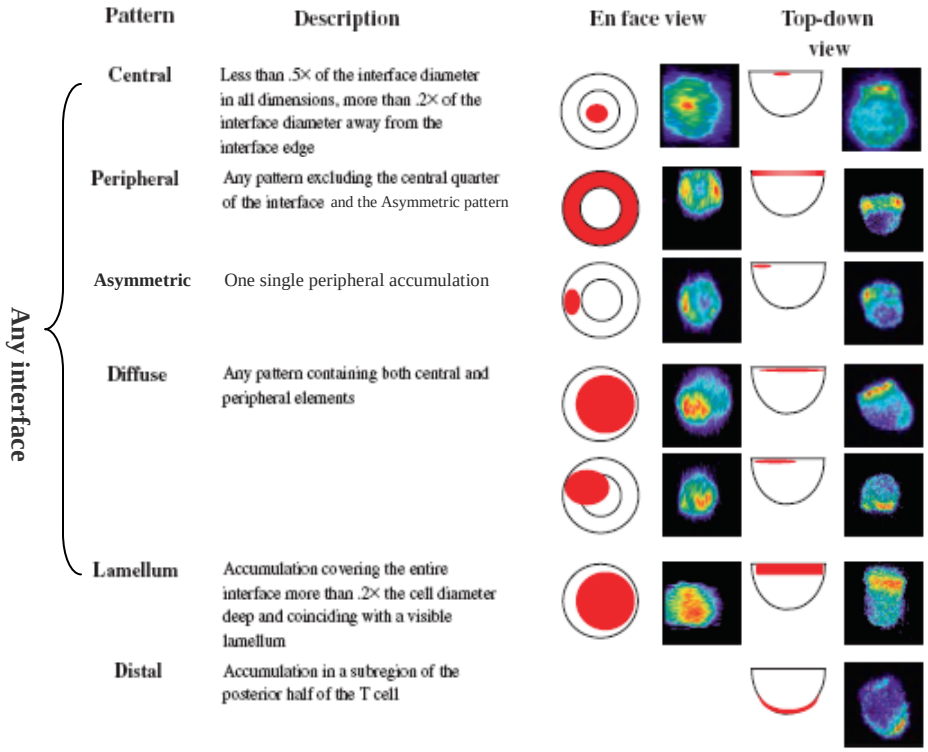
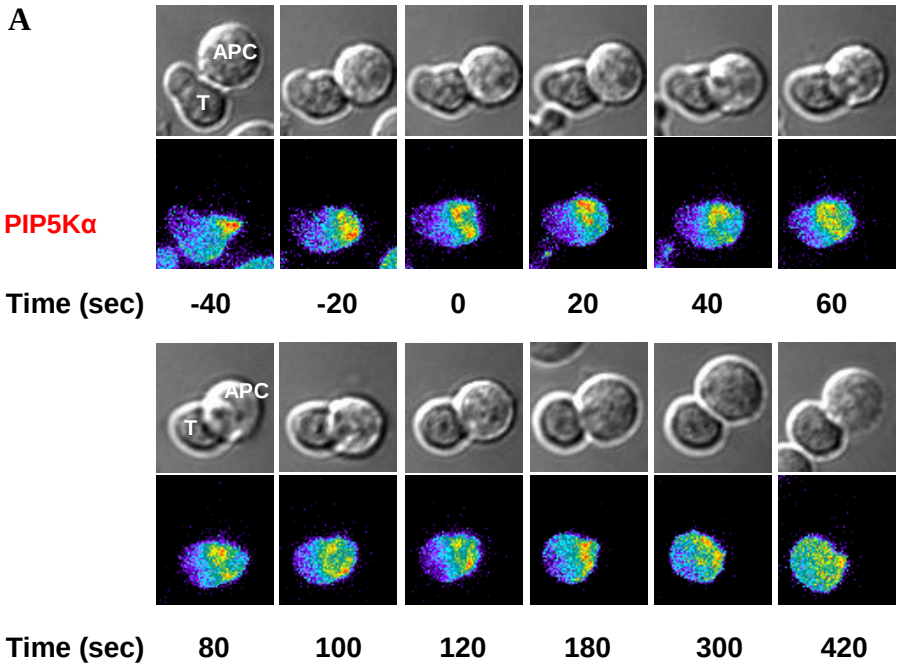


Figure 3-1. Real-time PCR analysis of the relative expression of PIP5K isoforms in primary mouse T cells. The expression value of each PIP5K isoform was normalized to that of a house keeping gene beta2-microglobuline, which was set to 1. The means were from three independent experiments. Error bars represent standard error.



From Singleton et al., Cell Signaling, 2009

Figure3-2. Spatiotemporal patterns of accumulation of GFP-tagged proteins. Definitions of patterns, schematic representations and representative images that prominently display the patterns are shown. The en face view looks at the T cell–APC interface in the same way that an APC would. In the schemes, the outer gray circle delineates the whole interface and the inner circle denotes its center. The top-down view looks down on the T cell from the top. In the schemes, the APC is not shown and would be on top of the T cell forming the T cell–APC interface. The pattern of any interface accumulation represents the total interface accumulation of central, peripheral, asymmetric, diffuse and lamellum.



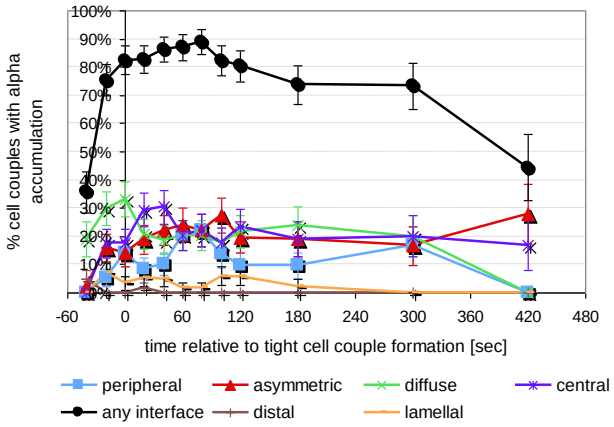


Figure 3-3. PIP5Kα rapidly accumulated to the T cell-APC interface upon T cell activation. A.Representative interactions of PIP5Kα-GFP transduced 5c.c7 T cells with CH27 B cell lymphoma APCs in the presence of 10 μM MCC antigenic peptide (full stimulus) are shown at the indicated time points relative to the time of formation of a tight cell couple. Differential interference contrast (DIC) images are shown in the top rows, with top-down, maximum projections of three-dimensional PIP5Kα -GFP fluorescence data in the bottom rows. The GFP fluorescence intensities are displayed in a pseudo-color scale (increasing from blue to red). B. The graph shows the percentage of cell couples with standard errors that displayed accumulation of PIP5Kα with the indicated patterns (Fig. 3-2) relative to tight cell couple formation for T cells transduced with PIP5Kα-GFP. 59 cell couples were analyzed.

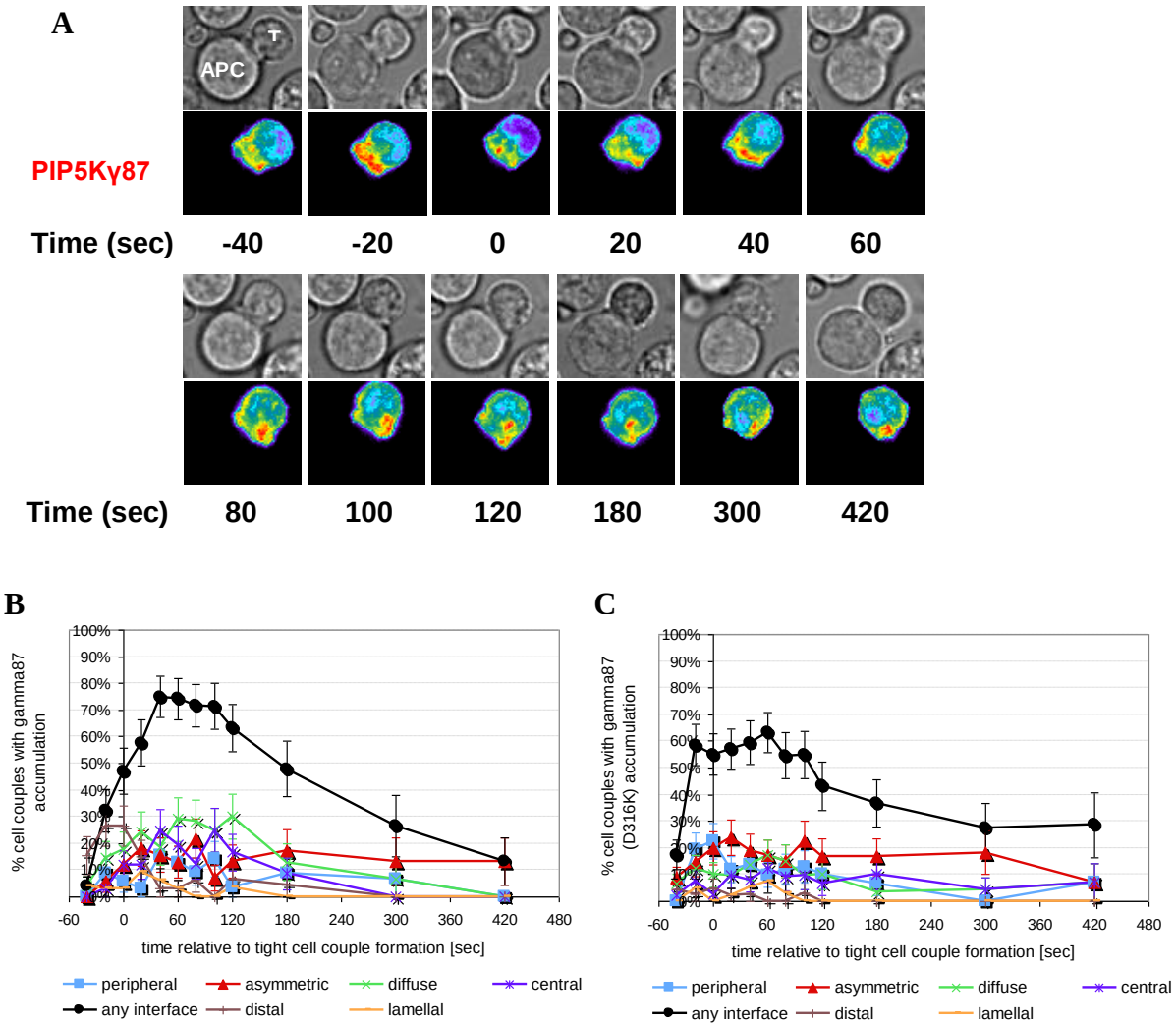


Figure 3-4. Localization of PIP5Kγ87 to the T cell-APC interface upon T cell activation. A.Representative interactions of PIP5Kγ87-GFP transduced 5c.c7 T cells with peptide loaded APCs at different time points upon forming a tight cell couple are shown (same as described in Figure 3-3A). B. The graph shows the percentage of cell couples with standard errors that displayed accumulation of PIP5Kγ87 with the indicated patterns for T cells transduced with PIP5K γ87-GFP upon coupling with APCs. 36 cell couples were analyzed. C. The graph shows the percentage of cell couples with standard errors that displayed accumulation of a kinase deficient mutant of PIP5Kγ87 (D316K) with the indicated patterns for T cells transduced with PIP5K γ87(D316K)-GFP upon coupling with APCs. 42 cell couples were analyzed.

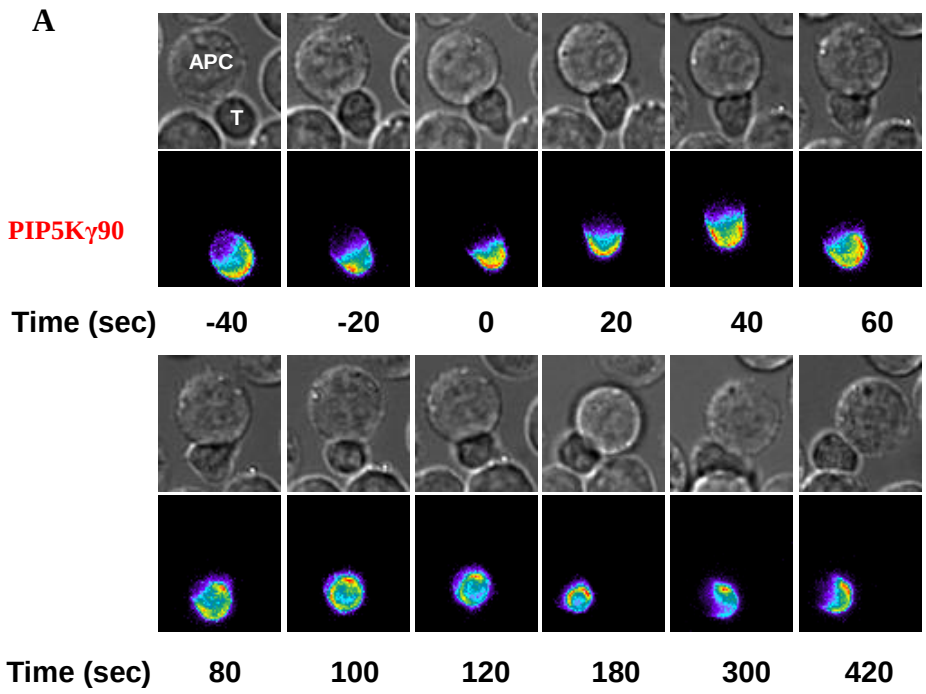
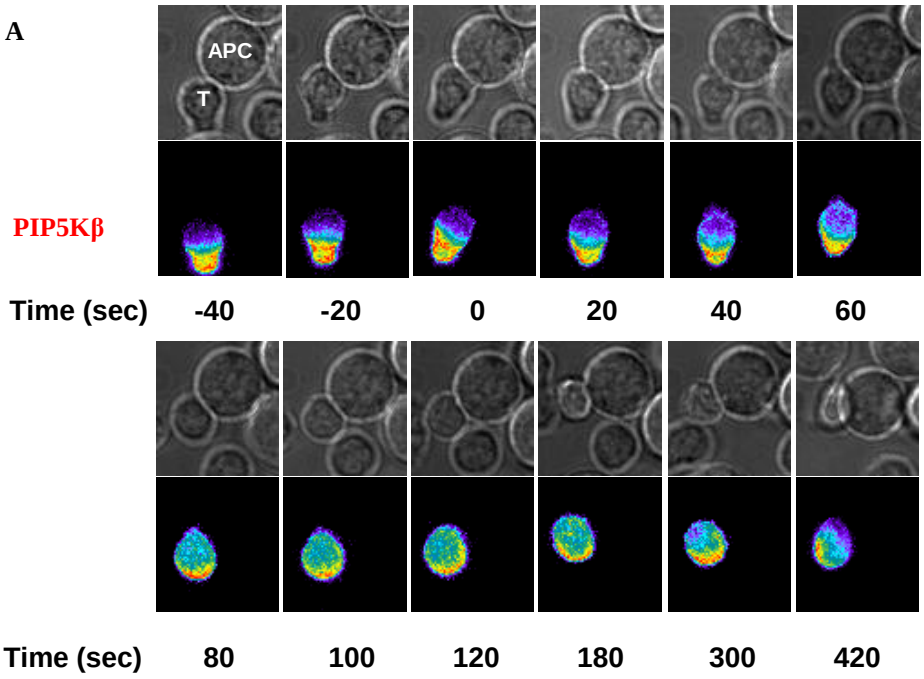


Figure 3-5. PIP5Kγ90 localized to uropod/distal pole region of APC activated T cells. A. Representative interactions of PIP5Kγ90-GFP transduced 5c.c7 T cells with peptide loaded APCs at different time points upon forming a tight cell couple are shown (same as described in Figure 3-3A).B. The graph shows the percentage of cell couples with standard errors that displayed accumulation of PIP5Kγ90 with the indicated patterns for T cells transduced with PIP5K γ90-GFP upon coupling with APCs. 34 cell couples were analyzed.



