Tim-1 signaling and localization during T cell activation

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Submitted to the Graduate Faculty of

University of Pittsburgh School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

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Transmembrane immunoglobulin and mucin 1 (Tim-1) belongs to a family of cell surface proteins with roles in immune regulation, among other functions. Tim-1 polymorphisms have been implicated in human asthma susceptibility, and antibody modulation of Tim-1 has been shown to modulate murine models of autoimmune disease and allograft tolerance. This ability of Tim-1 to influence disease progression has been attributed to its role in co-stimulating T cell function, inducing transcriptional activation, and skewing cytokine production.

Despite the emerging role of Tim-1 in immune modulation, the molecular mechanisms underlying Tim-1 function remain largely unidentified. We and others have demonstrated that Tim-1 is a co-stimulatory molecule with the ability to enhance transcriptional activation. However, it is unknown where Tim-1 localizes upon T cell activation, an avenue of investigation that has yielded important insights about other molecules involved in T cell activation. Using imaging, I demonstrate that in contrast to most co-stimulatory molecules, murine Tim-1 localizes away from the immunological synapse, and towards the distal pole complex in manner dependent on ezrin/radixin/moesin (ERM) family proteins. This localization is important for Tim-1 enhancement of cytokine production. In addition, a variety of molecular, pharmacological, and biochemical methods were used to examine the molecules and pathways induced downstream of Tim-1 activation. In particular, I discovered that Tim-1 can trigger NFAT/AP-1 activation in a PLC-γ1 independent, but TCR- and CD28-dependent, manner.

Overall, this dissertation reveals some of the complexity underlying Tim-1 function. Better understanding of where and how Tim-1 interacts with other molecules will provide greater insight into Tim-1 mediated T cell activation and disease modulation.

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LIST OF ABBREVIATIONS

DPC: distal pole complex

ERM: Ezrin, radixin, and moesin

IS: immunological synapse

Jγ: PLCg1 deficient Jurkat T cells

PS: phosphatidylserine

pY: tyrosine phosphorylation

SFK: Src Family Tyrosine Kinase

SMAC: Supramolecular Activation Complex

Tim-1: Transmembrane immunoglobulin and mucin 1

1.0 INTRODUCTION

1.1 TIM FAMILY OF PROTEINS

The Tim family of proteins consists of eight putative murine members with three known human orthologs. Although originally described as "T cell" immunoglobulin and mucin (Tim), a more accurate description is "transmembrane" immunoglobulin and mucin, since Tim family members have been found expressed on multiple immune cell types. All members of the Tim family have similar structural domains, starting with an N-terminal IgV domain with four conserved cysteines, a heavily glycosylated mucin domain, a single transmembrane stalk, and a cytoplasmic tail with conserved tyrosines (except for Tim-4). Murine Tim-1, Tim-3, and Tim-4 are orthologous to human TIM-1, TIM-2, and TIM-4. While the IgV domains of Tim family proteins are fairly well conserved (40% between different homologs), the mucin domains are dramatically different (1).

Tim-1 is a heavily glycosylated member of the Tim family. Although Tim-1 was initially described as being expressed predominantly on activated Th2 cells, expression and functions for Tim-1 have been identified on multiple other immune cells. Initial work described Tim-1 protein expression on activated Th2 cells within twelve hours of activation (and possibly even sooner) (2). However, Tim-1 is found on all activated T cells, as well as many other immune cells that will be described further. Tim-1 is considered to be a positive regulator of T cell responses, and

cross-linking the protein with specific antibodies can influence T cell proliferation and cytokine production both *in vitro* and *in vivo*.

Tim-2 is the most closely related family member to Tim-1 and was previously thought to be an ortholog of hTIM-1. Although low levels of Tim-2 are found on naïve and activated $CD4^+$ and $CD8^+$ cells, Tim-2 is preferentially expressed on T helper 2 cells that have undergone three rounds of polarization. However, despite their structural similarities, Tim-2 is expressed and functions differently from Tim-1. Thus, unlike Tim-1, Tim-2 is not found on B cells, macrophages, or dendritic cells. Tim2-Ig treatment resulted in delayed disease onset and milder symptoms in mice with experimental autoimmune encephalomyelitis (EAE). Furthermore, *in vitro* stimulation of splenic cells treated with Tim2-Ig resulted in enhanced Th2 cytokine production (IL-4, IL-10) as well as hyperproliferation. This suggest that blocking the Tim-2/Tim-2L interaction with Tim2-Ig can enhance Th2 responses, suggesting that Tim-2 is a negative regulator of Th2 T cell responses. (3)

Tim-3 is the third member of the Tim family and was discovered on the surface of terminally differentiated Th1, but not Th2, cells during a screen for Th1-specific markers. Since its initial discovery, Tim-3 has also been found on the surface of $CD8^+$, Th17 and regulatory T cells as well as monocytes, dendritic cells, and mast cells (4-8). While many studies support a model of Tim-3 as a negative regulator of T cell function, signaling work with ectopic Tim-3 overexpression supports a role for Tim-3 as a positive regulator of T cell signaling, at least in acute situations. Of interest, TCR and CD28 stimulation in the presence of Tim-3 enhances both NFAT/AP-1 and NF-κB dependent transcription. Furthermore, other molecules involved in T cell activation, including Fyn, pPLCγ1, pZap-70, p85 subunit of PI3K, have all been implicated in Tim-3 signaling (9). Recent work has implicated a role for Tim-3 in a variety of chronic viral

infections, including HIV and HCV, more specifically that Tim-3 is upregulated on exhausted T cells (10, 11). While treatment with certain Tim-3 antibodies can reverse the function of exhausted T cells, the precise function of Tim-3 in the acquisition or maintenance of an exhausted phenotype is not clear at this point.

Tim-4 is found on antigen presenting cells (APCs) and is best known as a receptor for both Tim-1 and phosphatidylserine (PS). Tim-4 expression appears to be relatively restricted to immune organs, since mRNA levels are highest in spleen and lymph nodes, which is consistent with its expression on antigen presenting cells ($CD11b^+$ and $CD11c^+$) (12, 13). In particular, Tim-4 is most highly expressed on mature dendritic cells that are double positive for CD8 and CD11c (12). Tim-4 binding to Tim-1 appears to require both the IgV and mucin domains of Tim-1(12) although it has been suggested that the Tim-1:Tim-4 interaction is mediated by their common ligand PS rather than as the result of a direct interaction (14). Stimulation with either Tim-4 Ig or Tim4-expressing CHO cells enhances T cell proliferation and cytokine production, which can be partially inhibited by Tim-4 specific antibodies. Tim-4 Ig appears to co-stimulate T cell proliferation even under conditions of suboptimal CD3 and CD28 activation (13). This enhanced T cell proliferation is due to the ability of Tim-4 to both increase the rate of cell division as well as to upregulate anti-apoptotic factors, including Bcl-2 (13). Intriguingly, Tim-4 treatment can inhibit proliferation of naïve T cells, which do not express detectable Tim-1 protein, suggesting that Tim-4 may have still-unidentified receptor(s) (15).

Recently, Tim-4 has received much attention due to its identification as a receptor for PS, which is exposed on the surface of cells early during the apoptotic program. The Tim-4 cytoplasmic tail does not appear to be important for signaling downstream of PS, but a recent paper indicates that the cytoplasmic tail is necessary for proper Tim-4 localization relative to

PS(16). The ability of Tim-4 to phagocytose apoptotic cells may be important for the prevention of autoimmunity. Peritoneal macrophages and B cells lacking Tim-4 cannot efficiently engulf apoptotic cells, and Tim-4 knockout mice have hyperactive T and B cells with a propensity to develop antibodies to double-stranded DNA(17). Thus, the ability to clear apoptotic cells by Tim-4 appears to be important for normal immune function and the prevention of autoimmunity.

1.2 TIM-1 AND DISEASE

1.2.1 Tim-1 and Disease Overview

Transmembrane immunoglobulin and mucin 1 (Tim-1), a co-stimulatory protein found on the surface of T cells, has been implicated in the regulation of a variety of immune conditions. Tim-1 was first identified as a Hepatitis A Virus Cellular Receptor (HAVCR1)(18). Tim-1 is also known as Kim-1 (kidney injury molecule), which serves as an early marker of renal injury/failure (19, 20). Interest in the field of immunology about this molecule built over the past decade with its discovery as a putative asthma susceptibility gene (21). However, ever increasing numbers of pathologic and physiologic effects have been linked to Tim-1, including atopic disease, rheumatoid arthritis, and systemic lupus erythmatosus (22-25) (26). This section will summarize the role of Tim-1 in asthma, multiple sclerosis, and transplantation. Truly understanding the function of Tim-1 in immune activation will require greater insight into Tim-1 localization, signaling, and impact on multiple immune cell types.

1.2.2 Tim-1 and asthma

Asthma is a multifactorial disease with both genetic and environmental components. Due to the complex interplay of factors contributing to disease, discovery of asthma susceptibility genes has been challenging. Thus, using a reductionist approach, a group used a genetically tractable mouse model to better study the genes underlying asthma susceptibility. It has long been known that BALB/c mice have higher Th2 responses and are more susceptible to allergic airway hyperreactivity (AHR) than C57Bl/6 or DBA/2 mice. In 2001 the Dekruyff and Umetsu groups identified a region on mouse chromosome 11B.1 as conferring susceptibility to asthma. The authors obtained congenic mice with specific segments of DBA/2 on a BALB/c background, to identify one line of mice with reduced Th2 responses. These mice have a segment of DBA/2 chromosome 11, which the investigators referred to as Tapr (T cell airway phenotype regulator). This region was found to be homologous to 5q33.2 and to be separate and distinct from the locus containing IL-4. Positional cloning revealed the Tim-1 and Tim-3 genes were present within the Tapr region. Comparison of the mouse genes revealed polymorphisms between Tim-1 in DBA/2 and BALB/c mice. Specifically, a 15 aa insertion exists in the BALB/c mucin domain but not the DBA/2 (or C57Bl/6) or congenic mice (27). Further, by co-culturing $CD4^+$ T cells and DCs from BALB/c and HBA, the authors suggested that Tim-1 on T cells were most important for the increased Th2 response of BALB/c mice (27).

Tim-1 is a very intriguing candidate gene with regard to asthma susceptibility because it is the first specific example of a gene that might link genetics and environment, providing a possible mechanism for the so-called "hygiene hypothesis." Over the past few decades, industrialized countries have witnessed a dramatic increase in incidence of asthma. Many have attributed the increased industrialization with similar increases in sanitation and subsequent

decreases in infectious disease. Thus, the hygiene hypothesis postulates that lack of exposure to certain infectious diseases during childhood, leads to an increase in inappropriate responses to typically benign allergens in certain genetically predisposed individuals, eventually leading to asthma and other atopic diseases in some people. HAV exposure is one of the infectious agents that has been inversely correlated with development of atopic asthma (28). Intriguingly, two years after the identification of Tim-1 as a putative asthma susceptibility gene, a fascinating study emerged inversely correlating a TIM-1 polymorphism with susceptibility to asthma, but only if the patient was seropositive for HAV. Specifically, patients with a six amino acid insertion in the TIM-1 mucin domain, and HAV sero-positivity, were protected from asthma (21). Subsequently, a Korean cohort study suggested a correlation between *TIM1* genotype and asthma, whereas some but not all studies with Chinese populations failed to find such an association (22, 29, 30). Any genetic association of TIM-1 genotype with asthma may be complex and dependent on both the specific polymorphism as well as on genetic background. Interestingly, the same six amino acid insertion also correlates with increased severity of HAV induced liver failure, possibly due to increased NKT activity in the longer forms of TIM-1(31). Thus, it has been suggested that in order to protect from this acute HAV liver damage, evolutionarily, the shorter form of TIM-1 has been conserved, which has the side effect of predisposing people to asthma and allergy.

These exciting findings have lead to a plethora of mouse and human studies, which mostly support a role for Tim-1 in asthma pathogenesis. Initial studies on Tim-1 and asthma focused on its role on T cells. Depending upon the specific Tim-1 antibody utilized as well as differences in specific animal models, modulation of Tim-1 can lead to either amelioration/prevention or aggravation of AHR. Many of these studies noted an increase in

Tim-1 expression during asthma development. For example, Tim-1 mRNA levels were shown to be increased in mice with AHR (32). Another study reported higher Tim-1 levels from activated T cells in lung draining lymph nodes (33). Further, the augmented Tim-1 expression was correlated with high levels of the transcription factor GATA3, the master gene regulator of Th2 development (32).

Various groups have generated different Tim1-specific antibodies to probe the effects of Tim-1 in immune modulation. In fact, one study suggested that Tim-1 might function as either a positive or negative co-stimulatory molecule, depending on the nature of its ligation (34). An initial *in vivo* study suggested that treatment with an anti-Tim-1 antibody before intranasal antigen challenge resulted in altered cytokine production (more IFN-γ and IL-10, but less IL-4) as compared to non-tolerized mice. Further, administration of anti-Tim-1 antibody during initial exposure to antigen prevented tolerance induction. As a result, subsequent antigen challenge of anti-Tim-1 treated mice led to AHR (2). However, not all Tim-1 antibodies modulate AHR similarly. Treatment with another anti-Tim-1 antibody ameliorated disease, with an accompanying decrease in cytokine production (35). Studies with other anti-Tim-1 antibodies revealed an ability to either augment or inhibit cell proliferation, cytokine production, and ultimately AHR (36). Further evidence for potential TIM-1 modulation of asthma in humans was demonstrated in a study using anti-human TIM-1 antibodies to decrease AHR in SCID mice adoptively transferred with peripheral blood mononuclear cells (PBMCs) from allergic/asthmatic patients (37).

1.2.3 Tim-1 and MS/EAE

Another autoimmune disease that TIM-1 may modulate is multiple sclerosis (MS), an immune-mediated demyelinating disease of the central nervous system. The first study to examine TIM-1 in MS found increased levels of TIM-1 mRNA in the mononuclear cells from the cerebral spinal fluid of MS patients with clinically inactive disease. Interestingly, levels of TIM-1 were inversely correlated to IFN-γ levels. Thus, high TIM-1 expression was correlated with low levels of IFN-γ, indicating that TIM-1 levels might be associated with regulating the different phases of MS (38). Despite this finding, an epidemiologic study from Western Austria did not demonstrate an association between TIM-1 SNPs and MS. This group discovered ten SNPs in the TIM-1 gene. However, the authors were unable to correlate any of these SNPs with MS (39). The difference between these studies could be due to many factors, including the populations studied and limited number of patients (n=272). Thus, the original study was conducted in Sweden (probably with Swedish patients) while the subjects in the second study were from West Austria. It is likely that more patients would be required to uncover possibly subtle effects of these TIM-1 SNPs on MS.

Animal studies of experimental autoimmune encephalomyelitis (EAE), a mouse model for MS, support a role for Tim-1 in the pathogenesis of MS. Anti-Tim-1 antibody treatment of SJL mice immunized with $PLP_{139\text{-}151}$ altered cytokine production and disease progression. Treatment with a high affinity anti-Tim-1 antibody resulted in an immune response skewed more towards Th1 and Th17, and disease exacerbation, while injection with a lower affinity anti-Tim-1 antibody resulted in a predominantly Th2 response with amelioration of disease symptoms (34). This was believed to be a T helper-mediated effect. However, further work by the same

group attributed the exacerbation of EAE by the high affinity Tim-1 antibody to effects on dendritic cells (DC) (40). Whether the effects of Tim-1 antibodies on immune responses are due to direct effects on T cells, or to indirect effects on APCs, will require further mechanistic insight into how these antibodies function on various cell types.

1.2.4 Tim-1 and immune modulation in transplantation

Tim-1 also has been shown to modulate immune responses associated with rejection of - or tolerance to - allografts. Taking advantage of different Tim-1 antibodies, two studies demonstrated that Tim-1 engagement on helper T cells can promote allograft acceptance or rejection, and that the effects are dependent on regulatory T cells. *In vitro* T cell stimulation with an "agonistic" (high affinity) Tim-1 Ab increased the number of IL-17- and IFN-γ-producing cells, but decreased the mRNA expression of FoxP3, GITR, and other markers of regulatory T cells (41). This same Tim-1 antibody also negated the protective effects of anti-CD154 (CD40L) treatment and resulted in allograft rejection. In contrast, an "antagonistic" (low affinity) anti-Tim-1 Ab was able to inhibit the Th1 cytokine IFN-γ and to promote Th2 cytokines, such as IL-5 (42). Thus, antagonist Tim-1 antibody treatment appeared to *prevent* chronic allograft rejection by a mechanism dependent on regulatory T cells. Interestingly, antagonistic anti-Tim-1 antibody treatment did not convert naïve CD4+ T cells into regulatory T cells, but rather appeared to inhibit the proliferation of allogeneic effector T cells. The mechanisms underlying the effects of Tim-1 antibodies on regulatory T cells (e.g. whether they cause expansion of existing Treg or conversion of new Treg) still remain to be discovered.

Aside from helper T cells, Tim-1 could be modulating transplant tolerance through $CD8^+$ and B cells. Treatment with a low affinity Tim-1 antibody, RMT1-10, prolonged allograft

survival, potentially by abrogating $CD8^+$ T cell production of IL-17 (43). In addition, Tim-1 has been found on a subset of regulatory B cells, and treatment with RMT1-10 in the presence of B cells promoted a Th2 phenotype with long-term graft survival (44).

Tim-1 modulates immune responses to AHR, EAE, and transplantation tolerance. While treatment with different affinity antibodies alters cytokine production and disease progression, we do not yet understand how this occurs. Better understanding of how these Tim-1 antibodies are mediating their effects may lead to better targeted approaches to modulate diseases states. One possibility is that Tim-1 is generating different signaling pathways downstream of its ligation, and we will examine this in Chapter 3.

Table 1-1: Effect of select Tim-1 antibodies on disease modification

Disease Model	Antibody	Effect	Ref
Asthma			
	222414 (R&D)	Decreased Th2	Journal of Allergy and Clinical Immunology, 2005, 116 (6)
	1H8.2	IL-4, IL-5, IL-10, IL-13 production	The Journal of Immunology, 2007, 178: 2249-2261
	4A2.2	Decreased IL-4, IL-10, IL-13, reduction lung inflammation	The Journal of Immunology, 2007, 178: 2249-2261
Influenza	222414, RMT1-4, Am1-005	IFN-γ	Clin Exp Immunol. 2006 July; 145(1): 123–129
EAE	3B3	IFN-γ and IL-17, exacerbation	J Exp Med. 2007 July 9; 204(7): 1691–1702
	RMT1-10	IL-4 and IL-10, amelioration	J Exp Med. 2007 July 9; 204(7): 1691–1702
Islet Allograft	3B3	IFN-γ and IL-17	J. Clin. Invest. 118(2): 735-741 (2008)
Cardiac Allograft	RMT1-10	IL-4	J Clin Invest. 2008 February 1; 118(2): 742–751

1.3 TIM-1 STRUCTURE

Solving the crystal structure of the Tim-1 IgV domain has led to interesting insights into this protein's function. The IgV domains of Tim-1 is composed of two anti-parallel β sheets (BED

and GFC) with an unusually high number of conserved cysteine residues. Furthermore, this structural analysis identified a possible Tim-1 homotypic (and possible Tim-1:Tim-2 heterotypic) interaction by Tim-1 expressed on adjacent cells. Two residues, His 64 and Glu 67, of the TIM-1 DE loop are thought to be important for this *trans*-Tim-1 binding, since a point mutation of histidine at position 64 to glutamic acid led to significant reductions in homophilic Tim-1:Tim-1 binding as well as partially decreased levels of Tim-1:Tim-4 binding (45). This could be of potential biologic relevance because the homophilic binding is conserved in humans. It was suggested that this Tim-1:Tim-1 homotypic interaction may play a role in the Tim-1 co-stimulatory function, and this will be examined in more detail in Chapter 2 of the thesis.

One highly conserved region between Tim-1 and Tim-4, but not Tim-2, is the folding of a CC' loop into the GFC β sheet. The Tim-1 and Tim-4 IgV domain crystal structures suggest that a metal-ion-dependent ligand binding cavity built by CC' and FG loops in the IgV domain is responsible for the recognition of PS. This loop creates a protected cleft for acidic compounds to enter. The hydrophilic phosphate head of PS can enter the cavity and interact with a metal ion, while the fatty acid tail interacts with the aromatic residues of the FG loop. Mutations of residues in the metal ion binding site also appear to increase localization of Tim-1 to the cell surface. Single mutations of the metal ion residues decreased Tim-1 and Tim-4 binding to liposomes containing PS, while a double mutation completely abolished PS binding. Individual mutations of the four amino acids of the CC' and FG loops dramatically decreased the ability of Tim-4 to mediate uptake of apoptotic red blood cells (45, 46). The consequences of Tim-1 and PS interaction in T cells will be further examined in Chapter 3.

1.4 TIM-1 LIGANDS

1.4.1 Tim-1 family ligands-overview

Multiple ligands for Tim-1 have been identified, and there is indirect evidence for additional as-yet-unidentified ligand(s). The first ligand described was HAV, and recently Tim-1 has also been shown to serve as a cellular receptor for other viruses, including Ebola and Marburg (18, 47). Tim-1 has also been shown to bind to the related family member Tim-4 (though some believe that this interaction is mediated by PS, as discussed above) and to form homotypic interactions (12, 45). Other ligands for Tim-1 include IgA (48), phosphatidylserine (49, 50), and the transmembrane protein LMIR5/CD300b (50). In this section, the various Tim-1 ligands and their effects on Tim-1 mediated function will be described in greater detail.

1.4.2 Tim-1 as a receptor for viruses

Primate (including human) TIM-1 serves as a cellular receptor for the Hepatitis A virus (HAV) (18, 51, 52), and many studies indicate that the interaction between HAV and different alleles of Tim-1 has the potential to impact asthma susceptibility. Sero-positivity for HAV and a six amino acid insertion in the Tim-1 mucin domain are associated with relative protection from asthma (21). Interestingly, while HAV binds to just the IgV domain of Tim-1 (45), uncoating of HAV requires the mucin domain of Tim-1 (53).

Tim-1 has been shown to influence viral infection. An early study with anti-Tim-1 antibodies suggested that Tim-1 might serve as an adjuvant to boost immunity during influenza vaccination. Specifically, treatment with anti-Tim-1 antibodies augmented antigen-specific

proliferation and IFN-γ production (54). However, Tim-1 may also have a more direct role in influencing viral infection. Recently, Tim-1 has also been identified as a cellular receptor for Zaire Ebolavirus and Lake Victoria Marburg virus. Expression of Tim-1 enhances the ability of ebolavirus glycoproteins to infect these cells. Also, since Ebola transmission is mostly through the aerosol route, Tim-1 expression in eye and airway epithelium provides a putative route of entry for these viruses. Finally pretreatment with a Tim-1 IgV-binding antibody, ARD5, prevented Ebola and Marburg virus infection (47). Due to the growing number of viruses being identified as Tim-1 ligands, understanding the mechanism(s) underlying Tim-1 activation may prove to be useful in identifying signaling pathways to modulate viral infection itself or to manipulate the immune response to viral infection.

1.4.3 Tim family members - Tim-4 and Tim-1

Tim-1 is able to bind two TIM family members. Interaction of Tim-1 with Tim-4, which is found on mature antigen presenting cells (APC), induces T cell proliferation, Tim-1 phosphorylation, and activation of downstream signaling pathways (12, 13). Thus, Tim-4 appears to increase activation of T cells and may also augment the strength and direction of a T helper response (12, 13). Some studies have suggested that the Tim-1:Tim-4 interaction is not direct but rather mediated by PS (55). However, this has yet to be definitively proven or ruled out.

Tim-1 has also been suggested to bind to itself in a homotypic fashion, in *trans*, i.e. with Tim-1 expressed by another cell (or in the case of treatment of cells with Tim1-Ig). This appears to be mediated by two residues (H64 and Glu67) and the BED loop, within the IgV domain, since a point mutation of H64E mitigated the homotypic Tim-1 interaction (45). Since the

impact of Tim-1 homotypic interactions on T cell function has not been determined, we were interested in investigating this, as described in Chapter 3.

1.4.4 Other Tim-1 ligands - IgA, LMIR5/CD300b, PS

Additional Tim-1 ligands have recently been discovered. In a search of more putative Tim-1 ligands, one group used an expression cloning screen with human lymph node cDNA to identify that IgA binds Tim-1 Ig. These investigators then demonstrated that although IgA binding to Tim-1 does not affect HAV binding, the presence of IgA enhances HAV neutralization (48). Another group, using retrovirus mediated expression cloning into Ba/F3 cells with an A20 cDNA library found that Tim-1 can bind to leukocyte mono-immunoglobulin-like receptor 5 (LMIR5)/CD300b. This interaction appears to occur near the PS-binding site on Tim-1. In addition, this group reported that Tim-1 expressed in the kidney (aka Kim-1) may interact with myeloid cells expressing LMIR5 and influence disease outcome (48). Thus, in a model of acute kidney injury, LMIR5 deficient mice had less kidney injury, possibly due to decreased neutrophil recruitment, despite normal levels of Tim-1 (50). While the LMIR5:Tim-1 interaction has not yet been demonstrated to modulate T cell function, the interaction can induce Erk phosphorylation and IL-6 production in mast cells (50). Thus, the novel ligands IgA and LMIR5 have potentially interesting but apparently disparate effects on Tim-1 mediated signaling.

One of the most intriguing Tim-1 ligands is phosphatidylserine (PS), which binds the IgV domain of Tim-1(46, 49, 56). On kidney epithelial cells, Tim-1 serves as a PS receptor and is involved in the phagocytosis and clearing of apoptotic cells (57). In addition, transfection of Tim-1 into the fibroblast –like NIH 3T3 cell line allows these cells to efficiently take up PS expressing cells (56). However, the Tim-1:PS interaction does not necessarily result in

phagocytosis of PS-expressing cells. For example, an association between PS and Tim1-expressing iNKT cells has been demonstrated to enhance cellular proliferation in a co-stimulation (anti-CD3) dependent manner. Furthermore, in mice, administration of anti-Fas antibody leads to increased airway hyperreactivity, which is dependent on Tim-1 and cytokine production (58). Unlike the role of the Tim-1:PS interaction in fibroblasts for engulfment of apoptotic cells, exposure to PS on immune cells, specifically iNKT cells, causes Tim-1 to work in costimulatory manner and augment immune function. However, not all ligands for PS boost immune function. For instance, CD300a expressed by mast cells also binds PS, which does not result in phagocytosis, but rather induces inhibitory signaling, in part by recruiting the phosphatase SHP-1 (59). In a mouse model of sepsis that releases apoptotic cells, CD300a-deficient mast cells recruited more neutrophils and enhanced bacterial clearance (60). Antibody blockade of the CD300a and PS interaction also prolongs survival, which suggests that the PS:CD300a interaction inhibits chemokine and cyokine production (60). While one of the better known roles for PS receptors is their ability to bind and phagocytose apoptotic cells, this is clearly not their only function. Tim-1 expression in epithelial cells has been shown to enable them to transform and engulf PS expressing cells and mediate immune function, but Tim-1 expression on other immune cells, like iNKT cells, does not result in phagocytosis of apoptotic cells but rather activation of the cells. Moreover, other PS receptors, such as CD300a, have been shown to inhibit immune responses. Thus, the functional outcome of PS:receptor interactions may depend on cell-specific and/or receptor-specific properties. The role of PS:Tim-1 binding in T cells has not been studied. Since Tim-1 has been suggested to be either a positive or negative regulator of T cell function, depending on the binding affinity of the particular antibodies used to modulate it (34), the ultimate effect of the Tim-1:PS interaction on T cell activation remains an

interesting and unanswered question. Thus, we will explore the localization of Tim-1 in relation to PS in Chapter 3 and the impact of the Tim-1:PS interaction on T cell signaling in Chapter 3.

1.5 TIM-1 ANTIBODIES

Tim-1 is a type I transmembrane protein composed of an IgV head, heavily glycosylated mucin domain, a transmembrane domain, and an intracellular cytoplasmic tail with a conserved tyrosine (1). Much of the work elucidating Tim-1's role in T cell activation and differentiation has been conducted using anti-Tim-1 antibodies. The best studied anti-Tim-1 antibody is the high avidity 3B3 generated by the Dekruyff/Umetsu group (2). This antibody has been used to investigate *in vitro* and *in vivo* effects on multiple cells of the immune system, including T cells. Administration of 3B3 appears to aggravate AHR and EAE as well as to accelerate islet cell transplant rejection. Many of these effects appear to be mediated through enhanced production of pro-inflammatory cytokines. However, the cytokines produced in these different disease models differ. For instance, in the AHR model, IL-4 production was enhanced, while 3B3 treatment in the EAE model resulted in greater IFN-γ and IL-17 production (2, 34). Conversely, another anti-Tim-1 antibody, RMT1-10, ameliorated EAE disease progression, possibly by skewing the immune response towards a more Th2 phenotype, with IL-4 production (34). Interestingly, while both 3B3 and RMT1-10 bind similar epitopes in the IgV domain of Tim-1, they have different binding affinities and have different effects on cytoskeletal movement. Thus, 3B3 has a higher affinity for Tim-1, causes co-capping with CD3, and significant cytoskeletal rearrangement. In contrast, RMT1-10 has a much lower binding affinity for Tim-1 and does not cause cytoskeletal-directed cellular movement (34). One hypothesis is that the affinity of a

particular antibody for Tim-1 might result in different effects on Tim-1 movement or generate different signals downstream of Tim-1 ligation. However, there is as yet no evidence to support this concept. Thus, in Chapter 3 we will investigate whether ligation with various Tim-1 antibodies results in changes in tyrosine phosphorylation downstream of TCR/CD28 co-stimulation.

While it might be tempting to attribute the various Tim-1 antibody effects solely to differing affinity, the full story is not so simple. Another study utilizing different anti-Tim-1 antibodies in the same AHR model demonstrates either enhanced or reduced Th2 type cytokine responses. The difference in disease progression and cytokine production in this case was attributed to differences in the epitope that the antibodies bind to (36). Anti-Tim-1 antibodies binding to the stalk and IgV domains appeared to ameliorate disease by inhibiting lung inflammation and reduced production of Th2 cytokines. Conversely, another antibody that binds near an N-linked glycosylation site on the Tim-1 stalk exacerbated disease and increased Th2 cytokine production. In this study the authors did not examine the affinity of antibody binding, but rather attributed the differences in cytokine production to differences in the epitopes bound by the various antibodies. Certainly this cannot be the case for all anti-Tim-1 antibodies, since 3B3 and RMT1-10 appear to bind identical or similar epitopes within the IgV domain. The ultimate effect of Tim-1 antibodies on disease pathogenesis and cellular cytokine production appears to be dependent on a combination of factors, including the specific epitope bound and the affinity (as well as subsequent cytoskeletal movement). Further work will need to be conducted in order to clarify the mechanism underlying these differences in effects of Tim-1 ligation by antibodies.

Many additional anti-Tim-1 antibodies have been generated but not fully tested.

Collaborators from the Kuchroo lab have generously donated antibodies including 5F12, 4F12 and 5G5. Agonistic mouse anti-Tim-1 antibodies include 5F12 and the well-studied 3B3, which bind the IgV domain. Antagonistic anti-Tim-1 antibodies include RMT1-10, which binds the IgV domain, and 5G5, which binds the mucin domain. A previous study indicated that induction or abrogation of respiratory tolerance may depend on the epitope recognized by the anti-Tim-1 antibody but did not provide a mechanism for these disparate actions (36). Thus, we hypothesize that the agonistic and antagonistic Tim-1 antibodies lead to the recruitment and phosphorylation of different downstream targets to modify cytokine production and modulate T helper subset differentiation.

Table 1-2: Tim-1 antibody properties

Antibody	**Binding to Tim-1**	**Properties**
3B3	IgV domain	"Agonistic," high affinity
RMT1-10	IgV domain	"Antagonistic," lower affinity
5F12	IgV domain	Similar to 3B3 in function
5G5	Mucin	unknown
4F12	Unknown	unknown

1.6 TIM-1 FUNCTION IN T CELLS

1.6.1 T cell Activation

T cell activation and polarization involves three signals. The first signal is delivered when the T cell receptor (TCR) recognizes peptide presented in the cognate major histocompatibility

complex (MHC), resulting in signaling through CD3/zeta. A second, co-stimulatory, signal augments T cell activation. For naïve T cells, this is usually provided by CD28 binding to its ligands CD80/CD86. Classical co-stimulatory molecules like CD28 are distinguished by the fact that their ligation alone does not result in T cell activation. Cytokines provide a third signal to direct T cell expansion and, eventually, polarization. Tim-1 is not a classical "co-stimulatory" molecule, since recent work suggests that Tim-1 can substitute for the initial signal or deliver either a positive or negative co-stimulatory signal (33, 34, 61). Furthermore, treatment with different Tim-1 monoclonal antibodies leads to the production of different cytokines and enhancement of specific subsets of T helper cells, which results in exacerbation or protection from disease in mouse models (2, 34, 41, 42, 54).

Initial studies implicated Tim-1 predominantly in modulating the Th2 response, particularly in regards to asthma (2, 32, 33, 35). However, emerging studies suggest that Tim-1 engagement can regulate multiple T helper subsets. Xiao *et al.* first demonstrated that treatment with an agonistic anti-Tim-1 antibody increases severity of experimental autoimmune encephalomyelitis (EAE) possibly by increasing the secretion of IFN-γ and IL-17. In contrast, the antagonistic anti-Tim-1 antibody ameliorates EAE and skews the immune response towards a Th2 phenotype (34). Work in allograft immunity revealed that mice treated with an agonistic anti-Tim-1 antibody rapidly rejected pancreatic islet allografts and have a Th1 and Th17 phenotype with reduction of regulatory T cells (41). In contrast, an antagonistic anti-Tim-1 antibody promoted cardiac transplant survival and skewed the immune response towards a Th2 phenotype with limitation of alloreactive T effector cell expansion (42). Determining the mechanisms by which Tim-1 ligation and antibody treatment influence T cell activation and

differentiation may provide valuable insight into T cell activation and lead to targeted therapeutics to promote tolerance or immunity.

1.6.2 Tim-1 and signaling/Tim-1 as a costimulatory molecule

Discovery of the TCR/CD3 complex eventually led to an enhanced understanding of the signaling molecules and pathways involved in T cell activation. It is now appreciated that Src family kinase (SFK) members Lck and Fyn, phosphorylate tyrosines on the CD3/zeta cytoplasmic tails. This allows for recruitment of ZAP-70 and the subsequent phosphorylation of adaptor proteins, such as SLP-76 and LAT, which serve as scaffolds for further signaling. In particular, activation of PLC-γ1 following its phosphorylation by ZAP-70 and Itk generates DAG and IP_3, which lead to MAP kinase activation and Ca^{2+} release, respectively (62). Much of the work uncovering these pathways has come from studying the human Jurkat leukemic T cell line, which will be used extensively our experiments.

Tim-1 has a conserved tyrosine in its cytoplasmic tail, and can function in a co-stimulatory manner with CD3 and CD28. In addition, both human and mouse Tim-1 have been suggested to physically interact with CD3 and ZAP-70 and Itk (34, 63). Thus, it is reasonable to hypothesize that shared proximal TCR signaling molecules phosphorylate the Tim-1 cytoplasmic tail. Although work from our lab has already demonstrated that Tim-1 activation of NFAT/AP-1 is dependent on phosphorylation of Y276 within Tim-1, the kinase(s) responsible for Y276 phosphorylation has not been completely elucidated. Src family kinase (SFK) member(s) may be responsible for this phosphorylation, since a SFK inhibitor blocks Tim-1 phosphorylation (64). Furthermore, subsequent phosphorylation of signaling molecules may be qualitatively or

quantitatively affected downstream of Tim-1 signaling. We have demonstrated recruitment of the p85 subunit of PI3K to the Tim-1 cytoplasmic tail and subsequent Akt phosphorylation (64).

Many of the signaling molecules listed above have been implicated in T helper differentiation. For instance, Tec kinase family members have been suggested to influence Th1/Th2 differentiation. While Itk appears to be important for Th2 development, Rlk (Resting Lymphocyte Kinase) may favor Th1 responses (65, 66). In addition, members of the MAP kinase family, particularly JNK and p38, appear to favor development of Th1, over Th2, responses (67). With regard to Akt, studies have demonstrated that enforced Akt expression decreases the ability of regulatory T cells to suppress effector cells, and that reduced Akt phosphorylation is necessary for effective regulatory T cell function (68-70). Recruitment or activation of specific targets downstream of Tim-1 ligation by different ligands and antibodies may therefore favor the development or maintenance of specific T helper subsets.

1.7 TIM-1 LOCALIZATION

1.7.1 Immune Synapse Formation

Intracellular interactions controlling T cell function and fate are of course far more complex than a linear cascade of signaling events. Visualizing the spatiotemporal movement of Tim-1 within the cell will provide valuable insight into its function. When a T cell forms a cognate interaction with an APC, a distinct arrangement of antigen receptor, co-stimulatory and adhesion molecules forms at the point of contact, often referred to as the immunological synapse (IS). The conventional immunological synapse has a "bulls-eye" arrangement. At the center of the bulls-

eye is the central supramolecular activation cluster (cSMAC) where many critical signaling and co-stimulatory molecules segregate, including TCR/CD3, ZAP-70, PKC-θ, and CD28. Surrounding the cSMAC is the peripheral supramolecular activation cluster (pSMAC), which includes talin and LFA-1(71). The presence of these larger and adhesive proteins is thought to stabilize the immunological synapse and allow for more prolonged interaction of the T cell and APC. Outside of the cSMAC is the dSMAC, which contains CD45 and talin.

The functional role of IS formation remains somewhat controversial. Initially, formation of the IS was thought to act solely to concentrate and to enhance signaling. One hypothesis was that formation of the cSMAC allowed many critical signaling molecules to be located in one area in order to amplify signaling upon T cell activation by antigen/APC. However, further investigation revealed that tyrosine phosphorylation could be detected well before the formation of a "mature" IS. For instance, while total Lck and ZAP-70 remain at the IS, the phosphorylated versions of these proteins lasted 5 and 30 minutes, respectively (72). This suggested that perhaps the IS was not a place for initiation of signaling, but rather for the concentration and subsequent down-regulation of signaling, i.e. for termination of signaling and recycling of receptors. Both signaling and degradation may occur at the IS, with the precise kinetics dependent on antigen dose (73). In addition to receptor endocytosis, another postulated role for the IS is that the interaction between T cell and APC allows for targeted exocytosis and release of lytic granules or cytokines. In this way the effects of activation would be limited to the cells involved and not result in widespread bystander activation or destruction. The development of new systems to better study the dynamic movement and the components of the IS may lead to important insights into the purpose of the structured immunological synapse.