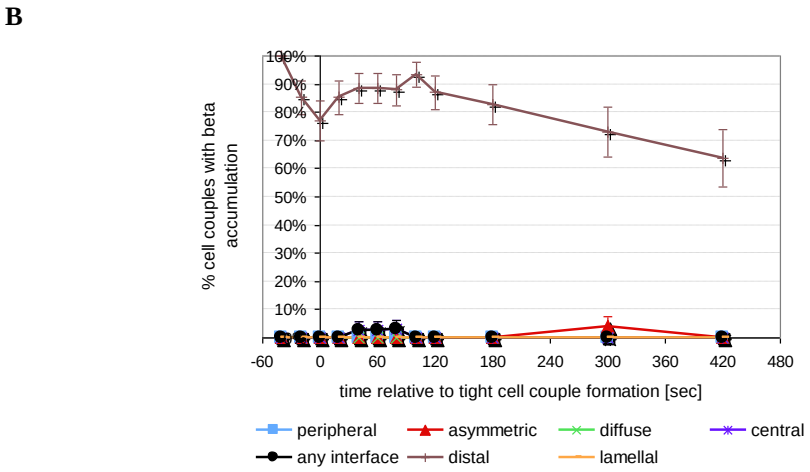


Figure 3-6. Accumulation of PIP5Kβ to the uropod/distal pole region of APC activated T cells. A. Representative interactions of PIP5Kβ-GFP transduced 5c.c7 T cells with peptide loaded APCs at different time points upon forming a tight cell couple are shown (same as described in Figure 3-3A). B. The graph shows the percentage of cell couples with standard errors that displayed accumulation of PIP5Kβ with the indicated patterns for T cells transduced with PIP5Kβ-GFP upon coupling with APCs. 35 cell couples were analyzed.

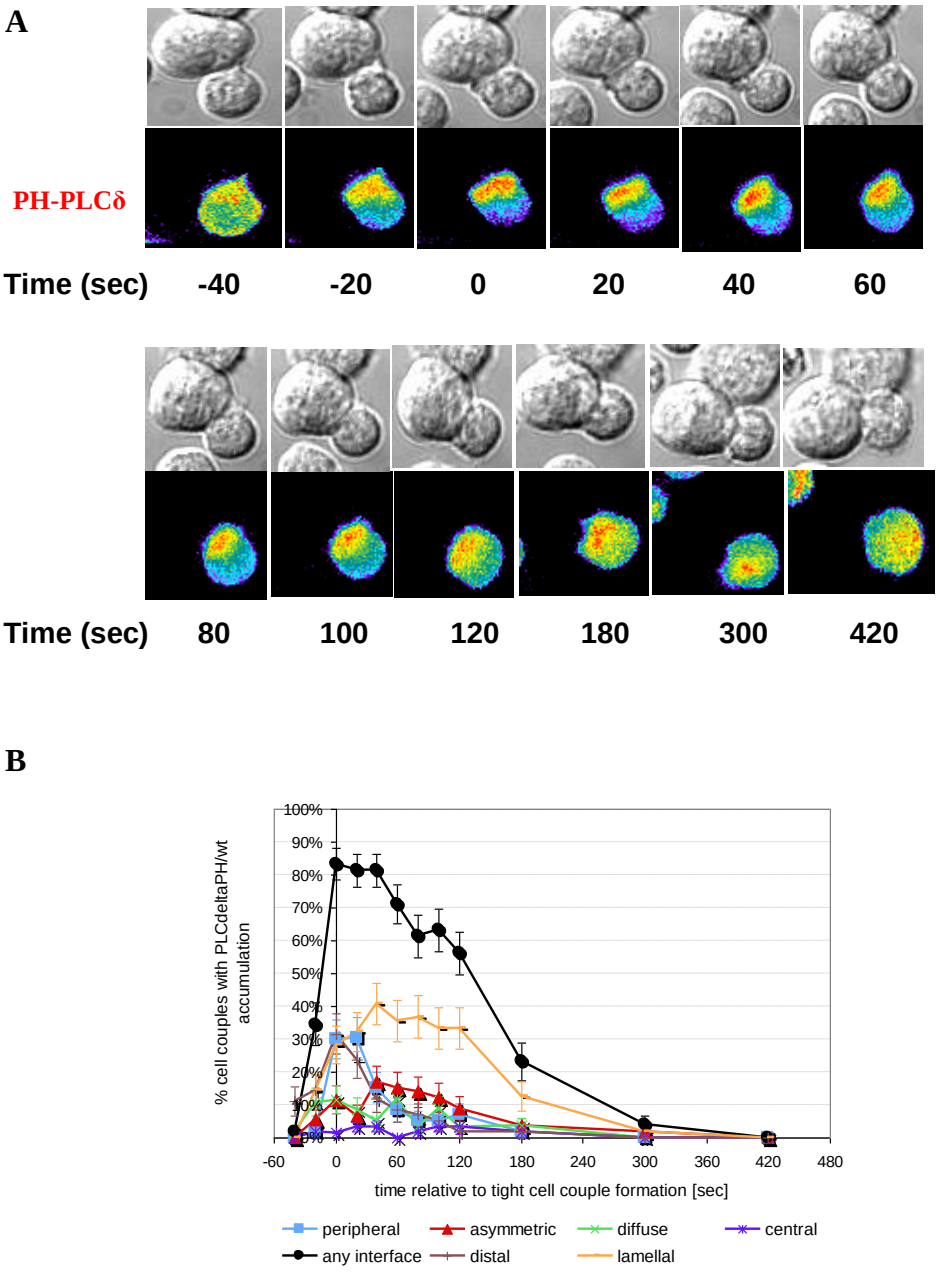


Figure 3-7. Dynamics of PLCδPH accumulation in early T cell activation.
A. Representative interactions of PH-PLCδ1-GFP transduced 5c.c7 T cells with peptide loaded APCs at different time points upon forming a tight cell couple are shown (same as described in Figure 3-3A).B. The graph shows the percentage of cell couples with standard errors that displayed accumulation of PH-PLCδ1 with the indicated patterns for T cells transduced with PH-PLCδ1-GFP upon coupling with APCs. 60 cell couples were analyzed.

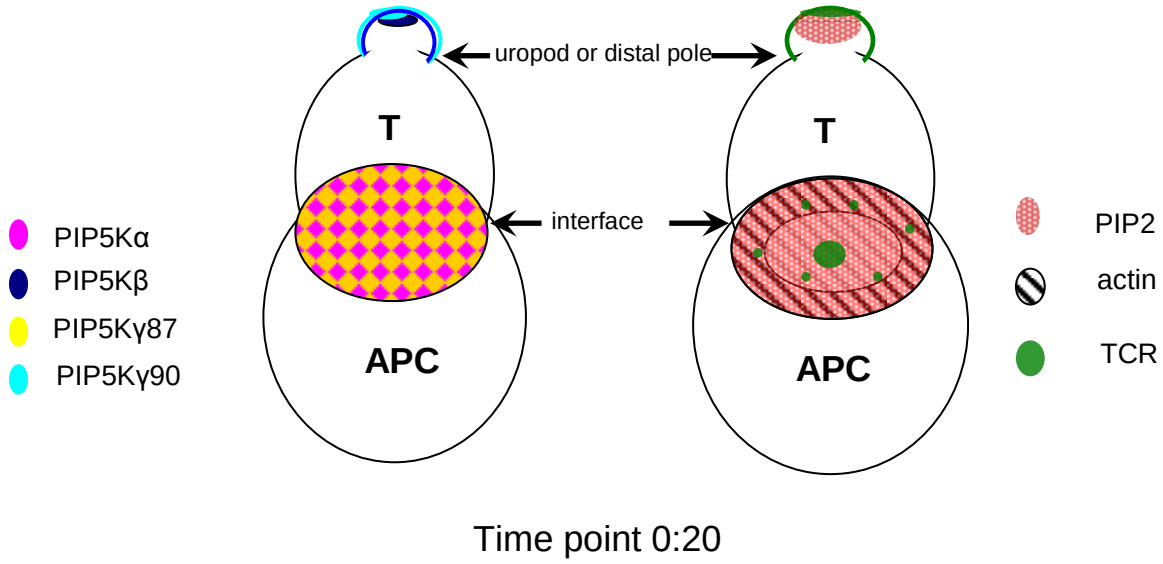


Figure 3-8. Differential accumulations of PIP5K isoforms in early T cell-APC coupling, as compared to that of PIP2, actin and TCRs. Immediate upon TCR triggering by antigen presenting cells, PIP5Kα and γ87 accumulate to the interface of the T cell and the APC, where they contribute to the majority of PIP2 production upon T cell activation. In contrast, β and γ90 isoforms mainly localize to the uropod or distal pole of the activated T cells, where a small amount of PIP2 is generated. While actin is mainly enriched at the periphery of the IS, PIP2 is diffusely accumulated there. TCR accumulation in the early time point (0:20) is mostly randomly distributed at the IS, with some distal accumulation as well.

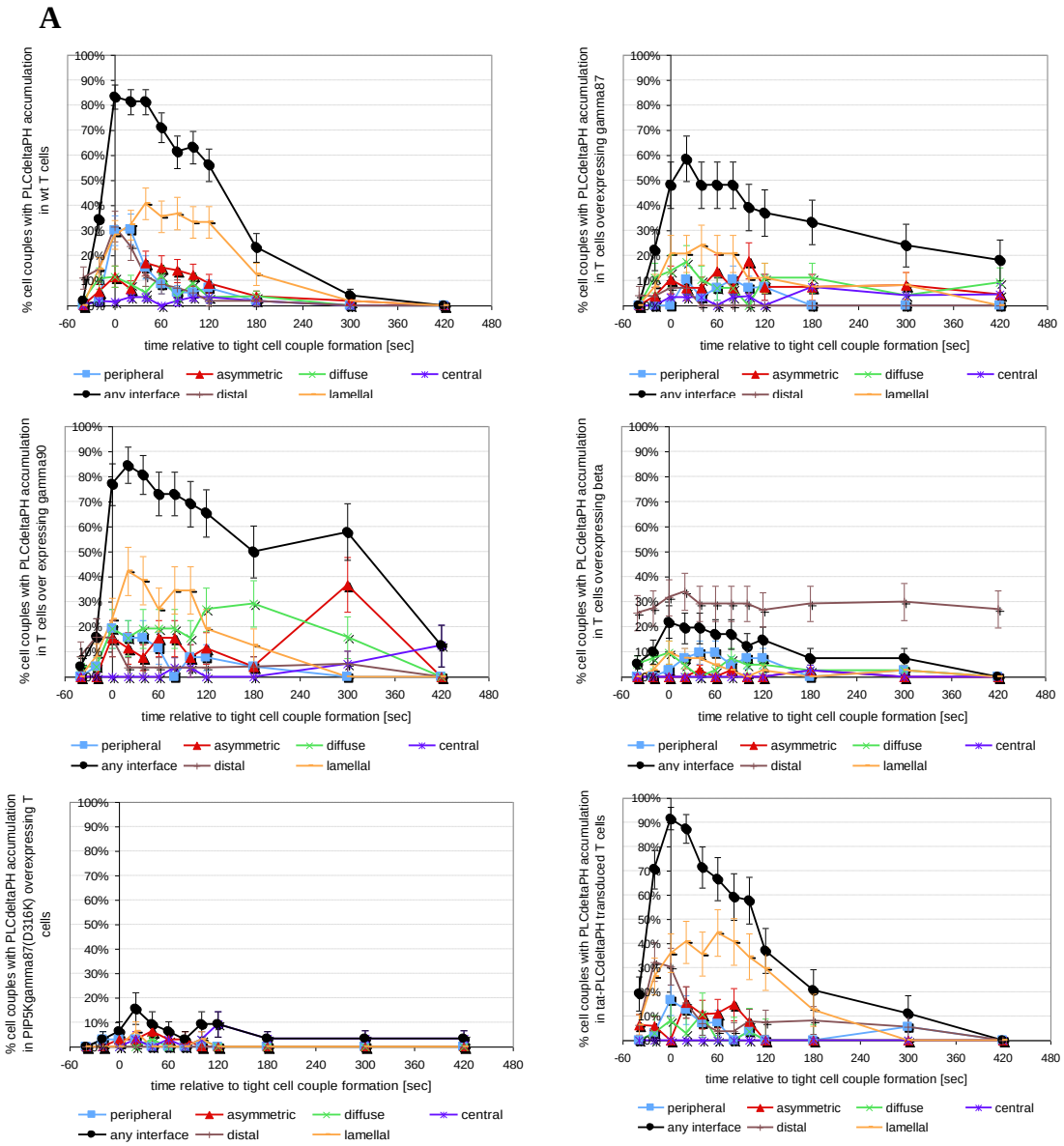


Figure 3-9. PLCδPH accumulation in PIP5K overexpressing or tat-PLCδPH tranduced T cells upon activation. Primary 5c.c7 T cells transduced with PLCδPH-GFP together each PIP5K isoform, or with treatment of 5μM tat-PLCδPH peptide, were mixed with antigen-loaded CH27 APCs, time-lapse images of both DIC and GFP were taken as described in materials and methods. PLCδPH-GFP accumulation was analyzed in each PIP5K isoform overexpressing T cell or tat-PH treated T cell forming a tight cell couple with an APC at different time points. A.The graphs show the percentage of cell couples with standard errors that displayed accumulation of PH-PLCδ with the indicated patterns for differently treated T cells upon coupling with APCs. 60 cell couples were analyzed for T cells transduced with PLCδPH-GFP alone, 41, 29, 26 cell and 32 couples were analyzed for T cells transduced with PLCδPH-GFP+PIP5Kβ, PLCδPH-GFP+PIP5Kγ87 and PLCδPH-GFP+PIP5Kγ90 and PLCδPH-GFP+PIP5Kγ87 (D316K) respectively. 36 cell couples were analyzed for T cells transduced with PLCδPH-GFP and treated with 5μM tat-PLCδPH peptide (Continued on next page).

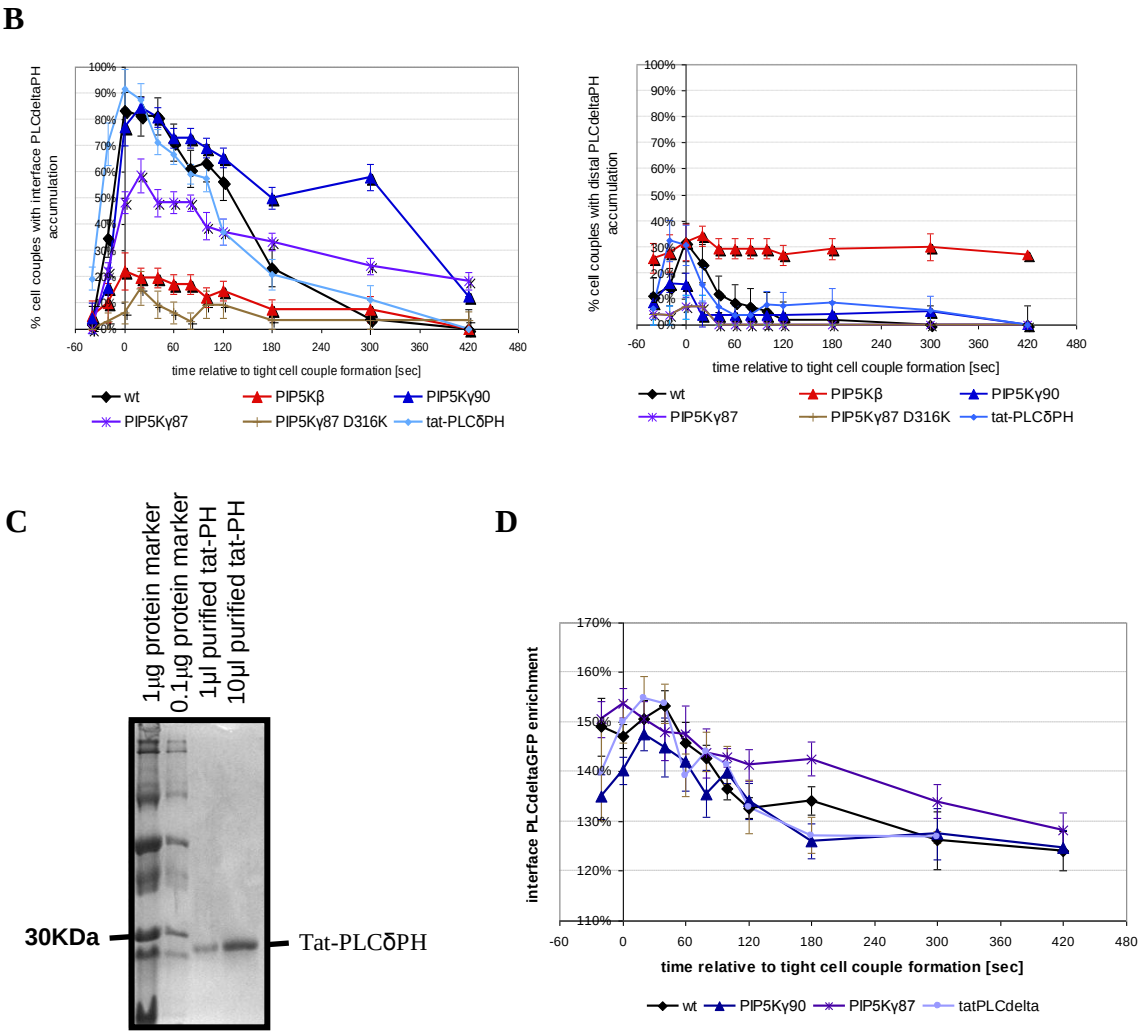


Figure 3-9. PLCδPH accumulation in PIP5K overexpressing or tat-PLCδPH tranduced T cells upon activation. (continued) B. The two graphs are alternative displays of the graphs in A, which show the the percentage of cell couples for differently treated T cells that displayed the same PH-PLCδ1 accumulation pattern (any interface or distal) upon APC stimulation, for direct comparison. C. A representative coomassie blue staining of purified tat-PLCδPH proteins (run on a SDS-PAGE gel) used for protein transduction experiment. D.The graph shows the interface enrichment of PH-PLCδGFP in differently treated T cells. Cell couples analyzed were 50, 21 and 19 for T cells overexpressing PH-PLCδGFP alone, PH-PLCδGFP+PIP5Kγ87 and PH-PLCδGFP+ PIP5Kγ90. 25 cell couples were analyzed for T cells overexpressing PH-PLCδGFP and treated with 5µM tat- PH-PLCδ protein.

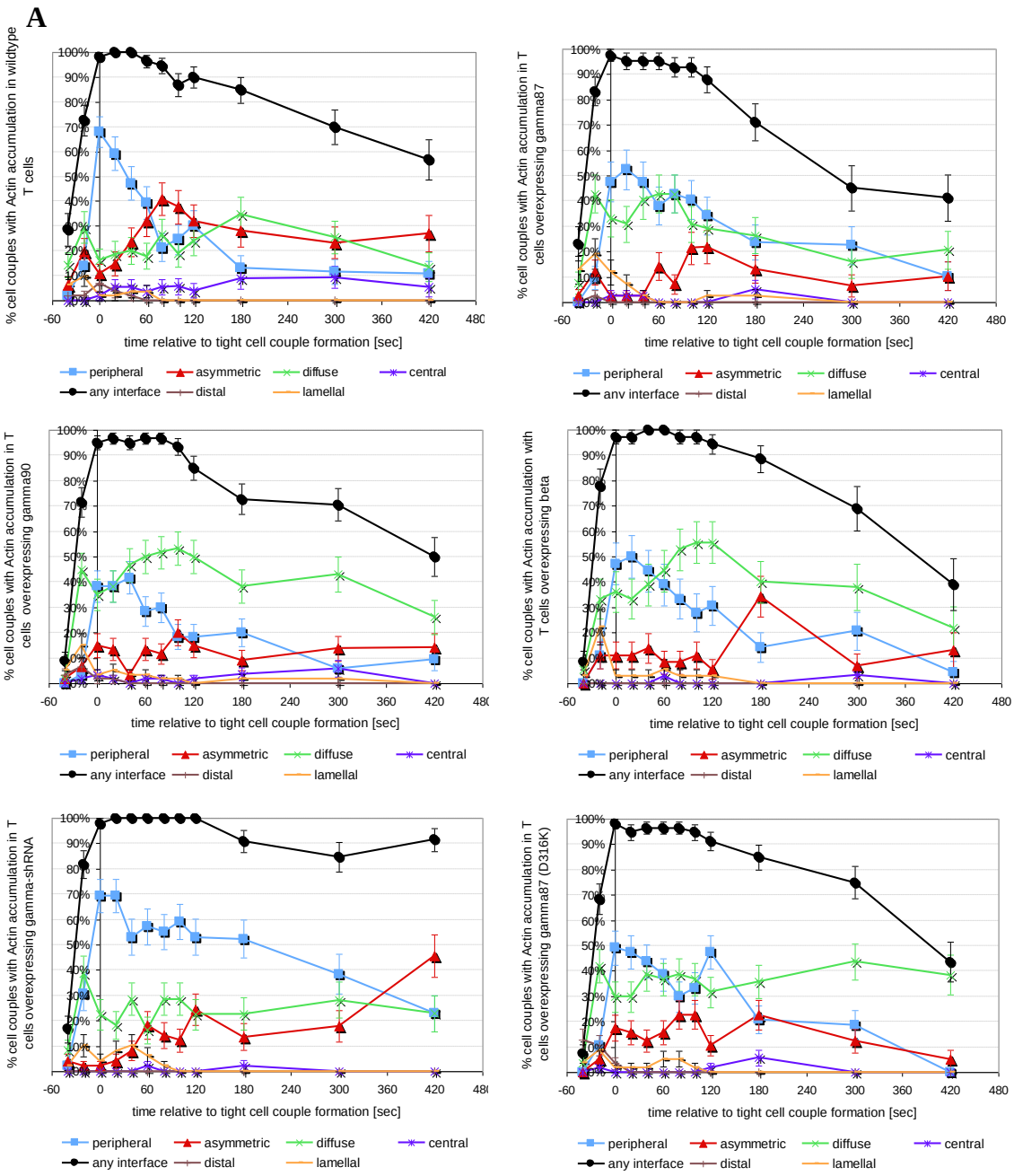


Figure 3-10. Actin accumulation in differently treated T cells upon APC activation. A. The graphs show the percentage of cell couples with standard errors that displayed accumulation of actin with the indicated patterns for T cells with different treatments upon coupling with APCs. 56, 36, 42, 60, 49, 56 and 29 cell couples were analyzed for T cells transduced with actin-GFP alone, actin-GFP+PIP5Kβ, actin-GFP+PIP5Kγ87, actin-GFP+PIP5Kγ90, actin-GFP+PIP5KγshRNA, actin-GFP+ PIP5Kγ87 (D316K) and tat-PH-PLCδ, respectively. (continued on next page)

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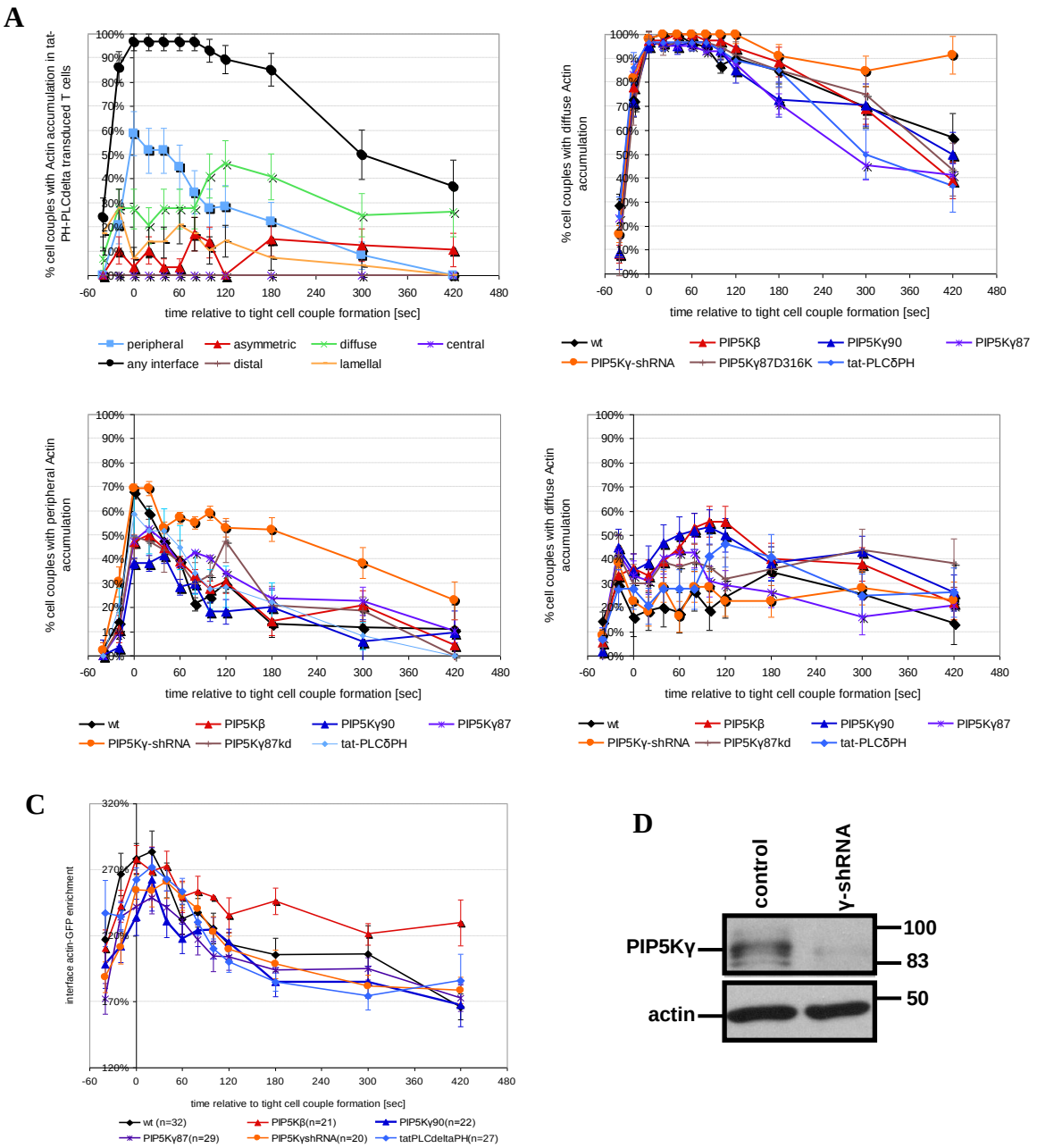


Figure 3-10. Actin accumulation in PIP5K overexpressing or knockdown T cells upon APC activation. (continued) B. The three graphs are alternative displays of the graphs in A, which show the percentage of cell couples with standard errors for PIP5K overexpressing or knockdown T cells that displayed the same actin accumulation pattern (any interface, peripheral or diffuse) upon coupling with APCs for direct comparison.C. The graph shows the interface enrichment of actin-GFP in differently treated T cells upon APC activation.D.A representative western blot using anti-PIP5Kpanγ Abs to detect PIP5Kγ protein levels in 5c.c7 T cell lysates with or without PIP5Kγ-shRNA overexpression. Three independent shRNA experiments showed an average 89±7% knockdown of endogenous PIP5Kγ protein levels (Steve Mao).