1.7.2 Distal Pole Complex

Opposite the immunological synapse is a less well-characterized region known as the distal pole complex (DPC). The DPC is similar to the uropod found on the back end of migrating cells, but there are differences, the most striking of which may be the presence of the MTOC at the uropod, while in T cell:APC conjugates, the MTOC polarizes toward the IS, rather than the DPC (74). The prototypical marker of the DPC is CD43, which was originally thought to localize towards the DPC based on its large size and charge that would make it unfavorable to localize at the IS. However, it is not the bulky extracellular domain and steric hindrance that causes CD43 to move to the DPC (75). Rather, the cytoplasmic tail, which mediates interaction of CD43 with ERM family proteins (ezrin, radixin, and moesin) is required (76, 77). The CD43-ERM interaction appears to be mediated through a juxta-membrane positively charged resides (KRR) (78). Since Tim-1 also contains a similarly charged motif (KRK) near its transmembrane domain, in Chapter 2 we examine whether Tim-1 might also interact with ERM proteins and whether this interaction influences Tim-1 localization and function. Other molecules located at the DPC include PKC-ζ, type I protein kinase A (PKA), SHP-1, ezrin/radixin, and multiple other proteins reviewed elsewhere (76, 77, 79-82)

The purpose of the distal pole complex is not fully understood. The prevailing hypothesis is that the DPC serves as a sink to segregate negative regulatory molecules away from active signaling at the IS. This is logical since many of the proteins found at the DPC, including SHP-1, CD148, PTP-BL, CD43, RhoGDI, and DLG1, could interfere with the transduction of positive early signals (82). For instance, the phosphatase SHP-1 is a negative regulatory protein that could antagonize the activation of the early TCR signals, possibly through its interactions with Lck (83). Another example is based on the fact that the ERM proteins bind ERM-binding

phosphoprotein 50, EBP50, and EBP50 binds PAG/Cbp. Although there is no direct link between ERM and PAG activity, overexpression of PAG at the cell surface prevents IS formation and subsequent T cell activation (84). Thus, sequestering specific proteins either directly or indirectly away to the DPC may allow for enhancement of signaling at the IS. However, not all molecules recruited to the DPC are strictly negative regulators. Although CD43 is considered to be a negative regulator of T cells, based on the enhanced proliferation and increased ability to combat viral infection in CD43 knockout mice (85), earlier studies suggested that CD43 augments T cell activation (86-88). Antibody engagement of CD43 activates PKC-θ, which leads to pERK1/2 and induces NFAT, AP-1, and NF-κB activation (89). Cross-linking CD43 can also lead to the phosphorylation of PLC-γ2 and p38 (90). In addition, the assembly of molecules associated with positive signaling, including PIP_3, ZAP-70, STIM-1 and Orai1, which concentrate at the DPC also indicates that the DPC may serve a site for enhancing signaling under some circumstances (91-93). In addition, these molecules may move to the DPC in order to escape internalization and degradation at the IS. A more recent hypothesis is that the DPC may be important for creating and/or maintaining cellular polarity. For instance, scribble and PKC-ζ, which are found in the DPC, have well-established roles in regulating cellular polarity in epithelial and neuronal cells. Furthermore, these proteins, particularly scribble, have been shown not only to localize opposite the IS but also to be important for establishing a uropod, forming stable conjugates, proper T cell migration, and allowing for the asymmetric division of proteins, including ezrin and DLG1 (94). This asymmetric division and establishment of T cell polarity may impact T cell function since work by Steve Reiner's lab has established that scribble and PKC-ζ segregate into the more "distal" daughter cell and that the "proximal" and "distal" daughter cells have different phenotypes (79). Another potential role for the DPC is in targeted

cytokine release. While some cytokines, such as IL-2, are released at the IS, other cytokines, including TNF-α, are secreted in a multidirectional manner and appear to be secreted from areas near the DPC (95). Greater understanding of not only which proteins localize at the DPC, and how they function there, can potentially offer novel insights into T cell function. Dissecting out the location-specific roles of proteins should offer new avenues to explore and may clarify and refine the role of the DPC.

We are very interested in characterizing where Tim-1 localizes, and how this impacts T cell function. Localization of a particular protein can vary between T cell subsets, and such differences in location can impact T cell function. For instance, PKC-θ localizes at the IS and is crucial for transducing signals in effector T cells. However, PKC-θ moves away from the IS (towards the DPC) in regulatory T cells (Treg), and this localization is vital for T_{reg} suppressive functions (96). Thus, understanding where a protein functions can often reveal previously unappreciated functions for the protein. Although the literature contains many reports of Tim-1 function in T cells, most of these studies utilize antibodies. Two studies identified an interaction between Tim-1 and CD3, either by co-IP or co-capping (34, 63); however, where Tim-1 localizes in an intact T cell in contact with an antigen presenting cell (APC) has not been reported. Based on insights from previous studies of T cell activation, understanding how Tim-1 might interact with APC could shed light on the mechanisms underlying Tim-1 function. Thus, we examine the localization of Tim-1 and consequences of altering Tim-1 localization on T cell activation in Chapter 2.

1.7.3 Microclusters

With the advent of more advanced imaging techniques, researchers began to visualize T cell activation on a smaller scale. Although the immunological synapse (IS) was identified in 1997, the relationship between the IS and early TCR signaling was not clear for some time. Signaling can occur within seconds and minutes after receptor ligation, but formation of the mature synapse usually takes minutes and can last for hours. Thus, it was logical to propose signaling molecules initially exist in small islands that aggregate into microclusters for rapid, kinetically favorable signaling, before merging together to form the IS/cSMAC (71, 97). Indeed, when T cells were allowed to adhere to anti-TCR/CD28 coated coverslips, small clusters formed within seconds of TCR interaction with the stimulatory antibody, clusters that co-localized with phosphotyrosine (98). A limitation to the antibody coverslip method is that the microclusters that form do not move and form mature synapses. The formation and movement of these 300-800 nm microclusters were confirmed using a more fluid lipid bilayer system, where T cells were allowed to gently settle in phospholipid bilayers coated with various co-stimulatory and adhesion molecules. From these studies, a better appreciation of the small differences in localization within the even the cSMAC was gained (99, 100). The kinetics and dynamics of microcluster movement and interacting proteins have offered insight into the mechanisms behind early signaling events. Recent work has also established even smaller units of activation called nanoclusters. For instance, using photoactivated localization microscopy (PALM), TCR/CD3 and LAT were shown to exist in separate, preformed "protein islands" that aggregate after antigenic stimulation (101). As technology progresses, studies are finding smaller and smaller units of signaling molecules that can aggregate into larger signaling units before ultimately becoming part of the cSMAC.

Over the past few years, research into the nature of microclusters has shed even more light, and sometimes confusion, on the role of these proteins. There are now reports suggesting that vesicles might contribute to microcluster signaling. For instance, Purbhoo *et al.* elegantly demonstrated that a subset of vesicular LAT appeared to interact with SLP-76, and that tyrosine phosphorylation was greatest in the microclusters with the longest interaction with vesicular LAT (102). In addition, Ron Vale's group demonstrated that some phosphorylated CD3 zeta was found in vesicles (103). These studies suggest that in addition to the traditional surface microclusters that form the cSMAC, there might be a fraction of endocytic vesicles that also contribute to TCR-proximal signaling. Thus, in Chapter 2 we will examine whether Tim-1 forms microclusters of signaling molecules to better understand the role of Tim-1 in T cell activation.

1.8 TIM-1 KNOCKOUT STUDIES

As described in previous sections, the bulk of the existing literature on Tim-1 concentrates on the effects of engaging Tim-1 with anti-Tim-1 antibodies. However, the effects of Tim-1 antibodies on disease exacerbation or amelioration likely depends on a combination of antibody properties, including affinity and epitope as well as the specific method of disease induction. To bypass the issues associated with specific antibodies, two groups have generated Tim-1 knockout mice to examine the role of Tim-1 in a variety of diseases, and one group has created a mouse lacking just the Tim-1 mucin domain. In the first published Tim-1 knockout mouse study, the authors did not observe an effect of either Tim-1 knockout or overexpression on *in vitro* Th2 cytokine production or *in vivo* disease, the latter with a Th2-biased model of *Schistosoma*. Indeed, the authors really only noted an increase in Tim-1 expression in germinal center B cells but found no

functional consequences of this expression (104). In a subsequent paper, utilizing the same mice, the authors concluded Tim-1 is not required for airway hyper-reactivity, although closer examination reveals greater cellular infiltrates in the Tim-1 knockouts as compared to littermate controls (105). However, investigation of the role of Tim-1 in a model of airway hyper-reactivity in another Tim-1 knockout mouse demonstrated increased airway hyper-reactivity, as measured by increased inflammatory infiltrate and decreased lung elasticity (106). In addition, this Tim-1 knockout displayed increased Th2 and Th17 cytokine production (106). In accordance with the previous study, this study also suggested that expression of Tim-1 on immune cells other than T cells influences immune responses. This is supported by work in the literature suggesting that DCs expressing Tim-1 are responsible for regulating T cell activation and cytokine production (40). While one group concluded that Tim-1 does not play a role in regulating immune responses in their model systems, another group suggested that Tim-1, especially on non-T cells, may play an inhibitory role in the generation of immune responses.

In support of the concept that Tim-1 may play a role in the generation of immune responses due to its effects on non-T cells, a third group generated a Tim-1 "mucin-less" mouse and examined its effects on autoimmunity. Polymorphisms in the mucin domain of both murine and human Tim-1 have been correlated to asthma susceptibility, especially in conjunction with HAV exposure. Thus, the authors were interested in examining whether a deletion of the mucin domain would affect immune function and disease progression. This mouse was generated by deleting exon three. Before six months of age, Tim-1 mucin-less mice had a mostly normal phenotype, but after ten months, they developed signs of activation and autoimmunity (107). In particular the T cells were mostly memory cells with high levels of CD44 and the B cells failed to produce IL-10. This is consistent with another report in the literature noting that Tim-1 is

expressed on regulatory B cells, and that Tim-1 ligation is important for their suppressive function (44). These studies are suggestive of roles for Tim-1 in non-T cells and the ability of Tim-1 to influence autoimmunity and immune function.

All four Tim-1 knockout/deletion studies offered insights into the role of Tim-1 in immune modulation. Although use of the Tim-1 knockout from the McKenzie group suggested no role for Tim-1 in T cell immune responses, certain caveats should be considered. The authors generated Tim-1 knockout mice on a BALB/c background that had been backcrossed for six generations. While in general this may appear to be sufficient, the Tim-1 gene is found on mouse chromosome 11, which is proximal to many other genes implicated in T cell activation, including the Th2 cytokine locus and Itk, so the remaining mixed genetic background might be of consequence. Also, despite the authors' claims that Tim-1 does not play a role in AHR, closer examination of the data suggests an upward shift in the dose-response curve for methacholine-induced AHR in the Tim-1 knockouts, which would indicate that Tim-1 does have a role in promoting asthma pathogenesis (105). Interestingly, this effect appears to be due to differential eosinophil recruitment, rather than T cell production of Th2 cytokines. This is consistent with other reports in the literature demonstrating that anti-Tim-1 antibody treatment affects the number of eosinophils in the lung and BAL after induction of experimental asthma (2, 36). Similarly, the other Tim-1 knockout and airway hyperreactivity study saw increased cellular infiltrates. However, this second study also noted non-T cell intrinsic increases in cytokine production. Rather than conclude that Tim-1 does influence AHR, this group concluded that Tim-1 is an inhibitory molecule influencing the expression of multiple cytokines and that loss of Tim-1 ameliorates the effects of AHR (106). Finally, the Tim-1 mucinless study indicates that Tim-1, particularly on B cells and in older mice, affects cytokine production of IFN-γ (increases)

and IL-10 (decreases) as well as enhances susceptibility to autoimmune disease (Tim-1-lpr mice have worse autoimmunity). It is possible that Tim-1 defects may not be readily apparent in younger mice, and longer survival without Tim-1 may reveal previously unknown defects in immune function, especially when combined with some factor of genetic predisposition. All of these investigations have exhibited some level of immune modulation in the absence of wild type or mucinless Tim-1. Further, since asthma is such a complex disease with multiple genetic and environmental factors, it would be surprising if Tim-1 completely accounted for AHR.

1.9 TIM-1 ON NON-T CELLS

1.9.1 Tim-1 expression

Tim-1expression is found on multiple organs and tissues in the body, suggesting that Tim-1 has functions that go beyond T cells. A quantitative PCR survey identified high levels of *Tim1* message in the kidney, consistent with its previous identification as the ischemia-associated molecule Kim-1, as well as lower levels of message in lung, lymph node, spleen, and thymus (2). In addition, Tim-1 has been found in many other non-immune tissues and organs, including mucosal membranes and epithelial cells of the respiratory tract (47). As Tim-1 expression in various tissues has been discovered, the potential roles for Tim-1 expand.

While most research in the field and this thesis has focused on the function of Tim-1 in T cells, a growing body of work has begun to uncover the importance of Tim-1 in other cells of the immune system. Much of the literature supports a role for Tim-1 influencing T cell function, especially under conditions of co-stimulation, but it is possible that the major effect seen by the

anti-Tim-1 antibodies on disease pathogenesis and progression may be attributed to other Tim-1 expressing cells. Thus, the Tim-1 knockout studies discussed above implicated a role for Tim-1 in regulating a variety of immune cells, most notably antigen presenting cells DCs, B cells, and more innate leukocytes (106, 107). However, very little is understood about how Tim-1 functions in these other cell types.

1.9.2 Tim-1 and non-lymphocytes

Tim-1 also appears to influence the function of mast cells and macrophages. Tim-1 can be detected on the surface of peritoneal mast cells and bone marrow-derived cultured mast cells. Interestingly, Tim-1 expression is down-regulated after IgE and antigen stimulation. Tim-4 treatment of mast cells promotes production of Th2 cytokines, such as IL-4, IL-6 and IL-13, without affecting degranulation (108). Macrophages also appear to be regulated by Tim-1, through its interaction with Tim-4 on the surface of the macrophages. Thus, addition of Tim-1 to a macrophage cell line resulted in increased production of TNF-α, IL-6 and IL-10, as well as increased levels of the co-stimulatory molecules B7-1, B7-H1, and PD-L2 (109). At least in the case of PD-L2, the level of expression after Tim-1 treatment was significantly higher than with LPS stimulation.

Tim-1 is also found on DCs and upregulated upon activation. The high affinity anti-Tim-1 antibody 3B3 induced NF-κB as well as production of the cytokines IL-1, IL-6, and IL-23. In the absence of APCs, Tim-1 antibody ligation in the presence of CD3 and CD28 on $CD4^+$ T cells could only produce a Th2 phenotype. However, in the presence of APCs, 3B3 augmented production of Th1, Th17 and Th2 type cytokines (40). This indicated that the ability of Tim-1 ligation to skew T helper differentiation and function requires APCs, and is not due solely to

Tim-1 on T cells.

Two studies have investigated the function of Tim-1 in NKT cells. Both of these studies demonstrated constitutive expression of Tim-1 on the surface of NKT cells and also found that ligation of Tim-1 influences NKT cell function. One study revealed that the Tim-1:PS interaction activates iNKT cells by inducing co-stimulation dependent IFN-γ and IL-4 production *in vitro* and that treatment with anti-Fas antibody in the lungs led to NKT recruitment and IL-4, IL-13, IL-17, and IFN-γ mediated airway hyperreactivity. This response could be blocked by inhibiting the Tim-1:PS interaction with an anti-Tim-1 antibody (58). Cross-linking Tim-1 with different antibodies also modulates NKT function and lung disease. In contrast to the previous study, TCR or α-GalCer co-stimulation appears to induce IL-4 production while suppressing IFN-γ production, although the effects on cytokine production varied, depending on the specific anti-Tim-1 antibody used, with higher affinity antibody inducing greater cytokine production. Adoptive transfer of α-GalCer and anti-Tim-1 treated NKT cells exacerbated a mouse model of idiopathic pulmonary fibrosis by enhancing TGF-β but suppressing IFN-γ (110). Both of these studies demonstrate that ligation of Tim-1 has the ability to influence NKT function in a co-stimulatory manner and ultimately disease pathogenesis through its effects on Tim-1.

1.9.3 Tim-1 on B cells

One of the most exciting recently described roles for Tim-1 has been in the context of B cells. The first paper characterizing a Tim-1 knockout mouse demonstrated upregulation of Tim-1 on germinal center B cells (104). Furthermore, a more recent study of Tim-1 knockout mice by the same group suggests that Tim-1 deficiency does not alter antibody production (105). However,

another group recently demonstrated that stimulation of B cells with an anti-Tim-1 antibody can result in the upregulation of CD138, a plasma cell marker, and production of antibodies of the IgG2b, IgG3, and IgE isotypes (111). In addition, Tim-1 was found to be expressed more highly on B rather than T cells, particularly in a subset of IL-10 producing $CD1D^{hi}CD5^{+}$, regulatory B cells. Treatment with the lower affinity anti-Tim-1 antibody, RMT1-10, promotes a Th1 phenotype and acceleration of allograft rejection in the absence of B cells. In contrast, in the presence of B cells treatment with the same antibody led to a Th2 phenotype and long-term graft survival (44). Further, mice in which the Tim-1 mucin domain had been deleted displayed a defect in B cell IL-10 production (107). These studies indicate that Tim-1 may modulate B cell function, and greater work will need to be conducted to clarify the role of Tim-1 in B cells.

1.10 SIGNIFICANCE:

Tim-1 engagement by antibody or ligands can modulate the immune response through effects on signaling pathways, localization, and regulation of immune cell function. Although Tim-1 has been implicated in immune conditions ranging from asthma, multiple sclerosis, arthritis, systemic lupus erythematosus, to allograft response modulation, the precise mechanisms by which Tim-1 regulates T cell function and fate remains largely unexplored. This work will determine the effect of Tim-1 ligands and antibodies on (1) localization with signaling molecules in microclusters and at the SMAC, and (2) proteins recruited to the Tim-1 cytoplasmic tail important for phosphorylation and proximal signaling as well as downstream pathways for Tim-1 induced T cell activation and T helper differentiation. Better understanding of the signaling pathways by which Tim-1 antibodies and ligands influence T cell activation and differentiation

will be important in the development of therapeutic agents to modulate the immune response.

2.0 TIM-1 FORMS MICROCLUSTERS AND LOCALIZES AWAY FROM THE IMMUNOLOGICAL SYNAPSE

2.1 ABSTRACT

The interaction between T cells and APCs bearing cognate antigen results in the formation of an immunological synapse (IS). During this process, many receptors and signaling proteins form microclusters that eventually segregate to regions proximal to the synapse. This microcluster movement and IS formation is thought to influence T cell function. However, some proteins are transported away from the IS, which is controlled in part by ERM family proteins. Tim-1 is a transmembrane protein with co-stimulatory functions that is found on many immune cells, including T cells. However, the formation of microclusters and the expression pattern of Tim-1 on T cells upon activation by APCs has not been explored. In this chapter we describe the arrangement of Tim-1 microclusters. Interestingly, we demonstrate that the majority of Tim-1 on activated T cells is excluded from the IS. Tim-1 predominantly resides outside of the IS, and structure/function studies indicate that the cytoplasmic tail influences Tim-1 polarization. Specifically, a putative ERM binding motif (KRK 244-246) in the Tim-1 cytoplasmic tail appears necessary for proper Tim-1 localization. Furthermore, mutation of the KRK motif results in enhanced Tim1-mediated early tyrosine phosphorylation downstream of TCR/CD28 stimulation. Paradoxically however, the KRK motif is necessary for Tim-1 induced NFAT/AP-1

activation and co-stimulation of cytokine production. This work reveals unexpected complexity underlying Tim-1 localization and suggests potentially novel mechanisms by which Tim-1 modulates T cell activity.

2.2 INTRODUCTION

Antigen receptor, co-stimulatory, and signaling proteins adopt distinct patterns of localization and segregation upon T cell stimulation by peptide antigen presented by antigen-presenting cells (APC). Current models suggest that these patterns are critical for proper regulation of T cell activation. T cell recognition of an APC bearing cognate peptide drives the formation of a structure termed the immunological synapse (IS) or supramolecular activation cluster (SMAC) (112). In a "mature" synapse, many proteins important for transduction of TCR signaling concentrate at the center of the contact site, the central supramolecular activation cluster (cSMAC), between the T cell and APC. These proteins include CD3, CD28, ZAP-70 and PKC-θ (112) (113, 114). At the IS, this concentration of signaling proteins may enhance signaling before engaged TCR's are internalized, possibly to terminate signaling (71).

Opposite the immunological synapse is a region known as the distal pole complex (DPC). Many large adhesion and glycosylated molecules, such as CD43, are transported to this region (71, 115). Formation of the DPC is thought to be driven by ERM (ezrin, radixin, and moesin) family proteins (76, 77). The prevailing hypothesis is that the DPC serves as a reservoir for sequestering negative signaling molecules, such as CD43, away from the IS to allow for greater T cell activation (74). However, the presence of a pool of active signaling molecules, including ZAP-70, PIP_3, and STIM-1 and Orai1 suggests an additional positive role for the DPC (91-93).

While the precise function of the DPC is disputed, formation of the DPC does appear to impact T cell activation. For example, disrupting localization of proteins to the DPC with an ERM dominant negative construct can disrupt specific functions, including transcriptional activation and cytokine production (76, 116).

The localization and interaction of proteins play key roles in T cell activation. Intracellular tyrosine phosphorylation, Ca^{2+} flux, and cytoskeletal rearrangements all begin within seconds of TCR ligation before the formation of a mature synapse (98, 117). It was discovered that microclusters of TCR and other associated signaling proteins, such as ZAP-70 and LAT, are formed upon stimulation and co-localize with tyrosine phosphorylation, and generation of these microclusters precedes calcium flux (98). Imaging has visualized tyrosine phosphorylated signaling microclusters that form around the edges of the cell that in turn aggregate and eventually coalesce at the SMAC (72, 100, 118). The data suggest that microcluster formation precedes formation of a mature synapse and that aggregation of microclusters at the periphery induce signaling that is terminated by the time they form the cSMAC.

Localization of microclusters also regulates co-stimulation of T cells. For instance, CD28 microclusters formed in the presence of their ligands initially move towards the cSMAC before separating from the TCR and sequestering themselves to a region around the periphery of the cSMAC where CD28 may continue to prolong signaling (99). Thus, understanding the movement and localization of microclusters has been crucial in greater understanding of their functions.

Microcluster formation has generally been studied with two methods. One method pioneered by the Samelson lab utilizes an elegant reductionist system of antibody coated glass

coverslips to study microclusters. The TCR microclusters become immobilized when binding to the anti-TCR coated glass, and this method has been useful for studying the early signaling events (119). Much of the work with antibody coated cover slides has been confirmed using a lipid bilayer system where ligands are inserted into a phospholipid bilayer (120). Using this system, investigators can observe movement of microclusters as they interact with the ligands and antibodies in the bilayer.

Transmembrane immunoglobulin and mucin 1 (Tim-1) is a co-stimulatory molecule found on the surface of many immune cells. It was first identified in primates as a Hepatitis A virus cellular receptor (HAVCR1), although the mouse homolog does not bind HAV (121). Variants in murine (and human) Tim-1 were later associated with asthma susceptibility (18, 21, 27). Early work on the immune function of Tim-1 also revealed a role for Tim-1 as a co-stimulatory molecule on $CD4^+$ T helper cells by enhancing inducible transcription, cytokine production, and proliferation (2, 33). Tim-1 has also been implicated in the regulation of B cells, $CD8^+$ T cells, dendritic cells, NKT cells, and mast cells (40, 43, 44, 58, 104, 108, 110, 111). Tim-1 antibodies have demonstrated efficacy in the modulation of immune function in different models of disease, including asthma and organ transplantation (2, 36, 37, 41-44).

Although much is known about the effects of Tim-1 on transcription factor induction and cytokine production, less is known about the sub-cellular localization of Tim-1, especially in T cells. The function of many molecules has not been fully appreciated until their localization was understood. For example, the role of PKC-θ in T cells was greatly enhanced by the discovery that it localizes at the SMAC in effector T cells and away from the IS in regulatory T cells (96, 122). Understanding Tim-1 localization in T cells may provide similar insights into its function, particularly since some controversy still exists about the role of Tim-1 in T cell signaling. While

previous studies have implicated Tim-1 in enhancing T cell activation (2, 33, 63, 64), one report has suggested that Tim-1 might function in either a positive or negative fashion, depending on the strength of antibody ligation (34). Another recent study demonstrated increased cytokine production by Tim-1 deficient T cells, suggesting that Tim-1 may also act as a negative regulator of T cell function, at least under some circumstances (106). Thus, defining Tim-1 localization on T cells under different conditions may yield novel insights that help to resolve these apparently disparate findings.

The localization of Tim-1 has not been extensively explored. A previous report suggested that Tim-1 exists in vesicles in the cytoplasm of human embryonic kidney cells (293) and 300.19 pre-B cells (45). Another study demonstrated that Tim-1 expressed on DO11.10 TCR transgenic T cells localized towards apoptotic thymocytes with exposed phosphatidylserine (PS), a Tim-1 ligand (58). One study has suggested that human TIM-1 interacts with ZAP-70 and PI3K (63). Another study demonstrated Tim-1 co-capping with CD3 (34). However, more data about Tim-1 localization and movement upon activation by antigen and APC's may aid the understanding the function of Tim-1 in T cells.

Despite previous studies implicating Tim-1 as a co-stimulatory molecule and modifier of disease pathogenesis, the molecular mechanism(s) underlying Tim-1 activity remain unclear. At this point, relatively little is known about the sub-cellular localization of Tim-1, especially in T cells. In particular, where Tim-1 distributes (or re-distributes) upon T cell activation is poorly characterized. In this study, we will determine whether Tim-1 exists in signaling microclusters. Then, we will define the patterns of Tim-1 localization before and after T cell recognition of antigen/MHC, as well as the functional consequences of altering Tim-1 localization. Our studies have revealed unexpected complexity in the regulation of Tim-1 localization and its function in T

cell activation. These findings may have implications for understanding the function of Tim-1 in regulating immune responses.

2.3 MATERIALS AND METHODS

Reagents and cell culture

Jurkat, D10, Raji, and CH27 cell lines were used and cultured as previously described (123). The following antibodies were used: pSrc Y416 and pZAP-70 Y319 (Cell Signaling), PKC-θ (C-18, Santa Cruz), CD43 (clone S7, BD Pharmingen), M2-Cy3 (Sigma Aldrich), EEA1 (BD Transduction), M2 anti-flag (Sigma Aldrich), anti-human CD3 (Becton Dickinson), mouse CD3 and CD28 (BD Pharmingen), human CD28 (Life Technologies), Tim-1 Fc (eBiosciences), anti-TCR antibody C305 (Harlan), anti-Tim-1 antibodies (3B3 and 5F12), anti-Tim-4 antibodies (3A1, 3H11 and 5G3). Alexa fluor-conjugated secondary antibodies were from Life Technologies. Conalbumin was from Sigma Aldrich, and SEE from Toxin Technology.

Microcluster formation

D10 and Jurkat cells transfected with Tim-1 were allowed to settle on antibody coated coverslips that were created as described previously (124). Briefly, T cells were allowed to settle on coverslips coated with anti-CD3, anti-CD28, anti-VCAM-1, and/or anti-Tim-1. The interface between the coverslip and spread cells was imaged either by confocal microscopy or by TIRF. For fixed images, cells were permeabilized with TX-100 and co-stained for anti-pY, pZAP-70, CD3 at 1:100 before secondary staining with Alexa 555 at 1:2000.

T cell:APC conjugates for confocal imaging

D10 cells were transiently transfected with up to 20μg total of plasmid DNA by electroporation at 250V/950mF and rested for 16 hours. Live cells were recovered the next day by spinning over a cushion of Lympholyte-M. D10 T cells ($0.3x10^6$) were combined with an equal number of conalbumin-loaded CH27 cells by centrifugation at 3000 rpm for 3 minutes, followed by incubation at 37 degrees for 5-40 minutes. The pellet was gently resuspended by pipetting 3 times with a large-bore 1 ml pipet tip. Cells were allowed to settle on a poly-l-lysine coated coverslip for 20 min before being fixed at a final concentration of 2% PFA. Cells were permeablized with 0.1% TX-100 and were blocked for 30 min in 10% anti-goat or anti-donkey serum. The following were used: M2-Cy3 (2 mg/mL), pSyk/ZAP-70 (1:100), PKC-q (1:100), and EEA1 (1:100). Secondary antibodies were used at the following concentrations: anti-rabbit Alexa-647 - 1:1000, anti-mouse Alexa-488 - 1:2000, anti-mouse Alexa-555 - 1:2000, anti-rat-Cy3 - 1:1000. Mid-plane images were captured on an Olympus FluoView 1000. Images were exported as bit TIFFs and analyzed with Image J. Figures were assembled in Canvas 8. For live cell imaging, Jurkat T cells were co-transfected with Tim-1 GFP and ZAP-70 Tag RFP. Equal numbers of Jurkat and SEE loaded Raji cells ($0.075x10^6$ cells) were maintained at 37^0 C in Matek dishes and imaged on an Olympus FluoView 1000 or a Nikon A1.

Mature conjugates were identified by morphology and localization of ZAP-70 or PKC-q at the interface between the B and T cell. Images were analyzed in Metamorph or Image J. When Tim-1 was localized opposite the IS, the cells were termed “anti-synapse.” Tim-1 in conjugates that appeared close to the IS were termed “front half” of the cell. Tim-1 that appeared to be both opposite and near to the IS were termed “unpolarized.” Finally, Tim-1 that had a predominantly intracellular and vesicular appearance were noted as “punctate.”

For a select number of imaged conjugates that expressed Flag-Tim-1, two parameters were measured using ImageJ. First, the distance of Tim-1 from the synapse was determined as the angle between the center of the IS to the center of the Tim-1 region with the vertex of the angle set at the center of the cell. The extent of spread of Tim-1 was measured as the angle between the two edges of the Tim-1 region.

Latex Beads

8.7micron aldehyde/sulfate latex beads (Life Technologies) were prepared according to manufacturer instructions. $80x10^6$/mL beads were coated with 50mg/mL anti-CD3 and 50 mg/mL anti-CD28.

DNA Constructs

Tim-1, Tim-1 Y276F, and Tim-1 cytoplasmic tail truncation were generated as described previously (33). Tim1-GFP was generated by inserting the C57Bl/6 Tim-1 into pEGFP-N1. Site-directed mutagenesis was utilized to mutate a Flag-Tim-1 construct, using the QuikChange system (Stratagene). The KRK at position 244-246 of the C57BL/6 allele of Tim-1 was mutated to QGQ using the following primers: Forward: cc aggta catac ttatg caagg gcagt cagca tctct aagcg; Reverse: cgctt agaga tgctg actgc ccttg cataa gtatg tacct gg. The sequence was verified by automated sequencing. The ERM DN-GFP construct was a gift from Dr. Janis Burkhardt. ZAP-70 cDNA and vector containing Tag RFP were gifts from Dr. Steven Bunnell.

Flow Cytometry

$0.5x10^6$ Jurkat and D10 T cells were stained with 1mg of M2 (anti-Flag) on ice and then stained with 1:200 Alexa-647 for surface staining. For intracellular staining cells were fixed in 1.5% paraformaldehyde at room temperature for 10 minutes. Cells were then permeablized on ice with ice cold methanol for 15 minutes before being washed and stained for M2 (anti-Flag). $0.5x10^6$ CH27 or Raji cells were stained with 1-4ug of anti-Tim-1 or anti-Tim-4 antibodies on ice and then secondarily stained with 1:200 Alexa-647 on ice. Samples were read on a BD LSR II; FlowJo software was used to analyze data.

Tyrosine Phosphorylation Western Blotting

$20x10^6$ Jurkat T cells were transfected with empty vector, Tim-1, or Tim-1^{QGQ}. $1.5x10^6$ cells were lysed using 1% NP-40 lysis buffer in addition with beta-glycerophosphate, sodium fluoride, sodium orthovanadate, AEBSF, aprotinin, leupeptin, pepstatin (Calbiochem/EMD Biochemicals). Lysates were run on a 10% SDS-PAGE gel before being transferred to PVDF membrane and blotted with anti-pY (4G10). Blots were developed with Super-Signal Pico ECL (Pierce) and imaged on a Kodak Image Station 4000MM.

TCR Internalization

Jurkat cells were transfected as described above with pCDEF3 (empty vector), WT Tim-1, Tim-1^{QGQ}, or Tim-1 lacking the cytoplasmic tail truncation (Tim-1^{DCyto}). Cells were re-suspended at $0.5x10^6$ in PBS and placed on ice in the presence of anti-TCR (C305) at a dilution of 1:250 for 30min. Cells were treated with 80 mM Dynasore for 20 minutes on ice to prevent clathrin-mediated TCR internalization. Cells were then incubated at 37^0 C for 0, 5, 10, 20, 30, 60, and 120 minutes. Immediately after the time points, cells were washed with ice cold PBS before

staining with anti-human CD3 and M2 (to detect Flag Tim-1). Samples were read on a BD LSR II; data were analyzed using FlowJo software.

Luciferase Assays

Jurkat T cells were co-transfected with empty vector, WT Tim-1, or Tim-1 QGQ, along with an NFAT/AP-1 luciferase reporter construct. Cells were allowed to recover for 16 hours before stimulating with the anti-TCR antibody C305 (1:1000) in the presence or absence of anti-CD28 (1:100) for 6 hours at 37^0. D10 T cells were co-transfected with empty vector, WT Tim-1, or Tim-1 QGQ, along with an NFAT/AP-1 luciferase reporter construct. Cells were allowed to recover for 16-18 hours before stimulating with 1 mg/mL biotinylated anti-CD3, -CD4 and -CD28, plus streptavidin for six hours at 37^0. Luciferase activity was determined as described previously (125).

ELISA

$0.5x10^6$ Jurkat cells were stimulated with anti-TCR antibody C305 (1:1000), with or without CD28 (1:200) for 24 hours. Supernatants were taken and production of IL-2 was determined by ELISA (BD OptIA). D10 T cells ($0.5x10^6$) were stimulated with 1mg/mL anti-CD3, -CD4 and -CD28 for 24 hours before supernatants were collected for measurement of IL-4 and TNF-a by ELISA. Comparisons were analyzed by paired Student's t test, performed with Prism.

Limitations to Data Interpretation

The chief limitation in these studies is the subjective nature of data collection and interpretation in the confocal imaging experiments. To determine true positive signal, cells that were stained

with comparable amounts of secondary antibodies alone were compared to staining with primary and then secondary antibodies. In addition, the laser voltage was adjusted so that pixels were not saturated. Protein localization was observed in two different T cell lines, using both epitope- and GFP-tagged constructs. The core observation of WT mTim-1 exclusion from the immune synapse was observed over the course of dozens of experiments. Quantitation of WT and mutant Tim-1 localization was pooled from conjugates obtained in multiple separate experiments. Other experiments were performed at least three times, with statistical analysis being performed on replicates within a representative experiment.

2.4 RESULTS

2.4.1 Tim-1 forms microclusters with pY proteins

Stimulation of T cells leads to activation of multiple kinases and subsequent signaling molecule organization into microclusters, and Tim-1 has been demonstrated to induce T cell activation. However, how Tim-1 mediates its co-stimulatory functions has not been fully elucidated. Further, understanding the localization and movement of various signaling molecules in relation to other signaling molecules has contributed greatly to enhancing knowledge of their function. Thus, we were interested in determining whether Tim-1 forms microclusters and whether these microclusters have similar patterns of localization as compared to other signaling molecules.

In order to study Tim-1 microclusters, we utilized a system of antibody coated coverslips to activate and to observe the formation of microclusters in T cells. We allowed T cells to settle on anti-CD3, anti-CD28, and/or anti-VCAM or anti-LFA coated coverslips in order to spread

and to become activated. Anti-CD3 and anti-CD-28 coated coverslips induce both spreading and activation while integrin binding with anti-VCAM or anti-LFA alone causes cell spreading but not T cell activation. Here we demonstrate that Tim-1 forms microclusters upon TCR stimulation with or without CD28 co-stimulation in both D10 and Jurkat cells (Fig. 2-1A and 2-2A/B). However, when D10 cells are stimulated with anti-LFA alone, the cells spread but do not form as distinct Tim-1 microclusters when compared to TCR or TCR/CD28 stimulation (Fig. 2-1A/B, Fig. 2-2A). Thus, Tim-1 microcluster formation requires activation and not just spreading.

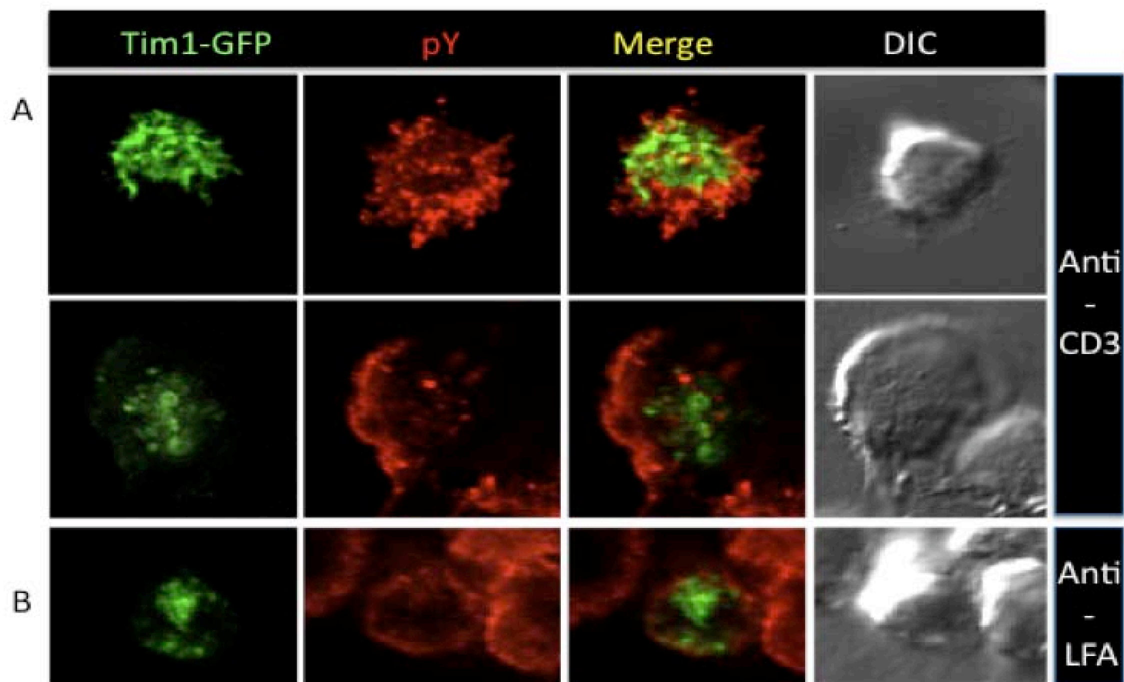


Figure 2-1. Tim-1 forms more MC upon activation than spreading

(A) D10 cells transiently transfected with Tim-1 GFP were allowed to settle on anti-CD3 coated slides. Cells were then co-stained with 1:100 4G10 followed by Alexa-555 secondary. (B) D10 cells transfected and stained as above were allowed to settle and spread on anti-LFA-1 coated slides.

Next, we sought to determine whether these Tim-1 microclusters might be signaling. Since tyrosine phosphorylation is often an early step and indication of active signaling, we co-stained Tim-1 transfected cells with anti-pY. Spreading of T cells by anti-LFA-1 alone is insufficient to induce tyrosine phosphorylated Tim-1, and similarly stimulation with only anti-CD3 does not induce much co-localization between Tim-1 microclusters and pY. However, more microclusters are undergoing pY signaling upon co-stimulation by anti-CD3/CD28 (Fig. 2-1A and 2-2A), which is consistent with reports in the literature that Tim-1 signaling is enhanced by co-stimulation (33).