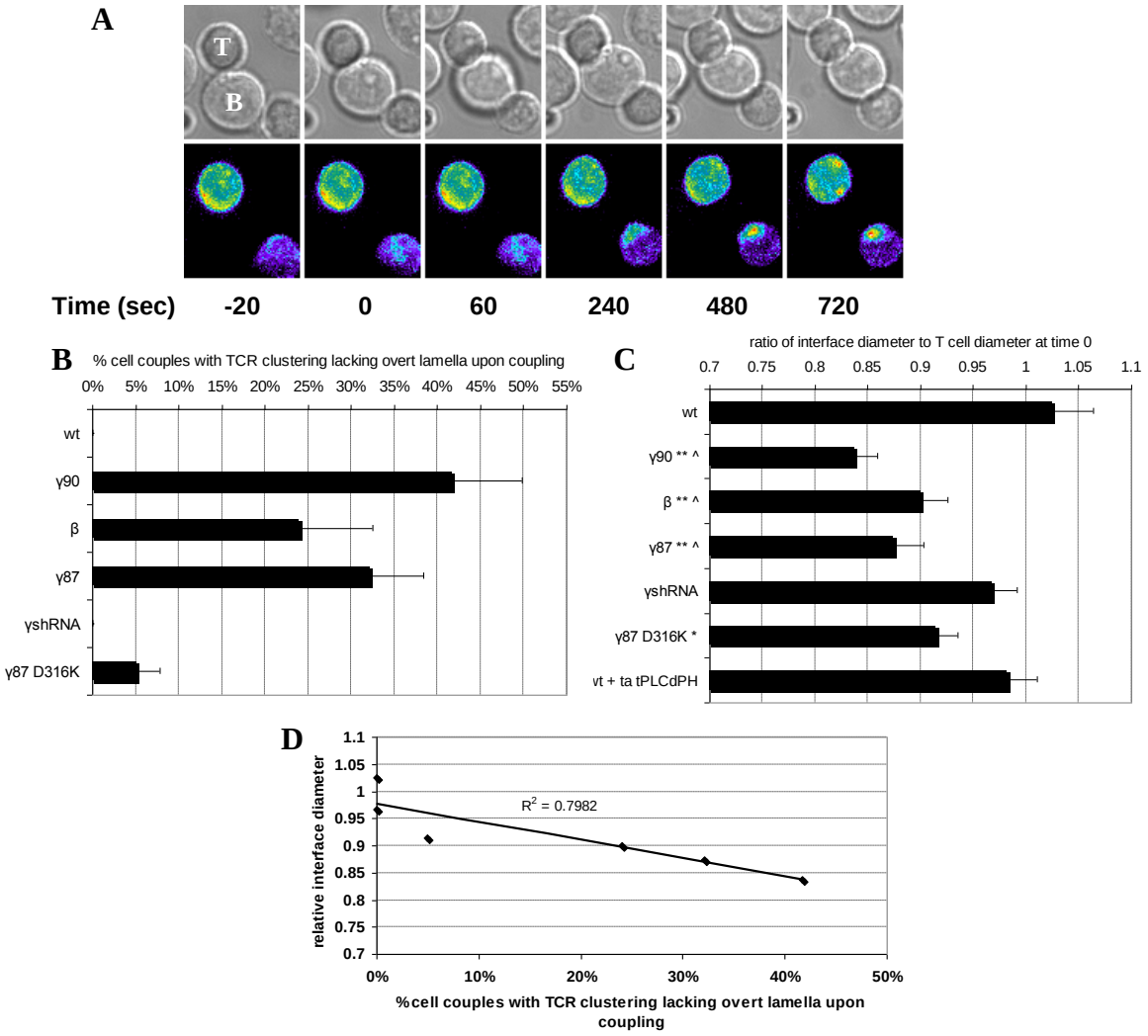


Figure 3-11. Morphological changes of PIP5K overexpressing T cells upon coupling with APCs. A. Representative images showing the absence of lamellapodium formation upon PIP5K overexpressing T cells forming cell couples with peptide loaded APCs. The T cells were transduced with PIP5K+TCRzeta-GFP. B. Statistics of the lamellipodium formation in PIP5K overexpressing T cells upon interacting with antigen-pulsed APCs. Lamellipodium formation was calculated by combining both DIC and GFP (TCRzeta) images. C. Decrease of interface diameter in PIP5K overexpressing T cell-APC couples. Data shown were the ratios of the interface diameters of T cell-APC couples to those of the T cell diameters upon tight cell couple formation (time 0). Interface diameters of T cell-APC couples and the diameters of the T cells were measured in DIC images using the region measurements function of the Metamorph software. The data are means ± S.E. of three independent experiments. (* $p<0.05$.** $p<0.01$. Two tails t test).D. Correlation between interface diameter and T cell lamellipodium formation. 27, 31, 39, 53 and 41 cell couples were analyzed for T cells tranduced with TCRzeta-GFP alone, TCRzeta-GFP+ PIP5Kβ, TCRzeta-GFP+ PIP5Kγ87, TCRzeta-GFP+PIP5Kγ90 and TCRzeta-GFP+PIP5KγshRNA, respectively.

A

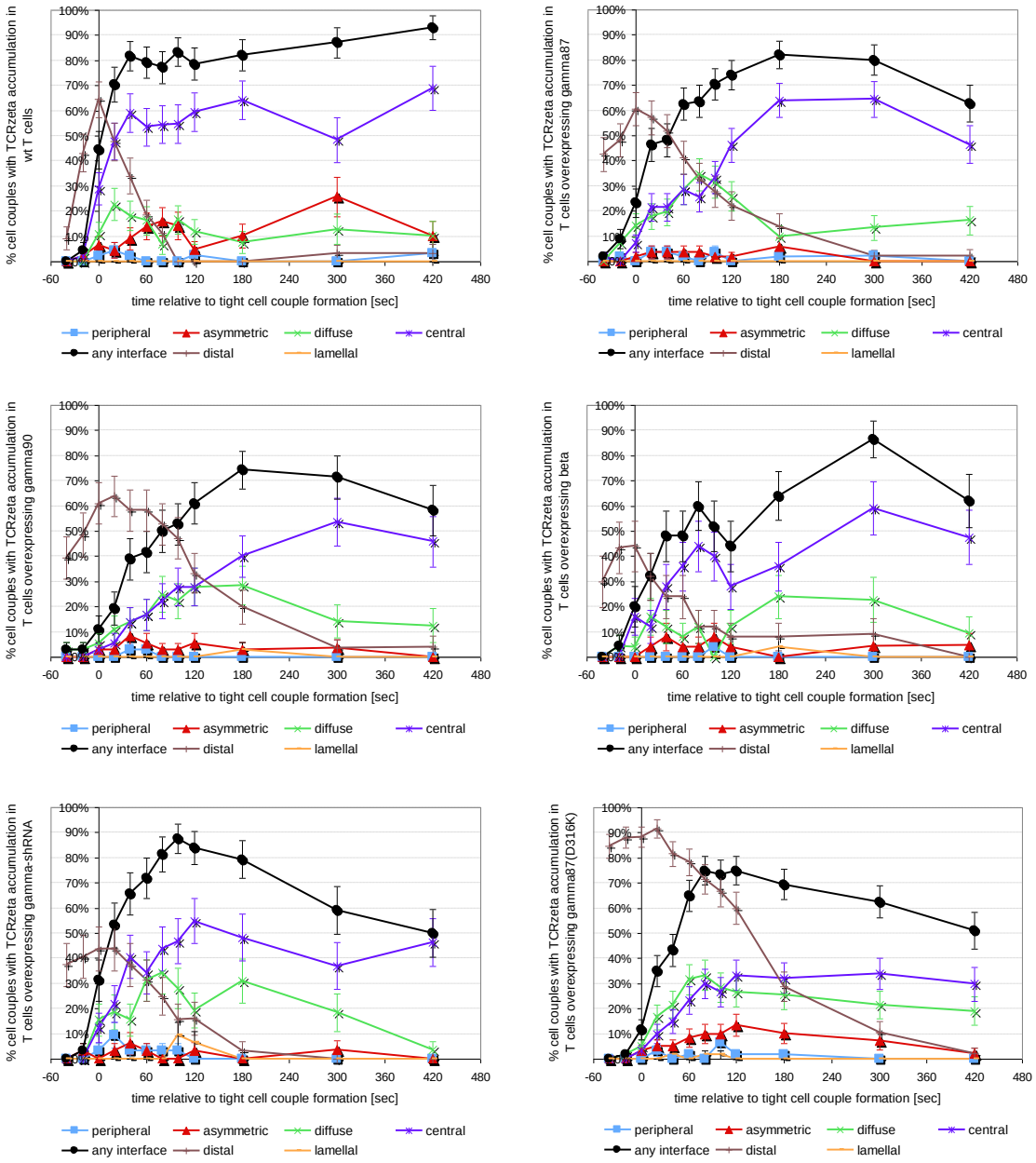


Figure 3-12. TCRzeta accumulation in PIP5K overexpressing or knockdown T cells upon activation. A.The graphs show the percentage of cell couples with standard errors that displayed accumulation of TCRzeta with the indicated patterns for T cells overexpressing or knockdown of PIP5K isoforms upon coupling with APCs. 45, 25, 56, 36, 32 and 60 cell couples were analyzed for T cells transduced with TCRzeta-GFP alone, TCRzeta-GFP+PIP5Kβ, TCRzeta-GFP+PIP5Kγ87, TCRzeta-GFP+PIP5Kγ90, TCRzeta-GFP+PIP5KγshRNA and TCRzeta-GFP+PIP5Kγ87 (D316K) respectively. (Continued on next page)

B

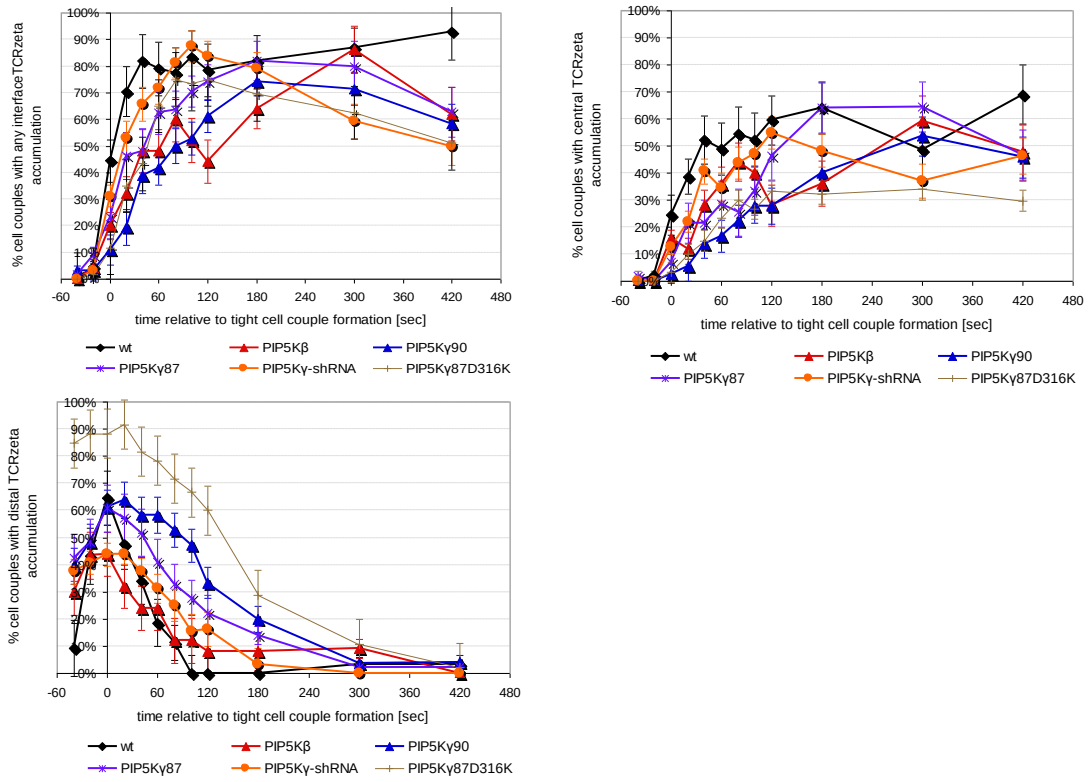


Figure 3-12. TCRzeta accumulation in PIP5K overexpressing or knockdown T cells upon activation. (continued) B. The graphs are alternative displays of the graphs in A, showing the percentage of cell couples with standard erros for PIP5K overexpressing or knockdown T cells that displayed the same TCRzeta accumulation pattern (any interface, central or distal) upon coupling with APCs for direct comparison.

Chapter IV Regulation of TCR proximal signaling and effector functions by PIP5Ks

Introduction

T cells are a central player in adaptive immunity. They fulfill their function by cytokine production or direct killing of foreign-antigen bearing cells. Appropriate T cell activation is required to prevent the development of infectious disease and cancer, whereas inappropriate T cell activation can lead to a variety of autoimmune diseases. Early steps in T cell activation occur through engagement of T cell receptors by peptide-MHC complexes on an APC, which initiate the signaling pathway leading to T cell proliferation and effector function (Smith-Garvin et al., 2009).

Though it is not exactly known how TCR signaling is triggered, the earliest event following TCR ligation is thought to be the activation of Src-related proximal tyrosine kinases, Lck and Fyn, which then phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) on the TCR/CD3 chains, resulting in the recruitment and activation of the ζ chain-associated protein of 70KDa (ZAP-70). ZAP-70 subsequently recruit and phosporylate numerous substrates, leading to a cascade of phosphorylation events (Billadeau et al., 2007; Burkhardt et al., 2008; Gomez et al., 2008). One of the most important ZAP-70 effectors is the 36KDa transmembrane adapter protein, linker for the activation of T cells (LAT). LAT is believed to be the scaffold protein that brings together the early tyrosine phosphorylation events with the distal TCR signaling pathway leading to T cell effector functions, such as IL-2 production and T cell proliferation (Zhang et al., 1998; Wange et al., 2000; Wonerow et al., 2001). Once phosphorylated, LAT recruits several SH2 domain containing proteins such as SLP-76, PLC-γ1, Grb2, Gads, c-Cbl, Vav, Itk and PI3K. This is known as the LAT signalosome, which is critical for TCR signaling. Both PLCγ and PI3K subsequently act on PIP2 on the inner leaflet of the membrane to produce the active signaling intermediates, IP3, DAG and PIP3. IP3 will be released from the membrane into the cytosol, activating the Ca^{2+} channels on the ER membrane to increase intracellular calcium. Calcium release then causes nuclear translocation of NFAT, a transcription factor important for transcription of several genes, most notably, IL-2, a cytokine that promotes long term proliferation of activated T cells. DAG stays on the membrane, binds to and activates several PKCs, among the most important is, PKCθ, which is responsible for activation of the transcription factors NF-κB

and AP-1. The PIP3/PI3K pathway has been implicated in T cell proliferation, survival and motility.

TCR ligation upon APC recognition also results in rapid actin remodeling at the contact area of the two cells, which is important for reorganization of signaling molecules and the formation the immunological synapse (IS). The structure and function of the IS has been subjected to intense study since its discovery in 1998. The 'original IS' was described as a highly ordered structure, with TCR and PKCΘ at the center, forming the central supermolecular activation cluster (cSMAC). Talin and LFA-1 surrounded the cSMAC, forming the peripheral supermolecular activation cluster (pSMAC) (Monks et al., 1998). Recent studies have shown, however, that IS organization is very variable and its formation is a dynamic process depending on many factors, such as antigen quality, stimulation type and T cell developmental status (Friedl et al., 2005; Fooksman et al., 2009). Though it is known that TCR engagement is required for actin reorganization, the exact signaling pathways that control TCR-mediated actin remodeling remain unclear. One of the best characterized molecules is Vav1, a guanine nucleotide exchange factor for small Rho GTPases. Upon TCR stimulation, Vav1 is recruited and activated by association with a signaling complex containing the adapter proteins LAT and SLP-76, and subsequently activates Cdc42 and Rac1, promoting actin polymerization by WASp and WAVE2, respectively (Holsinger et al., 1998; Fischer et al., 1998). Dysfunction of any of these TCR proximal signaling proteins have been shown to affect actin reorganization at the IS as well as T cell activation (Fuller et al., 2003).

Recent studies of individual actin binding proteins, have greatly advanced our understanding of how actin dynamics are regulated at the IS and how it would affect T cell signaling and effector functions. For example, ERM proteins are rapidly inactivated upon antigen stimulation. The inactivation relaxes the linkage between the cortical actin cytoskeleton and the plasma membrane. This decreases T cell rigidity, and subsequently promotes T cell-APC conjugate formation and T cell activation (Faure et al., 2004). HS1, the hematopoietic lineage-restricted homolog of the actin binding protein Cortactin, functions to stabilize existing branched actin filaments by bridging the Arp2/3 complex with actin. Upon TCR ligation, HS1 deficient T cells formed unstable and disordered actin-rich structures at the IS, with defective Ca^{2+} signaling and IL-2 promoter activity

(Uruno et al., 2003; Gomez et al., 2006). Cofilin, the actin severing and depolymerization protein, is dephosphorylated and activated upon TCR/CD28 costimulation, and then depolymerizes and severs the F-actin filaments. It has been shown that cell-permeable peptides that block Cofilin/F-actin interactions impair IS formation, and cytokine production, as well as T cell proliferation (Eibert et al., 2004).

PIP2 is an essential lipid signaling molecule involved in several key pathways leading to T cell activation. In addition, being a precursor for three second messengers, PIP2 regulates the activity of many actin-binding proteins, and thus is a critical regulator of the actin cytoskeleton in cells. The many functions of PIP2 suggest that production of PIP2 is locally regulated in response to different cellular stimuli. In the last chapter, we showed that different PIP5K isoforms, the major producers of cellular PIP2, were differentially targeted to the two poles of activated T cells, and contributed to different local PIP2 dynamics. In contrast to these differences, overexpression of different PIP5K isoforms caused similar defects in actin reorganization and increased cellular rigidity. In addition, knockdown of the major PIP5K isoform γ led to sustained actin accumulation at the periphery of the IS.

To better understand the roles of different PIP5K isoforms in T cell activation, in this chapter, we examined TCR proximal signaling as well as T cell effector function in PIP5K overexpressing or knockdown T cells. We also looked at the activity of the actin binding ERM proteins in the T cells with different PIP5K levels upon TCR stimulation. We found that overexpression of PIP5Ks caused defective TCR proximal signaling and IL-2 production, whereas knockdown of the PIP5Kγ in T cells led to enhanced proximal signaling and IL-2 production. In addition, T cells with increased PIP5Kγ exhibited significant reduction in ERM protein inactivation upon TCR stimulation. Together, these data suggest that T cell activation can be modulated by changing cellular PIP5K levels. PIP5K-mediated production of PIP2 inhibits ERM inactivation upon TCR triggering, which correlates with in increased cellular rigidity and impaired actin reorganization at the IS, as well as defective T cell signaling.

Results

1. Overexpression of PIP5Ks did not cause significant changes in cell couple formation

As PIP5K overexpressing T cells exhibited increased cellular rigidity upon coupling with APCs, we asked if PIP5K overexpression or knockdown would affect the ability of T cells to form cell couples with APCs (Figure 4-1). Cell couple formation was determined in bright field images. For every T cell in contact with an APC for ≥ 6 frames (i.e. > 90 sec) it was determined, whether a tight cell couple formed or not. For each condition, more than 50 cell couples were analyzed. Neither overexpression of PIP5Kβ, γ87 or γ90, nor knockdown of PIP5Kγ led to significant changes in T cell-APC couple formation. PIP5Kα overexpression instead caused increased cell couple formation as compared to wild-type controls (86±9% vs. 62±3%, $p<0.01$). These are not unexpected results, as it has been shown that PIP2 concentration positively regulates membrane adhesion energy (Raucher et al., 2000). Thus increased membrane adhesion may offset the effect of increased cellular rigidity on cell couple formation.

2. Overexpression or knockdown of PIP5Ks did not significantly change the T cell F-actin content both before and after TCR stimulation

Changes in cellular F-actin concentration have been documented in several cellular settings with PIP5K overexpression or knockdown/knockout, the results vary depending on the cell types and the stimulus applied (Mao and Yin 2007). To see if changes in cellular PIP5K levels affect actin polymerization in the primary mouse T cells, we stained the PIP5K overexpressing or knockdown T cells with fluorescently labeled phalloidin and analyzed the T cell F-actin staining by flow cytometry. The specificity of phalloidin staining was confirmed by fluorescent microscopy, which showed cortical actin distribution in resting T cells and polarized actin accumulation at the IS of fixed T cell-APC couples. Overexpression of none of the PIP5K isoform caused any significant changes in F-actin levels in resting T cells as compared to non-overexpressing T cell controls. As shown in Figure 4-2, the mean fluorescence intensity of phalloidin staining of PIP5K overexpressing and wt T cells was comparable. Knockdown of PIP5Kγ in T cells did not cause any significant changes in F-actin levels either, as compared to controls. To see if changes in PIP5K levels affect T cell actin polymerization upon T cell activation, we stimulated the PIP5K overexpressing or knockdown T cells with 10μg/ml anti-CD3/CD28 antibodies for 2 min, then fixed and stained the cells with phalloidin to look at their changes in F-actin concentrations. The reasons we did not use APCs to

stimulate the T cells were that, firstly, the APCs were indistinguishable from T cells when analyzed by flow cytotometry; Secondly, anti-CD3/28 stimulation is a widely used method for studying T cell activation. Stimulation of wt T cells with anti-CD3/CD28 antibodies (2 min) generally caused a 20-30% increase in phalloidin staining compared to non-stimulated controls, suggesting a rapid actin polymerization upon T cell activation (Figure 4-2). Similar to control cells, PIP5K overexpressing or knockdown T cells revealed increased F-actin staining after anti-CD3/CD28 stimulation, and the changes are comparable to that of control cells (Figure 4-2). These results are not unexpected, as similar studies on other cell types gave similar results. It is found that in some cell types, overexpression of PIP5Ks does not change the overall F-actin content, but changes the form of the F-actin assembled structures. For instance, it was found that overexpression of any of the three PIP5K isoforms in COS7 cells led to the production of massive amounts of short actin fibers while disrupting the actin stress fibers (Hisamitsu Ishihara 1998). Our phalloidin staining data was also consistent with our live cell imaging data, in which neither actin-GFP interface accumulation nor enrichment was significantly changed in T cells when PIP5Ks were overexpressed or knocked down (Figure3-10C), although the F-actin distribution patterns were changed at the IS.

3. TCR proximal signaling was negatively regulated by cellular PIP5Kγ levels.

To test if TCR proximal signaling is affected by changing cellular PIP5K levels, we used western blotting to detect the phosphorylation of one of the most important TCR proximal signaling protein, LAT, linker for the activation of T cells. LAT is a 36 KDa transmembrane adapter protein, which has four conserved LAT tyrosines 132, 179, 191, and 226, known to be important for LAT function (Zhang et al., 1998; Zhu et al., 2003). Once phosphorylated, these four conserved tyrosines serve as docking sites for SH2 domain containing proteins, including PLCγ1, Grb2 and Gads, which in turn associates with SLP-76, SOS and c-Cbl. These protein-protein interactions result in the formation of the LAT signalosome, vital for linking TCR proximal signaling to the downstream T cell effector functions. LAT 191 tyrosine specifically interacts with Gads and Grb2, and is known to be important for IL-2 gene expression (Wange et al., 2000; Wonerow et al., 2001). Thus we chose the specific anti-phosphoLAT 191 antibody to examine LAT activation in APC stimulated T cells expressing different levels of PIP5Ks. We first

tested the specificity of this antibody in the lysates of activated wt T cells. As expected, a 36 KDa band was only detected in T cell lysates that are activated by antigen loaded APCs, not in lysates containing APCs alone, T cells alone, or T cells with APCs but without antigen. Pilot experiments revealed that LAT phosphorylation can be detected as early as 30 seconds upon APC stimulation. The signal becomes strongest at around 2 minutes and is still detectable after 20 minutes of APC stimulation. To best match the biochemistry results with our imaging data, we chose 2 minutes APC stimulation for all of our western blotting experiments. Phosphorylation was detected at 1 min and 5 min APC stimulations. The results were comparable to 2 min stimulations. To minimize variations among samples potentially raised by small numbers of cells (100,000 T cells per sample), we FACS sorted equal numbers of GFP+ (transduced) and GFP- T cells (controls) and added equal numbers of APCs (~100,000) to stimulate the T cells for each condition. Due to the very low transduction efficacy for PIP5K β and α isoforms in primary mouse T cells (GFP+ cells were less than 0.5% of total live T cells), we were unable to sort enough β or α overexrpessing T cells for LAT phosphorylation western blots. Overexpression of PIP5Kγ87 and γ90 caused a consistent decrease in LAT phosphorylation in T cells activated by APCs (Figure 4-3). Quantification of the phosphorylated LAT intensity standardized to the loading control GAPDH from at least 3 independent experiments showed that γ87 overexpression resulted in a 15±5% decrease of LAT phosphorylation in APC activated T cells as compared to controls (Figure 4-3B). PIP5Kγ90 overexpression resulted in a 46±10% decrease ($p<0.05$) in LAT phosphorylation in activated T cells as compared to controls. In contrast, T cells with PIP5Kγ knockdown exhibited significantly increased LAT phosphorylation upon activaton. ($p<0.05$) (Figure 4-3B). To confirm the observed effects on TCR proximal signaling in response to PIP5K perturbations, we also tested the phosphorylation of another important proximal signaling protein, phospholipase C gamma 1(PLCγ1), whose phosphorylation activates PIP2 hydrolysis and increase intracellular calcium. We used an antibody that selectively recognize the endogenous form of PLCγ1 when phosphorylated on tyrosine 783, a site known to be vital for the in vivo function of PLCγ1 in T cells (Samelson et al., 2002, Irvin et al., 2000). Similar to LAT phosphorylation, overepxression of PIP5Kγ led to reduced PLCγ phosphorylation in T cells upon

activation: a consistent but not significant decrease (19±5%) for γ87 and a significant decrease for γ90 (46±12%, $p<0.05$), whereas knockdown of PIP5Kγ resulted a slight increase of PLCγ1 phosphorylation by 1.5±0.43 fold (Figure 4-3). Collectively, our results indicate that TCR proximal signaling is negatively regulated by cellular PIP5Kγ levels.