2.4.2 Tim-1 microclusters do not interact with CD3 or ZAP-70

Since Tim-1 has been demonstrated to induce T cell activation downstream of TCR/CD28 stimulation, we next sought to determine the specific tyrosine phosphorylated proteins interacting with Tim-1. Human TIM-1 binds important signaling molecules, including PI3K, ZAP-70, Itk, and CD3 (63), while mouse Tim-1 has been suggested to interact with CD3, the p85 subunit of PI3K, and PLCγ1 (34, 64). Thus, we postulated that Tim-1 might be interacting with pZAP-70 or CD3 microclusters; however, the majority of Tim-1 microculsters do not co-localize with CD3 or ZAP-70 in either D10 or Jurkat cells (Fig. 2-2B-C).

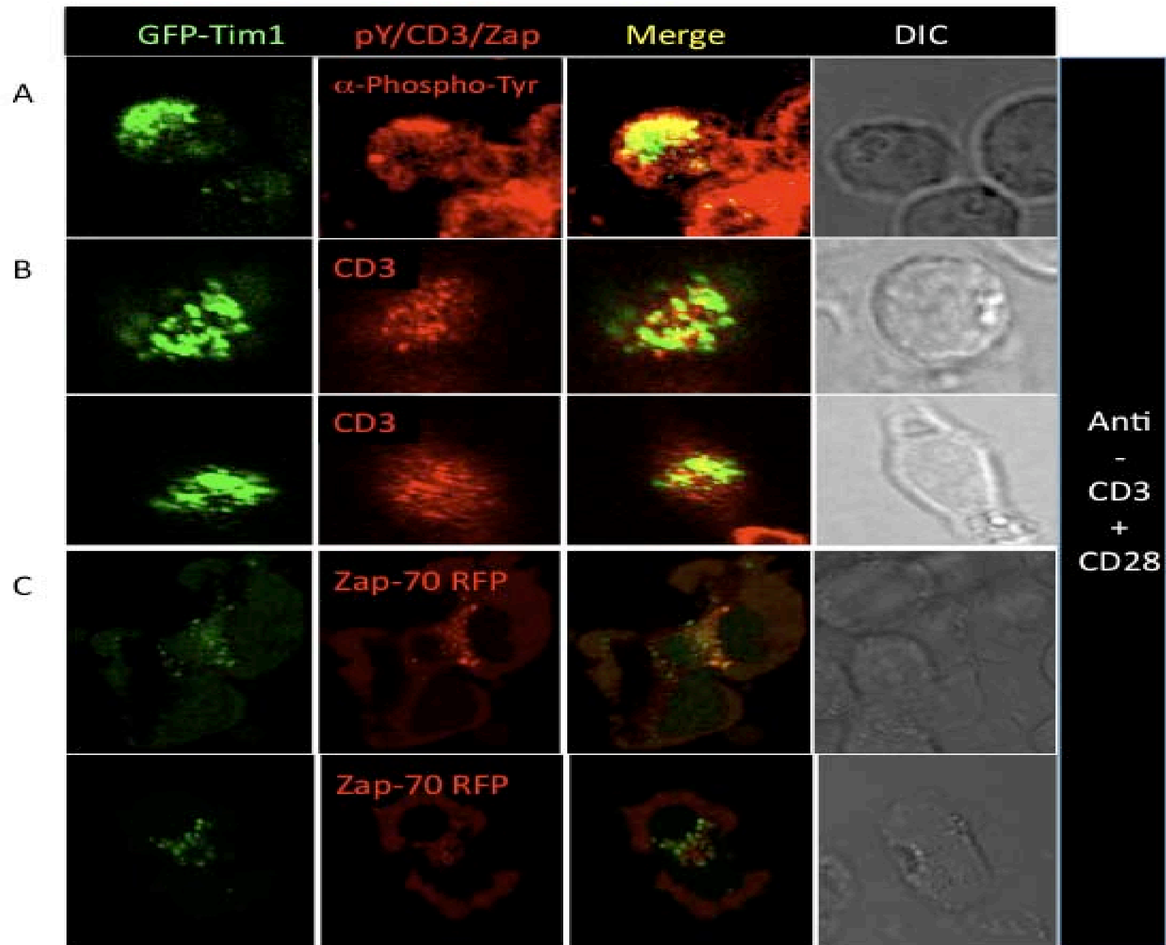


Figure 2-2 Tim-1 forms microclusters associated with tyrosine phosphorylated proteins. (A) Jurkat transfected with Tim-1-GFP were settled on anti-CD3/anti-CD28 coated slides for 30 minutes. Cells were then fixed and lysed with 0.1%Triton X-100 before staining for tyrosine phosphorylation (4G10) and secondarily with Alexa 555. (B) D10 cells transfected with GFP-Tim-1 were settle on anti-CD3/anti-CD28 coated slides for 15 minutes before fixation, lysing, and staining with CD3 followed by Alexa 555 secondary. (C) Jurkat T cells co-transfected with Tim-1-GFP and ZAP-70 RFP were settled on anti-CD3/CD28 coated slides.

Microcluster formation and signaling may depend on the stimulation conditions. For instance, CD28 microclusters require their ligand(s), particularily B7-2, in addition to TCR stimulation for

proper microcluster and IS localization (99, 126). Since Tim-1 is also a co-stimulatory molecule, we postulated that activation with a high affinity anti-Tim-1 antibody, 3B3, might impact its localization and interaction with other signaling molecules. D10 cells that were allowed to settle on slides coated with anti-CD3/CD28/Tim-1 did not appear to have increased co-localization with pZAP-70 (Fig. 2-3A). Interestingly, there did appear to be more peripheral ZAP-70 microclusters in anti-TCR/CD28/Tim-1 stimulated cells as compared to anti-TCR/CD28 (Fig. 2-3A and 2-2A).

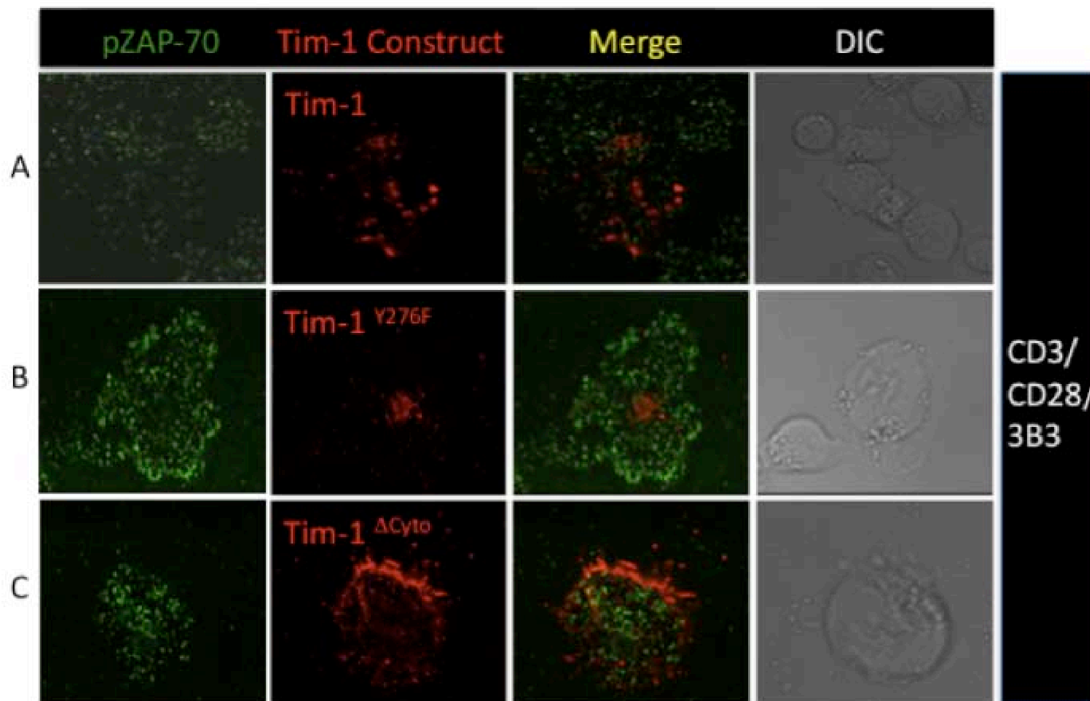


Figure 2-3: Altered Tim-1 microclusters in Tim-1 mutant constructs

D10 cells transfected with Tim-1 (A), Tim-1^{Y276F} (B), or Tim-1 $^{\Delta Cyto}$ (C) were allowed to settle on anti-CD3/CD28/Tim-1 (3B3) coated slides for 15 minutes. Cells were co-stained for pZAP-70.

Next, we examined the role of integrin co-stimulation on Tim-1 microclusters. Although integrin ligation alone does not induce signaling, it has been shown to sustain TCR mediated

signaling. Ligation of integrins increased the surface area for contact between the cell and antibodies, like CD3. Nguyen *et al* demonstrated that the use of VCAM-1 to ligate VLA-4 in the presence of anti-TCR (Okt3) enhances SLP-76 signaling. Specifically, co-stimulation with VCAM slowed centripedal actin flow, which allowed for longer interactions between SLP-76 with ZAP-70 at peripheral microclusters, where the greatest signaling is thought to occur (127). We postulated that stabilization of microclusters by integrin binding might allow better visualization of transient Tim-1 and ZAP-70 microcluster interactions. We utilized both LFA-1 and VCAM-1, which bind ICAM-1 and VLA-4, respectively to examine the interaction between Tim-1 and ZAP-70. We were unable to detect increased co-localization between Tim-1 and ZAP-70 microclusters with either LFA-1 or VCAM-1 ligation (Fig. 2-4A). At least at 15 minutes of stimulation, costimulation by integrin binding was insufficient to detect Tim-1 and ZAP-70 interactions.

2.4.3 Tim-1 may require Y276 for proper microcluster formation

Previous work from this laboratory has demonstrated that Tim-1 mediated signaling requires the cytoplasmic tail, particularly Y276. We wanted to characterize the effect of mutation or absence of Y276 on Tim-1 microclusters. We examined the formation of Tim-1 microclusters when Y276 (Tim-1^{Y276F}) is mutated or in the absence of the Tim-1 cytoplasmic tail, which would also lack Y276 (Tim-1$^{\Delta Cyto}$). Fewer microclusters were formed in the Y726F mutation. It is interesting that the type of co-stimulation may influence Tim-1 localization even in the absence of Tim-1 tyrosine phosphorylation. Stimulation by anti-TCR/CD28/Tim-1 seemed to concentrate Tim-1^{Y276F} microclusters at the center (Fig. 2-3B). In contrast, anti-TCR/CD28/LFA stimulation enhanced Tim-1^{Y276F} microcluster aggregation at the periphery (Fig. 2-4B).

Mutation or truncation of Tim-1 did not influence the ability of pZAP-70 to form microclusters regardless of stimulation conditions. More structure/function work will need to be conducted to identify the necessary Tim-1 components for microcluster formation and localization.

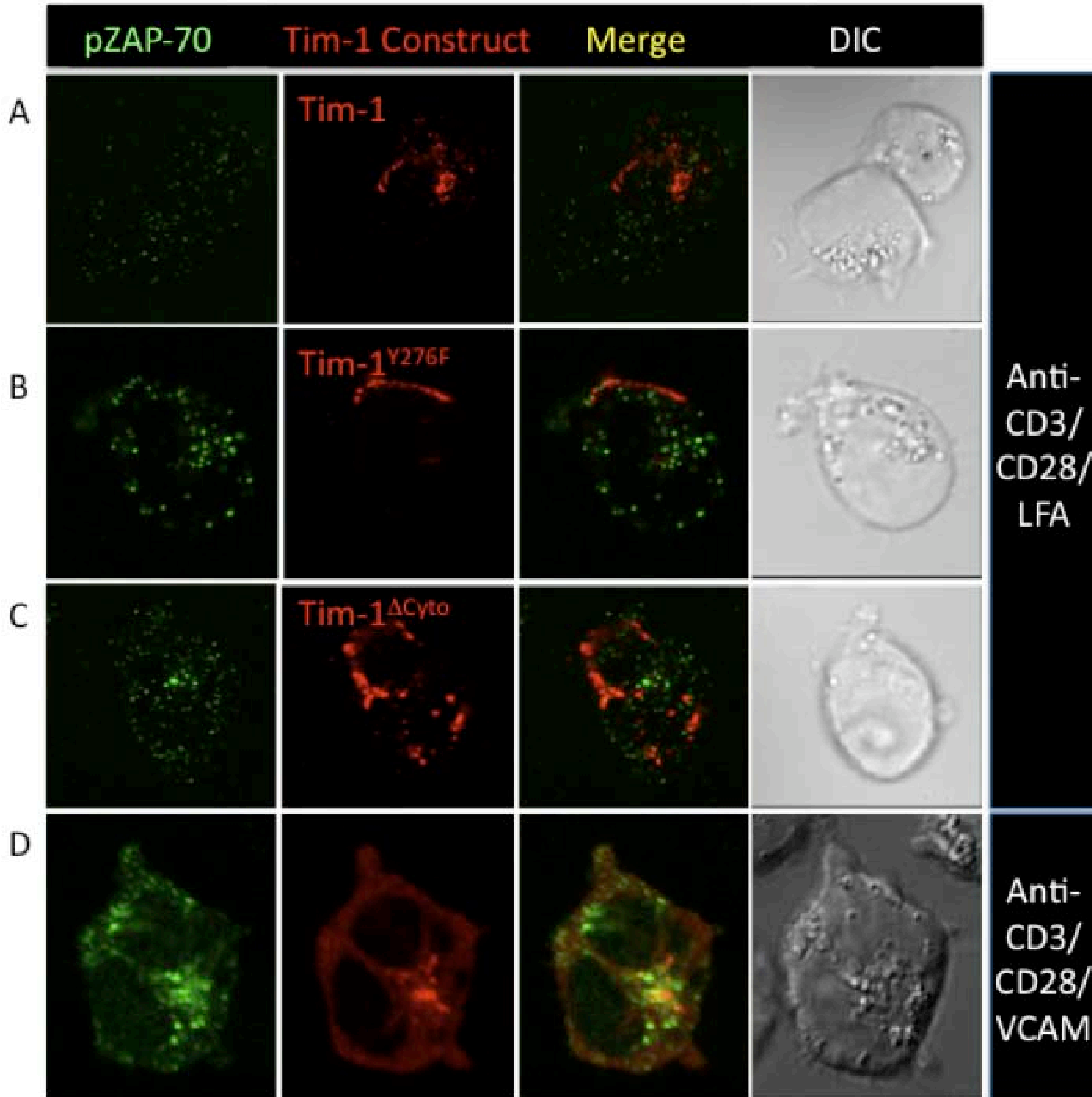


Figure 2-4: Tim-1 cytoplasmic tail may be involved in microcluster formation

D10 cells transfected with Tim-1 (A), Tim-1^{Y276F} (B), or Tim-1$^{\Delta Cyto}$ (C) were allowed to settle on anti-CD3/CD28/VCAM coated slides for 15 minutes. Cells were co-stained for pZAP-70.

Surprisingly, the effect of the cytoplasmic tail truncation of Tim-1 is less dramatic than the effect of the Tim-1^{Y276F}. The Tim-1$^{\Delta Cyto}$ construct forms more microclusters than the Tim-1^{Y276F}; however, the pattern of the microclusters is different than wild type and again does not co-localize with ZAP-70 microclusters (Fig. 2-3C and 2-4C). Co-stimulation with either Tim-1 or integrins appears to generate more microclusters at the cell's periphery.

2.4.4 Tim-1 does not co-localize with peripheral ZAP-70 microclusters

The previously described experiments were all fixed at specific time points, approximately 5-15 minutes. One reason we may not have detected an interaction between Tim-1 and ZAP-70 is that we were not catching the correct time. Thus, we co-transfected Tim-1 GFP with ZAP-70 TagRFP and utilized a live cell system using antibody coated coverslips and TIRF imaging in conjunction with the center for biological imaging (CBI) at the University of Pittsburgh. Using TIRF we would be able to detect at a depth of only 100nm from the coverslip, or where the microclusters would form with little background noise. The ZAP-70 microclusters were consistent with other reports in the literature with well defined, immobile peripheral microclusters and along the actin cytoskeleton. While there certainly might be some interaction between Tim-1 and ZAP-70 at the center of the cell, the majority of microclusters, particularly the peripheral microclusters where signaling is thought to occur, do not co-localize (Fig. 2-5A/B). Furthermore, the pattern of Tim-1 microclusters is not reminiscent of two well-characterized microclusters, ZAP-70 and SLP-76. Tim-1 microclusters do not have the same distinct peripheral microclusters that form along the lamellipodia as it spreads along the antibody coated slides (Fig. 2-5, Movie 2-1). Also, the Tim-1 microclusters did not appear to be consistent with the immobile pattern of ZAP-70 microclusters or the movement of SLP-76

microclusters (98). Rather, some Tim-1 clusters appeared to flit in and out of the field, which may be consistent with endocytosis (Movie 1).

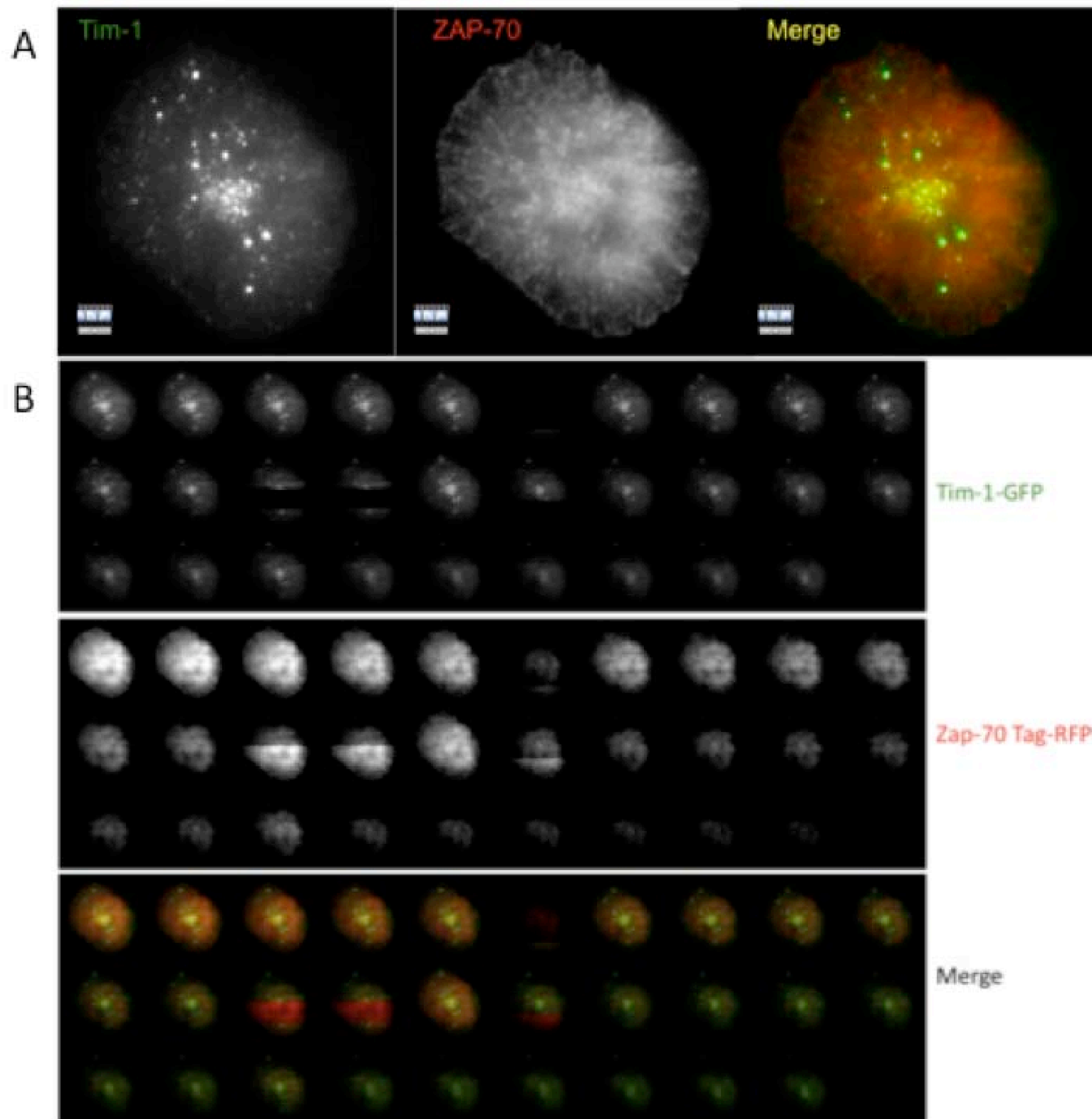


Figure 2-5. Tim-1 microclusters appears to be endocytic

(A) Still images from Jurkat T cells co-transfected with Tim-1-GFP and ZAP-70-TagRFP were allowed to settle on anti-TCR/CD28 coated slides and imaged with TIRF microscopy in a 37ºC chamber. (B) Montage of still images from the TIRF live cell imaging.

2.4.5 ZAP-70 microclusters move with anti-Tim-1 antibody co-stimulation

Since stimulation by ligands is required for CD28 localization to the IS, we postulated that co-stimulation with anti-Tim-1 could also influence Tim-1 microclusters. Using TIRF imaging, we imaged as Tim-1 GFP and ZAP-70 TagRFP co-transfected cells settled on coverslips coated with anti-TCR/CD28/Tim-1 (3B3). The Tim-1 microclusters were similar to stimulation without anti-Tim-1. Surprisingly, in this small subset of experiments (n=2), the ZAP-70 microclusters did not remain immobile on the actin cytoskeleton but rather appeared to flit in and out of focus (Fig. 2-6 and Movie 2). While co-stimulation with anti/TCR/CD28/Tim-1 does not stabilize Tim-1 microclusters, it does destabilize ZAP-70 microclusters.

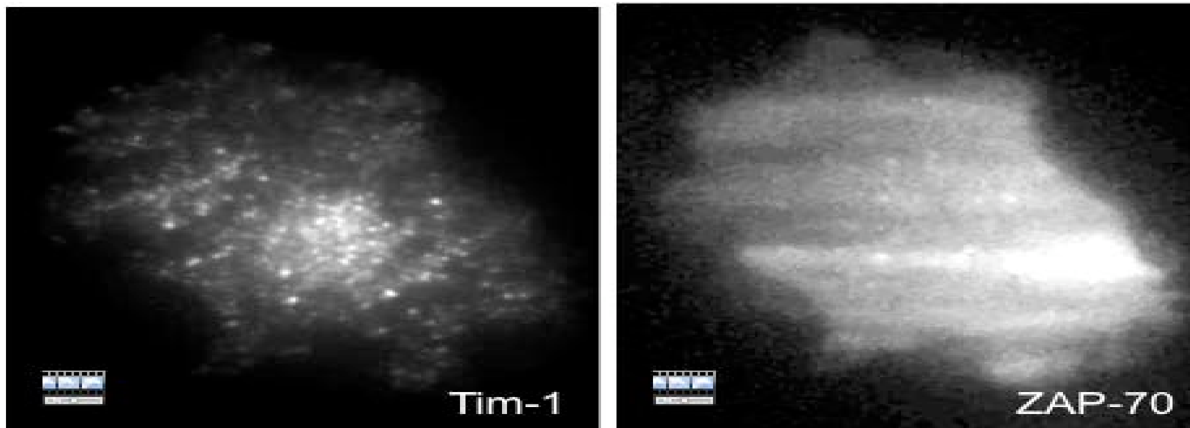


Figure 2-6: ZAP-70 microclusters do not remain immobile on anti-TCR/CD28/Tim-1 stimulation

Still images from Jurkat T cells co-transfected with Tim-1-GFP and ZAP-70-TagRFP were allowed to settle on anti-TCR/CD28/Tim-1 (3B3) coated slides and imaged with TIRF microscopy in a 37ºC chamber.

2.4.6 Tim-1 is excluded from the immunological synapse

To define patterns of Tim-1 localization on T cells, we transfected Tim-1 into the murine Th2 line D10, which does not expresses endogenous Tim-1 (33). In contrast to studies that reported predominantly intracellular Tim-1 in non-T cells (45), we found Tim-1 diffusely expressed on the surface of resting T cells (Fig. 2-7A). However, when Tim-1 expressing T cells are activated by antigen loaded APCs, the pattern of Tim-1 localization is altered. Surprisingly, Tim-1 concentrates in a region opposite the immunological synapse, with the latter represented by PKC-θ or pZAP-70 (Y319). This localization is not epitope tag-dependent, since both C-terminus tagged Tim1-GFP and N-terminus tagged Flag-Tim1 localize opposite, or at least outside, the immunological synapse (Fig. 2-7B). The majority of Tim-1 in T cell:APC conjugates (51.25% and 71% with Tim1-GFP and Flag-Tim1, respectively) appears in the "back" half of the cell, opposite, or at least away from, the immunological synapse ("anti-synapse"; Fig. 2-7C-E). Tim-1 was present within the IS in only 1 conjugate (1.03% of the total). This appears to be a general phenomenon, as Tim-1 localization on Jurkat T cells interacting with APC's is also found predominantly outside of the immunological synapse (57% of conjugates; Fig. 2-7F).

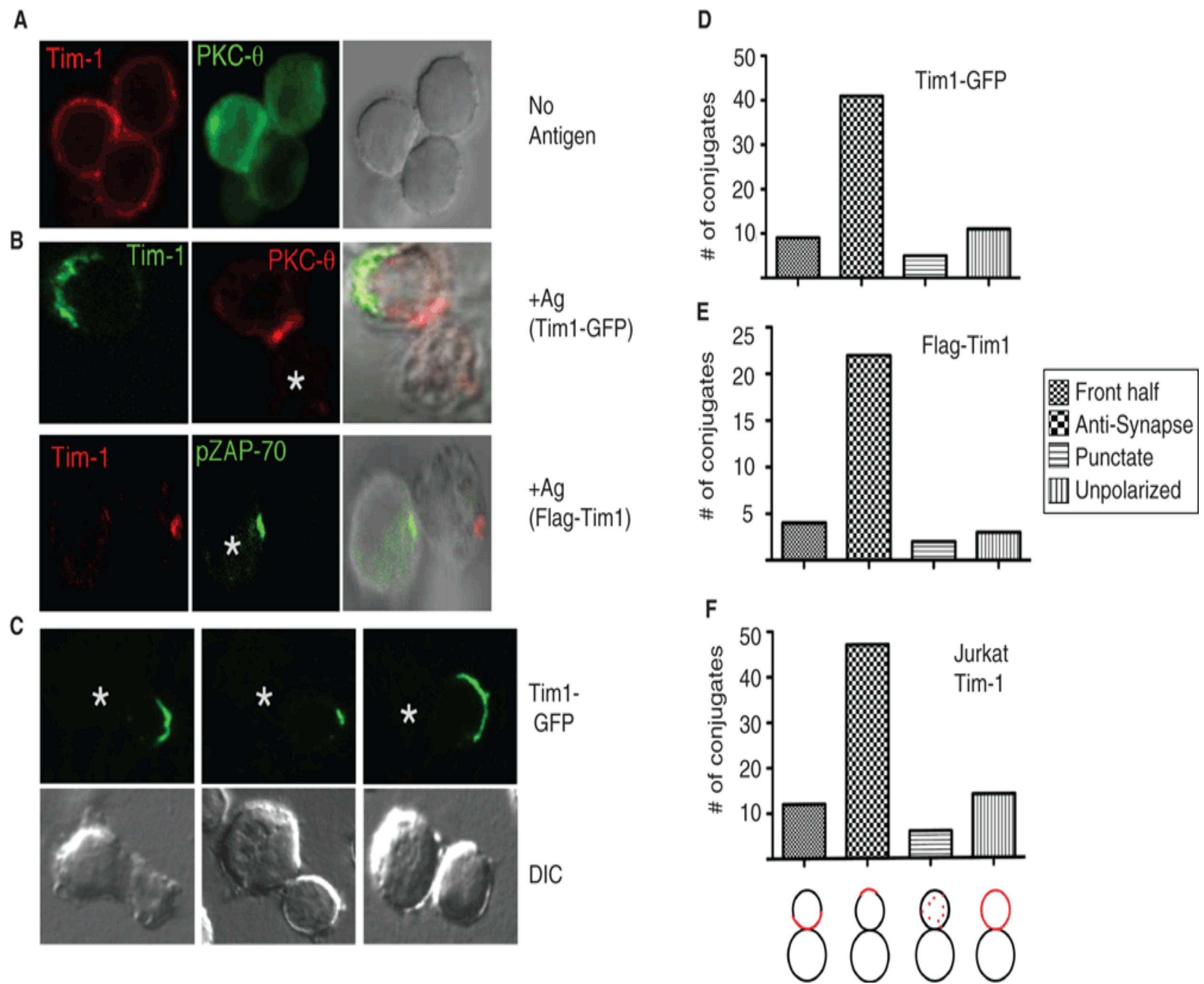


Figure 2-7: Tim-1 re-distributes away from the immunological synapse.

(A) Resting D10 T cells transiently co-transfected with Flag-Tim-1 and PKC-θ-GFP (green) were fixed, stained with anti-Flag-Cy3 (red), and visualized mid-plane by confocal microscopy. (B - upper panels) D10 T cells transiently transfected with Tim-1-GFP (green) were conjugated with conalbumin-loaded CH27 B cells. Endogenous PKC-θ was stained with PKC-θ (C-18) and Alexa-555-conjugated secondary antibody (red) as a marker of the IS/c-SMAC. (B - lower panels) D10 T cells transiently transfected with Flag-Tim1 (red) were conjugated with antigen loaded CH27 cells and stained with pZAP-70 and Alexa 488-conjugated secondary antibody (green) as a marker of the cSMAC. (C) Additional D10 T cell:APC conjugates showing the

exclusion of Tim1-GFP from the IS. (D) Quantitation of the phenotype of Tim-1 GFP localization in D10:CH27 conjugates (n=66) from 15 experiments or (E) Flag-Tim-1 in D10:CH27 conjugates (n=31) from 6 experiments. (F) Quantitation of Tim-1 localization on Jurkat T cells making synapses with superantigen-loaded Raji B cells. (F, bottom) Schematic of system used to score conjugate phenotypes.

To further demonstrate that Tim-1 localizes predominantly away from the cSMAC, we performed live cell microscopy. Again, Tim-1 moved away from the nascent ZAP70-containing immunological synapse (Fig. 2-8A and Movie 2-3). We also utilized a more reductionist system to examine the effect of anti-TCR and –CD28 on Tim-1 localization. Thus, Jurkat T cells expressing Tim1-GFP and ZAP70-TagRFP were mixed with latex beads coated with anti-CD3/CD28 antibodies. Here we observed that Tim-1 initially appears to concentrate near the bead along with ZAP-70. However, over time, most Tim-1 moves away from the beads (Movie 2-4). Overall, the pattern of Tim-1 localization is reminiscent of the distal pole complex (74).

Some proteins require the expression of their ligands on the APC in order to localize towards the IS. For instance, CD28 only localizes to the cSMAC in the presence of APCs expressing of one of its ligands - CD80 or CD86 (99). In agreement with the importance of ligands in receptor localization, it has been shown that Tim-1 faces apoptotic cells bearing one of its ligands, i.e. phosphatidylserine (58). We tested whether the APCs used in our system contain a ligand for Tim-1. Variable results were obtained when probing for expression of known ligands for Tim-1, including Tim-1 itself and Tim-4, on the APCs that we employed, CH27 and Raji (Fig. 2B and data not shown). While Tim-4 was consistently *not* detected on CH27 cells, Tim-1 staining was more variable. We did confirm that both types of APCs used in our studies express

one or more surface ligands for Tim-1, as evidenced by binding of Tim1-Fc to the surface of the APCs (Fig. 2C). Furthermore, Tim1- Fc binding to these cells was abolished in the presence of EDTA, demonstrating that Tim1-Fc binding to this/these still-undefined ligand(s), like the known Tim-1 ligands, requires divalent ions (46, 56). This finding is not entirely surprising since unidentified Tim-1 ligands have been suggested to exist in a previous study (128). Thus, although known Tim-1 ligands (Tim-1/Tim-4) may or may not be expressed on the surface of the APCs used in our studies, one or more Tim-1 ligand(s) are present. Interestingly, this still does not result in Tim-1 localization towards the IS.

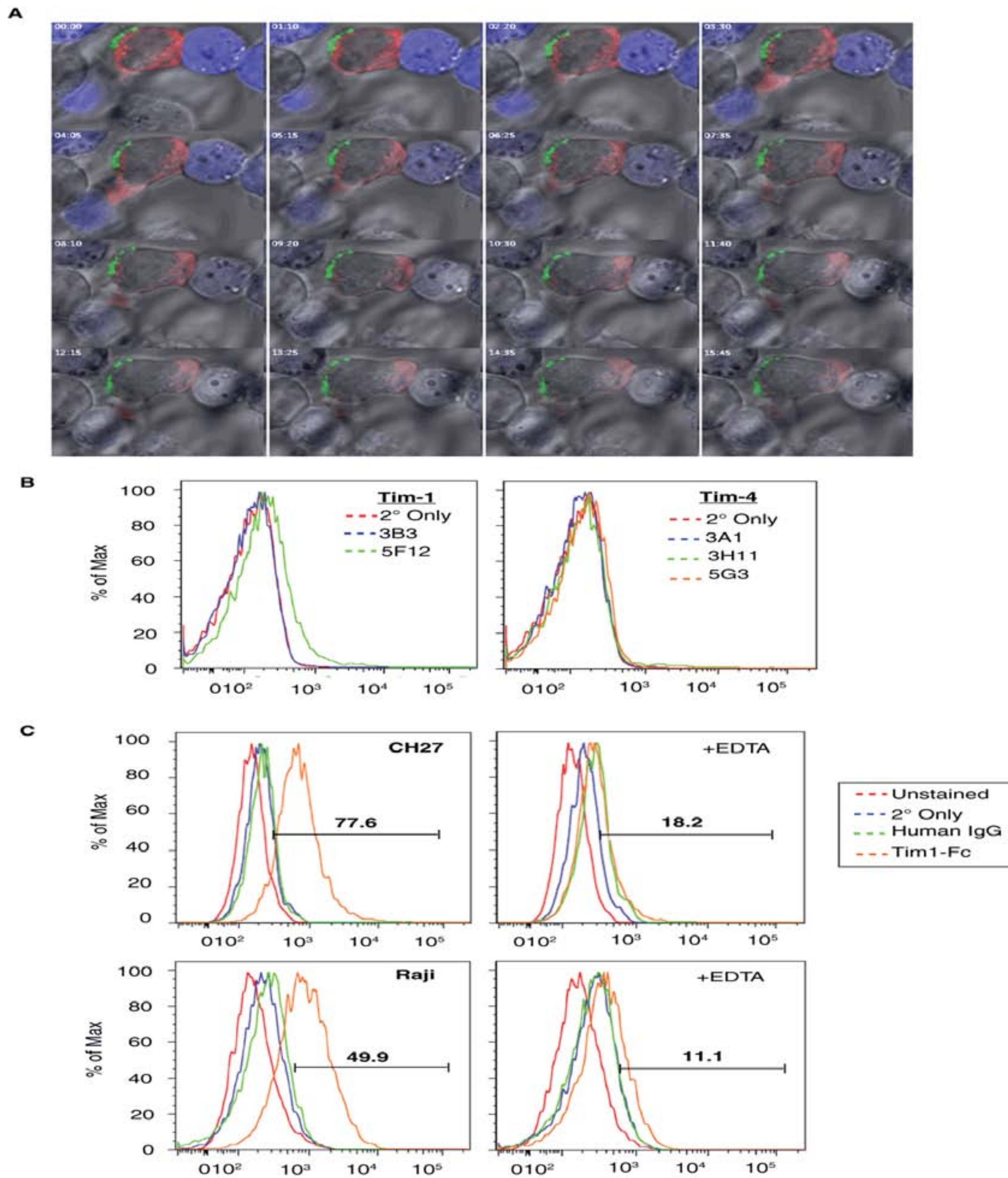


Figure 2-8: Tim-1 is excluded from the IS despite Tim-1 ligand(s) on the APC.

(A) Jurkat T cells transiently transfected with Tim-1-GFP (green) and ZAP70-Tag-RFP (red) were incubated with Raji cells pre-loaded with 1 mg/mL SEE and stained with Cell Tracker Blue (blue). Cells were incubated in a heated chamber for live cell imaging. (B) CH27 cells were stained with anti-Tim-1 (left) and anti-Tim-4 (right) antibodies and secondary antibody and analyzed by flow cytometry. (C) The presence of Tim-1 ligand(s) on CH27 (upper panels) and

Raji (lower panels) B cells was revealed by staining with Tim1-Fc and secondary antibody, in the absence (left) or presence (right) of EDTA.

2.4.7 Structural requirements for proper Tim-1 localization

Next, we determined the elements necessary for Tim-1 localization away from the IS. During conjugate formation, many proteins depend on motifs found in the cytoplasmic tail for proper localization. For instance, CD28 requires Y188 in its cytoplasmic tail for localization towards the IS (129). Likewise, CD43, which moves opposite the immunological synapse and to the distal pole complex, requires its cytoplasmic tail for this localization (76). Specifically, CD43 requires a membrane-proximal positively charged amino acid cluster (KRR) in its cytoplasmic tail for ERM binding and distal pole complex localization (78). ERM family proteins are necessary for driving certain proteins, such as CD43 and Rho-GDI, towards the DPC (76, 130). Intriguingly, we noted a similar sequence in the juxtamembrane region of the Tim-1 cytoplasmic tail – a KRK motif at residues 244-246.

To determine the intrinsic requirements for Tim-1 exclusion from the IS, we examined the effect of three constructs on Tim-1 localization. Specifically, we tested the effect of Tim-1^{Y276F}, a cytoplasmic tail truncation (Tim-$1^{del.cyto}$), and Tim-1 244-246 KRK-QGQ (Tim-1^{QGQ}) on Tim-1 localization (Fig. 2-9A). As shown previously by our group, Y276 is critical for Tim-1 co-stimulatory function (33). However, the Tim-1^{Y276F} mutant construct still appears to localize opposite the immunological synapse (Fig. 2-9B). To quantify the location and extent of spread of Tim-1 we examined two parameters. First, to determine the distance of Tim-1 in relation to the IS of T cell:APC conjugates, we measured the angle of Tim-1 from the center of the IS. Thus, if Tim-1 were concentrated directly opposite the synapse, Tim-1 would be 180° away from the IS.

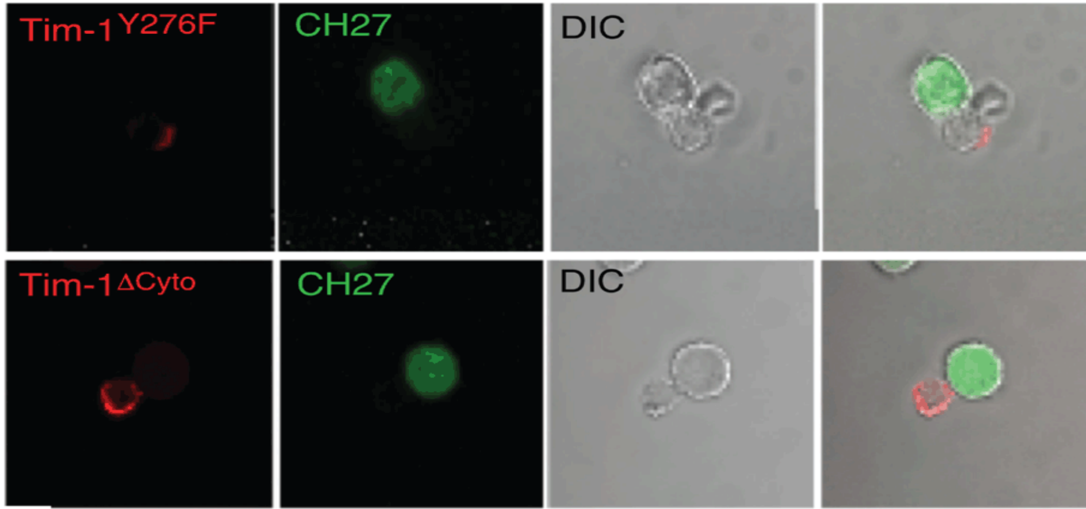
Second, we measured the extent of Tim-1 spreading on the cell surface. Wild type Tim-1 is predominantly found in the "back" half of the cell (>90 degrees away from the synapse with a median of 133.3º), opposite the immunological synapse, and is fairly tightly contained (spread of 20-180º with a median of 79.6º) (Fig 2-9B-C). Tim-1^{Y276F} localization is similar to wild type Tim-1, in that in a majority of conjugates the protein is found more than 90º (median 136.6º) from the synapse and is spread over 20-120º with a median of 58.1º (Fig. 2-9B-C). These findings suggest that the majority of Tim-1^{Y276F} is concentrated opposite the synapse. Next, a Tim-1 cytoplasmic tail truncation was utilized. In contrast to WT or Y276F forms of the protein, Tim-1 with a cytoplasmic tail truncation is more likely to be present in the front half of the cell, closer to the IS with a median distance from the IS of 106.5º (Fig. 2-9C). In about half of the conjugates analyzed, the Tim-1$^{del.cyto}$ construct was found in the front half of the cell (less than 90º from the IS), and in 28% of total conjugates Tim-1$^{del.cyto}$ even appears to cross into the IS (Fig. 2-9C).

A

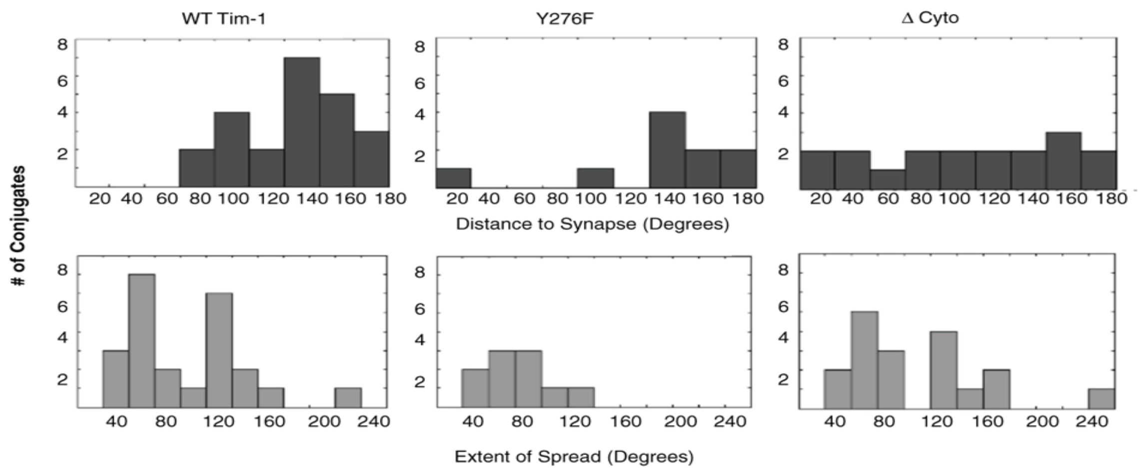
Y276

TRYIL|M<u>KR</u>KSASLSVVAFRVSKIEALQNAAVVHSRAEDNIYIVEDRP

B



C



D

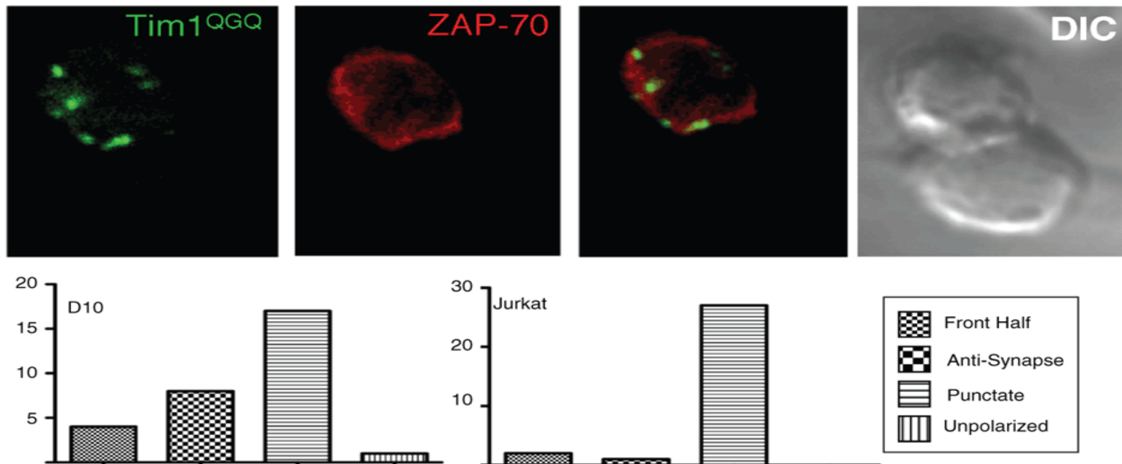


Figure 2-9: The cytoplasmic tail regulates Tim-1 localization relative to the IS.

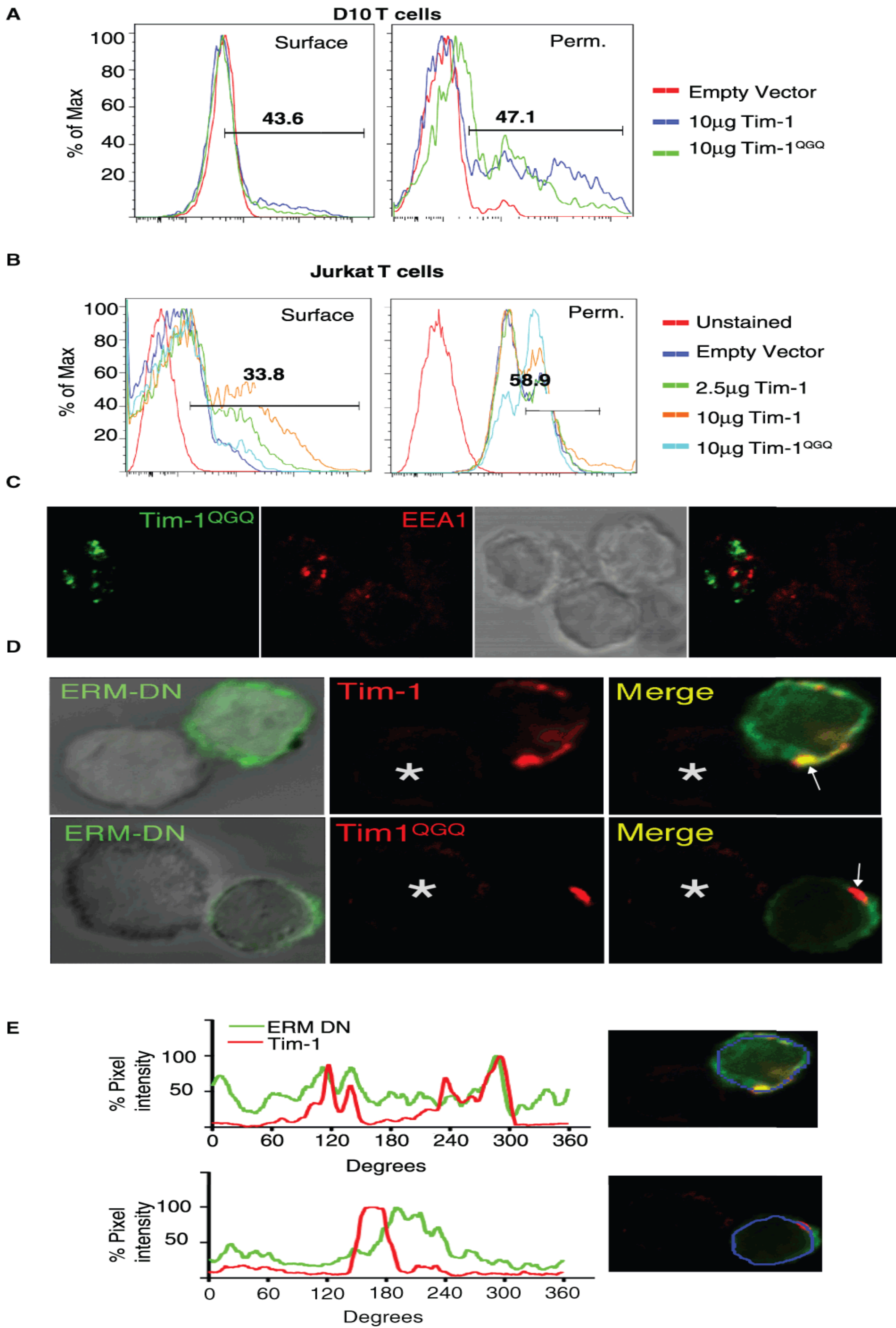
(A) Murine Tim-1 cytoplasmic tail sequence. The vertical line indicates the location of the truncation in the delta-cyto construct. KRK is the putative ERM binding domain; Y276 is underlined. (B) D10 T cells transiently transfected with either Flag-Tim1^{Y276F} (red) or Flag-Tim-1 cytoplasmic tail truncation (del.cyto; red) were mixed with conalbumin loaded CH27 cells (green). Cells were then stained with anti-Flag mAb directly conjugated to Cy3. (C) Quantitation of the angle from the IS to Tim-1 (top) and the extent of distribution of Tim-1 on the cell surface (bottom). (D - upper) Representative image of Tim-1^{QGQ}-GFP and ZAP-70 RFP expressing D10 cells interacting with antigen-loaded CH27 B cells. (D- lower) Quantification of Tim-1^{QGQ} localization in D10:CH27 and Jurkat:Raji conjugates from 12 and 13 experiments, respectively.

The greatest change in Tim-1 localization that we have observed thus far is seen when the positively charged, putative ERM-binding, motif in Tim-1 (244-246 KRK) is mutated. Rather than localizing diffusely on the surface of the T cells, Tim-1^{QGQ} has a predominantly punctate (56.7% of D10 conjugates and 90% of Jurkat conjugates) appearance, consisting of mainly intracellular Tim-1, with some of this mutant even present in the IS (Fig. 2-9 and Movie 2-4). Thus, the ability of Tim-1 to bind ERM proteins appears to be important for Tim-1 localization distal to the IS and within the DPC.

2.4.8 Tim-1 co-localizes with ERM proteins

Given the dramatic effect on Tim-1 localization, we further characterized the Tim-1^{QGQ} mutant. We observed that the Tim-1^{QGQ} construct has lower surface expression than wild type Tim-1, even when higher concentrations of Tim-1^{QGQ} plasmid are transfected. Although the total

amount of plasmid transfected is the same (10μg total), 10μg of Tim-1^{QGQ} plasmid yields less surface expression than 2.5μg of WT Tim-1 plasmid (along with 7.5μg empty vector). However, the total amount of Tim-1^{QGQ} protein appears to be equivalent to WT when cells are permeablized (Fig. 2-10 A-B). This is consistent with our imaging, wherein Tim-1^{QGQ} is not highly expressed on the cell surface but appears to distribute into intracellular pools within the cell (Fig. 2-10 D). Further, Tim-1^{QGQ} does not co-localize with early endosomal antigen 1 (EEA1), suggesting that this pool of vesicular Tim-1 is not found in early endosomes (Fig. 2-10 C). The significant amount of intracellular Tim-1^{QGQ} suggests either that Tim-1 ^{QGQ} is rapidly recycled from the cell surface or that Tim-1^{QGQ} is retained in a vesicular compartment within the cell.


A
D10 T cells
100
80
60
40
20
% of Max
Surface
43.6
Perm.
47.1
Empty Vector
10µg Tim-1
10µg Tim-1QGQ
B
Jurkat T cells
% of Max
Surface
33.8
Perm.
58.9
Unstained
Empty Vector
2.5µg Tim-1
10µg Tim-1
10µg Tim-1QGQ
C
Tim-1QGQ
EEA1
D
ERM-DN
Tim-1
Merge
ERM-DN
Tim1QGQ
Merge
E
ERM DN
Tim-1
% Pixel intensity
100
50
0
60
120
180
240
300
360
Degrees
% Pixel intensity
100
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0
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120
180
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Degrees

Figure 2-10: A putative ERM-binding motif in the cytoplasmic tail regulates Tim-1 localization.

Anti-Flag staining of EV (empty vector), Flag-Tim-1, or Flag-Tim-1^{QGQ} transfected D10 (A) or Jurkat T cells (B) as determined by flow cytometry. Surface staining of non-permeablized cells is on the left. Methanol permeabilization of T cells for intracellular Flag expression of EV (empty vector), Flag-Tim-1, or Flag-Tim-1^{QGQ} transfected T cells, as determined by flow cytometry (right). (C) Representative image of Jurkat T cells transiently transfected with Tim-1^{QGQ}-GFP (green) and co-stained for EEA1 and Alexa-555 (red) after conjugation to antigen loaded Raji cells from three experiments. (D) D10 T cells co-transfected with Flag-Tim1 or Flag-Tim1^{QGQ} (red) and FERM-GFP ("ERM-DN") constructs and conjugated to antigen-loaded CH27 cells were stained with anti-Flag-Cy3 antibody and imaged by confocal microscopy. (E) To quantify the ERM DN and Tim-1 localization, a ten pixel line scan was drawn along the surface of the cell, with the intensity of staining represented as a percentage of the maximal pixel intensity.

Since the KRK sequence in the Tim-1 cytoplasmic tail represents a putative ERM binding motif, we wanted to determine whether Tim-1 might interact with ERM proteins. Here, we used an dominant negative (DN) ERM construct, containing the N-terminal FERM domain (from ezrin) that binds proteins with ERM-binding motifs, along with a GFP moiety, but not the C-terminal actin-binding domain (76). When cells are co-transfected with both Tim-1 and the ERM-DN, there is partial Tim-1 co-localization with the FERM-GFP (Fig. 2-10 C-D). This is consistent with a role for WT Tim-1 interacting with ERM proteins in the regulation of Tim-1 localization. However, mutation of the Tim-1 KRK motif diminishes the ability of the mutant to interact with the FERM-GFP construct, as compared to WT Tim-1 (Fig. 2-10 C-D), providing further validation of a possible interaction between Tim1- and ERM family proteins.

2.4.9 Altering Tim-1 localization impacts its effects on early tyrosine phosphorylation

We next determined whether Tim-1 localization affects Tim-1 co-stimulatory activity in conjunction with TCR and CD28. Interestingly, we were surprised to find that Tim-1^{QGQ} promotes enhanced cellular tyrosine phosphorylation, as compared to wild type Tim-1 (Fig. 2-11 A). One of the tyrosine phosphorylated substrates induced in the Tim-1^{QGQ} expressing cells is a band slightly above 50 kD. Since this would be consistent with Src family kinases (SFK), we were interested in determining whether this band was a phosphorylated SFK member. Using antibodies against the activating tyrosine (Y416 in Src), we were able to detect increased phosphorylation in Tim1^{QGQ}- expressing cells within minutes of TCR/CD28 stimulation (Fig. 2-11 A). Although not all tyrosine phosphorylation results in positive signaling, the increased phosphorylation at the activating tyrosine (Y416 in Src) in T cells expressing the Tim-1^{QGQ}-expressing cells suggests that Tim-1^{QGQ} may enhance early T cell signaling. We were also able to visualize an increase in the inhibitory tyrosine of Lck, Y505 (Fig. 2-11 B). Tim-1 may increase phosphorylation of both the activating and inhibitory tyrosines of Lck. The net effect of the phosphorylation remains to be determined but could impact Tim-1 induced T cell activation.

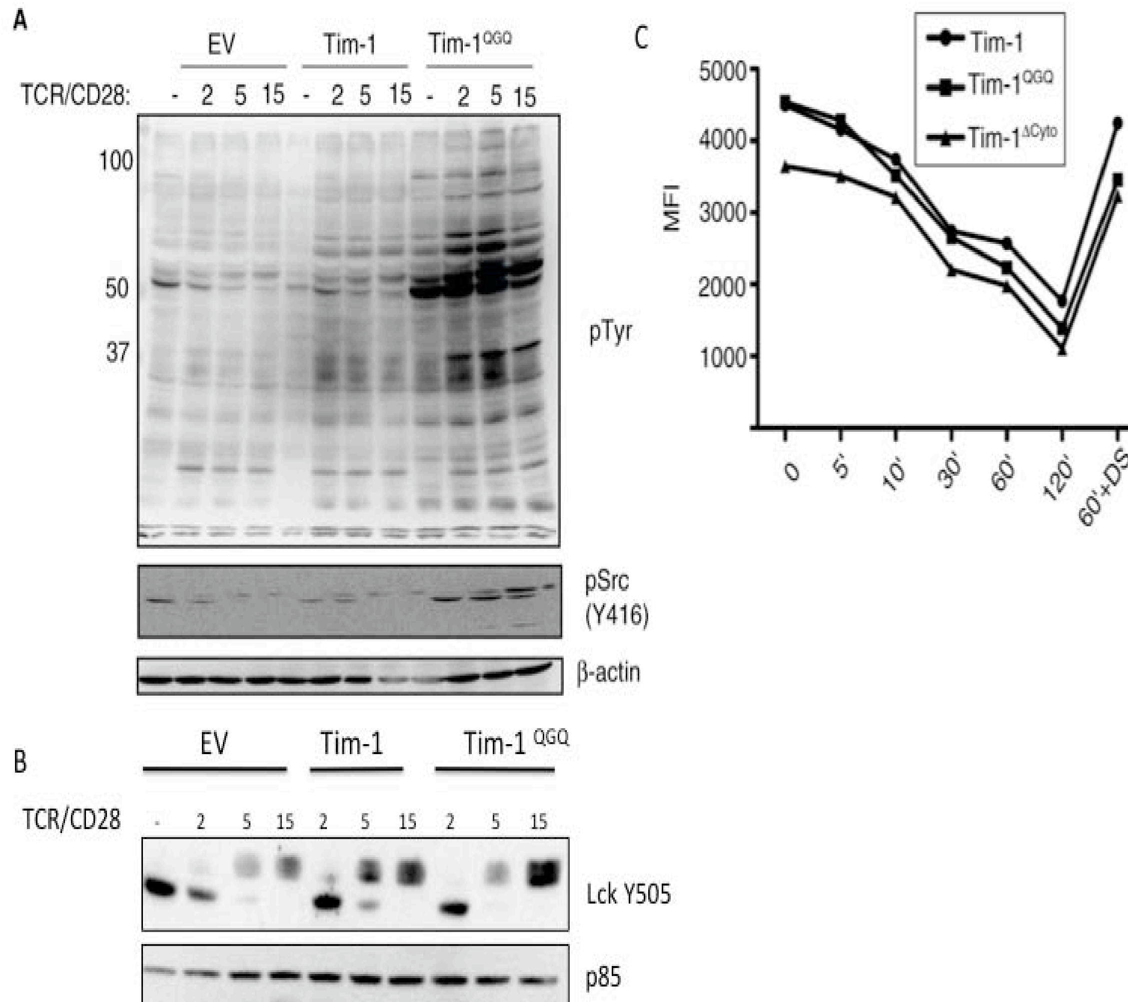


Figure 2-11: Tim-1^{QGQ} enhances early signaling events downstream of TCR/CD28 independent of the rate of TCR internalization.

(A) Jurkat T cells transfected with empty vector (EV), Flag-Tim-1, or Flag-Tim-1^{QGQ} were stimulated with anti-TCR and anti-CD28 antibodies for the indicated times. Lysates were analyzed by SDS-PAGE and western blotting for pY (4G10), pSrc (Y416; analogous to Y394 in Lck), and b-actin. (B) Jurkat T cells were stimulated with anti-CD3 mAb for the indicated times. (C) CD3 expression was measured with flow cytometry and mean fluorescence intensity (MFI) was determined in FloJo. Dynasore (DS, 80 mM) was used to prevent clathrin-mediated endocytosis after TCR/CD3 crosslinking.

Since two previous reports demonstrated an association between Tim-1 and CD3 (34, 63), another possible explanation for the enhanced tyrosine phosphorylation in Tim-1^{QGQ} expressing cells was that Tim-1^{QGQ} might increase the levels of surface TCR/CD3 and/or slow the rate of TCR/CD3 internalization. To address this possibility, we stimulated Jurkat cells expressing WT or mutant Tim-1 with anti-CD3 antibody, and measured the levels of CD3 surface expression by flow cytometry. As expected, after antibody crosslinking, CD3 surface expression decreased over time (Fig. 5B). T cells expressing WT Tim-1 or Tim-1^{QGQ} displayed equivalent rates of TCR internalization, although starting levels of TCR/CD3 varied somewhat (Fig. 2-11 B). Thus, impairment of TCR/CD3 down-regulation does not appear to be the mechanism behind the increased tyrosine phosphorylation in T cells expressing Tim-1^{QGQ} after CD3 crosslinking.

2.4.10 Tim-1^{QGQ} is impaired in co-stimulation of inducible transcription and cytokine production

Next, we examined the effects of altering Tim-1 localization on its ability to modulate inducible transcription and cytokine production. As we demonstrated previously, WT Tim-1 is able to co-stimulate the activity of an NFAT/AP-1 transcriptional reporter (33, 64). However, Tim-1^{QGQ} was not able to enhance NFAT/AP-1 activation in D10 cells (Fig. 2-12 A). Furthermore, while WT Tim-1 can enhance cytokine production, Tim-1^{QGQ} cannot (Fig. 2-12 B-D). Consistent with a role for ERM protein binding to the KRK motif in Tim-1, a dominant negative ERM construct also suppresses the ability of WT Tim-1 to enhance transcription or cytokine production (Fig. 2-12). These findings suggest that Tim-1 interaction with ERM proteins, with proper subsequent

localization of Tim-1, plays a role in Tim1-mediated transcriptional activity and cytokine production.

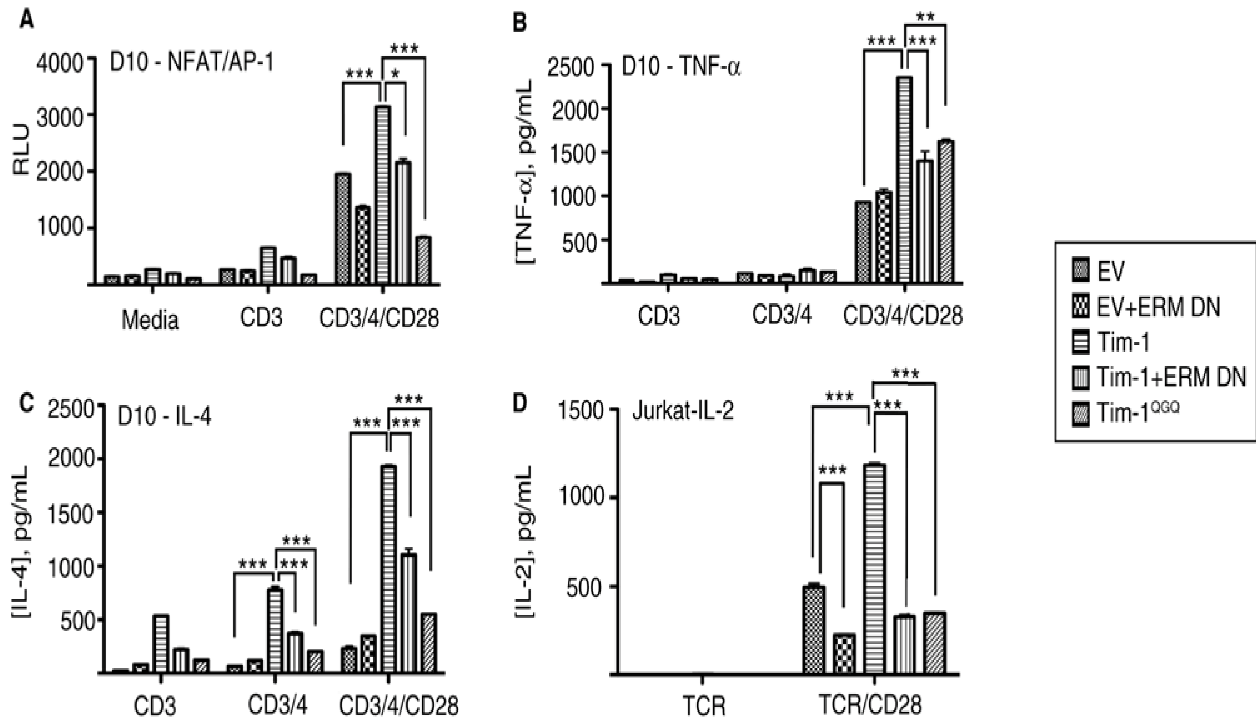


Figure 2-12: Altered Tim-1 localization impacts inducible transcription and cytokine production.

(A) D10 cells were transfected with an NFAT/AP-1 reporter, along with empty vector, WT or mutant Flag-Tim-1 in the presence or absence of ERM-DN. The next day, cells were cultured for six hours in the presence or absence of CD3/CD4/CD28 stimulation before assaying for luciferase activity. (B) D10 T cells were transfected with empty vector, WT Tim-1, ERM-DN, or Tim-1^{QGQ}. Cells were stimulated with anti-CD3 or anti-CD3/CD28 antibodies for 24 hours. Cell-free supernatants were collected and assayed for TNF-a production by ELISA. (C) D10 T cells were transfected with empty vector, Tim-1, ERMDN, or Tim-1^{QGQ}. Cells were stimulated with anti-CD3 or anti-CD3/CD28 antibodies for 24 hours and IL-4 production was determined by ELISA. (D) Jurkat T cells were transfected with empty vector, Tim-1, ERMDN, or Tim-1^{QGQ}.

Cells were stimulated with a-TCR or a-TCR/CD28 for 24 hours before IL-2 production was determined by ELISA. Data are presented as average values, +/- standard deviation, of duplicate samples from an individual experiment.

2.5 DISCUSSION

Here we have shown that, in contrast to the majority of known co-stimulatory molecules and TCR associated signaling molecules, Tim-1 does not form typical microclusters and does not localize towards the immunological synapse. Tim-1 microclusters are not localized at the periphery or stabilized by additional co-stimulation with integrins or anti-Tim-1 antibodies. Rather, surprisingly, Tim-1 is excluded from the immunological synapse in an ERM-dependent manner. Our structure/function studies suggest that Tim-1 exclusion from the immunological synapse is an active process requiring more than one step. First, the Tim-1 cytoplasmic tail appears to be necessary for exclusion from the immunological synapse, since a cytoplasmic tail truncation results in greater amounts of Tim-1 in the SMAC. Second, specific residues in the cytoplasmic tail (i.e. KRK) are required for proper Tim-1 localization towards the distal pole complex. Furthermore, concentration of Tim-1 opposite the immunological synapse towards the anti-synapse, or distal pole complex, appears to influence both early signaling and Tim-1 induced enhancement of T cell function.

Tim-1 forms tyrosine phosphorylated microclusters that are not associated with CD3 or ZAP-70. This is a bit surprising considering reports in the literature demonstrating an interaction between these proteins (34, 63). However, these punctate Tim-1 structures are consistent with microclusters since they are dependent on TCR/CD28 activation but cannot be stimulated by

integrin spreading. The formation of Tim-1 microclusters may be dependent on Y276 in the cytoplasmic tail since mutation of this residue alters and restricts Tim-1 localization. Tim-1^{Y276F} localizes differently depending on antibody stimulation. Under co-stimulation by LFA, Tim-1^{Y276F} microclusters localize around the edge of the cell whereas co-stimulation by anti-Tim-1 results in Tim-1^{Y276F} microclusters that appear to be mostly at the center of the cell. One possible explanation for this is that under conditions of co-stimulation by anti-CD3/CD28 and LFA, the LFA-1 slows actin arrangement and traps some microclusters at the periphery. In contrast, in the presence of anti-Tim-1 co-stimulation, the Tim-1^{Y276F} microclusters are at the center of the cell, reminiscent of the cSMAC. The Tim-1^{Y276F} microclusters may be held centrally in order to be internalized and degraded since the Tim-1 ^{Y276F} does not signal. More structure/function mutants will have to be created in order to better study the minimal domains necessary for signaling.

Another interesting finding is the movement of Tim-1 and ZAP-70 with live cell imaging. Tim-1 does not have the same pattern of localization as other well studied signaling proteins. Instead of being located along the actin cytoskeleton like ZAP-70 or SLP-76, Tim-1 appears to have microclusters of different sizes, a central pool of microclusters, and a subset of smaller microclusters darting in and out of the field, which is very reminiscent of endocytosis. This may align with recent reports that there is intracellular and endocytic signaling. While some LAT exists in microclusters at the IS, there is also a pool of vesicular LAT that merges with SLP-76 microclusters to enhance signaling. The flitting Tim-1 microclusters could be working in a similar manner to feed into SLP-76 microclusters and enhance early signaling. Alternatively, Tim-1 itself could also be influencing intracellular signaling. Previously most if not all signaling was thought to occur at the cell surface and internalization was considered to be for the

termination of signaling. However, signaling can be endocytic, and this endocytic signaling may be generating different pathways than surface interactions. For instance, the BCR signals both at the cell surface and in intracellular vesicles. Prevention of BCR internalization by dynasore creates a state of hyperphosphorylation of MAP Kinases and hypophosphorylation of Akt with subsequent disruptions in transcriptional activity (131). Tim-1 may be working in a similar manner where surface signaling induces one set of signals while vesicular Tim-1 signaling activates a different signaling pathway. This also fits with the Tim-1 KRK-QGQ mutation where this vesicular form of Tim-1 has greater tyrosine phosphorylation than wild type Tim-1. It will be interesting to see whether blocking the internalization of Tim-1 with dynasore influences T cell activation.

The majority of the data with stimulation by anti-TCR and/or CD28 suggests that ZAP-70 microclusters become immobilized and form along the actin cytoskeleton. Surprisingly, the addition of anti-Tim-1 to CD3 and CD28 stimulation causes the ZAP-70 microclusters to lose its distinct pattern of localization. One reason for this could be that the concentration of anti-Tim-1 antibody used to coat the slides was enough to interfere with the ability of ZAP-70 to interact with the anti-TCR. This could be tested by either decreasing the concentration of Tim-1 antibody used or by coating with equivalent amounts of an isotype control. Alternatively, stimulation with Tim-1 could be causing the cytoskeleton to move and influencing the movement of ZAP-70. Stimulation with the high affinity Tim-1 antibody, 3B3, triggers cytoskeletal rearrangement whereas activation with the lower affinity antibody, RMT1-10, does not mobilize actin (34). Thus, determining the effect of the lower affinity anti-Tim-1 antibody on ZAP-70 localization may also offer insights into how Tim-1 affects ZAP-70 and the ensuing signaling.

We find that Tim-1 is found mostly on the cell surface of T cells in the steady state. This is in contrast to previously published reports suggesting that Tim-1 is maintained in a mostly intracellular store and only becomes localized to the cell surface upon activation (45). These discrepancies could be due to differences in cell type. Thus, the previously published report used HEK 293 cells and 300.19 pre-B cells. Also, since Tim-1 is a transmembrane protein, it is also possible that WT Tim-1 might reside in an intracellular compartment before being inducibly cycled to the surface, similar to CLTA-4. On T cells, Tim-1 localizes towards the interface with PS-expressing apoptotic thymocytes, a finding we were also able to confirm (data not shown) (58). However, in our studies Tim-1 does not localize towards the interface with APCs bearing antigenic peptide and an unidentified Tim-1 ligand. This suggests that different Tim-1 ligands may have distinct effects on localization. Further examination of known Tim-1 ligands, such as Tim-4 and HAV, may help to clarify this issue. In addition, it will also be of interest to determine the identity of the as-yet-unknown ligand(s) expressed on the B cell lines that we have used as APC's in our studies.

Regarding the relationship of Tim-1 to TCR/CD3, there is some discrepancy between our findings and the recent literature. Thus, it has been suggested that hTIM-1 co-localizes with CD3 and ZAP-70 and that CD3 can be co-capped with mTim-1 (34, 63). These findings suggest that Tim-1 should be found at the IS with CD3 and ZAP-70. However, none of the previous studies investigated the kinetics of Tim-1 localization or the localization on T cells in conjugates with antigen-bearing APCs. We have shown that Tim-1 may at least partially co-localize with ZAP-70 in the presence of TCR/CD28 coated beads (and absence of any ligand for Tim-1). However, at later time points Tim-1 relocates away from the antibody coated beads. In addition, we have obtained preliminary data indicating that Tim-1 and ZAP-70 microclusters may co-localize (data

not shown). This suggests that Tim-1 and ZAP-70 might interact at some early time point during T cell activation but that the interaction may not persist.

The question then arises of the functional importance of Tim-1 exclusion from the IS, possibly at the distal pole complex in T cells, and how it might relate to Tim-1 enhancement of NFAT/AP-1 activation and cytokine production. If Tim-1 is truly a co-stimulatory molecule, then why would it be excluded from the IS? While the predominant view in the field is that the region opposite the IS, or distal pole complex, serves as a reservoir for molecules that inhibit signaling, there is evidence that the DPC may also serve as an area for active signaling. Multiple reports in the literature have shown that certain active signaling molecules, including PIP_3, ZAP-70, STIM1/Orai, and CD46, reside at least in part in the DPC (91-93, 132). Thus, Tim-1 may localize in the DPC in order to avoid being internalized and degraded at the immunological synapse. This may allow for extended time to interact with other signaling molecules, and in this way enhance signaling. Alternatively, Tim-1 may also enhance signaling by binding inhibitory molecules and moving them towards the DPC and away from the positively acting signaling molecules found at the immune synapse. This would be in agreement with one of the Tim-1 knockout studies suggesting that the Tim-1 deficient mice develop worse lung inflammation in a model of airway hyper-reactivity, although another knockout study did not demonstrate this (105, 106).

Also intriguing is the paradoxical difference between early signaling events in cells expressing WT Tim-1 or Tim-1^{QGQ}. Surprisingly, Tim-1^{QGQ}-expressing cells displayed enhanced tyrosine phosphorylation at early time points downstream of TCR and CD28 stimulation, compared with the effects of WT Tim-1. This may represent phosphorylation of inhibitory molecules and/or increased tyrosine phosphorylation of positive signaling molecules. Also, the

punctate appearance of Tim-1^{QGQ} could result from localization in endosomal compartments. Recent studies have highlighted the importance of endosomal vesicles carrying signaling molecules (e.g. LAT) into microclusters, in order to enhance the very earliest signaling events at the microclusters (102). Thus, vesicular Tim-1^{QGQ} localization could enhance early signaling before being rapidly transmitted to the SMAC for degradation. In this way, Tim-1^{QGQ} may enhance very early signaling and be degraded before having an opportunity to enhance later events, such as cytokine production and transcriptional activity. Alternatively, the Tim-1^{QGQ} mutant may be rapidly internalized, which would explain the reduced levels of surface expression. Proximal to the Tim-1 KRK motif is a YILM motif that is very similar to the CTLA-4 clathrin adaptor-binding motif (YVKM) (133). It is therefore possible that the KRK-QGQ mutation (and subsequent reduced ERM protein binding) exposes this YILM motif and causes increased internalization. This would also be consistent with the fact that the Tim-1$^{del.cyto}$ construct, in which part of this motif is truncated (before the M), is not found in an intracellular, vesicular, compartment. WT Tim-1 may also briefly cycle through these endosomal compartments before being expressed more stably at the cell surface. Since a recent report has suggested that internalized/endosomal TCR can signal, it is possible that the increased early tyrosine phosphorylation in cells expressing Tim-1^{QGQ} arises from this internal compartment (103).

Relevant for this discussion, recent reports have also implicated signaling from endosomes as contributing to signaling (102, 103, 134). Tim-1^{QGQ} displays a predominantly punctate pattern, which is consistent with possible endosomal localization. Thus, another intriguing possibility is that during early signaling events, Tim-1^{QGQ} in endosomes can enhance early signaling events downstream of the TCR.

The movement of proteins during T cell interaction with antigen presenting cells impacts T cell function. Here we have demonstrated that Tim-1 on T cells preferentially localizes opposite the immunological synapse when conjugated to antigen-bearing APCs. Our studies have begun to unravel the motifs and complexities involved with regulating Tim-1 localization. These findings may provide insight into the mechanism underlying the effects of Tim-1 on the immune response.

Reference: Much on the immunological synapse work can be found published in F1000 Reports: Jean Lin, *et al* (2012) Murine Tim-1 is excluded from the immunological synapse. [v1; ref status: Indexed, http://f1000r.es/OaEfdg] *F1000 Research*, **1**:10 (doi: 10.3410/f1000research.1-10.v1)

3.0 TIM-1 SIGNALING

3.1 ABSTRACT

T cell immunoglobulin and mucin 1 (Tim-1) is upregulated on all activated T cells and delivers a co-stimulatory signal to influence intracellular signaling and ultimately T cell fate. While a number of studies have examined the effect of Tim-1 ligation by antibodies or ligands in animal models of disease, the precise mechanism(s) by which Tim-1 influences T cell activation remains unknown. We and others have demonstrated that Tim-1 antibody cross-linking leads to interaction with important signaling molecules, including CD3 and p85, as well as induction of NFAT/AP-1 transcription, which is dependent on Tim-1 tyrosine 276 phosphorylation. We hypothesize that Tim-1 influences T cell activation by selectively recruiting different proteins and directing a unique signaling network after tyrosine phosphorylation. Preliminary data suggests that BALB/c Tim-1 has lower levels of NFAT/AP-1 and Elk-1 activation as compared to C57Bl/6 levels. However, while interfering with homotypic interactions does influence NFAT/AP-1 activation, Elk-1 activation may be decreased in the Tim-1^{H64E}-expressing cells.

Tim-1 appears to have previously unrecognized phosphorylated substrates and interacting proteins. We have shown that Tim-1 may bind BAT2, translation factors, ACC1, and PLCγ1. We demonstrate that although Tim-1 binds PLCγ1 in a phospho-tyrosine dependent manner, Tim-1-expressing T cells bypass PLCγ1 to induce NFAT/AP-1 activity in a calcium-dependent manner. Chemical inhibition of PLCγ decreased transcriptional activation, inhibited

upregulation of early markers of activation, and reduced cytokine production in D10 T cells and primary T cells. This work reveals that Tim-1 has the ability to bind and activate previously unknown pathways.

3.2 INTRODUCTION

Transmembrane immunologlobulin and mucin-1 (Tim-1) is expressed by multiple cells of the immune system and has the ability to modulate immune function. Tim-1 is the first member of a family of Tim genes, which consists of eight putative members in mice with three human orthologs (1). Tim-1 initially garnered interest as a putative asthma susceptibility gene and multiple studies have demonstrated a role for Tim-1 in the AHR response (2, 32, 35-37, 40). However, the role of Tim-1 has expanded to include modulation of EAE, influenza, rheumatoid arthritis, atopic disease, and allograft tolerance (25, 34, 54, 135, 136). Most of these studies attribute the ability of Tim-1 to mediate disease and immune function to its effects on T cells, specifically by influencing T cell activation and cytokine production.