4. TCR mediated IL-2 production was impaired in PIP5Kγ overexpressing T cells

One of the most important cytokines produced by TCR-stimulated T cells is interleukin-2 (IL-2), which controls T cell proliferation. To test if IL-2 production is affected by changing cellular PIP5K levels in T cells, we stimulated equal numbers of PIP5K overexpressing, knockdown, or control T cells with antigen preloaded APCs for 16h in IL-2 free medium, and supernatants were assayed for IL-2 production by ELISA. We found that T cells overexpressing the PIP5K isoforms, γ87, γ90 or β produced significantly reduced levels of IL-2, lowering on average, to 74±8%, 44±4%, and 30±3% of those produced by control cells, respectively (Figure4-4A). The experiments were repeated more than three times on each condition and consistent decreases were found. The decreases in IL-2 production was not a result of increased cell death, as less than 5% cell death was observed in each condition by Trypan blue staining at the time when the supernatants were collected. In contrast, T cells with PIP5Kγ knockdown showed a 26±9% increase in IL-2 production compared to control cells ($p<0.05$). To see if overexpression generates an artifact in IL-2 production, we also tested IL-2 production in T cells with GFP overexpression alone. As expected, no significant changes were found in IL-2 production in GFP overexpressing T cells compared to controls. As another control, we examined IL-2 production in T cells overexpressing the kinase deficient PIP5Kγ87(D316K), again, IL-2 secretion in these cells were comparable to controls, suggesting that decreased IL-2 production correlates with PIP5K dependent PIP2 synthesis. Interestingly, we found that overexpression of the PH-PLCδ domain, the PIP2 marker that has been shown to bind PIP2 and decrease free PIP2 levels, significantly increased IL-2 production in T cells (Figure 4-4A). Taken together, these results suggest that PIP5K mediated PIP2 synthesis negatively correlates with IL-2 production in activated T cells. PIP2 masking by PH-PLCδ overexpression enhances IL-2 production further indicate there is a pool of PIP2 on the T cell membrane that negatively regulate

IL-2 secretion. These are unexpected results, as in general, increase in PIP2 production is supposed to generate more IP3 and increase intracellular calcium, which in turn should lead to increased IL-2 gene transcription. One explanation is that PIP5K overexpression impaired PLCγ1 activation by affecting LAT phosphorylation (Figure 4-3), which is required to hydrolyze PIP2 for IP3 and DAG production. Another explanation is that PIP5K overexpression caused defective cortical actin reorganization and proximal signaling, which can lead to decreased IL-2 secretion. Manipulating cellular PIP2 levels by other means, such as lowering PIP2 by overexpressing a membrane targeted 5-phosphatase or a constitutively active PLCγ should help to further delineate the role of cellular PIP2 in regulating T cell IL-2 production.

5. Latrunculin B treatment did not rescue IL-2 production in PIP5K overexpressing T cells

As PIP5K overexpressing T cells exhibited defective actin reorganization and rigid cellular morphology, we asked if the observed defect in IL-2 production was due to an increase in local actin polymerization that could not be detected by total cellular phalloidin staining. Though it is generally believed that Latrunculin (Latr.) treatment inhibits T cell IL-2 secretion, the Latr. concentration used in the literature is relatively high(>=1μM). It was shown that 0.05-0.2 μM Latr. B treatment restored particle binding to PIP5Kγ-/- bone marrow derived macrophages (which had increased F-actin), whereas application of 0.5 μM Latr. B that inhibited binding to wt cells, failed to rescue particle binding to PIP5Kγ-/- BMMs (Mao et al., 2009). This biphasic requirement for polymerized actin was also found in regulated exocytosis (Muallem et al., 1995). A similar mechanism might apply for IL-2 secretion in T cells. To find out the optimal range of Latr.B concentration that does not interfere with normal IL-2 secretion in wt T cells but would potentially increase IL-2 secretion in PIP5K overexpressing T cells (if there is a local increase in F-actin in these cells), we treated wt T cells with a series of Latr.B concentrations (from 8nM to 4 μM). These cells were then stained with phalloidin and their F-actin levels were analyzed by flow cytometry. By so doing we aim to narrow down the concentration range of Latr. B that has no observable effect on cellular F-actin and would be applied to the IL-2 secretion-rescue assay. As shown in Fig 4-5, only when Latr.B was used at >=125nM Latr. B, did we observe a consistent decrease in F-actin

levels in T cells. So we tried Latr. B concentrations at 10nM-500nM. However, PIP5K overexpressing T cells produced similar levels of IL-2 when treated with Latr. B within this range, as compared to those produced without Latr.B treatment (Figure 4-5C). Thus decreased IL-2 production does not appear to be due to increased actin polymerization in these PIP5K overexpressing T cells.

6. TCR triggered ERM inactivation was inhibited in PIP5K overexpressing T cells

We reasoned that the inability of the T cells to form a F-actin enriched peripheral lamellapodium at the IS when PIP5K was overexpressed may be due to the inhibition of ERM inactivation caused by increased membrane PIP2 production. ERM proteins are thought to be the crosslinkers between the cortical actin cytoskeleton and the plasma membrane. ERM activation is achieved by binding to PIP2 and phosphorylation at a conserved Threonine site. Our hypothesis is that, increased PIP2 generation by PIP5Ks should bind to the ERM proteins and prevent them from being inactivated, and as a consequence, the cortical actin cytoskeleton can not be detached from the plasma membrane, resulting in disorganized F-actin at the IS and increased T cell rigidity. To test our hypothesis, we used a phospho-ERM antibody and analyzed the levels of ERM phosphorylation in T cells expressing different levels of PIP5Ks before and after stimulation by western blotting. As the APCs (CH27 B lymphoma cells) used to stimulate the 5c.c7 T cells contain comparable levels of phosphorylated ERM proteins to those in the T cells at resting state, we stimulated the T cells with anti-CD3/CD28 antibodies. As previously published, the phosho-ERM antibody detected a major band at around 78 KDa corresponding to phosphorylated Moesin and a faint slower migrating band representing phosphorylated Ezrin in lysates prepared from unstimulated wt T cells. Upon TCR costimulation by anti-CD3/CD28 antibodies, a rapid decrease (70-80%) of ERM phosphorylation was found (1min) in wt T cells (Figure 4-6). In contrast, T cells overexpressing PIP5Kγ87 or γ90 exhibited reduction in ERM dephosphorylation upon activation (Figure 4-6B). In addition, T cells with PIP5Kγ knockdown showed similar degree of ERM dephosphorylation to that of control cells upon CD3/CD28 stimulation (Figure 4-6). These data suggest that PIP5K mediated PIP2 production prevented ERM from dephosphorylation and inactivation upon TCR-triggering, and strongly support our

hypothesis that the cytoskeletal defects in PIP5K overexpessing T cells upon activation is due to the inability to inactivate the ERM proteins.

Discussion

Previous imaging data demonstrated that although different PIP5K isoforms showed different accumulation patterns and caused different changes in PIP2 dynamics, similar defects were observed in actin reorganization. In this chapter we show how changes in PIP5K levels affect T cell function biochemically. We found that:
1.Overexpression of PIP5K isoforms did not significantly change T cell F-actin levels, both before and after TCR stimulation, compared to controls, suggesting that the disorganization of actin cytoskeleton observed microscopically might be a local rather than a global effect. Another possibility is that there are changes in the forms of actin filaments assembly. Although the prevailing thought is that increased PIP2 favor actin polymerization, literature searches on how changes in PIP5K levels affect cellular PIP2 levels and actin polymerization gave variable and complicated results in different cell types. For example, in bone marrow derived macrophagess (BMMs), γ87 is the most abundant isoform, which is 14 fold more than α at the mRNA level. Knockout of γ in BMMs showed little effect on ambient PIP2 content, whereas greatly increased actin polymerization which impedes FCγR clustering and particle attachment. On the other hand, α-/- BMMs showed a slight decrease of PIP2 (20%). There were no obvious changes in cellular F-actin staining, though less actin polymerization was observed at the phagocytic cup (Mao et al., 2009). Megakaryocytes derived from PIP5Kγ-/- embryos had normal basal level of PIP2 and showed no defect in total F-actin levels by phalloidin staining in the absence or presence of integrin stimulation (Wang et al., 2008). It is also shown that overexpression of PIP5K isoforms in COS7 cells did not increase cellular PIP2 levels however it led to the production of massive short actin fibers while disrupting actin stress fibers (Ishihara et al., 1998). Yamamoto et al. found that overexperssion of PIP5K in CV1 cells induced robust actin-stress fiber formation, whereas PIP5K overexpression in REF52 cells and Swiss 3T3 cells generated actin comets predominantly and dissolve stress fibers (Yamamoto et al., 2001; Rozelle et al. 2000). They suggested that the dominance of a unique subset of regulatory proteins within each cellular context may determine the final outcome. Together, these results suggest that cellular PIP2 levels

are relatively stable and finely balanced, and that they do not undergo dramatic changes even when missing a major producer. Changes in local PIP2 concentrations may be functionally more important in affecting local actin dynamics. In addition, PIP5K-mediated PIP2 production does not promote universal actin dynamics and reorganization in different cellular backgrounds.2. PIP5K negatively regulated TCR proximal signaling. T cells with increased PIP5K expression showed significant decreases in LAT and PLCγ phosphorylation upon APC stimulation as compared to control cells. In contrast, T cells with PIP5K knockdown exhibited increased LAT phosphorylation in early activation. How could this happen? One possibility is that PIP5K overexpression causes defective actin reorganization that affects the assembly and recruitment of signaling molecules at the IS and leads to defective signaling initiation and transduction. Alternatively, enhanced actin polymerization at the IS upon PIP5K knockdown promoted signaling molecule assembly and signal transduction. Recent studies on TCR regulation suggest another possibility. It was found that the cytoplasmic tale of the T cell receptor signaling subunits ITAMs contain basic residues that interact with acidic phospholipids on the plasma membrane such that the key signaling tyrosines of the ITAMs are buried in the bilayer at resting state. Receptor ligation is supposed to lead to unbinding of the TCR ITAMs from the membrane and render these tyrosines accessible to phosphorylation by Src kinases (Xu et al., 2008). T cells from basic residue-modified mice exhibited reduced TCR-mediated signaling responses to peptide-loaded APCs, and delayed CD3 epsilon localization to the immunological synapse (Deford-Watts et al., 2009). These results combined with ours, suggest that overexpression of PIP5Ks, by increasing membrane PIP2, an acidic phospholipid, may sequester the basic residues on the TCRs and prevent them from being phosphorylated by Lck. As a consequence, TCR proximal signaling is impaired. In contrast, knockdown of PIP5K, by reducing membrane PIP2, would favor TCR phosphorylation, and thus enhance signaling transduction. 3. Similar to their effects on TCR proximal signaling, cellular PIP5K levels oppositely regulate T cell IL-2 production. IL-2 secretion is one of the most widely used marker for T cell effector function. T cell IL-2 production can be affected by many factors including proximal signaling efficiency, the actin cytoskeleton, Ca^{2+} signaling, T cell proliferation and viability. The last two possibilities are excluded as decreases in IL-2 production found in

PIP5K overexpressing T cells can be detected as early as 4h after APC stimulation and that all cell cultures showed >=95% cell viability at the time when the supernatants were collected for IL-2 ELISA. Both defective proximal signaling and actin reorganization can impair IL-2 production in PIP5K overexpressing T cells, although increase in PIP2 synthesis theoretically should cause more production of IP3 and thus increased Ca2+ signaling. However, we found less phosphorylation of PLCγ in PIP5K overexpressing T cells upon activation, which would lead to reduced PIP2 hydrolysis and less IP3 production. Together, these data suggest that defective TCR signaling and actin reorganization resulted in reduced IL-2 production in PIP5K overexpressing T cells. We also noticed that PIP5Kβ overexpression caused the most dramatic defect in IL-2 production in T cells (lowered to 30% of controls). Overexpression of PIP5Kβ exhibited sustained PH-PLCδ accumulation at the distal pole of the activated T cells. Is it possible that PIP2 produced at the distal pole negatively regulates T cell activation? Another PIP5K isoform γ90, located at the uropod/distal pole, resulted in more pronounced defects in T cell function when overexpressed than overexperssion of its splice variant, γ87; this includes effects on actin reorganization, cellular rigidity, TCR proximal signaling and IL-2 production. These two isoforms differ only in a C-terminal 26aa in γ90, but they target to different poles of activated T cells. This further supports the possibility that distal PIP2 production may further dampen T cell activation by an unknown mechanism. It would be interesting to study T cell activation in PIP5Kβ-/- cells and in PIP5Kβ-/- cells with specific γ90 knockdown. 4. T cells with PIP5K overexpression revealed remarkable reduction in ERM protein inactivation upon TCR costimulation. This blockade of ERM inactivation is likely the cause of increased cellular rigidity and defective actin reorganization at the IS observed in the PIP5K overexpressing T cells. ERM proteins are key players in controlling the cytoskeletal organization and have been demonstrated to regulate the formation of many F-actin containing protrusive structures such as microvilli, filopodia, lamellipodia and microspikes in a wide range of cell types (Denker et al., 2000; Takeuchi et al., 1994; Bretscher et al., 2002; Belkina et al., 2009). The ERM proteins exist in two forms, an ‘inactive form’ in which the proteins are folded through the intramolecular interaction of the FERM domain and the C-terminal end of the protein and an “active” form, in which the protein is unfolded and capable of

interacting with membrane partners and with the actin cytoskeleton. The activation of ERM proteins requires binding of the FERM domain to membranes rich in PIP2 and by the phosphorylation of a conserved threonine residue in the C-terminal domain. In resting T cells, a pool of active phosphorylated ERM proteins is present at the cell cortex. Upon TCR engagement, ERM proteins undergo rapid dephosphorylation and inactivation, an event shown to be critical for cytoskeleton relaxation and enhanced T cell-APC conjugation (Faure et al., 2004). Considering the importance of PIP2 in ERM activation, we suggest that inhibition of ERM inactivation in T cells overexpessing PIP5K can be explained by increased membrane PIP2 production. Several reports have provided evidence for the *in vivo* regulation of ERM function by PIP5Ks though PIP2 production. For example, in NIH3T3 cells, overexpression of PIP5K induced significant recruitment of ERM proteins to the plasma membrane to form microvilli, where the phosphorylated (activated) ERM were concentrated. Overexpression of a kinase-inactive mutant of PIP5K did not induce such changes, suggesting that the induction of ERM phosphorylation and microvillus formation *in vivo* require the kinase activity of PIP5K. In addition, it was found that overexpression of PIP5K in HeLa cells induced a ~sixfold increase in the level of ERM phosphorylation as compared to control cells (Matsui et al., 1999). One study has shown that shrinkage-induced Ezrin translocation and activation in ELA cells is dependent on increased PIP2 level on the plasma membrane. Transfection of a membrane targeted 5-phosphatase domain caused depletion of membrane PIP2 and prevented the shrinkage-induced translocation of GFP-Ezrin to the cortical region (Rasmussen, 2008). Another study demonstrated that microinjection of Neomycin, an aminoglycoside antibiotic that binds to PIP2 with high affinity and thus reduces its availability, induced rapid dephosphorylation of ERMs and translocation of ERM proteins from microvilli to the cytoplasm in L cells (Yonemura et al., 2002). Skittles (Sktl), a PIP5K homologue in C.elegans has been shown to be crucial for the synthesis and maintenance of PIP2 at the plasma membrane. Impairing Sktl function leads to disorganization of the microfilament scaffold along the oocyte cortex and the detachment of cortical actin. Reduction of Sktl activity also impairs the activation of Moesin, a member of the ERM protein family, at the plasma membrane (Gervais et al., 2008). A recent report showed that reduction of membrane PIP2 concentration by either activating

PLC signaling or acute targeting of phosphoinositide-5-phosphatase to the plasma membrane induce the dephosphorylation and release of ERM proteins from the membrane of Jurkat T lymphocytes. The authors suggest that PIP2 regulates both activation and inactivation of ERM proteins by inducing conformational changes in the proteins (Hao et al., 2009). Other than affecting the cortical actin cytoskeleton, dysfunction of ERM proteins influence other aspects of T cell activation as well. After a few minutes of TCR triggering, ERM proteins are rephosphorylated, and bind to membrane proteins such as CD43 and move together with them to the distal pole of the T cells. This ERM mediated CD43 movement out of the center of immunological synapse is thought to be crucial for PKCΘ polarization and TCR clustering at the IS as well as T cell activation (Allenspach et al., 2001). Ezrin and Moesin double-deficient T cells exhibited diminished phosphorylation of PLC-γ1, Ca2+ signaling and IL-2 production (Shaffer et al., 2009). ERM proteins are key components of the uropod of polarized T cells. They have been shown to regulate T cell polarity during migration by a Rho-mediated signaling pathway. Although very little is known about the role of ERM proteins in antigen triggered intracellular signaling pathway in T cells, it is speculated that ERM proteins could play a role in the spatiotemporal positioning of key signaling molecules that polarize upon TCR engagement (Charrin and Alcover, 2006).