Ligation of Tim-1 either by anti-Tim-1 mAb or by its ligands activates T cells, enhances T cell proliferation, and may skew T cell differentiation. Tim-4 stimulation of T cells, either by Tim-4 Ig or Tim-4 coated beads, is also able to boost T cell proliferation by increasing T cell division, phosphorylating Akt, and by upregulating the anti-apoptotic factor Bcl-2 (12, 13). However, treatment with very low doses of Tim-4 can lead to inhibition of T cell proliferation (12). Treatment with a high affinity anti-Tim-1 antibody, 3B3, results in hyperproliferation of T cells *in vitro* as well as the production of specific cytokines *in vivo and ex-vivo.* For instance, in an airway hyperreactivity model, treatment with 3B3 enhances IL-4 production *in vitro* but IL-4, IL-10, and IFN-γ *ex vivo* (2). In a cardiac transplant model treatment with the same antibody resulted in enhanced IFN-γ and IL-17 production, but decreased IL-4 and "deprogramming" of regulatory T cells (41). Further, in another transplant model of islet cells, use of a lower affinity

anti-Tim-1 antibody, RMT1-10, led to increased IL-4 production and enhanced Treg function (42). These paradoxical effects by both Tim-1 ligands and antibodies demonstrate the need for a better understanding of the mechanistic basis underlying Tim-1 function. Better understanding of protein-protein interactions after Tim-1 ligation may lead to important information on Tim-1 mediated signaling, and greater knowledge of the biology underlying Tim-1 function and immune modulation.

The extracellular domain of Tim-1 contains an IgV domain, a heavily glycosylated mucin domain, and a stalk domain. This portion of Tim-1 is where ligand binding occurs and polymorphisms in the mucin domain have been associated with altered susceptibility to asthma. Characterization of the TIM family structure has led to new avenues for investigation of downstream biochemical pathways. Specifically, Santiago *et al.* revealed that neighboring cells can have homophilic TIM-1:TIM-1 binding from intermolecular surface reactions between the cell-surface receptors on opposite cell surfaces.[6] This could be of potential biologic relevance because the homophilic binding is conserved in humans. Further, it was found that His67 and Glu67 of the TIM-1 DE loop were important for TIM-1:TIM-1 and TIM-1:TIM4 binding. A mutation of the histidine at position 64 to glutamic acid led to significant reductions in TIM-1:TIM-1 binding as well as decreased levels of TIM-1:TIM-4 binding. [6] Based upon these observations and previous work from the laboratory, we investigated whether structural mutations in the TIM-1 DE loop, particularly from histidine at position 64 to glutamic acid (H64E), will result in functional alterations in signaling pathways through NFAT/AP-1 and Elk-1. Furthermore, polymorphisms in the mucin domain of mice and humans has been shown to influence susceptibility to asthma. Thus, we examined the effect of Tim-1 with differences in the mucin domain on transcriptional activity.

Despite the plethora of knowledge on Tim-1's effects on T cell function, the mechanisms by which Tim-1 is able to modulate T cell function is limited. In particular, the intracellular signaling pathways responsible for mediating signaling downstream of Tim-1 activation are relatively unknown. One study with hTIM-1 demonstrates that TIM-1 associates with CD3 and PI3K and induces phosphorylation of ZAP-70 and Itk (63). Work from our lab has shown that overexpression of Tim-1 is capable of enhancing NFAT/AP-1 activity and IL-4 production downstream of TCR stimulation (33). Indeed, Tim-1 appears to function in a co-stimulatory manner since activation of NFAT/AP-1 is further enhanced in the presence of both TCR and CD28 (33). Structure/function studies have demonstrated that the cytoplasmic tail of Tim-1 is necessary for signaling, and specifically that Y276 contained therein is required for NFAT/AP-1 activation(33). We have demonstrated that the cytoplasmic tail of Tim-1 is phosphorylated in an Lck-dependent manner and that the p85 subunit of PI3K binds the Tim-1 cytoplasmic tail (64). Tim-1 signals through PI3K in order to enhance early markers of activation, including CD25 and CD69(64). Supporting a role for PI3K in Tim-1 function, a study using human Tim-1 demonstrates an association between Tim-1 and PI3K (63). Tim-4 Ig treatment also resulted in increased phosphorylation of Akt as well as of Erk1/2 and LAT (13). However, not all Tim-1 ligands have the same effect on T cell activation. In contrast, Hepatitis A Virus (HAV) binding of TIM-1actually inhibits Akt phosphorylation and blocks Treg function (121). Discerning the different effects on T cell signaling induced by the various anti-Tim-1 antibodies and ligands might enhance our understanding of Tim-1 function and lead to better avenues to pursue research and treatment options.

Proximal T cell signaling is enhanced by the formation of a "signalosome," or complex of molecules recruited to the plasma membrane. Previous work has described the importance of

the conserved Y276 residue in the Tim-1 cytoplasmic tail in signal transduction downstream of TCR/CD28 co-stimulation(33). PI3K and CD3 have been shown to be associated with Tim-1 in both murine and human systems (34, 63, 64). However, more of the specific signaling proteins interacting with the Tim-1 cytoplasmic tail forming the Tim-1 signalosome upon T cell activation remain to be unidentified. Thus, we sought to identify and validate proteins interacting with the Tim-1 cytoplasmic tail in order to determine their impact on Tim-1 mediated signaling.

In this chapter we examined signaling downstream of Tim-1 after its ligation by antibodies or ligands. We also identified molecules interacting with the Tim-1 cytoplasmic tail that might be involved in mediating signaling downstream of TCR and CD28 stimulation. We determined that different anti-Tim-1 antibodies can induce different patterns of phosphorylation and that incubation of Tim-1-expressing cells in the presence of co-stimulation by CD3 and CD28 with a Tim-1 ligand, PS, may enhance phosphorylation of Akt and ERK1/2. We identified several potential binding partners, including translation factors and PLCγ1. Surprisingly, we determined that ectopic Tim-1 expression in Jurkat and D10 T cells enhances NFAT/AP-1 and Elk-1 transcriptional activation in a TCR/CD28-dependent but PLCγ1-independent manner. This effect requires Ca^{2+} but does not require Akt and Vav1. Interestingly, Jurkat T cells express PLCγ2, which may help compensate for loss of PLCγ1. Consistent with this, blocking total PLCγ activity with pharmacological inhibitors inhibited transcriptional activation, blocked markers of early activation, and decreased cytokine production in D10 and primary T cells. This study demonstrates the complexity underlying Tim-1 signaling and suggests new avenues to explore non-traditional mechanisms/molecules for inducing T cell activation.

3.3 MATERIAL AND METHODS

Cell lines and reagents

Jurkat, J.Vav, and D10 T cell lines were used and cultured as previously described (123). Jγ and JγWT cells were purchased from ATCC and maintained in RMPI supplemented with 5% BGS and 1% penicillin and streptomycin.

The following antibodies and reagents were used: anti-Tim-1 antibodies-3B3 (R. Dekruyff), RMT1-10 (e-bioscience), 5G5, 5F12, 4H10, 4F12 (V. Kuchroo), anti-phosphotyrosine 4G10 and PY20 (Millipore), Horseradish peroxidase (HRP)-conjugated anti-mouse and protein A (GE Healthcare), M2 anti-flag (Sigma Aldrich), pAkt (Biosource), pERK1/2 t202/Y204 (BD biosciences), pp38 (BD biosciences), EIF4G (Cell Signaling Technologies), RasGap (Millipore), Fyn (Millipore), PLCγ1 (Millipore), phospho-PLCγ1 Y783 (BD biosciences), pSrc Y416 (Cell Signaling Technologies), anti-human CD3 (Becton Dickinson), mouse CD3 and CD28 (BD Pharmingen), human CD28 (Life Technologies), Vav1 (Millipore), PLCγ2 (Santa Cruz), CD25-Fitc (ebiosciences), CD69-APC (ebiosciences), anti-TIM-1 (Telos) directly conjugated to a Cy2 donkey anti-rat secondary

Reagents: PMA, ionomycin, sodium orthovanadate, aprotinin, leupeptin, pepstatin, 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF), and U73122 were all from (Calbiochem/EMD Biosciences), neomycin (Sigma), , Tim-1 Fc (eBiosciences), anti-TCR antibody C305 (Harlan), PLCγ1 smartpool siRNA (Dharmacon), Vav1 shRNA (Steve Bunnell), indo-1 am (Life

Technologies), Akti and Ly294002 (EMD Biosciences), IL-2, IL-4, TNFα ELISA (BD Optia), luciferin (Pierce), 4G10 beads (Millipore)

DNA constructs.

Histdine-64 of TIM-1, Flag-TIM-1 (B6), and Flag-TIM-1 (Balb/c) was mutated to a glutamic acid with the QuikChange site-directed mutagenesis kit (Stratagene). All DNA constructs were verified by automated DNA sequencing.

Western Blot

$20x10^6$ D10 or Jurkat T cells were transfected with empty vector or Tim-1. Cells were rested for 16-18 hours before $1.5x10^6$ cells were treated with anti-Tim-1 antibodies in the presence or absence of CD3/TCR and CD28 co-stimulation. Cells were then lysed using 1% NP-40 lysis buffer in addition to protease and phosphatase inhibitors: beta-glycerophosphate, sodium fluoride, sodium orthovanadate, AEBSF, aprotinin, leupeptin, pepstatin (Calbiochem/EMD Biochemicals). Lysates were run on a 10% SDS-PAGE gel before being transferred to PVDF membrane, blocked in 4% BSA in TBS-Tween for one hour, blotted with primary antibody overnight, washed three times, and incubated with secondary antibody for one hour. Super-Signal Pico ECL (Pierce) was used to develop the blots and images captured by a Kodak Image Station 4000MM or by film.

Peptide pull down

$10 x10^6$ Jurkat cells were lysed with NP-40 lysis buffer with protease and phosphatase inhibitors, including sodium orthovanadate, aprotinin, leupeptin, pepstatin, 4-(2-aminoethyl)benzene

sulfonyl fluoride, NF, and BGP. These lysates were then incubated with a biotinylated 13aa peptide (RAEDNIYIVEDRP) of the Tim-1 cytoplasmic tail or a biotinylated control peptide with or without five minutes of pervanadate stimulation. The biotinylated 13 aa Tim-1 peptide was generated as described (64). Interacting proteins were immunoprecipitated by tumbling with streptavidin beads for 4 hours and run on an SDS-PAGE gel. Bands pulled down by the Tim-1 peptide but not the control peptide were excised and the proteins were indentified by mass spectrometry by ProtTech.

IP (4G10 or Flag) and Silver Staining

10-20x10^6 empty vector or Tim-1 transfected D10 cells stimulated by CD3/4/28 (TCR) or anti-Tim-1 antibodies. Cells were then lysed as described above, and lysates were precleared with 20ul of Protein G beads for one hour before being immunoprecipitated for four hours with 20ul of directly conjugated 4G10 beads or for two hours with 15ul of M2 beads. Lysates were gently washed three times, 2X sample buffer (biorad) with β-mercaptoethanol was added, proteins were released from the beads by boiling for 5 minutes in a sand bath, and lysates run on SDS-PAGE gels and blotted for protein.

Apoptotic Thymocyte induction

Thymocytes were harvested from the thymi of C56Bl/6 mice. Cells were mashed through a 0.4μm strainer and spun down. Red blood cells lysis (Sigma) was used to eliminate the red blood cells. “Viable” thymocytes were placed on ice for four hours, and “apoptotic” thymocytes were incubated with dexamethasone for 4 hours at 37ºC. PS expression was verified with

Annexin V staining by flow cytometry on an LSR II. “Viable” cells had less than 5% annexin v staining whereas “apoptotic” thymocytes were over 90% positive for annexin v.

Empty vector or Tim-1 with or without NFAT/AP-1 luciferase reporter transfected D10 or Jurkat T cells were rested for 16-18 hours before lympholyting to remove dead cells. Then, different ratios of T cells and thymocytes were gently spun together for 5 minutes to allow for conjugation. The cells were then used in assays for western blotting, phospho-flow, or NFAT/AP-1 luciferase assays.

Luciferase assays

Luciferase assays were conducted as described previously (33). In certain cases, cells were pre-treated with 1mM neomycin or 0.2-2μM U73122 before stimulation with anti-CD3/TCR and anti-CD28.

Early Markers of Activation

$CD4^+$ T cells were isolated by negative selection ($CD4^+$ mouse T cell isolation kit Miltenyi) from spleen and lymph nodes of 8-12 week old female C57Bl/6 mice. CD4 purity was determined by flow cytometry and was greater than 95%. Cells were pre-treated with anti-Tim-1 antibodies and/or U73122 before they were crosslinked with varying concentrations of plate bound CD3 and CD28 as described previously (64). 16 hours after stimulation, cells were stained for CD25 and CD69 expression.

Calcium flux

Jγ or JγWT cells were labelled with Indo-1 (Life Science Technologies) according to manufacturer instructions. Briefly, 5-10x10^6 cells were respuspened in 1mL of media with in 2.5μM of Indo-1 and allowed to incubate at 37ºC for 45 minutes. Cells were then washed two times with excess media and samples were resuspended at 1x10^6 cells/mL. To co-stain with Flag, cells were stained with M2 Ab (Sigma) on ice for 20 minutes and then stained with secondary for 20 minutes. Samples were read on a LSR II and data analysis completed with FloJo.

PLCγ1 and Vav1 Knockdown in D10 cells

PLCγ1 smartpool siRNA was purchased from Dharmacon. D10 cells were transfected with 5μg Tim-1, 5μg PLCg1 siRNA, and 15μg of NFAT/AP-1 reporter. Cells were allowed to rest for 24 hours. Knockdown of PLCγ1 was determined by Western Blot. Varying concentrations of Vav1 shRNA were transfected with an NFAT/AP-1 luciferase reporter into Jγ and JγWT T cells. Knockdown was verified by Western Blot. Luciferase assay was performed as described previously.

ELISA

Primary $CD4^+$ T cells or Tim-1 transfected D10 cells were stimulated with varying concentrations of CD3 and CD28 in the presence or absence of anti-Tim-1 antibodies with or without U73122. Supernatants were collected at 24 and 48 hours.

3.4 RESULTS

3.4.1 Tim-1 cross-linking results in inducible tyrosine phosphorylation of downstream targets.

Multiple reports have demonstrated that cross-linking Tim-1 by various anti-Tim-1 antibodies can result in skewing of T helper cell subsets and subsequent differential cytokine production (34, 36, 41, 42). However, the specific proteins mediating these effects have not been elucidated. To identify proteins induced specifically by Tim-1 in a non-biased manner, various anti-Tim-1 antibodies were used to cross-link Tim-1 on Tim-1 transfected D10 T cells in the presence or absence of anti-CD3, anti-CD4, and anti-CD28 co-stimulation, followed by western blotting for tyrosine phosphorylation (pY). 3B3 is the best studied anti-Tim-1 antibody, with high affinity binding to Tim-1, and is often considered to be "agonistic" (34). 4F12, 4H10, 5F12, and 5G5 are all monoclonal anti-Tim-1 antibodies that are less well characterized. 5F12 is believed to act in a similar manner to 3B3, i.e. serve in an "agonistic" fashion (personal communication L. P. Kane and V. Kuchroo).

The pattern of Tim-1 co-stimulation-induced tyrosine phosphorylation varies among the different antibodies. For instance, blotting with PY20, an antibody specific for tyrosine phosphorylated proteins, revealed three bands selectively induced by anti-Tim-1 mAb 3B, a doublet between 204-250 kD and a single band around 75kD not found with TCR stimulation alone (Fig. 3-1a). The higher molecular weight double bands are further enhanced with Tim-1 and TCR co-stimulation. In addition, other tyrosine phosphorylated proteins (bands between 75 and 100kD) are also enhanced upon 3B3 plus TCR stimulation as compared to TCR stimulation

alone (Fig. 3-1a). In general, anti-Tim-1 3B3 stimulation in the presence of TCR co-stimulation increases overall tyrosine phosphorylation.

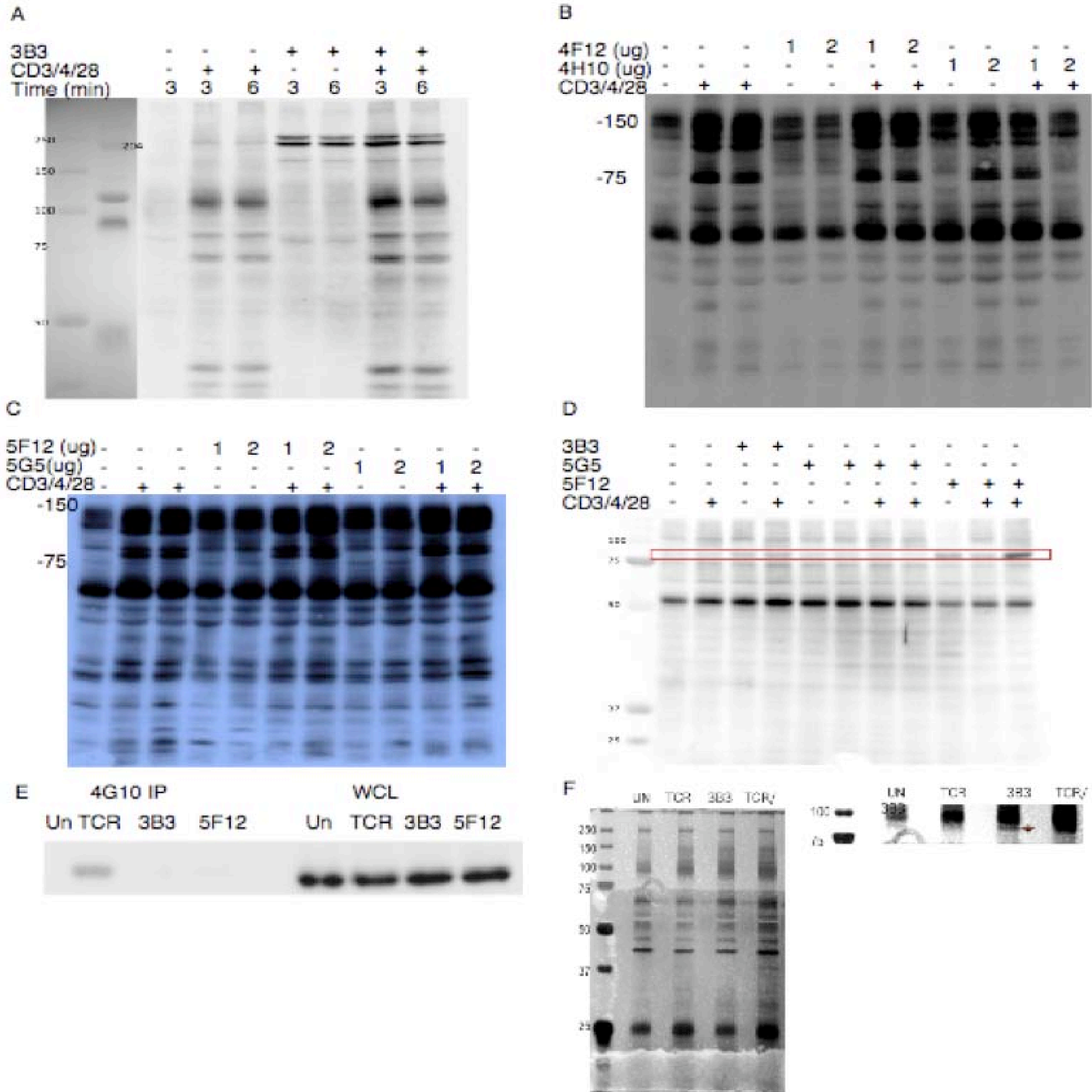


Figure 3-1: Differential tyrosine phosphorylation downstream of Tim-1 antibody cross-linking

One million D10 cells cells were transiently transfected with Tim-1 and treated with anti-Tim-1 antibodies 3B3 (A), 4F12 or 4H10 (B), or 5F12 or 5G5 (C) in the presence or absence of CD3/CD4/CD28 co-stimulation. Tyrosine phosphorylation was visualized by anti-P-Tyr mAb PY20. (D) One million activated T cells from C57Bl/6 spleens and lymph nodes were stimulated for 7 minutes with anti-Tim-1 antibodies at different concentrations as indicated (mg/mL) in the presence or absence of CD3/CD4/CD28 co-stimulation before blotting with anti-P-Tyr mAb 4G10. (E) Whole cell lysates from Tim-1 transfected D10 cells stimulated by CD3/4/28 (TCR) or anti-Tim-1 antibodies were immunoprecipitated with 4G10 beads. Lysates were run on SDS-PAGE gels and blotted for SLP-76. (F) Whole cell lysates from Tim-1 and CD3/CD4/CD28 stimualted D10 cells were immunoprecipitated with 4G10 beads to purify tyrosine phosphorylated proteins. The lystaes were run on SDS-PAGE gel and proteins were visualized by silver staining. The selectively induced band (star) was excised and sent to ProTech for masss spectrometry.

Reports from the literature indicate that anti-Tim-1 antibodies can impact T cell activation in different ways. The best characterized cases are differences in the type of cytokines (Th1, Th2, or Th17) expressed from murine T cells isolated from mouse models of airway hyperreactivity, EAE, or transplantation that were treated with anti-Tim-1 antibodies of varying binding affinities and epitopes (34, 36, 42, 44, 137). Thus, it is not surprising that the pattern of pY induced downstream varies with the specific anti-Tim-1 antibody used (Fig. 3-1a-d). For instance, 4H10 induces bands that 4F12 does not (Fig. 3-1b). Similarly, the pY pattern induced by 5F12 cross-linking is similar but not identical to treatment with 5G5 (Fig. 3-1c). In further

support roles for Tim-1 in differential tyrosine phosphorylation, activated primary $CD4^+$ T cells were stimulated with Tim-1 specific antibodies and blotted with 4G10, another antibody specific for tyrosine phosphorylated proteins. Of particular interest was the observation that "agonistic" Tim-1 antibodies, 3B3 and 5F12, selectively induced a band between 75-100kD, while another antibody, 5G5, which binds to the mucin domain of Tim-1, attenuates the signal (Fig 3-1d). This suggests that characteristics of the anti-Tim-1 antibodies, such as binding affinity or specific epitope on Tim-1, may lead to differential downstream signaling, which may in turn impact T cell activation.

We were particularly interested in identifying a particular band selectively induced by some, but not all, anti-Tim-1 antibodies. Based on the molecular weight of the band at slightly above 75kD, we hypothesized that Tim-1 might induce phosphorylation of the adaptor protein SLP-76, which is known to be crucial for transducing signals downstream of TCR activation that leads to IL-2 production (138, 139). Thus, we wanted to determine whether Tim-1 might directly recruit SLP-76 upon activation. We were unable to detect a direct physical interaction between Tim-1 and SLP-76 by co-IP (Fig. 3-1e). This could indicate that binding is weak or that Tim-1 and SLP-76 do not directly interact.

In an attempt to determine the identity of the selectively phosphorylated proteins in an unbiased manner, we activated cells using anti-Tim-1 antibodies in the presence or absence of anti-TCR and anti-CD28 stimulation. These lysates IP'ed with 4G10 beads before being run on a gel and silver stained in order to visualize proteins (Fig. 3-1e). We were able to detect a band selectively induced between 75-100kD similar to the original anti-pY blot (Fig. 3-1d, f). This band was excised, and the identity of the protein was determined by mass spectrometry. This method identified Lrch3 (leucine-rich repeats and calponin homology domain containing 3),

which appears to have high transcript levels in T cells (Fig. 3-1f) (140). These data suggest that Tim-1 may selectively induce specific proteins, such as Lrch3. While little is known about mammalian Lrch3, it is highly homologous to drosophila *dLrch* and has been implicated as a cytoskeletal scaffolding protein important for cellular division (141). Cell division is a necessary part of T cell activation and expansion.

3.4.2 PS expressing thymocytes in the presence of co-stimulation may enhance pAkt and pERK in Tim-1 expressing T cells

Tim-1 ligands have also been implicated in influencing cellular function. For instance, the Tim-4:Tim-1 interaction has been suggested to stimulate T cell proliferation and phosphorylation of signaling proteins (13). However, since Tim-4 can influence the function of naïve T cells, which express little if any Tim-1, Tim-4 may also bind to receptors other than Tim-1. Thus, we were interested in determining how another natural Tim-1 ligand, phosphatidylserine (PS), might impact Tim-1 enhancement of T cell activation. Ligation of Tim-1 on NKT cells by anti-Tim-1 antibodies, co-stimulation with α-GalCer, and PS expressing erythrocytes enhances NKT proliferation and cytokine production (58). Further, blocking the PS interaction with excess amounts of annexin V can mitigate these effects. In addition, although Tim-1 (Kim-1) on kidney cells has been shown to bind and engulf PS-expressing apoptotic cells (57), a function for Tim-1 and PS ligation on conventional T cells remains to be elucidated. Since Tim-1 has the ability to enhance NFAT/AP-1 activity, we first determined whether the Tim-1:PS interaction might also impact reporter activity this transcriptional response. We stimulated D10 or Jurkat T cells with different ratios of either viable or apoptotic thymocytes, the later of which express high levels PS. We were unable to detect enhanced NFAT/AP-1 activity in cells stimulated with anti-Tim-1

(3B3) alone (Fig. 3-2 A-B). Ectopic expression of Tim-1 in the presence of anti-TCR/CD28 co-stimulation enhanced NFAT/AP-1 activation in D10 and Jurkat T cells as has been shown before (Fig. 3-2 A-B). However, surprisingly, apoptotic thymocytes incubated with anti-CD3/CD4/CD28 stimulation in the presence of Tim-1 expressing D10 cells resulted in a drastic decrease in NFAT/AP-1 activation (Fig. 3-2 A). A slight decrease in NFAT/AP-1 activation was seen in Tim-1 expressing Jurkat T cells incubated with apoptotic cells at the lower ratio (1 million Jurkat T cells to 2 million apoptotic thymocytes) as compared to T cells incubated with viable thymocytes (Fig. 3-2 B). However, this effect was not observed when Tim-1 expressing Jurkat T cells were incubated with the higher ratio of apoptotic thymocytes (Fig. 3-2 B). These data suggests that Tim-1 expression on T cells in the presence of apoptotic thymocytes-PS and co-stimulation may affect NFAT/AP-1 transcriptional activation, depending on the ratio of T cells to ligand expressing thymocytes.

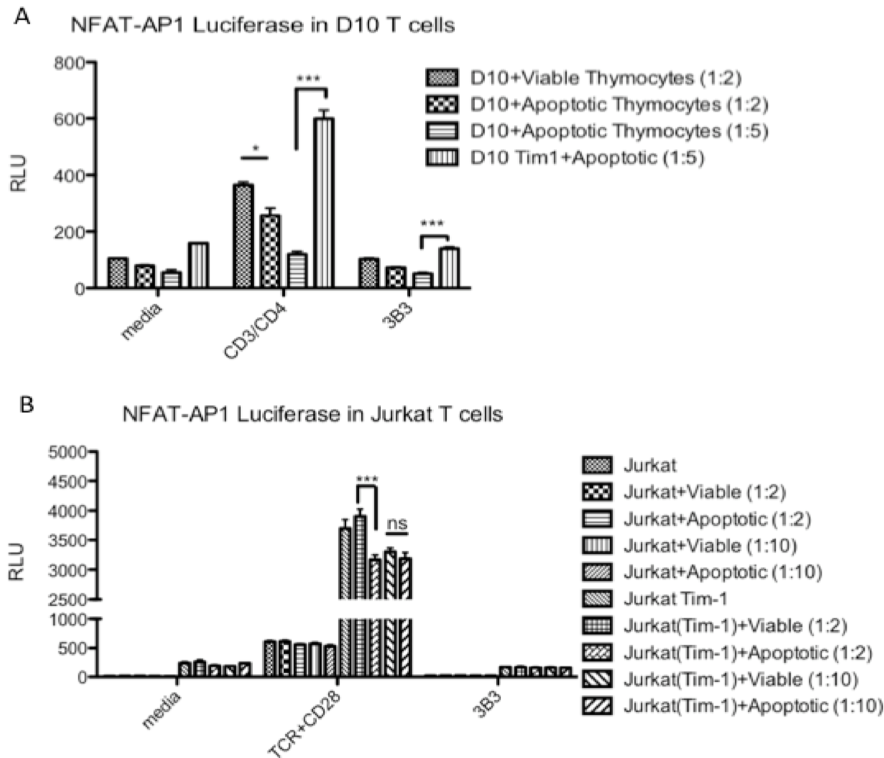


Figure 3-2: PS-expressing apoptotic thymocytes do not affect Tim-1 induce T cell activation

D10 (A) or Jurkat (B) T cells were transfected with empty vector or Tim-1 in the presence of an NFAT/AP-1 reporter. The next day, one million T cells were cultured for six to eight hours with the indicated ratio of viable or apoptotic thymocytes before assaying for luciferase activity.

We examined the ability of Tim-1-expressing D10 T cells to broadly induce pY downstream in the presence of viable or apoptotic thymocytes. Viable and PS expressing apoptotic thymocytes alone were unable to induce much pY. The addition of Tim-1-expressing D10 cells and anti-CD3/4/28 were also unable to induce signficant pY, suggesting that activation may have been suboptimal. We were unable to detect any specifically tyrosine phosphorylated proteins downstream of PS-expressing apoptotic thymocytes on Tim-1-expressing D10 cells, as compared to the viable thymocytes (Fig. 3-3 A). Thus, unlike antibody cross-linking, the Tim-1

interaction with PS-expressing cells did not clearly induce a particular pattern of tyrosine phosphorylation.

Ectopic Tim-1 expression has been shown to augment Akt phosphorylation upon TCR activation. Thus, we examined the role of PS-expressing thymocytes cultured with Tim-1 expressing D10 cells on pAkt levels. D10, viable, and apoptotic thymocytes alone were unable to induce pAkt. Incubation of D10 cells with apoptotic thymocytes, particularly at five minutes, was able to induce pAkt. However, pAkt levels do not appear to correlate with PS expression, since both viable and apoptotic thymocytes induce phosphorylation (Fig. 3-3 B). To examine Akt phosphorylation at the level of the individual cell, we also performed phospho-flow cytometry. Similar to the western blot, incubation with thymocytes slightly enhanced pAkt levels. However, this increase in pAkt did not appear to specifically depend on the presence of PS since both apoptotic and viable thymocytes increased phosphorylation (Fig. 3-3 C). Since Tim-1 enhancement of pAkt is increased by co-stimulation(64), we wanted to determine the effect of PS on Tim-1-expressing cells with co-stimulation. Another Tim-1 ligand, Tim-4, has been shown to induce pERK1/2 (13). Thus, we investigated whether Tim-1 and PS-thymocyte expression in the presence of anti-CD3 and anti-CD28 co-stimulation could influence pAkt and pERK1/2 levels. Interestingly, incubation of Tim-1-expressing D10 cells with PS-expressing apoptotic thymocytes and TCR co-stimulation resulted in enhanced pAkt and pERK1/2 (Fig. 3-3 D-F). Thus, the data suggest that interaction of Tim-1 with PS-expressing thymocytes might enhance pY of specific proteins.

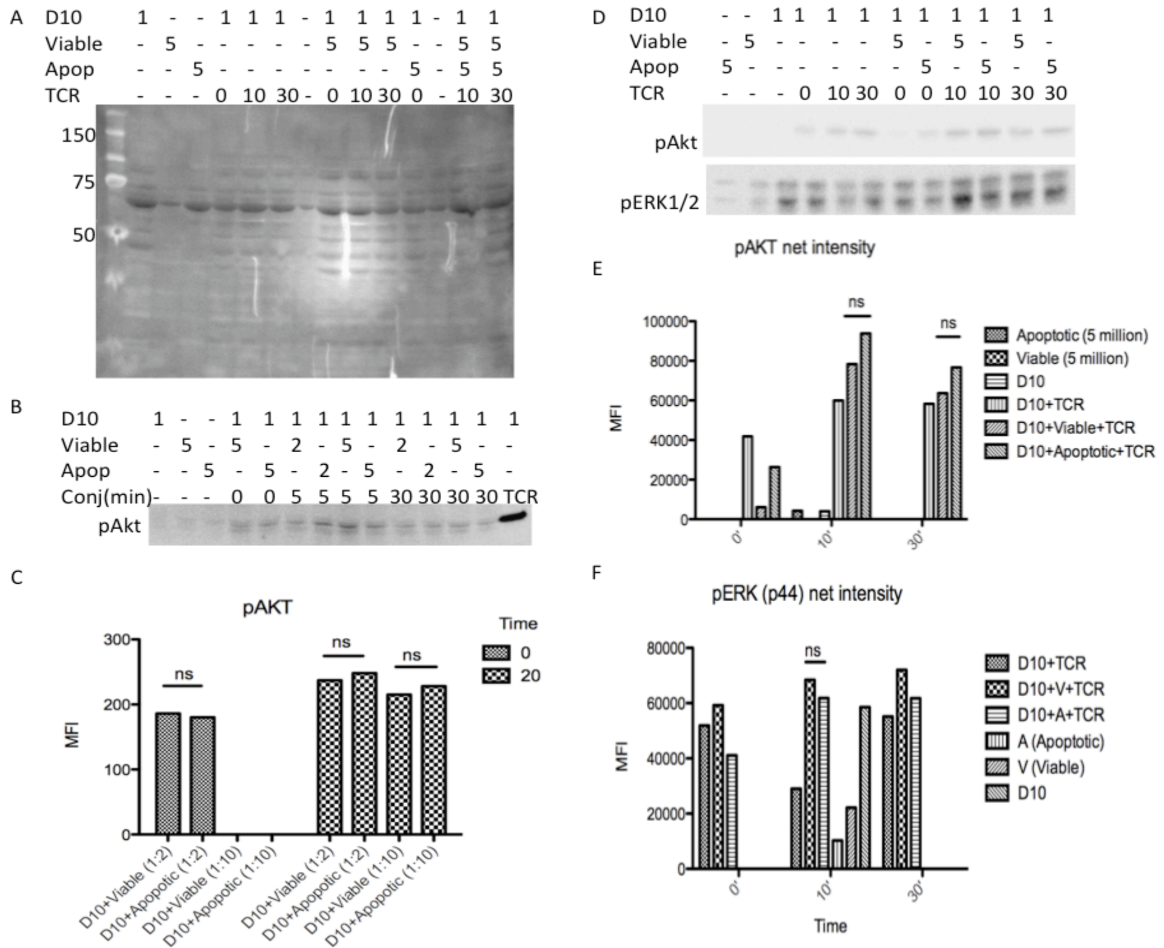


Figure 3-3: PS-expressing apoptotic thymocytes do not affect Tim-1 induce T cell activation

(A) Tim-1 (Balb/c) transfected D10 cells were briefly centrifuged with viable or apoptotic thymocytes in the presence or absence of TCR (CD3/CD4/CD28) stimulation for the indicated times. Lysates were separated by SDS-PAGE electrophoresis before transferring and blotting for anti-pY (4G10). Tim-1 transfected D10 cells were incubated with viable or apoptotic thymocytes for the indicated times before phosphorylation of Akt was determined by immunoblotting (B) or phosphoflow (C). (D) Tim-1 transfected D10 cells were incubated with viable or apoptotic thymocytes in the presence or absence of TCR (CD3/4/28) stimulation. Lysates were blotted for pAkt (E) and pERK1/2 (F) and net intensity of the bands was quantified by densitometry on the Kodak image station. Cells numbers are indicated in the millions.

3.4.3 Homotypic Tim-1 interactions do not affect NFAT/AP-1 but enhance Elk-1 activation

To determine whether homotypic Tim-1 interactions influence NFAT/AP-1 activation, Jurkat and D10 T cells were transfected with vector, TIM-1, or TIM-1^{H64E} in the presence or absence of TCR/CD28 co-stimulation. The D10 and Jurkat T cells did not demonstrated significant differences in reporter activity between wild type and Tim-1^{H64E} mutants, although there may be a slight (but not statistically significantly) increase in NFAT/AP-1 activation in the C57Bl/6 Tim-1 (Fig. 3-4 A/B). Interestingly, Elk-1 activation did not recapitulate NFAT/AP-1 activity. In Jurkat T cells, although higher than empty vector transfected cells, the Elk-1 reporter activity was decreased in the presence of the Tim-1^{H64E} mutant as compared to wild type in the presence of TCR/CD28 co-stimulation (Fig. 3-4 B). This suggests that homotypic interactions are dispensable for NFAT/AP-1 activation but may be involved in enhancing Elk-1 activity.

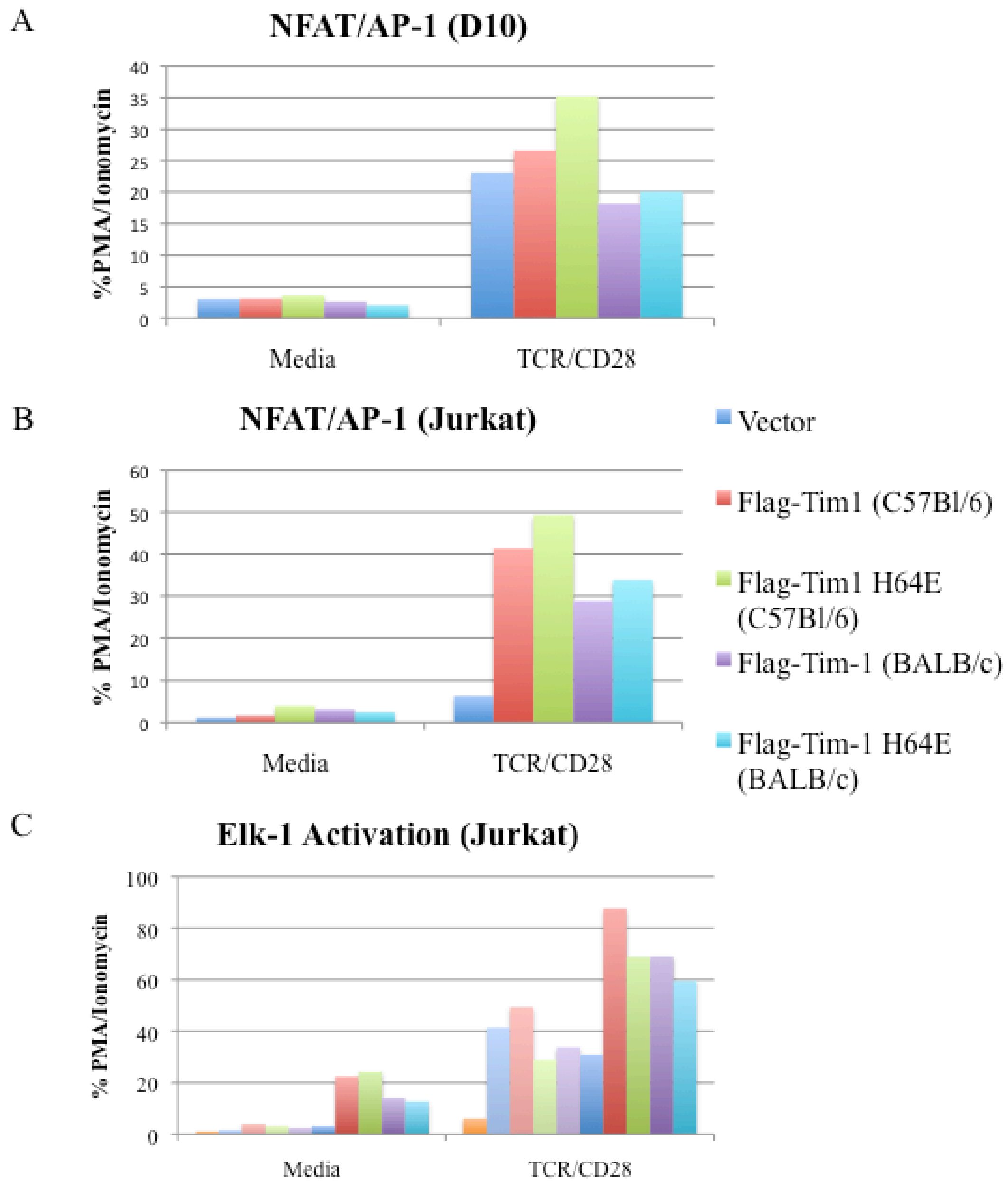


Figure 3-4: Homotypic interactions do not effect NFAT/AP-1 activation

Tim-1 and Tim-1 H64E constructs were transfected with NFAT/AP-1-luciferase into (A) D10 (n=2) and (B) Jurkat (n=2) T cells. (C) Tim-1 and Tim-1 H64E were transfected for Elk-1 into Jurkat cells (n=1).

3.4.4 Tim-1 on BALB/c cells have reduced transcriptional activation

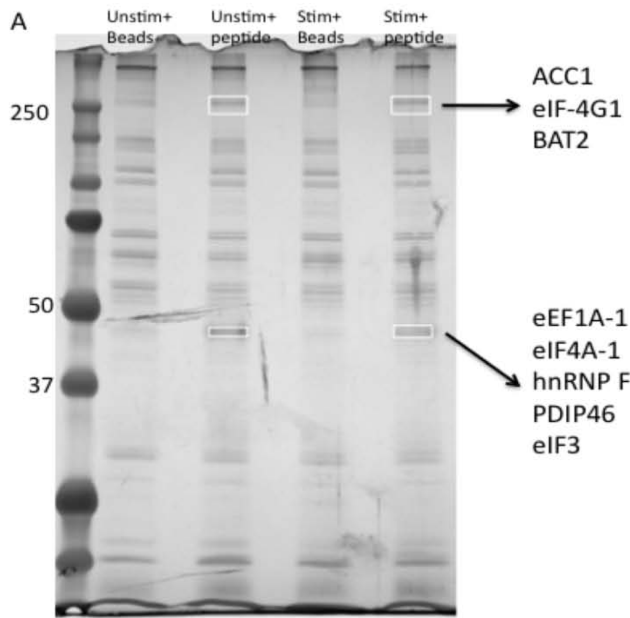
The original description of the TAPR region suggested that polymorphisms in Tim-1 might account for the differences noted in AHR susceptibility. It is well accepted that BALB/c mice are more prone to Th2 responses and exacerbation of AHR, whereas C56Bl/6 mice are more resistant to AHR. These mice have differences in their Tim-1 sequences. In particular, Tim-1 in HBA mice, where the TAPR region was cloned, is identical to Tim-1 in C57Bl/6 and contains a 15 amino acid deletion as compared to BALB/c mice (27, 33). Thus, we postulated that strain dependent differences in Tim-1 function might exist. In support of this hypothesis, although not yet statistically significant, BALB/c T cells trended towards decreased Tim1-dependent NFAT/AP-1 and Elk-1 activity as compared to C57Bl/6 T cells cells (Fig. 3-4 C). More experiments will be necessary to confirm this finding.

3.4.5 Identification of potential Tim-1 binding partners

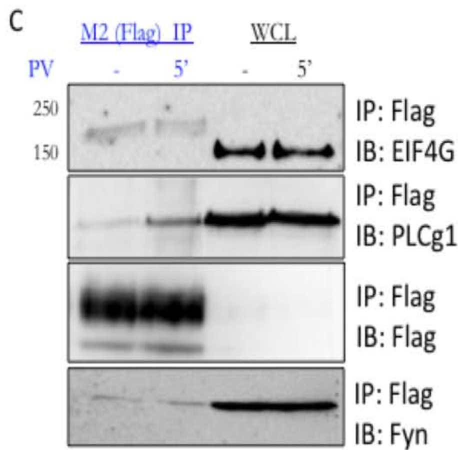
We have hypothesized that additional signaling proteins interacting with the Tim-1 cytoplasmic tail to form the Tim-1 "signalosome" upon T cell activation remain unidentified. Initial work from this lab utilized an SH2 domain array to identify binding partners, and previous work has described the importance of Y276 in the Tim-1 cytoplasmic tail for signal transduction downstream of TCR/CD28 co-stimulation (33, 64). While published work has confirmed that Tim-1 binds p85 and Tim-1 signaling is mediated through PI3K, other proteins were identified but not validated, including Fyn and RasGap (64). Further, we used the scansite program to indicate possible Tim-1 binding partners based motifs and sequences of the cytoplasmic tail

(142). This method identified PLCγ1 as a potential binding partner (Fig. 3-5 B). PLCγ1 is an attractive target due to its established role in transducing early T cell signals (62, 143, 144).

To define additional binding partners, we employed a peptide pull down approach using the same Tim-1 cytoplasmic tail peptide used in the SH2 domain array (64). Using a previously described biotinylated 13aa segment of the Tim-1 cytoplasmic tail containing a phosphorylated Y276, we detected two protein bands associated with the Tim-1 peptide but not a control biotinylated peptide (Fig. 3-5 A). These bands were excised, and proteins were identified by mass spectrometry. These proteins included HLA-B associated transcript 2 (BAT2), Acetyl-CoA carboxylase 1 (ACC1), and a number of translation factors (Fig. 3-5 A/B). This suggests that Tim-1 could potentially play a role in activating cellular processes within T cells in preparation for T cell activation.



Protein	Method Identified
Fyn	SH2 domain array
RasGAP	SH2 domain array
p85 α and β	SH2 domain array
Acetyl-CoA carboxylase 1	Peptide pull down / Mass. Spec.
Eukaryotic translation initiation factor 4 gamma 1	Peptide pull down / Mass. Spec.
BAT2	Peptide pull down / Mass. Spec.
Nuclease sensitive element binding protein 1	Peptide pull down / Mass. Spec.
Eukaryotic translation elongation factor 1 alpha 1	Peptide pull down / Mass. Spec.
Eukaryotic translation initiation factor 4A isoform 1	Peptide pull down / Mass. Spec.
Heterogeneous nuclear ribonucleoprotein	Peptide pull down / Mass. Spec.
DNA polymerase delta interacting protein 3 isoform 2	Peptide pull down / Mass. Spec.
Eukaryotic translation initiation factor 3, subunit 6	Peptide pull down / Mass. Spec.
PLCγ1	Binding Motif (ScanSite®)



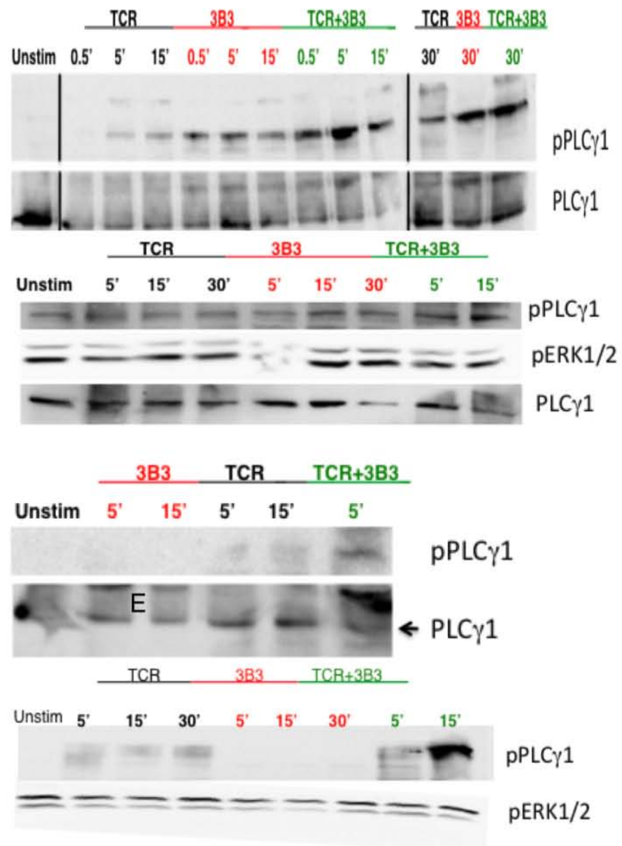


Figure 3-5: Identification of binding partners to Y276 region of the Tim-1 cytoplasmic tail

(A) Jurkat cell lysates were incubated with a biotinylated control peptide or a biotinylated 13 aa peptide of the Tim-1 cytoplasmic tail containing a phosphorylated Y276. Cells were stimulated with or without pervanadate for 5 minutes. Interacting proteins were immunoprecipitated with streptavidin beads and run on an SDS-PAGE gel. The indicated bands were excised and proteins sent to ProTech for identification by mass spectrometry. (B) Chart detailing putative Tim-1 interacting partners and method of identification. (C) 15-25 million Flag-Tim-1 transfected D10 cells were lysed, IP'ed with anti-M2 (Flag), run on gel, and blotted with the indicated antibodies. (D-E) Determination of PLCγ1 phosphorylation (Y783) or ERK1/2 phosphorylation in primary cells stimulated with TCR (CD3/4/28), anti-Tim-1, or TCR and anti-Tim-1. (D-top) pPLCγ1 in first round Th1 induced Balb/c T cells. (D-bottom) pPLCγ1 and pERK1/2 induced within 48 hours of TCR/CD28 activated splenic T cells. (E) Immunoblotting for pPLCγ1 or pERK1/2 from restimulated T cells isolated from Balb/c spleens and lymph nodes.

3.4.6 Validation of Tim-1 binding partners

We next sought to validate the interaction between Tim-1 and its putative binding partners based on the predictions above. We performed Co-IPs on promising candidates to confirm their interaction with Tim-1. D10 cells transfected with Flag-Tim-1 were immunoprecipitated with M2 (α-Flag) antibody and blotted to detect interacting proteins. Flag Tim-1 is challenging to detect in whole cell lysates but clearly visible after IP (Fig. 3-5 C). Among the proteins identified included multiple translation factors and a protein involved in cellular metabolism. Specifically the mass spectrometry revealed translation factors, such as EIF4A and EIF4G, and

the metabolic factors ACC1 (Fig. 3-5 A). Although most research has focused on transcription factors and the genes they regulate in lymphocyte activation, T cells must also undergo increased aerobic and anaerobic glycolysis and translation of proteins to support cellular activation (145). Thus, we were interested in the potential interaction between Tim-1 and the translation factor EIF4G. However, we were unable to reliably detect an interaction between Tim-1 and EIF4G (Fig. 3-5 C).

Several putative Tim-1 interacting proteins were identified by SH2 domain array. A particularly intriguing candidate was Fyn, which is member of the Src family of tyrosine kinases (SFK) and is important for early phosphorylation events downstream of TCR/CD3 (146). Our lab previously demonstrated the importance of another SFK, Lck, in signal transduction downstream of Tim-1 in T cells (64, 147). Further, another lab demonstrated that Fyn interacts with phosphorylates Tim-1 in B cells (147). Thus, we hypothesized that Fyn might also interact with Tim-1 in T cells and aid in the transduction of downstream signals. However, we were unable to consistently observe Tim-1 and Fyn binding by Co-IP. Fyn appears to weakly interact with Tim-1 but this interaction is not stimulation dependent (Fig. 3-4 C). Other experiments did not suggest an interaction between Tim-1 and Fyn (data not shown). However, lack of detectable physical interaction does not necessarily rule out the ability of PS through Tim-1 to activate or phosphorylate of Fyn. Another signaling protein identified by SH2 domain array was RasGap, a negative regulator of the Ras small G protein. However, we were unable to confirm interaction of RasGap with Tim-1 by co-IP or co-localization, possibly because of the quality of the RasGap antibody (data not shown). These data indicate that there is not a strong interaction between Tim-1 and either Fyn or RasGap, although we cannot rule out weak or more transient interactions.

Potential interaction of Tim-1 with PLCγ1 was suggested through sequence homology by Scansite (142). PLCγ1 is the predominant PLCγ isoform in T cells and is an important part of the early T cell signalosome. PLCγ cleaves PIP_2 into IP_3 and DAG (62). This allows for NFAT/AP-1 transcription factor activation, which Tim-1 has been shown to enhance in a Y276-dependent manner. Thus, we hypothesized that Tim-1 might interact with PLCγ1 in order to mediate Tim-1 downstream effects. We were able to demonstrate basal Tim-1 binding to PLCγ1, which was enhanced by stimulation with pervanadate (Fig. 3-5 C). This is indicative of a Tim-1 and PLCγ1 interaction that is strengthened by phosphorylation.

We were interested in whether ligation of Tim-1 in activated primary T cells could induce phosphorylation and activation of PLCγ1. In two experiments, stimulation with anti-Tim-1 antibody 3B3 induced increased phosphorylation of PLCγ1. This was further enhanced by co-stimulation with anti-CD3/CD28 (Fig. 3-5 D). In the majority of experiments (n>3), we were unable to induce PLCγ1 phosphorylation by anti-Tim-1 stimulation alone, although there might still be increased PLCγ1 phosphorylation in anti-TCR/CD28 and anti-Tim-1 co-stimulated cells (Fig. 3-5 E). Alternations in pERK1/2 levels are more difficult to detect in these experiments, since the baseline levels are so high (Fig. 3-5 D/E). This all suggests that Tim-1 enhances PLCγ1 phosphorylation in a CD3/CD28-costimulation dependent manner.

3.4.7 Tim-1 interaction with PLCγ1 depends on the Tim-1 cytoplasmic tail

Next, we further characterized the structural elements necessary for the Tim-1 and PLCγ1 interaction. Using Tim-1^{Y276F} and Tim-$1^{\Delta Cyto}$ constructs, we observed that binding was at least partially abrogated by truncation of the cytoplasmic tail and by mutation of Y276 (Fig. 3-6).

While there still appears to be some binding of PLCγ1 to the Y276 mutant, the levels are lower, particularly when compared to the amount of total Tim-1 protein pulled down. This suggests that the majority of Tim-1 binds to PLCγ1 in a cytoplasmic tail dependent manner.

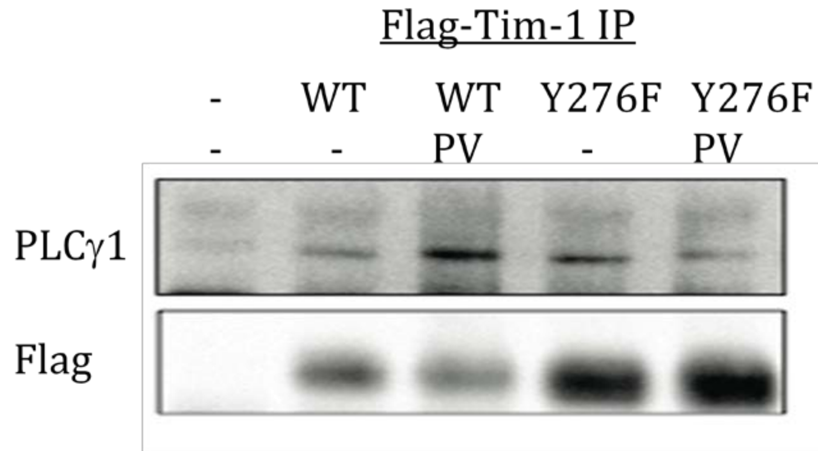


Figure 3-6: Tim-1 binding is decreased in when Y276 in the cytoplasmic tail is mutated

15-25 million Flag-Tim-1 transfected D10 cells were lysed, IP'ed with anti-M2 (Flag), run on gel, and blotted for PLCγ1 and Flag. This is a representative blot from three separate experiments.

3.4.8 Tim-1 induction of transcriptional activity depends on TCR and CD28 co-stimulation but is independent on PLCγ1

To determine the functional consequences of Tim-1 signaling through PLCγ1, we tested the ability of Tim-1 to modulate transcriptional activity in the absence of PLCγ1. PLCγ1 is an important signaling molecule in transducing signals downstream of TCR/CD28 ligation through its products DAG and PI(4,5)P_2. DAG activates PKC-θ and through various steps leads to AP-1 activation while PI(4,5)P_2 mobilizes intracellular Ca^{2+} signaling leading to the induction of

NFAT activity. Therefore, we hypothesized that Tim-1 would enhance NFAT/AP-1 activation through its interaction with PLCγ1. First, we examined the ability of Tim-1 to signal in PLCγ1-deficient cells. We expressed Tim-1 in Jurkat T cells completely lacking PLCγ1 (J.γ1) and J.γ1 T cells reconstituted with PLCγ1 (J.γ WT) together with an NFAT/AP-1 luciferase reporter. Surprisingly, Tim-1 expression in the presence of anti-TCR and anti-CD28 resulted in similar levels of NFAT/AP-1 activity in Jurkat, J.γ, and J.γ WT cells (Fig. 3-6 A). This induction was dependent on co-stimulation by both TCR and CD28, since stimulation by either TCR or CD28 alone was insufficient to induce transcriptional activity in the presence of Tim-1 (Fig. 3-7 A). We previously published that the ability of Tim-1 to induce NFAT/AP-1 transcriptional activity requires the cytoplasmic tail, specifically Y276 (33). To better define the structural elements necessary for this Tim-1-dependent, but PLCγ1-independent, effect, we utilized a Tim-1 cytoplasmic tail truncation and Y276F point mutant. Expression of the Tim-1 cytoplasmic tail truncation as well as the Tim-1 Y276F mutant abrogated Tim-1-mediated induction of NFAT/AP-1, even in the presence of TCR and CD28 (Fig. 3-7 B). This is consistent with the fact that the Tim-1 cytoplasmic tail, particularly one or more proteins interacting with Y276, is necessary for Tim-1 mediated enhancement of NFAT/AP-1. Interestingly, PLCγ1 does not appear to be the required protein for this Tim-1 mediated transcriptional activation. This might be due to compensation by other PLC isoforms or due to other compensating proteins found within the J.γ T cells.

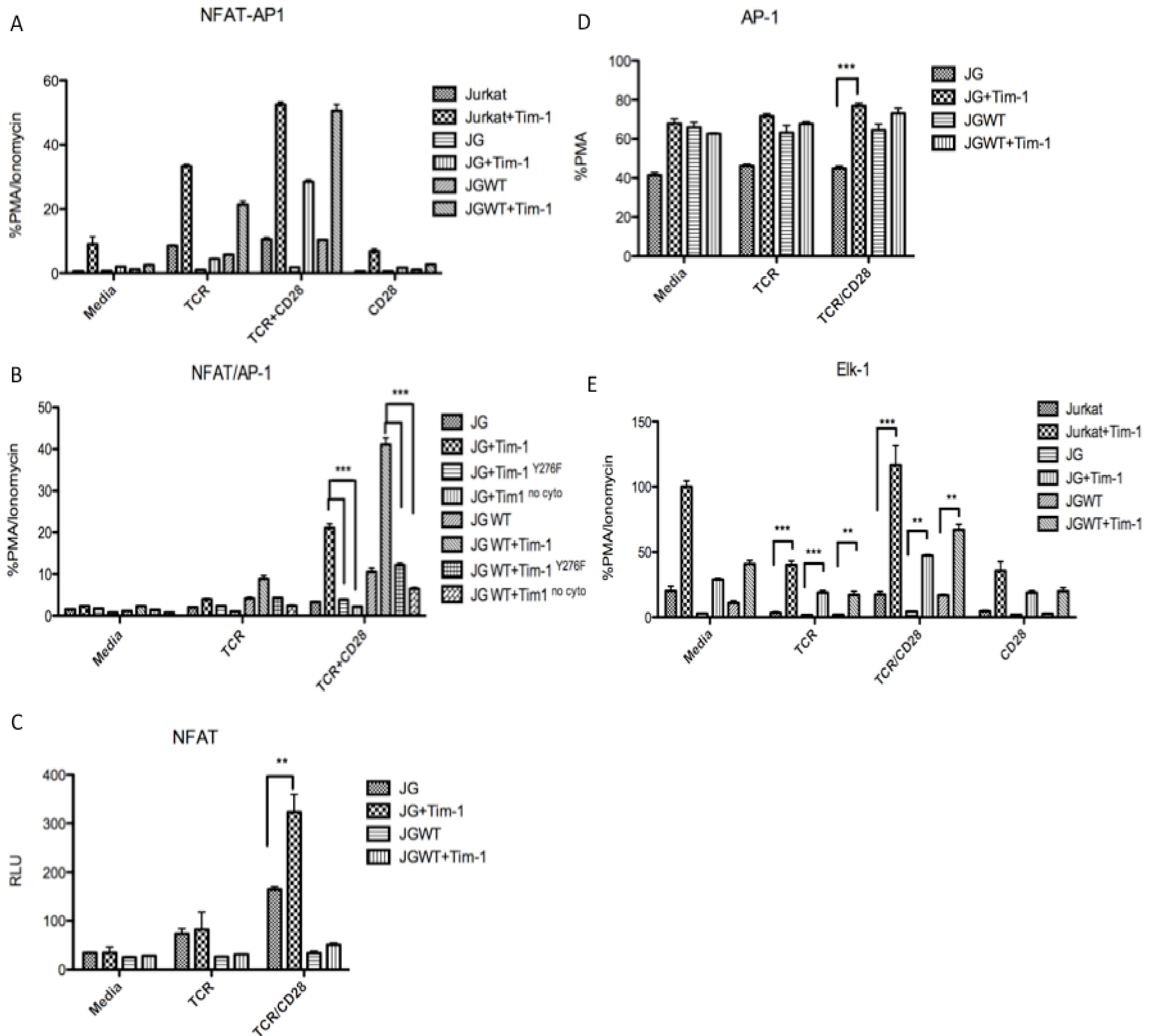


Figure 3-7: Effect of Tim-1 through PLCγ1 on transcriptional activation

(A) NFAT/AP-1 luciferase reporter activity in Jurkat, Jγ1 (Jurkat cells lacking PLCg1), or Jγ1 WT (Jγ1 cells reconstituted with PLCg1) transfected with Tim-1 before activating with anti-TCR, anti-TCR/CD28, anti-CD28, or PMA/ionomycin. Reporter activity is expressed as a percentage of PMA+/-ionomycin. (B) NFAT/AP-1 reporter activity in Jurkat, Jγ1, or Jγ1 WT cells transfected with empty vector, Tim-1, Tim-1^{Y276F}, or Tim-1$^{\Delta Cyto}$ and stimulated in the

presence or absence of TCR and CD28. NFAT (C), AP-1 (D), Elk-1 (E), CD28 RE (F) luciferase reporter activity in Jγ1 or Jγ1 WT cells stimulated with anti-TCR, anti-TCR and CD28, or PMA+/-ionomycin.

To further assess the Tim-1 dependent but PLCγ1 independent pathways, we examined the effect of Tim-1 signaling through PLCγ1 on the individual components of the pathways. First, we examined the ability of Tim-1 to induce activation of NFAT in a PLCγ1 independent manner. We overexpressed Tim-1 in PLCγ1 deficient Jurkat T cells with a pure NFAT reporter, which is dependent on only Ca^{2+} signaling. Similar to the case with NFAT/AP-1 activation, we were observed PLCγ1-independent induction of NFAT in Tim-1-expressing T cells stimulated with both anti-TCR and anti-CD28, but not with anti-TCR alone (Fig. 3-7 C). Next, we determined the effect of Tim-1 through PLCγ1 on activity of a pure AP-1 reporter. This was more difficult to interpret since signal-to-noise was not optimal. The presence of Tim-1 in the absence of PLCγ1 is still able to slightly enhance AP-1 activation, as compared to empty vector alone (Fig. 3-7 D). Thus, the TCR/CD28 dependent, but PLCγ1 independent, effect of Tim-1 appears to play a role in both NFAT and AP-1 separately, although the NFAT effect is more robust and is also more consistent with the NFAT/AP-1 effect.

Tim-1 expression or ligation does not appear to induce NF-κB in T cells, but a role for Elk-1 has been previously demonstrated (Anjali de Souza's thesis). Thus, we investigated the role of PLCγ1-independent Tim-1 signaling on the MAP kinase pathway in T cells. Ectopic expression of Tim-1 in Jurkat, J.γ, and J.γ WT cells resulted in induction of an Elk-1-gal4 transcriptional reporter, which is a read-out for MPK-mediated Elk-1 phosphorylation. This increase in Elk-1 reporter activity was greatest in cells stimulated with both TCR and CD28.

However, unlike NFAT/AP-1 transcription, the increased Elk-1 reporter induction is less dependent on co-stimulation, since either TCR or CD28 stimulation alone is also able to induce some Tim-1 dependent Elk-1 activity (Fig. 3-7 E). This is not entirely surprising since MAP Kinase activation is less strictly dependent on PLCγ1 expression than NFAT/AP-1, *ie.* other pathways might compensate for Elk-1 activity, such as the Ras GTPases.

3.4.9 Role of PLCγ1 in Tim-1 co-stimulation of D10 T cells

We utilized pharmacological inhibition and genetic silencing to validate our work in the PLCγ1 deficient Jurkat T cell line discussed above. To confirm these findings, we were interested in the effect of PLCγ inhibitors on Tim-1 induced NFAT/AP-1 activation. Both U37122 and neomycin have been shown to inhibit PLCγ activity (148-150). While the mechanism for U73122 has not been fully elucidated, neomycin is believed to inhibit PLC due to its phosphatidylinositol 4,5-bisphosphate binding affinity (150, 151). Thus, we pre-treated Tim-1 or Tim-1$^{\Delta Cyto}$ (a control) expressing Jγ or JγWT cells with U73122 (0.1μM and 1μM) or Neomycin (1mM) followed by anti-TCR and anti-CD28 co-stimulation. In both the Jγ and the JγWT cells, PLCγ inhibitors did not abrogate the ability of Tim-1 to induce NFAT/AP-1 activation upon CD3/CD28 co-stimulation (Fig. 3-8 A). Thus, in these experiments, chemical inhibition of PLCγ was not sufficient to attenuate Tim-1 and co-stimulation induced NFAT/AP-1 activity in Jurkat T cells.

Jurkat T cells are known to have abnormalities in signaling, mostly notably increased pAkt due to lack of PTEN and SHIP (152, 153). Thus, we examined the role of PLCγ1 in the D10 T cell line, which possesses apparently normal signaling through the PI3K pathway (123, 125). As anticipated, Tim-1 expression enhances NFAT/AP-1 activation in the presence of anti-

CD3/CD4/CD28 co-stimulation. However, treatment with the U73122 inhibitor decreases this effect and results in comparable levels of reporter activity between empty vector and Tim-1 expressing cells despite CD3/CD4/CD28 co-stimulation. Similarly, neomycin treatment also attenuates much of the Tim-1 and anti-CD3/CD4/CD28 co-stimulation dependent NFAT/AP-1 activation (Fig. 3-8 B). Thus, in contrast to Jurkat cells, ectopic Tim-1 expression in D10 cells even in the presence of co-stimulation cannot compensate for blockade of the PLCγ activity.

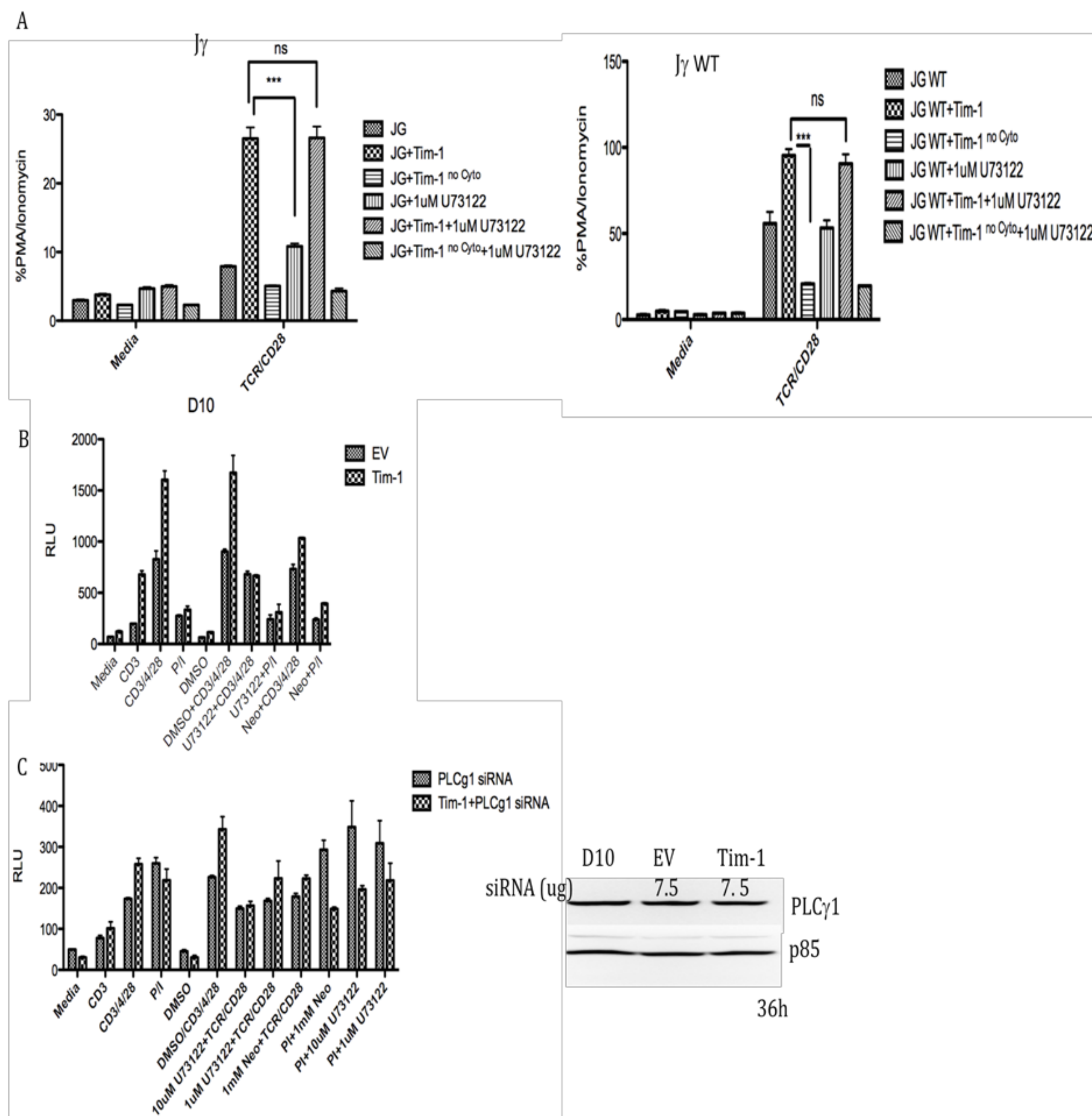


Figure 3-8: Effect of PLCγ inhibition on Jurkat and D10 cells

(A) NFAT/AP-1 luciferase reporter activity in Jγ1 (left), or Jγ1 WT (right) transfected with Tim-1 before activating with anti-TCR, anti-TCR/CD28, anti-CD28, or PMA/ionomycin. Reporter activity is expressed as a percentage of PMA+/-ionomycin. (B) NFAT/AP-1 reporter activity in D10 T cells transfected with empty vector and Tim-1 and stimulated in the presence or absence of CD3 and CD28 with or without U73122 or Neomycin treatment. (C) NFAT-AP-1 luciferase

reporter activity in D10 T cells transfected with empty vector or Tim-1 and PLCγ1 siRNA was stimulated with anti-CD3 and CD28, or PMA+/-ionomycin in the presence or absence of U73122 or Neomycin. Western blot for PLCγ1 and p85 expression in the transfrected D10 cells.

To specifically examine the role of PLCγ1, we also attempted to knockdown PLCγ1expression in D10 cells and to determine its effect on Tim-1 influenced transcriptional activation. Cells transfected with Tim-1 cDNA and the PLCγ1 siRNA were still able to induce NFAT/AP-1 activation, in a CD3/CD4/CD28 dependent manner, as compared to empty vector controls. A caveat with the experiment was that the overall levels of reporter activity were not optimal, and PLCγ1 knock down was only partial (Fig. 3-8 right). Tim-1-expressing PLCγ1 knockdown D10 cells treated with the higher dose of U73122 had reduced NFAT/AP-1 reporter activity to levels comparable to empty vector. Treatment with the lower dose of U73122 (1uM), or with neomycin, also decreased NFAT/AP-1 luciferase activity as compared to vehicle treated cells (Fig. 3-8 C). The luciferase signal after inhibitor treatment could be lower because the inhibitor is blocking downstream signaling pathways or because treatment effects cellular viability. To clarify whether the effects of the inhibitors on NFAT/AP-1 activation are on Tim-1 signaling and not due to inhibitor-induced cell death, we also examined the effect of inhibitor treatment on luciferase activity after PMA and ionomycin stimulation. PMA, a DAG mimic, and ionomycin, a calcium ionophore, work downstream of PLCγ1 and should bypass any Tim-1 mediated effects on NFAT/AP-1 activation. Any cells that are alive should be stimulated to induce transcriptional activity by PMA and ionomycin treatment, so inhibition of this stimulation by U73122 would suggest impaired viability of the cells. The amount of luciferase activity induced by P/I stimulation is similar between empty vector and Tim-1 expressing cells.

However, Tim-1-expressing-PLCγ1 knockdown cells had decreased reporter activity as compared to empty vector-control siRNA expressing cells in the presence of chemical PLCγ inhibiton. This may indicate that a double block with PLCγ1 knockdown in conjunction with PLCγ inhibition decreases cellular viability. In general, Tim-1-expression despite PLCγ1 knockdown in D10 T cells, similar to Tim-1 expression in PLCγ1 deficient Jurkat T cells, can still enhance NFAT/AP-1 activation. However, effects of the PLCγ inhibitor treatment remain to be clarified, since the Tim-1-induced NFAT/AP-1 reporter activation is not affected by U73122 treatment in Jurkat T cells but is decreased in D10 T cells.

3.4.10 Tim-1 upregulation of early markers of activation and cytokine production requires PLCγ

Next, we wanted to determine the importance of Tim-1 signaling through PLCγ1 for further downstream functions, such as early markers of T cell activation. We activated primary $CD4^+$ T cells with varying concentrations of CD3 and CD28 in the presence of the PLCγ inhibitor (U73122), anti-Tim-1 (3B3), or both for 18 hours. Stimulation with the anti-Tim-1 antibody slightly enhances both CD25 and CD69 expression at the highest dose of TCR and CD28 co-stimulation (Fig. 3-9). This could be in part due to the fact that the highest level of Tim-1 expression is found at the highest doses of anti-CD3 and anti-CD28 co-stimulation, so the anti-Tim-1 antibody is able to better crosslink and activate surface Tim-1 (Fig. 3-9 bottom). In contrast, PLCγ inhibition blocks upregulation of these early markers of activation at all concentrations of anti-CD3 and anti-CD28. Treatment with both anti-Tim-1 and the PLCγ inhibitor resulted in low levels of CD25 and CD69 (Fig. 3-9). This suggests that while Tim-1

may enhance early markers of T cell activation, this effect is dependent on signaling through PLCγ.

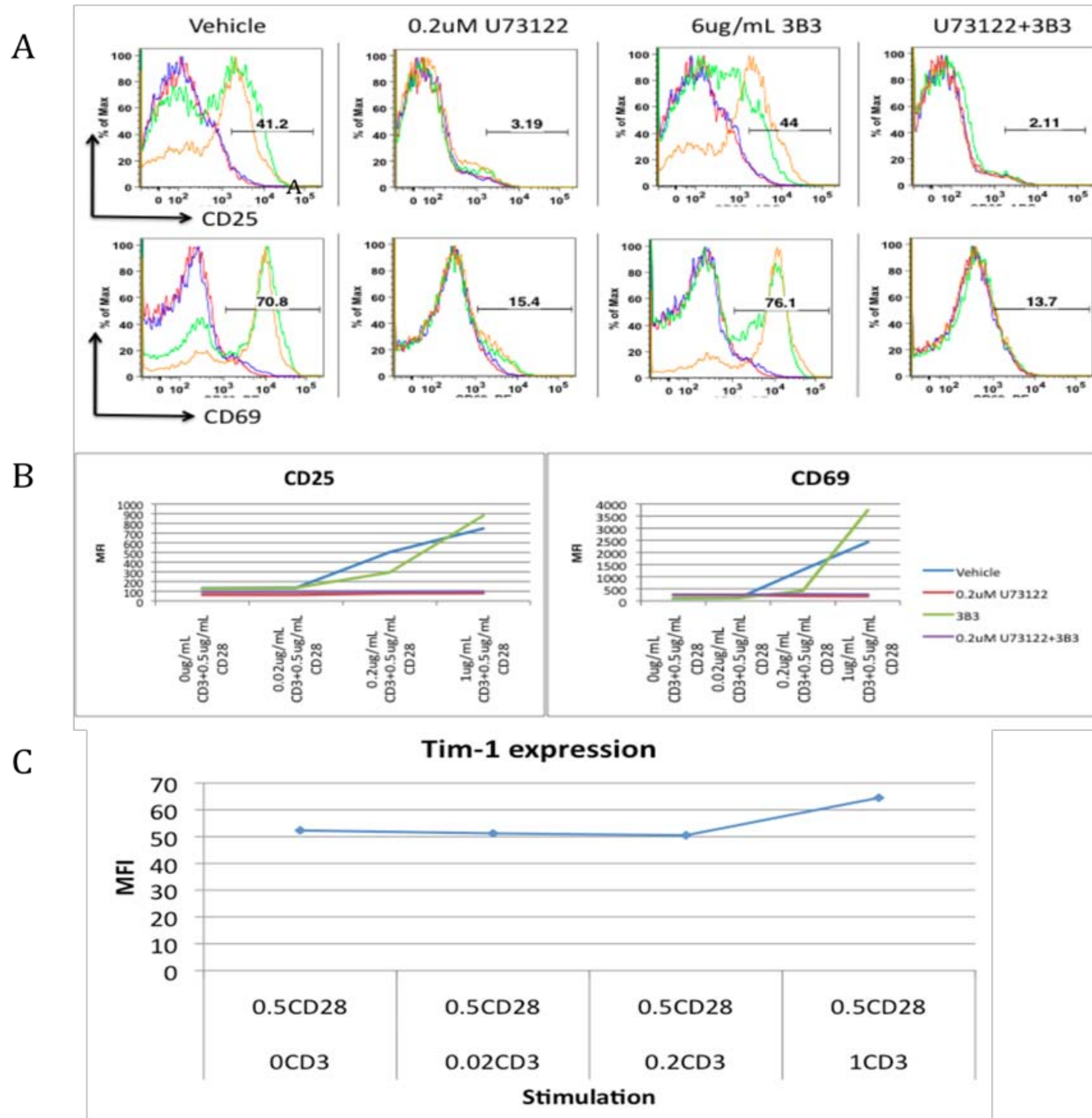


Figure 3-9: PLCγ inhibition blocks the Tim-1 enhancement of early markers of activation

(A) $CD4^+$ T cells isolated from C57Bl/6 spleens and lymph nodes were stimulated with varying concentrations of CD3 and a fixed concentration of CD28 for 16 hours after isolation. (B) Tim-1, CD25, and CD69 levels were determined by flow cytometry and represented by MFI. (C) MFI of Tim-1 expression as measured by flow cytometry.