In summary, our data suggest that PIP5K mediated PIP2 generation negatively regulate T cell activation through a mechanism that involves impaired ERM activity, which contributes to increased T cell rigidity and impaired actin rearrangement. This in turn may lead to defective TCR signaling and IL-2 production. A working model was proposed in Figure 4-7. In addition, PIP5K isoforms play redundant but not overlapping roles in T cell activation. The PIP2 produced at the uropod/distal pole region of T cells by PIP5K β and γ90 are likely negative regulators of T cell activation.

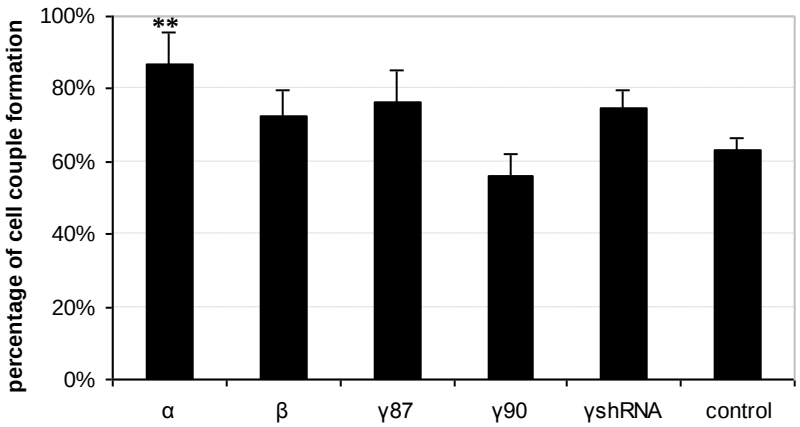


Figure 4-1. Overexperssion of PIP5K isoforms on T cell-APC couple formation. 5c.c7 T cells overexpressing different PIP5K isoforms were mixed with antigen-pulsed CH27 cells. Cell couple formation was determined in bright field images. For every T cell in contact with an APC for ≥ 6 frames (i.e. > 90 sec) it was determined, whether a tight cell couple formed or not. The data are means ± S.E. of at least three independent experiments. The numbers of cell couples analyzed for T cells transduced with PIP5Kα, β, γ87 or γ90, γshRNA and wt controls were 56, 51, 64, 86, 83 and 231 respectively. (**$p<0.01$, as compared to controls. paired t test, 2 sided)

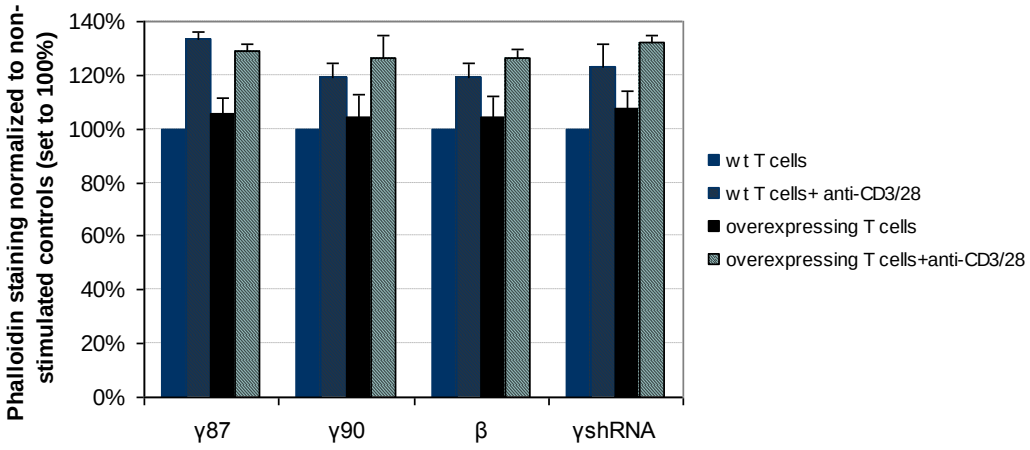


Figure 4-2. Effects of PIP5K overexpression or knockdown on F-actin concentrations in T cells. A. Phalloidin staining in T cells expressing different levels of PIP5Ks with or without stimulation**.** Wt, PIP5K or γshRNA overexpressing 5c.c7 T cells were unstimulated or stimulated with 10ug/ml CD3/CD28 for 2min. Cells were fixed, stained with Alexa-phalloidin 594 and analyzed by flow cytometry. The results were mean fluorescence intensity normalized relative to non-stimulated wt controls (which was set to 100%) to allow for direct comparison. The data are means ± S.E. of three independent experiments.

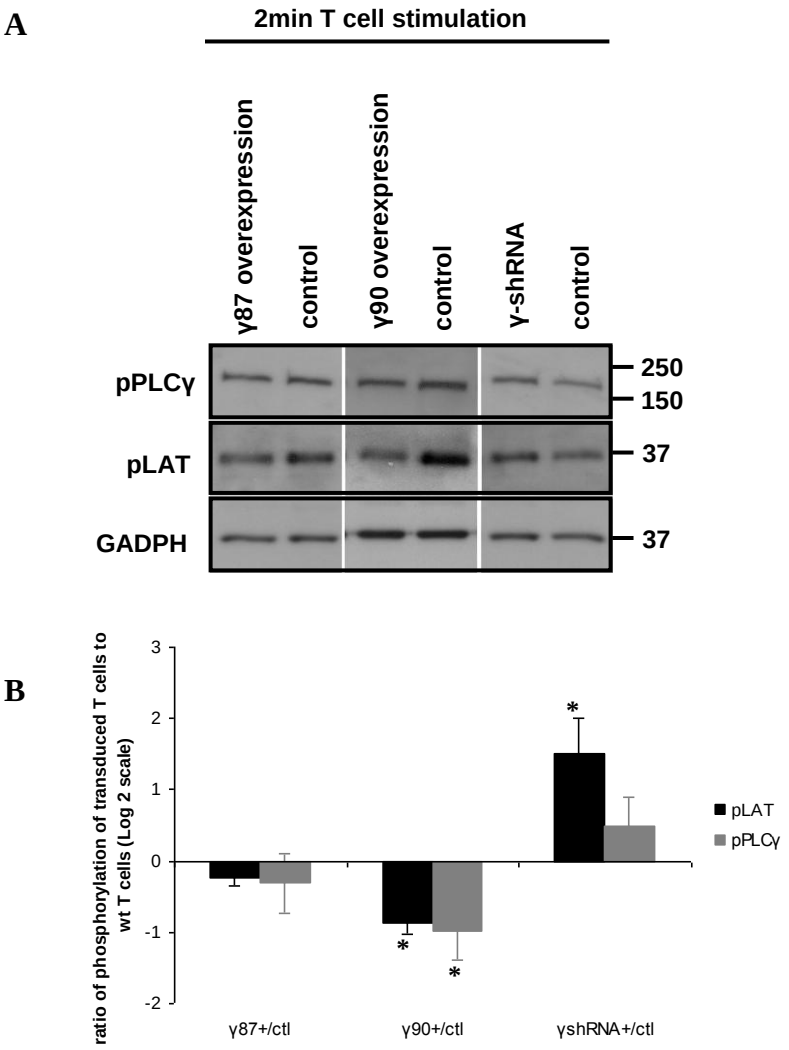
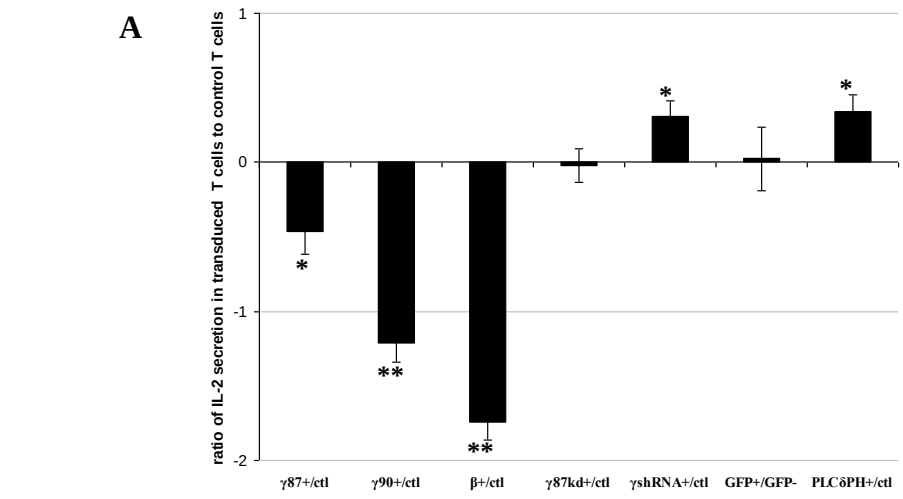


Figure 4-3. Regulation of TCR proximal signaling by PIP5Ks. A. Western blots of phosphorylation of LAT and PLCγ1 in T cells expressing different levels of PIP5Ks. 5c.c7 T cells overexpressing γ87, γ90 or γ-shRNA were stimulated with MCC peptide pulsed CH27 cells for 2min at 37°C. Tyrosine phosphorylation of LAT and PLCγ1 was determined by immunoblotting. GADPH was used as a loading control. A representative blot from three independent experiments was shown. B. Quantitative analysis of western blots of pLAT and pPLCγ1 in PIP5K overexpressing or knockdown T cells upon APC stimulation, as compared to control cells. The densitometry values were normalized relative to non-transduced controls to allow for direct comparison. The data were means ±S.E. of at least three independent experiments (* $p<0.05$. Two tails paired t test).



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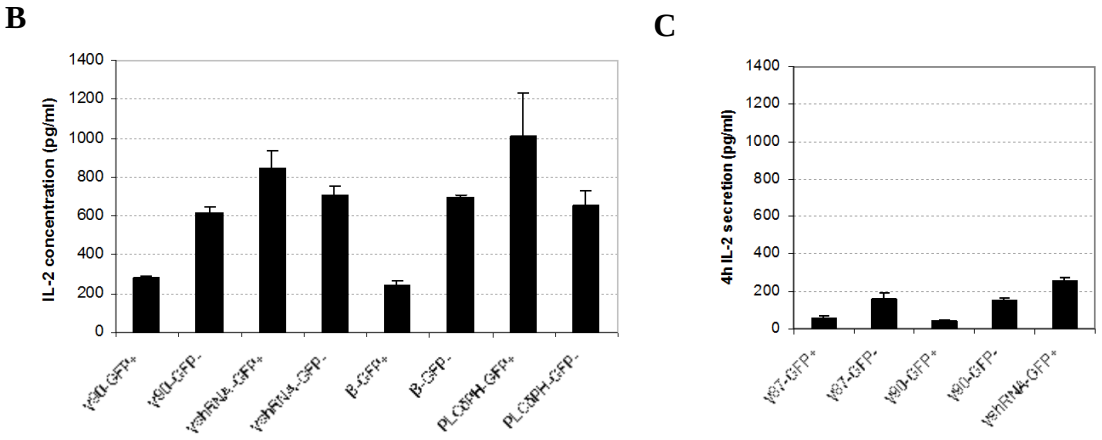

Figure 4-4. Regulation of IL-2 production by PIP5Ks. A. Ratio of IL-2 secretion between Transduced (GFP+) T cells and non-transduced (GFP-) controls. 5c.c7 T cells overexpressing or knockdown of PIP5Ks were incubated with peptide-loaded CH27 cells for 16h. Supernatants were assayed for IL-2 secretion by ELISA. IL-2 production from T cells overexpressing GFP alone or a kinase dead γ87 were used as controls. The results were normalized relative to non-transduced controls to allow for direct comparison. The data are means ±S.E. of at least three independent experiments. (* $p<0.05$.** $p<0.01$. Two tails paired t test.) B.16 h IL-2 secretion (pg/ml) from one representative experiment. In each condition, the GFP+ T cells were T cells tranduced with indicated PIP5K isoforms or PLCδ-PH. GFP- T cells were the non-transduced control T cells sorted from the same culture. The values were normalized to 15,000 T cells. The data are means ± S.E. of three replicates. C. 4 h IL-2 secretion from one representative experiment. The values were normalized to 15,000 T cells. The data are means ± S.E. of three replicates.