Next, we examined the effect of PLCγ inhibition on Tim-1 induced cytokine production. IL-2 cytokine production is enhanced after NFAT/AP-1 activation. Thus, we examined the effect of Tim-1 through PLCγ1 in IL-2 expression. Primary $CD4^+$ T cells were activated by anti-CD3/CD28 in the presence of anti-Tim-1 antibodies, with or without PLCγ inhibition. Here, we observe that lower concentrations of TCR activation resulted in little IL-2 production. Interestingly, we note that the higher affinity anti-Tim-1 antibody, 3B3, decreases cytokine production. In contrast the lower affinity anti-Tim-1 antibody, RMT1-10, may enhance IL-2 production at the higher concentrations of anti-CD3/CD28 stimulation (Fig. 3-10 A). Regardless of which anti-Tim-1 antibody was used, inhibition of PLCγ with U73122 abrogated any detectable IL-2 production.

We also studied the effect of PLCγ1 knockdown on Tim-1 co-stimulation of cytokine production. D10 cells were transfected with empty vector or Tim-1 in the presence of control or PLCγ1-specific siRNA. Unlike the control, PLCγ1 siRNA partially decreased expression of PLCγ1. In the presence of co-stimulation from CD3, CD4, and CD28, Tim-1 enhances IL-4 production, and even partial knockdown of PLCγ1 attenuates IL-4 levels. This decrease in IL-4 expression in PLCγ1 knockdown cells cannot be rescued by ectopic Tim-1 expression (Fig. 3-10 B right). Tim-1 does not appear to greatly influence TNF-α expression, and PLCγ1 knockdown reduced overall levels of TNF-α. Potentially intriguing is the fact that PLCγ1 reduction completely abrogated TNF-α production while similar PLCγ1 knockdown in Tim-1 expressing cells only partially reduced TNF-α expression (Fig. 3-10 B-right). Overall, these results suggest that Tim-1's effects on cytokine production are at least in part, if not mostly, dependent on signals generated downstream of PLCγ1.

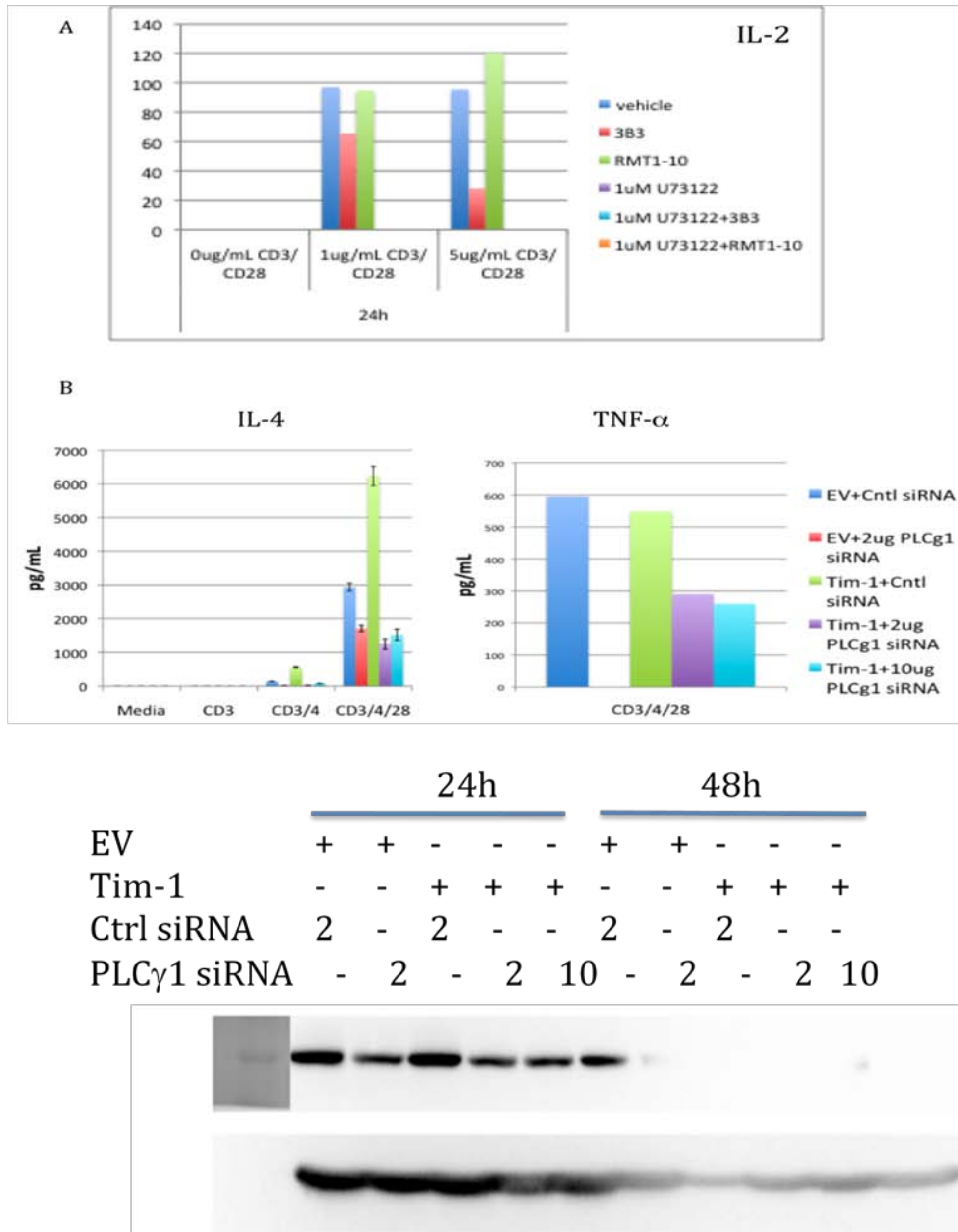


Figure 3-10: Tim-1 cytokine induction is inhibited in the absence of PLCγ

(A) $CD4^+$ T cells isolated from C57Bl/6 spleens and lymph nodes were activated with anti-Tim-1 antibodies (3B3 or RMT1-10) in the presence or absence of varying concentrations of CD3 and CD28 with or without U73122. (B) D10 cells transfected with Tim-1 and control or PLCγ1 siRNA were rested overnight. The next day $0.5x10^6$ cells per condition were stimulated with

CD3, CD4, and/or CD28. 24 and 48 hours after TCR stimulation, supernatants were collected and IL-4 and TNF-α concentrations were determined by ELISA.

3.4.11 PLCγ1 deficient Jurkat have reduced tyrosine phosphorylation induced by Tim-1

We next wished to determine the mechanism underlying PLCγ1 independent, but CD3, CD28, and Tim-1 dependent, NFAT/AP-1 activation in Jurkat T cells. First, since the PLCγ1-independent effect required co-stimulation by anti-TCR and anti-CD28 together, we investigated the patterns of pY in Tim-1-expressing T cells stimulated with anti-TCR alone as compared to both anti-TCR and anti-CD28. Overall, the pY pattern in Jγ cells did not differ much regardless of type of stimulation, though more numerous bands were apparent at 20 minutes. Interestingly, there was a slight induction of a band below 75kD in the TCR/CD28 co-stimulated cells that is less prominent in the anti-TCR alone lanes (Fig. 3-11 A left). In addition, the Jγ stimulated cells displayed weaker pY induction as compared to the Jγ WT, Jurkat, and J.vav cells (Fig. 3-11 A).

We next studied differences in pY of specific proteins in Jγ cells transfected with empty vector or Tim-1, after anti-TCR, anti-CD28, and anti-Tim-1 stimulation. In general, pY induction is reduced in Jγ cells as compared to Jγ WT cells (data not shown). Very surprisingly, a small amount of pPLCγ1 was induced, and this band was even more prominent in anti-TCR/CD28/Tim-1 stimulated cells (Fig. 3-11 B). According to the manufacturer, their pPLCγ1 antibody does not cross react with other PLCγ members, such as pPLCγ2. However, these Jγ cells are completely deficient in PLCγ1, which might suggest that there is some cross reactivity with PLCγ2, or even cross-reacting with other proteins of the same size. There are small differences in tyrosine phosphorylation between anti-TCR/CD28 stimulated control or Tim-1-

expressing cells. One such difference is the induction of pERK1/2 found in Tim-1 expressing cells co-stimulated with anti-TCR/CD28 (Fig. 3-11 B). The greatest induction of pPLCγ1, pZAP-70, and pp38 is found in Tim-1 expressing cells stimulated with anti-TCR/CD28/Tim-1 (Fig. 3-11 B). This suggests that cross-linking Tim-1 in the presence of TCR/CD28 co-stimulation might rescue some important pY proteins and thus contribute to the enhanced NFAT/AP-1 activation seen in Jγ cells.

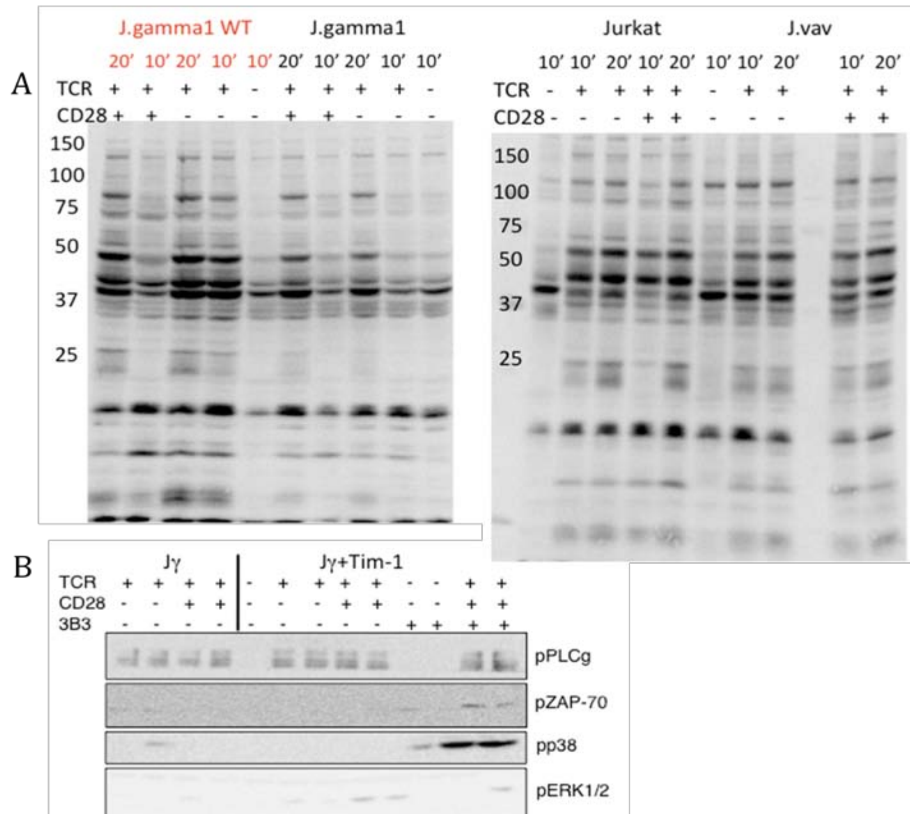


Figure 3-11: PLCγ1 deficient Jurkats have decreased phosphorylation

(A) Jγ1 or Jγ1 WT cells transfected with Tim-1 were stimulated with anti-TCR with or without anti-CD28 for varying times. Lysates were run on SDS-PAGE gels and immunoblotted with anti-pY. (B) Jγ1 cells were transfected with empty vector or Tim-1. Cells were stimulated with anti-TCR or anti-CD28 in the presence or absence of anti-Tim-1. Lysates were separated on SDS-PAGE gel and blotted for a variety of phosphorylated proteins.

3.4.12 Tim-1 requires Ca^{2+} for NFAT/AP-1 reporter activity

Due to the dramatic effect of Tim-1 on NFAT/AP-1 and pure NFAT activation and the dependence of NFAT induction on calcium, we examined the relationship between Tim-1 and calcium. First, we asked whether the Tim-1, TCR, and CD28 induction of NFAT/AP-1 transcriptional activity is dependent on the presence of extracellular calcium. We incubated Tim-1 expressing Jurkat cells in the presence of 2mM EGTA to chelate extracellular calcium. While Tim-1-transfected PLCγ1-deficient Jurkat T cells are able to induce NFAT/AP-1 activation, treatment with EGTA abrogates this effect (Fig. 3-12 A). This suggests that Tim-1 activation of NFAT/AP-1 in the absence of PLCγ1 is still dependent on increased intracellular calcium.

Given the above findings, we hypothesized that Tim-1 could potentially enhance calcium flux in a PLCγ1-independent manner. Using the ratiometric dye indo-1, we compared the calcium flux between Jγ cells transfected with empty vector or Tim-1. After analyzing the ratio of bound to free calcium, we were unable to detect a significant difference between empty vector and Tim-1 expressing Jγ cells. Surprisingly, stimulated Jγ cells that did not express Tim-1 had slightly enhanced calcium flux in the presence of TCR and CD28 stimulation (Fig. 3-12 B). Since these studies were conducted in transiently transfected Jγ cells, it was possible that the inability to detect differences in Ca^{2+} flux depends on the varying levels of Tim-1 expression from cell to cell. Thus, we gated the Tim-1 transfected Jγ cells for Tim-1 expression (Flag staining), and separated these into two populations-those with high levels of Tim-1 and those with low levels. The level of Flag (i.e. Tim-1) expression had no effect on modulation of calcium flux (Fig. 3-12 C). Thus, while calcium is required for the Tim-1-dependent but PLCγ1-

independent, stimulation of NFAT/AP-1 activation, ectopic expression of Tim-1 itself is not sufficient for enhancing calcium flux. Another molecule must therefore be required for the Tim-1 and TCR/CD28 co-stimulation dependent effects on transcriptional activation.

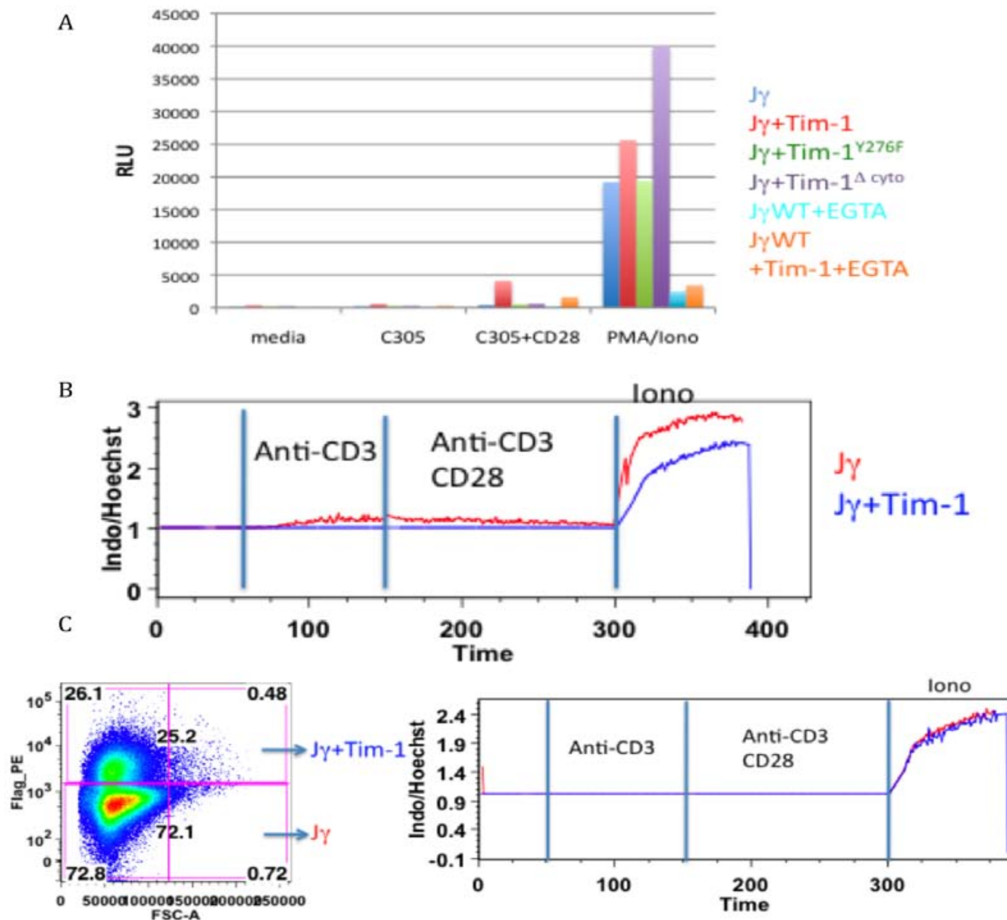


Figure 3-12: Tim-1 induction of NFAT/AP-1 activation requires Ca^{2+} but does not influence Ca^{2+} flux

(A) Jγ1 or Jγ1 WT cells were transfected with an NFAT/AP-1 luciferase reporter and empty vector, Tim-1, Tim-1^{Y26F}, or Tim-$1^{\Delta Cyto}$ construct in the presence or absence of 2mM EGTA. (B) Jγ1 cells were transfected with empty vector or Tim-1. The next day cells were loaded with Indo-1 and stimulated with anti-CD3, CD28, or ionomycin. The Ca^{2+} flux was determined by flow cytometry. Indo-1 ratio of bound to free Ca^{2+} was analyzed using FloJo software. (C) Tim-1 transfected Jγ1 cells were stained for Flag-Tim-1 (M2) expression after being loaded with

indo-1. Ca^{2+} flux was determined as above. Cells were gated based on Flag expression and analyzed using FloJo.

3.4.13 Akt inhibition of Tim-1 expressing cells enhances NFAT/AP-1 activation

Tim-1 enhancement of transcriptional activity independent of PLCγ1 has been most consistent in the PLCγ1 deficient Jurkat line, Jγ. One of the best understood defects in Jurkat T cells is the increased phosphorylation of Akt due to lack of the phosphatases PTEN and SHIP (153). In addition, work from this lab and others has demonstrated that Tim-1 overexpression and ligation by one of its ligands, Tim-4, is able to induce phosphorylation of Akt (13). In particular, our lab has shown that the p85 subunit of PI3K binds the Tim-1 cytoplasmic tail in an Lck-dependent manner. This binding leads to the phosphorylation of Akt and induction of IL-2 expression (64). T cells from p85 α/β double knockout mice are unable to induce IL-2 in a Tim-1 dependent manner (64). We hypothesized that Tim-1 binding and activation of Akt might help compensate for loss of PLCγ1 to enhance NFAT/AP-1 activation in Jγ cells. To test this hypothesis, we treated Tim-1 transfected Jγ and JγWT cells with the Akt inhibitors Ly294002 and Akti. Surprisingly, treatment with either Ly294002 or Akti in the presence of TCR and CD28 co-stimulation enhance Tim-1 mediated NFAT/AP-1 activation (Fig. 3-13 A-C). This effect is not dependent on the dose used since concentrations from 0.5-5μM of Akti do not decrease the reporter activity. This is especially unanticipated because work from our lab has previously demonstrated that p85 and Akt activity are necessary for Tim-1 induced NFAT/AP-1 activation in the parental Jurkat line.

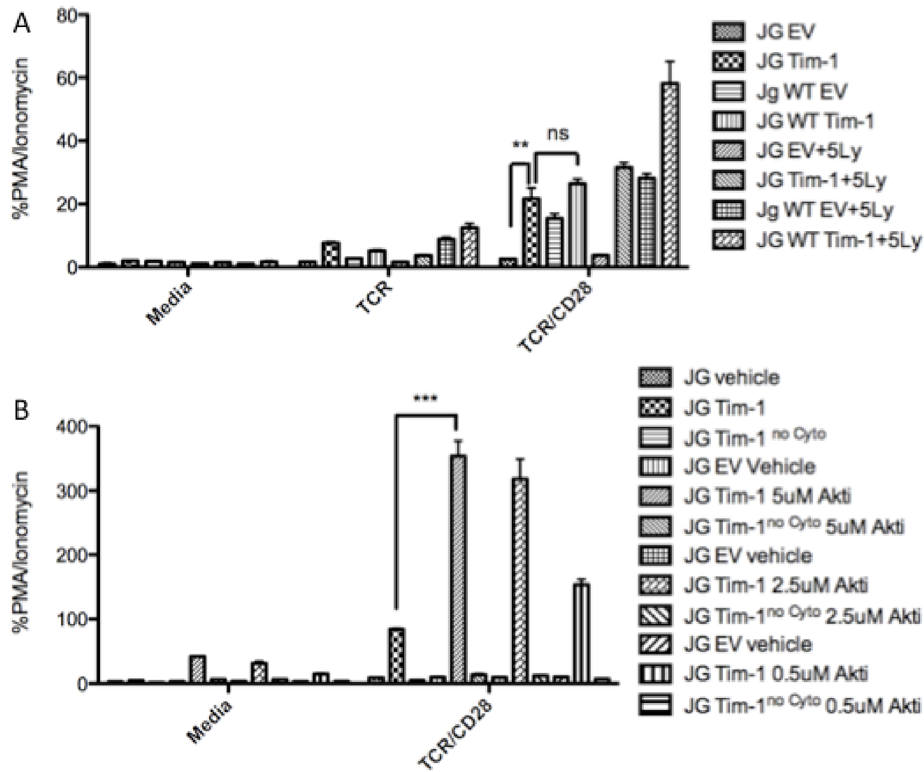


Figure 3-13: Inhibition of the PI3K pathway enhances Tim-1 induced NFAT/AP-1 activation

Jγ1 and Jγ WT cells were transfected with NFAT/AP-1 reporter with or without Tim-1 in the presence of a Ly (A) or varying concentrations of Akt*i* (B). Luciferase activity was assayed as described previously.

Since some Akt inhibitors have been shown to paradoxically increase activation, we utilized another approach to better delineate a role for Akt in these Jγ and JγWT cells. The p85β subunit of PI3K is the dominant subunit in primary mouse T cells. Thus, we attempted to knockdown p85β and assess its effects on NFAT/AP-1 activation. In Jγ cells, transfection of p85β siRNA in conjunction with Tim-1 and anti-TCR/CD28 did not alter NFAT/AP-1 activation (Fig. 3-14 top). In contrast, in JγWT cells, p85β knockdown decreased NFAT/AP-1 activity

(Fig. 3-14 middle). It was challenging to determine the amount of p85β knockdown, since it was difficult to separate out the p85 α and β bands well enough to distinguish between the two isoforms. However, there does appear to be some knockdown, especially in the JγWT when 10μg of shRNA were transfected (Fig. 3-14 bottom). It is possible that the failure to reduce NFAT/AP-1 activity in the Tim-1 expressing Jγ cells could be due in part to incomplete p85 knockdown. Another possibility is that partial or complete knockdown of both p85 isoforms is required for inhibition of Tim-1 and co-stimulation induced reporter activity.

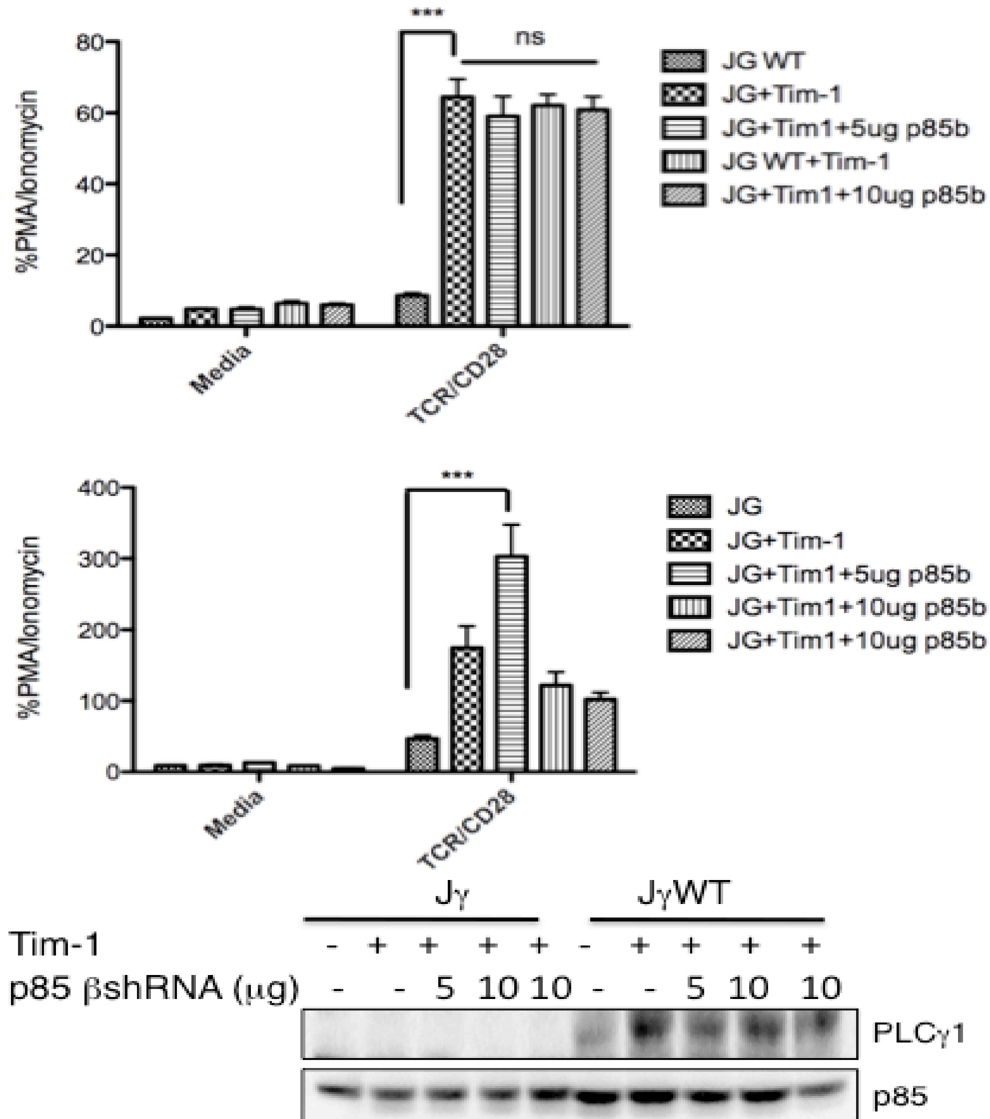


Figure 3-14: p85β is not required for Tim-1 induced NFAT/AP-1 activation

Jγ1 (top) and Jγ WT (middle) cells were transfected with empty vector or Tim-1 in the presence or absence of p85β siRNA. Luciferase activity was determined as done previously. Expression levels of PLCγ1 and p85 were assessed by immunoblotting (bottom).

3.4.14 Vav1 is not sufficient for Tim-1 mediated signaling

Vav1 is another intriguing candidate for mediating PLCγ1-independent but Tim-1 and co-stimulation dependent transcriptional activity. Vav1 has well established roles in enhancing NFAT/AP-1 activation, and multiple pathways have been implicated (154). Vav1 is necessary for the phosphorylation of PLCγ1 and PLCγ2 as well as Ca^{2+} mobilization in mast cells (155). Signaling between Vav1, possibly through interactions mediated by SLP-76 and Nck, and Pak may also lead to NFAT/AP-1 activation (156, 157). Vav1 has even been shown to facilitate NFAT movement to the nucleus to become transcriptionally active (158). Considering these important signaling pathways requiring Vav1, we hypothesized that the effect of Tim-1 on transcriptional activity might be mediated through Vav1. To investigate the requirement for Vav1 in Tim-1 induced NFAT/AP-1 activation, we transfected Tim-1 or Tim-$1^{\Delta Cyto}$ into Vav1-deficient Jurkat cells (J.Vav) and assayed for NFAT/AP-1 luciferase activity. Similar to PLCγ1 deficiency, Tim-1-expressing J.Vav cells are still able to induce NFAT/AP-1 activation in the presence of TCR and CD28 co-stimulation. Of interest, this enhancement of reporter activity appears to be less dependent on co-stimulation, since activation by anti-TCR alone also increases the induction of NFAT/AP-1 (Fig. 3-15 A). This does appear to be a Tim-1 mediated effect since the cytoplasmic tail truncation of Tim-1 is unable to enhance transcriptional activation.

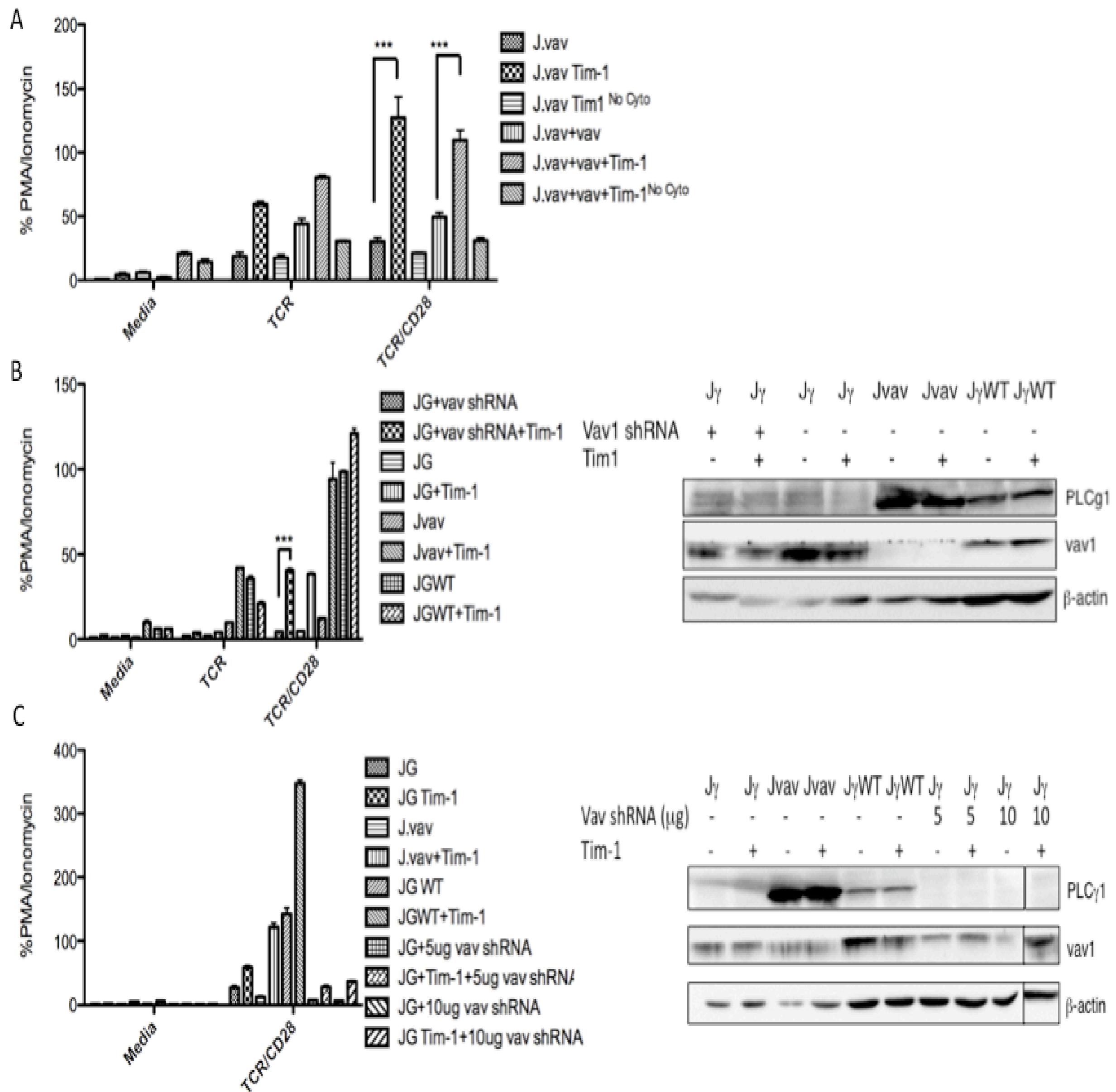


Figure 3-15: Vav1 is not necessary for Tim-1 induced NFAT/AP-1 activation

(A) Jvav cells were transfected with an NFAT/AP-1 reporter in the presence of empty vector, Tim-1, or Tim-1$^{\Delta Cyto}$. Luciferase activity was determined as done previously. (B) Jvav, Jγ1, and Jγ WT were transfected with and NFAT/AP-1 reporter in the presence or absence of Tim-1 with or without Vav1 shRNA. Expression of PLCγ1, vav1, and β-actin were determined by immunoblotting. (C) Jvav, Jγ1, and Jγ WT were transfected with and NFAT/AP-1 reporter in the

presence or absence of Tim-1 with varying concentrations of Vav1 shRNA. Protein levels were determined by immunoblotting. Each luciferase assay is a representative image from at least three separate experiments.

Since lack of Vav1 alone is insufficient to abrogate the Tim-1 and co-stimulation induced NFAT/AP-1 activity, we next examined the possibility that this transcriptional upregulation requires both PLCγ1 and Vav1. Towards this goal, we knocked down Vav1 in the PLCγ1 deficient line, Jγ, and determined the necessity of both molecules for Tim-1 induced NFAT/AP-1 activity. Knockdown of Vav1 in the absence of PLCγ1 but presence of Tim-1 and co-stimulation demonstrated similar levels of NFAT/AP-1 induction as the lack of PLCγ1 alone (Fig. 3-15 B). It is interesting to note that the J.Vav cells express higher levels of PLCγ1, which might contribute to enhance NFAT/AP-1 activation. Thus, partial loss of Vav1 and absence of PLCγ1 are not sufficient to attenuate Tim-1 mediated NFAT/AP-1 activity.

We next determined whether greater Vav1 knockdown was necessary for the Tim-1 mediated increased transcriptional activity. Co-transfection with higher levels of Vav1 shRNA in the control (empty vector) cells induced more efficient Vav1 knockdown. Interestingly, co-transfection of Vav1 shRNA with Tim-1 resulted in less Vav1 knockdown in the Jγ cells (Fig. 3-15 C). The presence of Tim-1 appears to rescue Vav1 expression or to interfere with Vav1 shRNA expression. This increased Vav1 in the presence of Tim-1 was observed in three out of four experiments. Overall, even in the absence of PLCγ1, increased Vav1 knockdown was insufficient to abrogate the Tim-1 mediated NFAT/AP-1 transcriptional activity. PLCγ1-deficient-Vav1 reduced Jurkat T cells are still able to enhance NFAT/AP-1 activation.

3.4.15 Tim-1 does not bind PLCγ2 but induces a tyrosine phosphorylated band around 150kD

PLCγ2 shares many similarities with PLCγ1. Although PLCγ1 is the predominant isoform found in T cells, PLCγ2 can also be detected in T cells (159). Reports from the literature have demonstrated that sometimes loss of multiple family members are necessary for loss of function in different systems. For instance, the phenotype of Lck knockout mice were not as dramatic as anticipated. This was because Fyn, another Src family kinase, can partially compensate for the loss of Lck (160, 161). Thus, we hypothesized that in the absence of PLCγ1, its closely related family member, PLCγ2, might serve a similar function and rescue downstream signaling. Jγ cells do not express PLCγ1 but do express PLCγ2 (Fig. 3-14b). We were not able to detect a direct interaction between PLCγ2 and Tim-1 by Co-IP nor were we able to detect tyrosine phosphorylation of a band around the size of Tim-1 (Fig. 3-14c). However, we were able to visualize pY of a band around 150kD that was induced by anti-TCR and anti-TCR/CD28 co-stimulation (Fig. 3-14c). This band could potentially be phosphorylation of PLCγ2 since Jγ cells do not express PLCγ1 (Fig. 3-14c and a). In addition, the pattern of pY in the Tim-1 transfected, TCR/CD28 stimulated cells is different than cell transfected with empty vector and Tim-1 cytoplasmic tail truncation mutant. In particular, there is greater induction of some lower molecular weight bands (Fig. 3-14c). These data suggest that while Tim-1 may not directly interact with PLCγ2, it may still induce some signaling downstream of PLCγ2 to impact T cell activation.

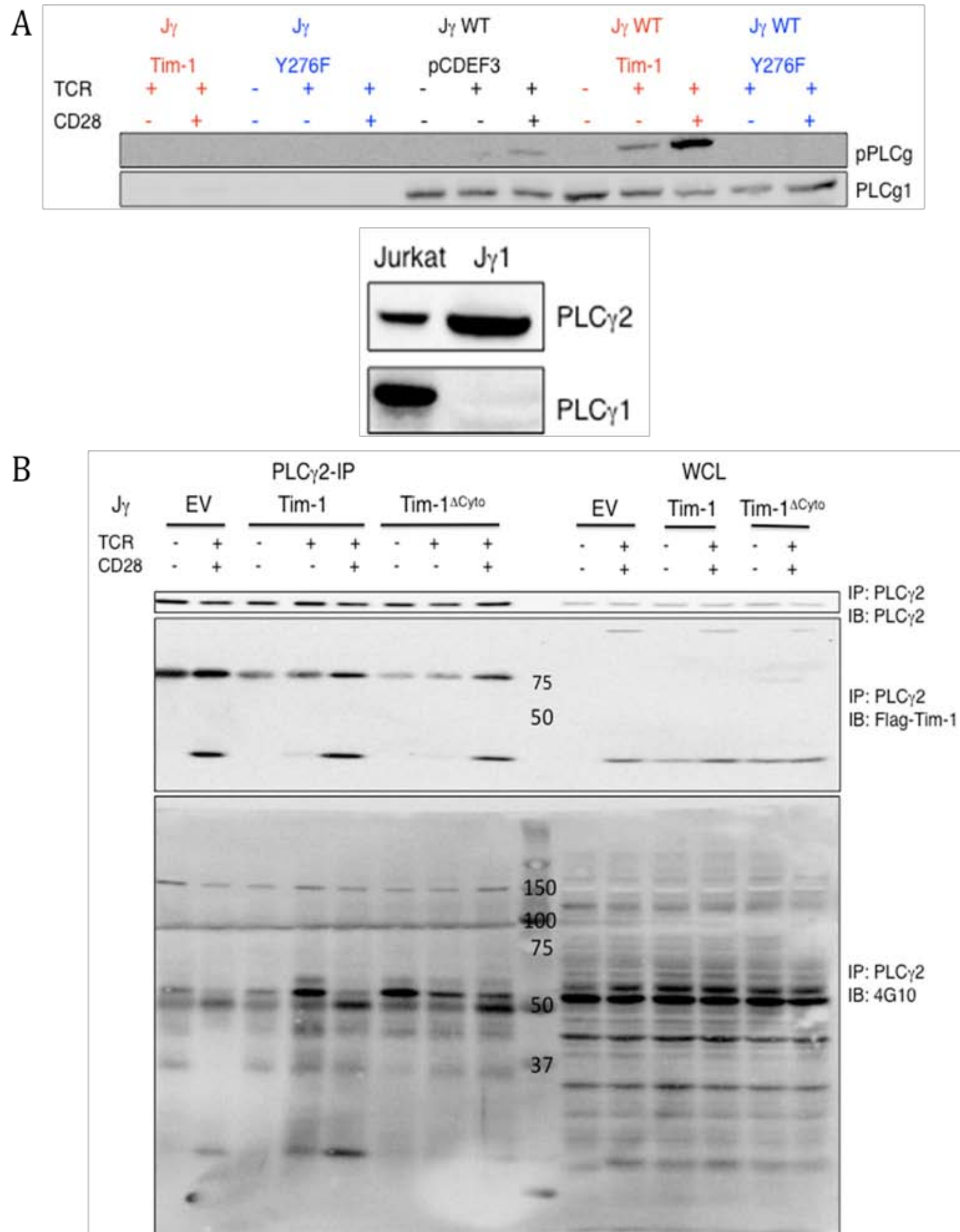


Figure 3-16: PLCγ2 is expessed in Jγ cells

(A) One million Jγ1 and Jγ WT cells transfected with empty vector, Tim-1, or Tim-1^{Y276F} were stimulated with anti-TCR or anti-CD28. The lysates were separated on SDS-PAGE gel and blotted for pPLCγ1 as well as total PLCγ1. (B) One million Jγ1 and Jurkat T cells were lysed and blotted for PLCγ2 before stripping and reprobing for PLCγ1. (C) Jγ1 cell lysates were IP'ed with

2μg of PLCγ2. The lysates were separated by SDS-PAGE gel electrophoresis and blotted for PLCγ2, Flag-Tim1, and pY.