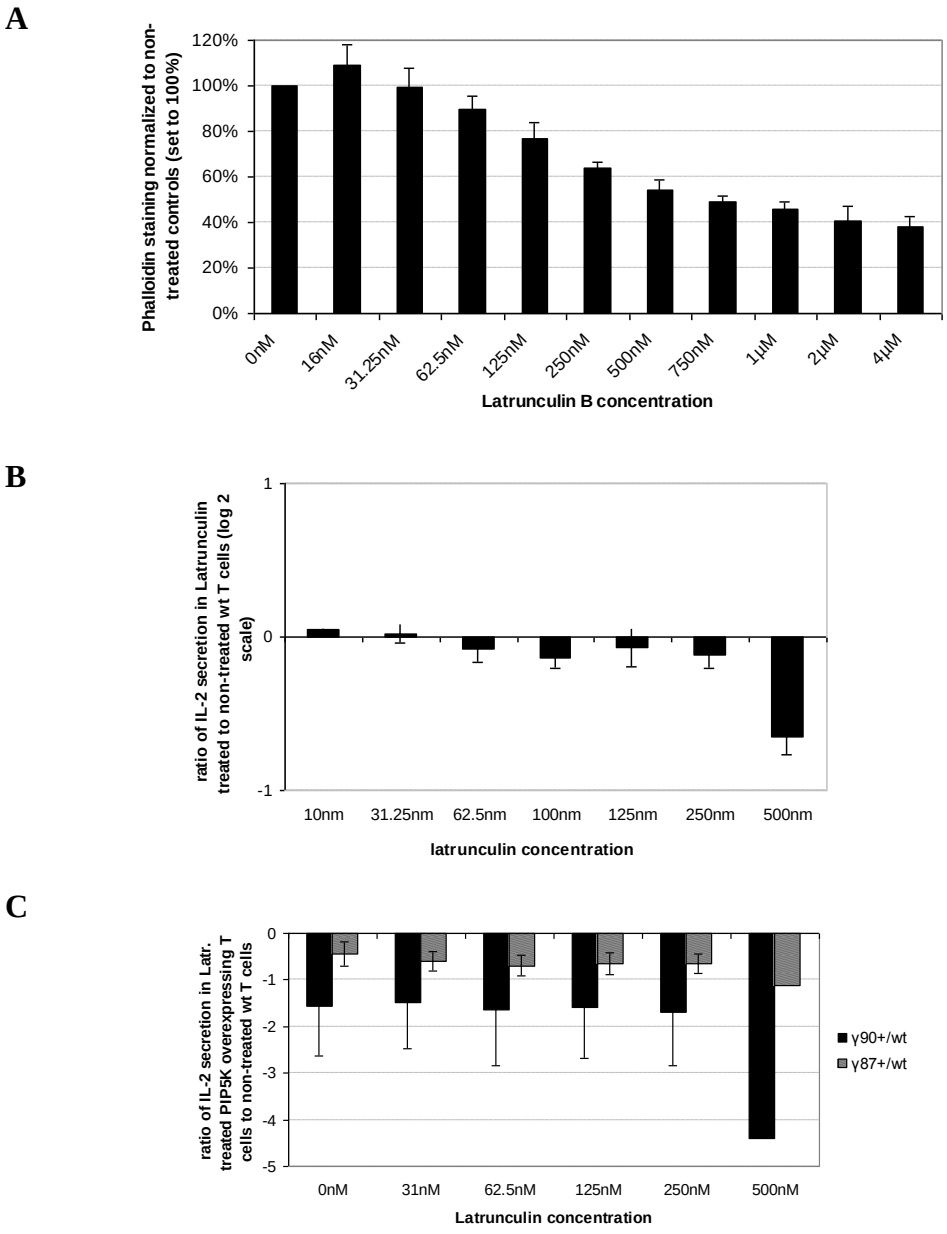

Figure 4-5. Latrunculin B treatment did not rescue of IL-2 secretion in PIP5K overexpressing T cells. A. Effects of Latrunculin B (Latr B) on F-actin concentrations in 5c.c7 T cells. 5c.c7 T cell blasts were treated with different concentrations of Latr. B for 1h before fixation. Intracellular F-actin was stained with fluorescently labeled phalloidin and analyzed by flow cytometry. The results were mean fluorescence intensity normalized relative to untreated controls to allow for direct comparison. The data are means ± S.E. of three independent experiments. B-C. Ratios of IL-2 secretion in different concentrations of Latrunculin B treated wt, PIP5Kγ87 and PIP5Kγ90 overexpressing T cells to non-treated wt T cells.

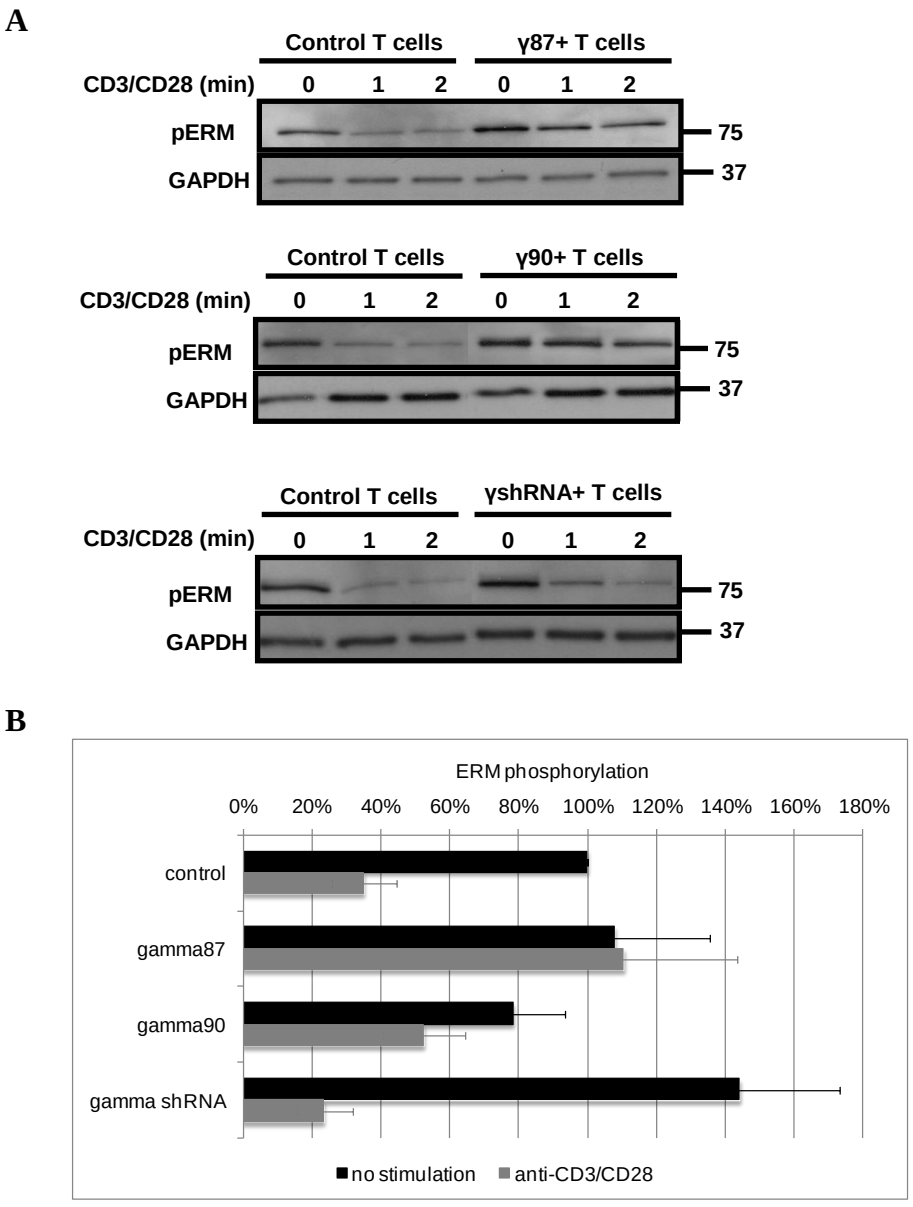

Figure 4-6. Overexpression of PIP5Kγ inhibited ERM protein inactivation upon T cell activation. A. Western blots of phosphorylation of ERM proteins in unstimulated and stimulated T cells expressing different levels of PIP5Ks. 5c.c7 T cells overexpressing γ87, γ90 or γ-shRNA were stimulated with anti CD3/CD28 antibodies at 37°C for the indicated times. Threonine phosphorylation of ERM proteins was determined by immunoblotting. GADPH was used as a loading control. A representative blot from three independent experiments was shown. B. Quantitative analysis of western blots of ERM phosphorylation in PIP5Kγ overexpressing or knockdown T cells with or without 2min CD3/CD28 stimulation, as compared to wt T cells. The densitometry values were standardized to the internal NAPDH control. ERM Phosphorylation in non-stimulated wt T cells from the same culture was set to 1.The data were means ±S.E. of three independent experiments.

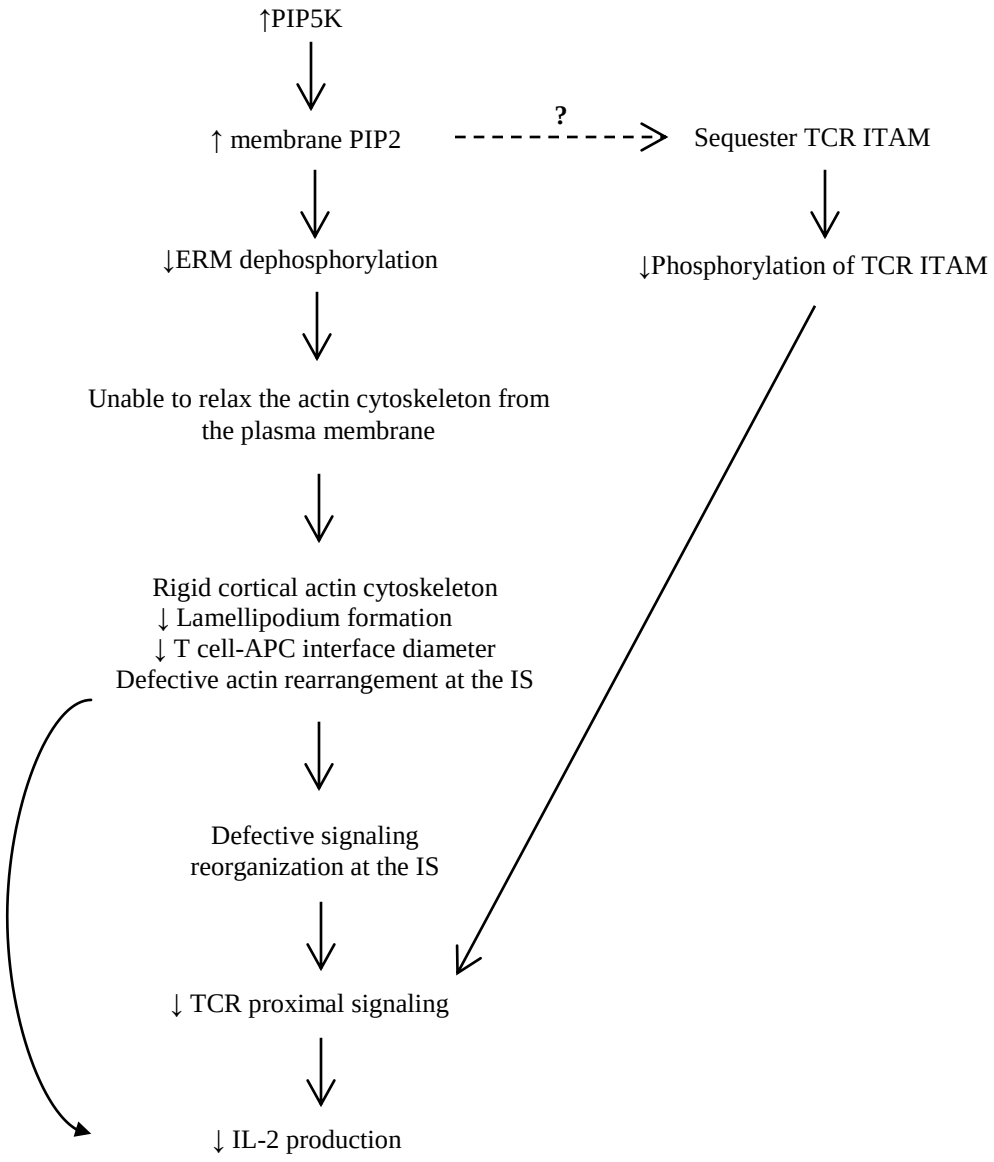

Figure 4-7. Working model for PIP5K-dependent PIP2 generation in T cell activation. Overexpression of PIP5Ks increases membrane PIP2, which binds and keeps the ERM in its active conformation. As a consequence, TCR stimulation fails to dephosphorylate and inactivate the ERM protein, resulting in increased cellular rigidity, defective actin rearrangement and T cell activation. Increase of membrane PIP2 might also binds and prevents the phosphorylation of the basic residues on the TCR ITAM, leading to impaired TCR proximal signaling. In contrast, decrease of membrane PIP2 by knocking down of PIP5K should favor TCR ITAM phosphorylation and increases TCR signaling.

Chapter V Summary and future directions

PIP2 is an important lipid signaling molecule that regulates a variety of cellular functions. Aberrant PIP2 signaling has been shown to be related to many human diseases, such as Lowe Syndrome, Alzheimer's disease, Bipolar disorders, Hypertrophy and Type 2 Diabetes (Halstead et al., 2005). Little is known about how PIP2 levels are regulated in cells of the immune system. In this thesis, we studied the roles of PIP5K-mediated PIP2 production in regulating T cell activation. We found that different PIP5K isoforms targeted to the different poles of activated T cells and contributed to different PIP2 dynamics. However, overexpression of different PIP5K isoforms led to similar defects in actin reorganization and increased T cell rigidity, which correlates with impaired TCR proximal signaling and IL-2 production. Knockdown of PIP5Kγ instead caused enhanced TCR signaling and IL-2 production. In addition, we found that the activity of the key regulator of the cortical actin cytoskeleton, the ERM proteins, was impaired in response to PIP5K overexpression. Based on these findings, we propose that PIP5K-mediated PIP2 production negatively regulates T cell activation by rigidifying the cortical actin cytoskeleton.

Though several findings are made in this research, more questions emerge and remained to be answered. For example, what is the physiological role of the distally located PIP5Kγ90/β/PIP2 in T cell activation? What are the *in vivo* interactions between the PIP5K isoforms and the ERM proteins? Small GTPases RhoA, Rac1 and Cdc42 play a general role in actin-based cellular motility and have been shown to directly regulate PIP5K activity *in vitro.* Are they the signaling intermediates linking PIP5Ks to actin remodeling and TCR signaling? How are the other PIP2-regulated actin-binding proteins affected in the proposed pathway? How are the IP3/Ca^{2+} signaling and membrane trafficking processes affected in response to PIP5K mediated PIP2 produciton? Future experiments will be focused on these interesting and important questions.

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