3.5 DISCUSSION

Here we identify multiple Tim-1 binding partners and patterns of differentially phosphorylated proteins downstream of Tim-1 signaling. Specifically, we demonstrated that although Tim-1 can bind PLCγ1, Tim-1 does not necessarily mediate its signaling events through PLCγ1. Ectopic expression of Tim-1 in conjunction with TCR and CD28 co-stimulation leads to induction of NFAT/AP-1 activity even in the complete absence of PLCγ1 in Jurkat T cells. Knockdown of PLCγ1 in the D10 line was also able to recapitulate this finding. This PLCγ1-independent effect requires the Tim-1 cytoplasmic tail and Ca^{2+} but does not require Akt and Vav1, although PLCγ2 may be involved.

However, these are data that still need to be reconciled. While D10 cells also have Tim-1 mediated NFAT/AP-1 induction, treatment with the PLCγ inhibitors, U73122 and neomycin, abrogated this reporter activity, suggesting that some PLCγ (possible PLCγ2) is required for enhanced reporter activity. In contrast, treatment with U73122 in Tim-1 expressing Jγ and JγWT cells (derived from Jurkat T cells) was not able to attentuate NFAT/AP-1 activation indicating that PLCγ family members are not necessary for Tim-1 signaling and that additional or different molecules are involved in Jurkat T cells but not D10 T cells.

Further, the PLCγ-dependent expression of early markers of activation and cytokine production does not correlate with the enhanced NFAT/AP-1 transcriptional activity. When

PLCγ1 was knocked down in Tim-1 expressing D10 cells, cytokine production of IL-4 decreased. Conversely, while treatment with anti-Tim-1 antibodies can enhance cytokine production and expression of early markers of activation, inhibition of PLCγ by U73122 attenuates the Tim-1 induced IL-2 production as well as CD25 and CD69 surface expression. Tim-1 signaling through PLCγ1 appears to be more critical for cytokine expression in D10 and primary $CD4^{+}$T cells than in Jurkat cells. NFAT/AP-1 is not the only transcription factor induced to regulate early markers of activation, including CD25 and CD69, or to enhance cytokine production, IL-2 or IL-4. One possibility is that while Tim-1 can compensate for lack of PLCγ1, other proteins that still remain to be identified influence other transcription factors and T cell activation. It would be interesting to perform a transcriptional microarray analysis on the different T cell lines in the presence or absence of Tim-1 and CD3/CD28 co-stimulation with or without PLCγ inhibition to determine a profile of genes differentially regulated under these conditions. Transcriptional microarray could identify novel targets downstream of Tim-1-dependent and PLCg-independent signaling.

One intriguing candidate protein that might confer some functional compensation on T cell activation in the absence of PLCγ1 is its close family member, PLCγ2. While a role for PLCγ1 in T cell activation is well accepted, a role for PLCγ2 in T cell signaling was unknown, and PLCγ2 has often been considered to be more important for BCR signaling. However, recent work has begun to establish a role for PLCγ2 in TCR mediated activation. A recent report demonstrated that PLCγ2 can associate with LAT and SLP-76 and that PLCγ1/PLCγ2 double deficient mice have more defects in T cell activation than PLCγ1 deficient mice (159). These papers suggest that PLCγ2 may have a greater role in T cell activation than previously appreciated. Also, it has been reported that even a when 90% of PLCγ1 is knocked down, there

is relatively normal T cell function as described by normal transcriptional activation and calcium flux (144). This suggests that perhaps even a small amount of PLCγ2, if it functions similar to PLCγ1, may be sufficient to compensate and induce transcriptional activity. PLCγ1 deficient Jurkat T cells express PLCγ2, so in these experiments PLCγ2 may be interacting with Tim-1 and compensating for loss of PLCγ1 in Jγ cells. Although we were not able detect a direct interaction between Tim-1 and PLCγ2 by co-IP, this does not mean that there is no interaction. There could be an indirect interaction whereby Tim-1 binds an intermediate protein that in turn activates PLCγ2. In this case we would not be able to detect a direct interaction between Tim-1 and PLCγ2. Alternatively, the interaction between Tim-1 and PLCγ2 may be very weak. In this case, the low affinity interactions between the proteins might be destroyed upon lysis during the co-IP process. There is a band phosphorylated at approximately 150kD, which is suggestive of PLCγ2 is phosphorylation in the Tim-1 expressing and co-stimulation induced Jγ cells. Detection of pPLCγ2 with a phosphospecific antibody would be a more specific indication of PLCγ2 activation. Further, determining the effect of knockdown of PLCγ2 in Tim-1 expressing Jγ cells on NFAT/AP-1 activation would also be critical in determining its importance in this signaling pathway. A caveat with this hypothesis is the effect of chemical inhibition of PLCγ by U73122 or neomycin. In my data, it appears that U73122 and neomycin are not able to abrogate Tim-1 and co-stimulation induced NFAT/AP-1 activation in Jγ or JγWT cells though the inhibitors can block reporter activity in D10 T cells, as well as cytokine expression and early activation marker upregulation in primary $CD4^+$ T cells. One possibility is that these inhibitors might have off target effects and block more than just PLCγ. The mechanism behind U73122 inhibition of PLCγ remains poorly understood, and at least one study in smooth muscle cells has

shown that U73122 blocks Ca^{2+} release through its effects on the sarcoplasmic reticulum calcium ATPase pump rather than through PLC (162). Hence, U73122 may not be the most specific inhibitor and may be interfering with an unknown factor impacting activity in the Jγ T cells. Alternatively, the paradoxical effects on transcriptional and cytokine activity might be due to the different levels of PLCγ1 and PLCγ2 in these cell types, i.e. D10 cells may express less PLCγ2 than Jγ cells, which may have functional consequences. Better characterization of the expression and activation of PLCγ2, as well as the downstream pathways in these different cell types, Jγ, JγWT, D10, and primary $CD4^+$ cells, will be necessary to clarify the role of PLCγ1 and PLCγ2 in Tim-1 function. While the above effects might be due to the different levels of PLCγ1 and PLCγ2 found within these cells types and the ability of PLCγ isoforms to compensate in function, more work will be necessary to uncover the specific mechanism(s) underlying the effect on transcriptional and cytokine activity.

Further investigation of the role of Vav1 in Tim-1 mediated signaling may also be warranted. While Vav1 deficiency is not sufficient to attenuate Tim-1 and co-stimulation mediated NFAT/AP-1 activation, this may in part be due to the increased levels of PLCγ1 found in the J.Vav cells or the ability of different Vav isoforms to compensate for Vav1. Similarly, knockdown of Vav1 in PLCγ1 deficient Jurkat T cell is not able to abrogate the Tim-1 co-stimulation of NFAT/AP-1 activation. This could suggest that complete deficiency of Vav1 is necessary to observe a decrease in reporter activity. To really address this issue, it would be necessary to either completely knockdown both PLCγ1 and/or PLCγ2 in the Vav1 deficient cells or to knockdown more of the Vav isoforms in the PLCγ1 deficient Jurkat T cells. Alternatively, if deficiency of all the Vav isoforms is necessary to abrogate all Tim-1 mediated signaling effects, then one might assay for cytokine production in $CD4^+$ T cells from Vav triple knockout

mice stimulated with varying concentrations of CD3/CD28 and anti-Tim-1 antibodies and compare these to $CD4^+$ T cells from littermate controls treated under the same conditions.

Another puzzling aspect is the discrepancies on the role of PI3K and IL-2 production between this data and previously published work. For instance, previous work by this laboratory has demonstrated that inhibition of Akt by Ly294002 in Jurkat T cells is able to abrogate the Tim-1 and co-stimulation induced NFAT/AP-1 activation and that in primary T cells deficient in both p85α and β Tim-1 was unable to induce IL-2 production (64). However, in this work, chemical inhibition of the PI3K pathway resulted in enhanced NFAT/AP-1 reporter activity. This could be due to some intrinsic difference in the Jγ cells after somatic hypermutation as compared to their parental Jurkat T cells. Also, perhaps less surprising, p85β knockdown alone was insufficient to abrogate Tim-1 and co-stimulation induced NFAT/AP-1 activation. It is likely that knockdown of both p85 α and β would be required to observe an effect on reporter activity. Another divergence from the literature is that the high affinity anti-Tim-1 antibody, 3B3, has been shown to induce IL-2 production (33), whereas my data suggest that 3B3 attenuates IL-2 production while the lower affinity RMT1-10 enhances IL-2 levels. This could be possibly due to differences in the batch of antibody or differences in CD3 and CD28 stimulation conditions. The differential effects of the Tim-1 antibodies is at least consistent with the literature suggesting that treatment with these antibodies have diverse effects on cytokine production and disease progression.

The extracellular domain of Tim-1 has been suggested to be important for asthma susceptibility and ligand binding. However, whether mutations in the extracellular domain contribute to differences in signaling has not been extensively studied. Tim-1 structural predictions suggest that Tim-1 may interact in a homotypic manner, and that this binding

depends on motifs found in its IgV domain, specifically H64. To investigate the functional consequences of interfering with this binding, we mutated the histidine to a glutamine. While there is no difference in NFAT/AP-1 activation between the WT Tim-1 and Tim-1^{H64E} constructs, there may be a decrease in Elk-1 activation. Thus, homotypic Tim-1 interactions may only transduce a specific subset of signaling pathways that would allow for finer tuning of the Tim-1 response. The mucin domain has also been implicated in affecting T cell function, and the C57Bl/6 form of Tim-1 with a longer mucin domain appears to have greater transcriptional activity than the BALB/c form.

Although the majority of this work focused on the physical and functional interactions between Tim-1 and PLCγ1, multiple other putative binding proteins were identified that might also play roles in Tim-1 induced T cell activation. One protein identified, BAT2, may be of interest based on its potential association with Grb2, a well known signaling adaptor molecule (163). These putative interactions could be exciting for a few reasons. One possibility is that Tim-1 might interact with BAT2 and Grb2 and lead to activation of MAPKinase pathways. This could be one of the mechanisms by which Tim-1 induces increased elk-1 activation. Another option is a role for Tim-1/BAT2/Grb2 in microcluster formation. As described in the previous chapter, Tim-1 forms microclusters upon activation by anti-TCR/CD28. A 2011 paper on BCR microclusters demonstrated that Grb2 is found in BCR microclusters and important for coupling to dynein and proper movement (but not formation) of the micrclusters (164). Less is known about the importance of the Grb2 adaptor protein and movement. An appealing possibility is that Tim-1 may bind BAT2 and Grb2 and subsequently influence dynein mediated TCR microcluster movement.

Another intriguing and logical binding partner is Fyn, a member of the Src family of

tyrosine kinases, which are important for inducing early TCR signals. Further, Fyn has been demonstrated to bind, phosphorylate, and interact with Tim-1 in B cells (147). While my work did not consistently demonstrate Tim-1 binding to Fyn, occasionally one could observe some interaction that was not always dependent on pervanadate stimulation. This could indicate that there is only weak binding affinity between Tim-1 and Fyn that was not well preserved during the co-IP process. Even if there is only a transient physical interaction, Fyn could still be phosphorylating Tim-1. A kinase assay would be necessary to definitively rule include or out a role for Fyn in phosphorylating Tim-1.

Other interesting binding partners are the multiple translation factors. T cell activation also requires co-stimulation to undergo selective metabolic changes to expand and proliferate after activation. CD28, one of the best-known T cell co-stimulatory molecules, regulates survival, translation, and metabolism via PI3K (145). Tim-1 also signals through PI3K and may work in a similar manner (64). Thus far Tim-1 has only been shown to regulate T cell activation by influencing cytokine expression. However, CD28 promotes T cell survival by regulating protein translation of Bcl-XL via PI3K phosphorylation of 4E-binding protein-1 phosphorylation, which allows for initiation of the eIF4F translation complex (165). Since Tim-1 also signals through PI3K and enhances mRNA of anti-apopotic molecule Bcl-2 (41, 64), Tim-1 may have a role in T cell activation and survival by signaling through PI3K to induce anti-apoptitc factors, Bcl-XL and Bcl-2. Further, the association between the cytoplamsic tail of Tim-1 and translation initiation factors, eIF4G and eIF4A, suggest that Tim-1 may also induce expression of anti-apoptotic factors by initiation of the eIF4F complex.

T cells must also initiate metabolic changes in preparation for expansion. CD28 supports aerobic glycolysis in T cells by upregulating GLUT1 via PI3K (166). Tim-1 may potentially

bind the important metabolic factor, ACC1, may work in a similar manner to allow for glucose accumulation. It could be interested to determine whether Tim-1 in the presence of co-stimulation signals through PI3K and ACC1 to mediate any changes in GLUT1 expression.

While the effects of anti-Tim-1 antibodies and ligands in T cell differentiation have been studied in animal models of disease, the underlying intracellular signaling pathways mediating these effects are unclear (13, 34, 35, 41, 58). Our data contributes to these findings by demonstrating differential tyrosine phosphorylation of various proteins downstream of anti-Tim-1 cross-linking in the presence or absence of CD3/CD28 co-stimulation. Specifically, we identified a protein, Lrch3, around 75kD that is induced by treatment with some but not all anti-Tim-1 antibodies. While little is known about Lrch3 except for a possible role in *E. coli* susceptibility in pigs (167), more is known about *dLRCH*, the drosophila homolog. There is a great deal of sequence homology between the murine and drosophila Lrch proteins(141). It has been suggested that Lrch binds to ERM proteins by yeast two hybrid but this binding has not been confirmed (141, 168). Lrch proteins have been suggested to be cytoskeletal scaffolding proteins. In particular, *dLRCH* appears to be important for cellular division, particularly proper localization of the mitotic spindle. Further *dLRCH* has been implicated fertility and fitness under conditions of stress (141). Cross-linking Tim-1 by anti-Tim-1 antibodies has also been implicated in enhanced cellular proliferation and survival (41). Since Tim-1 co-localizes with ERM proteins and Lrch putatively binds ERM, Tim-1-Lrch-ERM may form a complex that influences cellular division and to promote T cell activation and differentiation.

Tim-1-expressing cells incubated with PS have increased Akt and ERK1/2 phosphorylation upon CD3/CD28 co-stimulation. However, the Tim-1:PS effects on T cell activation appear to be less striking than the effects on iNKT activation (58). This could be due

to differences cell type, systems utilized, or activity measured. The iNKT study used primarily primary cells, stimulation with α-GalCer, PS stimulation by PS-coated liposomes, and measured cytokine activity (58). These data used T cell lines, anti-CD3/CD28 stimulation, apoptotic PS-expressing thymocytes, and measured NFAT/AP-1 activity and phosphorylation. Since thymocytes have a tendency to rapidly undergo apoptosis, it is possible that the "viable" thymocytes had started expressing PS, especially at the later time points, and this could contribute to the smaller differences between phosphorylation dectected in Tim-1 cells incubated with apoptotic or "viable" thymocytes. Thus, another, reductionist approach to study the Tim-1 interaction would be to incubate PS-coated liposomes or related control phospholipid that Tim-1 does not bind, i.e. phosphatidylcholine (49), to Tim-1-expressing T cells and to measure intracellular phosphorylation or cytokine production. This might yield insights to clarify the role of PS interactions with Tim-1 in T cells.

Taken together these data refine our understanding of the pathways and mechanisms underlying Tim-1 mediated signaling. We have provided mechanistic insight into how differential Tim-1 ligation may lead to diverse outcomes, possibly through the selective tyrosine phosphorylation of select proteins. We have demonstrated that Tim-1-expression can bypass the requirement for PLCγ1 for the induction of transcriptional activity. Investigating the role of these Tim-1 interacting proteins could lead to greater insight into how Tim-1 functions *in vivo*.

4.0 SUMMARY/FUTURE DIRECTIONS

Tim-1 is a member of a family of transmembrane receptors with roles in immune regulation. Original work from this lab demonstrated that Tim-1 functions as a co-stimulatory molecule to enhance T cell activation downstream of TCR/CD28 co-stimulation in a Y276 dependent manner(33, 169). Although initial work suggested that activation of Tim-1 resulted in increased Th2 phenotype, a plethora of work has arisen suggesting that Tim-1 may more broadly regulate T cell activation in a both positive and negative manner (34, 170, 171). At the initiation of this thesis work, the mechanism by which Tim-1 mediates these activities was unclear. In addition, where Tim-1 localizes in response to activation by APCs was not understood. Thus, this work has focused on identifying Tim-1 binding partners and characterizing its localization upon activation by APCs.

In this thesis, I have added to our knowledge of Tim-1 signaling and localization. In Chapter 2, multiple putative Tim-1 binding partners were identified. In particular, Tim-1 was shown to bind PLCγ1, but could activate NFAT/AP-1 transcription in a PLCγ1-independent, but still TCR/CD28-dependent, manner. Chapter 2 demonstrated that Tim-1 forms unconventional microclusters and, in contrast to most co-stimulatory molecules, ultimately localizes opposite the immunological synapse upon activation by APCs. Furthermore, this DPC localization is mediated in part by ERM binding proteins and influences transcriptional activity and cytokine

production. These data suggest that Tim-1 does not function or localize like other co-stimulatory molecules and reveals alternative ways to consider Tim-1 function.

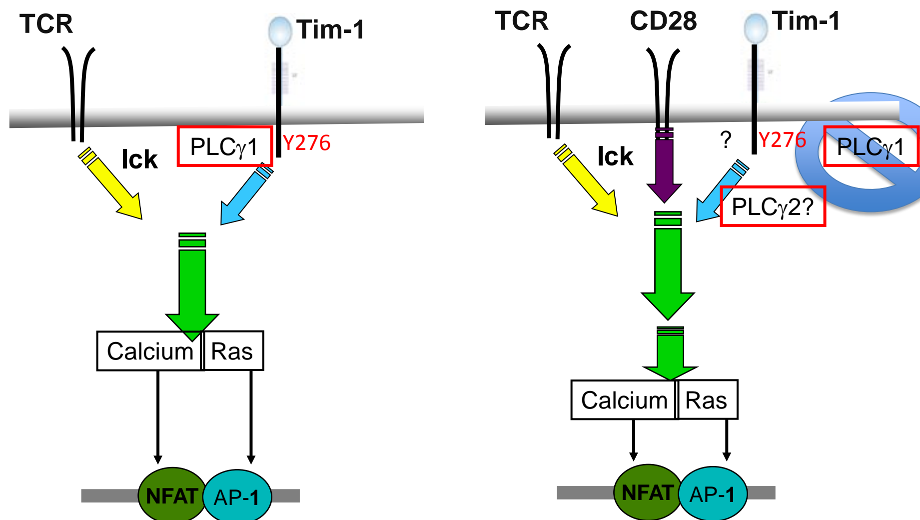


Figure 4-1: Model of Tim-1 Signaling

In the presence of TCR alone, Tim-1 induction of NFAT/AP-1 activation is dependent on PLCγ1 (left). However, in the absence of PLCγ1, Tim-1 is still able to induce NFAT/AP-1 activation in a TCR and CD28-dependent manner (right).

4.1 TIM-1 MAY HAVE BROADER CO-STIMULATORY FUNCTIONS

The majority of Chapter 3 focuses on a physical interaction between Tim-1 and PLCγ1, and the ability of Tim-1-expressing cells to enhance NFAT/AP-1 activation in a PLCγ1-independent, but TCR/CD28 co-stimulation-dependent, manner. Although binding to PLCγ1 is decreased in the

absence of Y276 in the Tim-1 cytoplasmic tail, we were surprised to find that Tim-1 does not require PLCγ1 to induce NFAT/AP-1 activation. The PLCγ1-independent but Tim-1 and TCR/CD28 co-stimulation-dependent effect on NFAT/AP-1 activation requires extracellular Ca^{2+}, but does not require Akt phosphorylation or Vav1. Thus, at this point we hypothesize that at least in this system PLCγ2 may be compensating for PLCγ1.

Tim-1's ability to co-stimulate T cell activation has mainly been studied in the context of enhancing transcriptional activation and modulating cytokine production. However, aside from inducing signaling pathways that lead to inducible transcription, T cell activation also requires rapid enhancement of translational and metabolic processes (145). A quiescent T cell is small and utilizes relatively little energy, whereas an effector T cell must quickly increase in size and undergo glycolysis to meet the energy demands required for activation (172). Co-stimulation by CD28 enhances T cell activation, not only by generating signals that complement those emanating from CD3, but also by preparing the cell to meet the metabolic needs of becoming an effector T cell (145). Specifically, via PI3K, CD28 promotes cell survival by initiating the eIF4F translation complex to increase translation of Bcl-XL (165), and CD28 assists in aerobic glycolysis by upregulating the glucose transporter GLUT1(166). Although I did not observe a consistent interaction between Tim-1 and EIF4G by co-IP, pull-down with a peptide based on the Tim-1 cytoplasmic tail revealed other putative binding partners that regulate metabolism and translation, including ACC1 and EIF4F. Thus, verification of other interacting proteins may also expand our understanding of Tim-1 function. The putative Tim-1 binding partners that I have uncovered suggest that Tim-1 may have a more global role in T cell activation than previously appreciated.

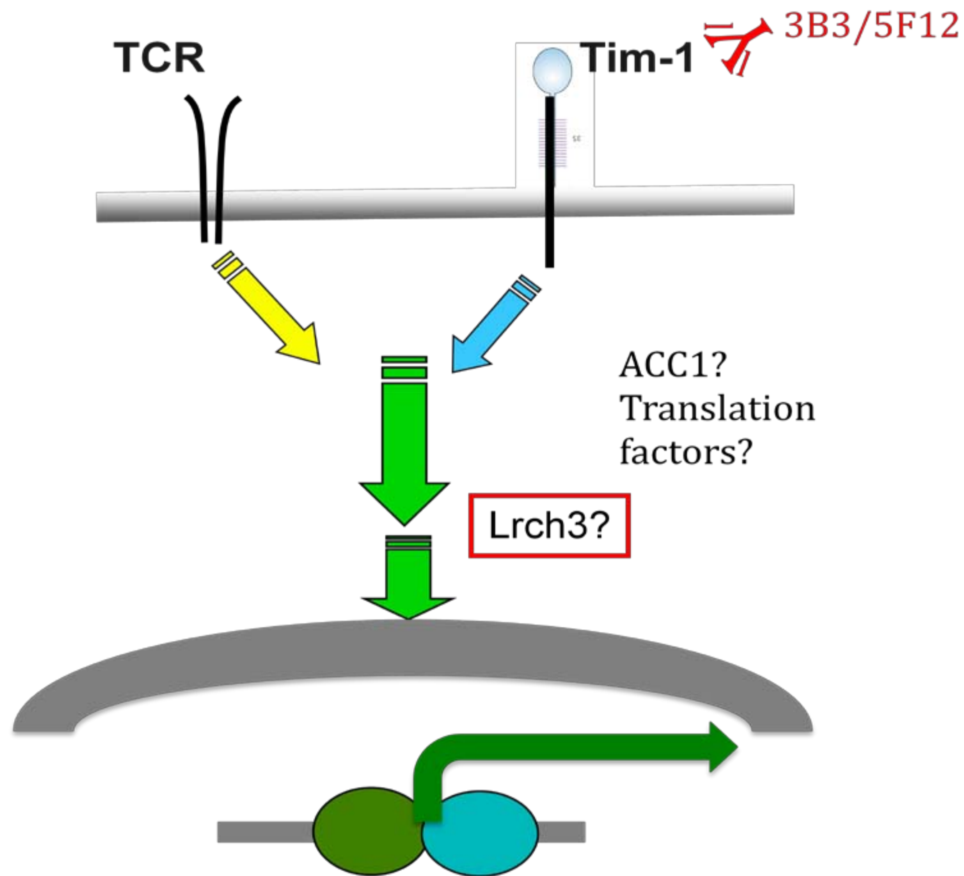


Figure 4-2: Tim-1 signaling.

Tim-1 activation by some (3B3 and 5F12) but not all antibodies can lead to the tyrosine phosphorylation of select proteins. One putative protein induced downstream of Tim-1 ligation is Lrch3. Further, a 13 amino acid peptide of the Tim-1 cytoplasmic tail is thought to bind translation and metabolic factors. This suggests that Tim-1 may have more roles in T cell activation than previously appreciated.

4.2 TIM-1 LOCALIZATION AWAY FROM THE IS

We demonstrate that Tim-1 movement towards the DPC requires one or more ERM proteins and that mutating putative ERM binding residues (KRK-QGQ) in the juxtamembrane Tim-1 cytoplasmic tail results in altered localization and decreased cytokine production but, paradoxically, enhanced tyrosine phosphorylation downstream of TCR/CD28 co-stimulation. Likewise, interference with its proper localization with an ERM-DN construct impaired the ability of Tim-1 to enhance transcriptional activation or cytokine production. The seemingly paradoxical increase in early tyrosine phosphorylation, but reduced cytokine production, induced by the Tim-1$^{KRK\text{-}QGQ}$ mutant could be due to a number of factors. First, not all tyrosine phosphorylation leads to T cell activation, as evidenced by the increase phosphorylation at inhibitory Y505 of Lck. Thus, the increased tyrosine phosphorylation could be due to phosphorylation at inhibitory sites, leading to impaired transcriptional activation and cytokine production. Second, there is evidence that cell surface vs. intracellular/vesicular BCR induces phosphorylation of distinct substrates (131). WT Tim-1 is found mostly on the cell surface, while Tim-1$^{KRK\text{-}QGQ}$ has extracellular as well as intracellular pools, which may generate two different and distinct pools of tyrosine phosphorylated proteins. A third possibility is that Tim-1$^{KRK\text{-}QGQ}$ shifts the localization of other tyrosine phosphorylated proteins, such as Lck or Fyn, and increases tyrosine phosphorylation either by prolonging their localization in peripheral signaling microclusters or by altering their interacting proteins. The enhanced tyrosine phosphorylation detected in presence of the Tim-1$^{KRK\text{-}QGQ}$ mutant happens early (within 2 min), which precedes the formation of a mature immune synapse. Thus, examining the localization of microclusters on a lipid bilayer system, where one can see the movement and formation of the SMAC, could be instrumental to understanding how early signaling differs from later signaling.

Determining the pattern of tyrosine phosphorylation at later time points, on the order of hours, may also be enlightening. For instance, while there is apparently a transient increase of tyrosine phosphorylation at between 2-15 minutes, this increased phosphorylation may not persist. Identifying specific molecules that are differentially tyrosine phosphorylated downstream of Tim-1 or Tim-1$^{KRK\text{-}QGQ}$ and TCR/CD28 co-stimulation will contribute to our understanding of the importance of Tim-1 localization on T cell function.

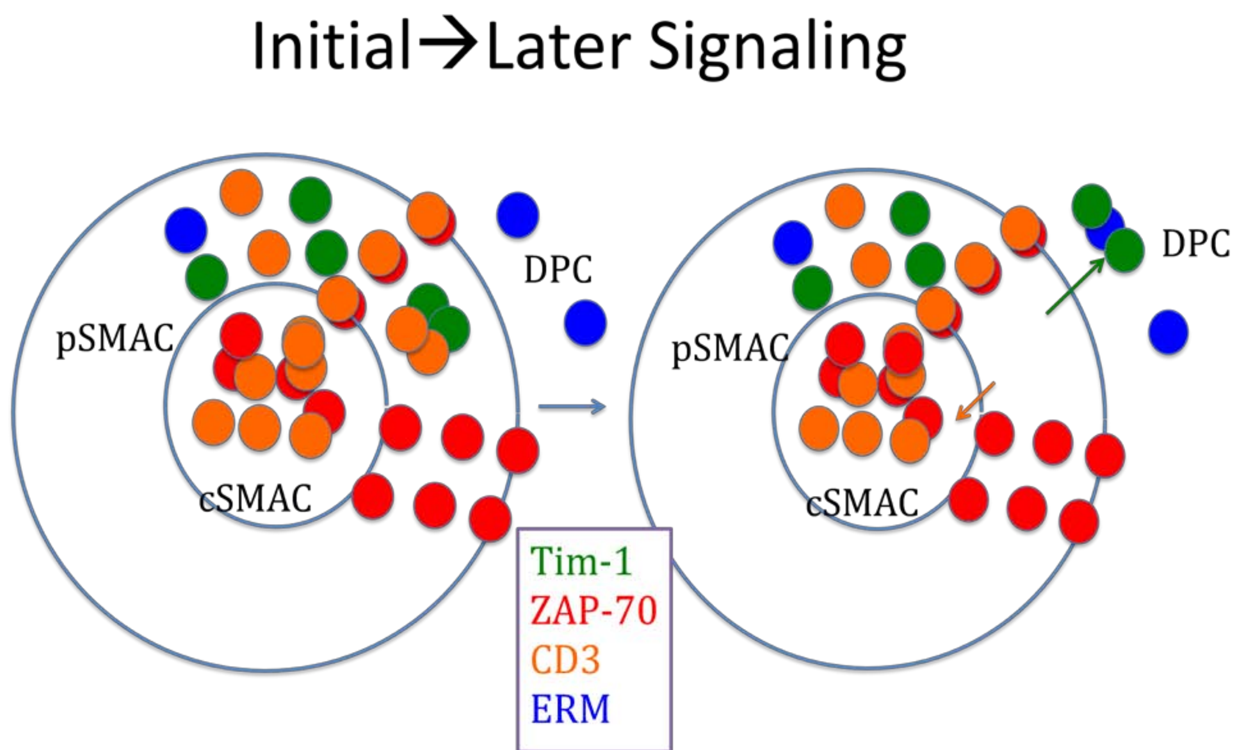


Figure 4-3: Tim-1 localizes away from the IS in an ERM dependent manner.

Tim-1 forms microclusters. Many signaling microclusters move towards the immunological synapse and cSMAC, such as ZAP-70 and CD3. However, Tim-1 moves towards the distal pole complex (DPC), and this movement appears to require binding to the ERM family of proteins.

Another question for future exploration is whether WT Tim-1 *ever* localizes at the immunological synapse. Some proteins that ultimately reside in the DPC at least transiently visit the IS. One could speculate that Tim-1 might briefly move to the IS in order to interact with ligands, such as Tim-4. However, where Tim-4 localizes and how its interaction with Tim-1 is spatially regulated are issues that have not been addressed. A second possibility is that Tim-1 requires its IgV domain for proper localization. Other receptors, such as Ly49A, have been shown to be masked be proteins interacting in *cis* with its extracellular domains, and disruption of this interaction allows the NK cell to localize towards the IS (173). Thus, Tim-1 could be binding another protein in cis that prevents its localization towards the IS. More structure/function work will be necessary to clarify the importance of the different Tim-1 domains on Tim-1 localization and function.

One of the most interesting questions arising from this thesis work is why Tim-1 localizes away from the IS. One way to address the importance of Tim-1 localization towards the DPC would be to force Tim-1 to localize at the immunological synapse, for instance, by creating a chimeric fusion protein with the extracellular domain of CD28, which should drive localization in the immune synapse, and the Tim-1 cytoplasmic tail, which should transmit Tim-1 downstream signaling. How forced Tim-1 localization at the IS affects T cell activation or effecter function could yield valuable knowledge about the importance of its regulated localization and compartmentalization in T cell activation. One caveat to this approach is that Tim-1 signaling may be influenced by interaction of its IgV domain with its natural ligands.

Tim-1 localization towards the DPC may also be important for other still-unexplored possible roles for Tim-1, including T cell migration or T cell polarization during asymmetric division. The DPC and uropod share many features, and a recent paper has implicated CD43,

which is concentrated at the DPC and at the uropod, in T cell migration in response to chemokines. Specifically, CD43 undergoes enhanced cytoplasmic tail serine phosphorylation in response to chemokine signals, and this migration can be blocked by interfering with ERM protein binding (174). Hence, determining the velocity and distance of Tim-1 or Tim-1$^{KRK-QGQ}$ movement in response to Tim-1 antibodies or to chemokines may offer interesting insights into Tim-1 function. Chemokine dependent T cell migration is important in mediating transplant tolerance or rejection. Thus, a role for Tim-1 in migration could potentially be reveal why certain Tim-1 antibodies result in allograft acceptance or rejection. Another fascinating role for Tim-1 could be in asymmetric cell division. Work from Steve Reiner's lab has suggested that T cells undergo asymmetric division after activation and that the "proximal" daughter cell has different functions than the "distal" daughter cell (79). Tim-1 could be localization away from stimulation by the APCs in order to be separated with the more distal "memory" cell precursor during asymmetric division. Understanding whether Tim-1 participates in asymmetric division and identifying the functional consequences could contribute to our knowledge of T cell polarization.

Another intriguing question is whether Tim-1 localization away from the IS is universal to all T cells. Tim-1 is found on all activated CD4^{+} cells, but antibody cross-linking of Tim-1 induces very different responses in *in vivo* mouse models of disease, and these differences are often attributed to the effect of Tim-1 on regulatory T cells (42). In this regard, we know that PKC-θ protein localization impacts T cell function. Specifically, PKC-θ localizes at the cSMAC in effector T cells but away from the cSMAC in regulatory T cells (96, 112). Work presented in this thesis shows that Tim-1 moves away from the IS in a Th2 cell line; however, where Tim-1 localizes in regulatory T cells, and how this might impact T cell function, has not been

examined. Tim-1 could, similar to PKC-θ, concentrate at different areas of the cell in response to activation by APCs, and this localization could have implications for signaling and function, including influencing cytokine production or suppressive activity (96). This could be particularly interesting, since TIM-1 is highly expressed on regulatory T cells, and its interaction with hepatitis A virus (HAV) inhibits, rather than enhances, TIM-1 mediated co-stimulatory functions (121). It is possible that HAV ligation alters Tim-1 localization in regulatory T cells to inhibit its functions. For instance, perhaps interaction with HAV promotes aggregation of Tim-1 on the surface of the T cell, away from specific signaling proteins, to mediate its suppressive functions.

4.3 TIM-1 RECEPTOR INTERACTIONS

One of the biggest questions currently in the field is how ligation of Tim-1 results in disparate signals, which result in induction of different cytokines and T cell activation or inhibition. For instance, ligation of Tim-1 with a high affinity antibody, 3B3, leads to IFN-γ and IL-17 production and exacerbation of EAE, while the low affinity antibody RMT1-10 promotes enhanced IL-4 and IL-10 production and inhibits EAE development (34). Although difference in the epitopes recognized and binding affinity of the Tim-1 antibodies have both been implicated (34, 36), neither can fully explain their incongruent functions on cytokine production and disease modulation. One possibility is that cross-linking with different antibodies generates different signaling pathways downstream of Tim-1 ligation. In Chapter 3, we demonstrate that treatment with different Tim-1 induces diverse patterns of tyrosine phosphorylation. Another relatively

unexplored possibility is the orientation of receptor binding, which may influence the downstream signaling. For instance, at least two families of immune receptors, the NK receptors and HVEM, can enhance or inhibit signaling based on whether binding is in *cis* or in *trans*. In these receptors, binding in *cis* appears to generate inhibitory signals since *cis* binding competes with *trans* binding, while binding in *trans* leads to cellular activation (175). In accordance with this would be the enhanced T cell activation produced by Tim-1 ligands, Tim-4 and PS, which should be binding in *trans* (13, 58). Therefore, cross-linking by the Tim-1 antibodies may lead to differences in signaling, depending on their ability to enhance or inhibit signaling generated in *cis* or interfere with binding in *trans*. Alternatively, gonadotropin receptors can generate specific but different activating signals in *cis* and in *trans* (176). Understanding whether Tim-1 engages in *cis* binding, and identifying how these binding partners are regulated may provide insights into how Tim-1 signaling is regulates. In addition, *trans* binding often requires flexibility and folding of the stalk domain (177, 178), so investigating the effects of deletion or elongation of the stalk domain may also offer insights into Tim-1 receptor function.

Binding affinity may still play a role in the *cis/trans* binding. Similar to CD22 (179), Tim-1 on a resting T cell may be constitutively interacting in *cis*, but high affinity antibodies, such as 3B3, may bypass this *cis* interaction to transduce specific downstream signals and induce actin remodeling (perhaps through Vav1). Lower affinity Tim-1 antibodies, including RMT1-10, may only partially overcome *cis* interactions, and, therefore, may generate weaker, or different, downstream signals that result in alternative transcriptional activation and cytokine production. Strength of TCR signaling has been implicated in skewing T helper subset differentiation, with higher TCR signal strength enhancing T bet expression and a Th1 phenotype, while weaker TCR signal strength result in more of a Th2 phenotype (180). Tim-1 may cooperate with TCR signal

strength to influence this process. For instance, in a murine cardiac transplantation model, treatment with 3B3 resulted in production of the Th1 type cytokine IFN-γ and rejection of allograft, while in an islet allograft model, treatment with the weaker RMT1-10 led to a more Th2 type phenotype and allograft acceptance. This is not a complete explanation, since 3B3 has also been shown to induce Th1, Th2, and Th17 type cytokines (2, 34, 36, 63, 171, 177), but factors in the extracellular milieu may also be contributing the cytokine production. Visualizing the pattern of Tim-1 localization upon Tim-1 ligation by these antibodies with different epitopes and affinities may clarify the role of the receptor in T cell function.

While Tim-1's effects on immune modulation has been attributed directly to T cells, a growing body of knowledge suggests indirect effects may be key, *i.e.* effects of Tim-1 on T cell activation may be the result of Tim-1's effects on other cells. As Tim-1 knockout studies and other recent work have demonstrated, Tim-1's influence on immune modulation may depend more on its effects on B cells or recruitment of innate immune cells than on T cells (105, 106). Therefore, understanding Tim-1 localization and signaling in other cells may clarify some of the controversy in the field. Non-CD4$^+$ expressing cells have distinct tyrosine phosphorylated bands induced downstream of Tim-1 antibody ligation (data not shown). Another consideration is how ligation of Tim-1 may bi-directionally influence signaling. Tim-1 expression has been found on multiple cells of the immune system, and Tim-1 has the potential to engage in homotypic *trans* interactions (45). Therefore, Tim-1 ligation may generate signaling in both the T cell and its APC. Signaling bi-directionally has been shown to generate different signaling pathways in different cells (181). Thus, Tim-1 ligation on T cells may predominant lead to a Th2 type cytokine production while Tim-1 engagement on DCs may result in differential activation and the production of distinct cytokines as has been demonstrated (40).

It may be necessary to reevaluate our thinking about Tim-1 receptor ligation in order to better understand how Tim-1 may either activate or inhibit T cell activation. Many publications have determined that Tim-1 is able to modulate immune function. However, Tim-1 may have greater complexity and more functions than previously appreciated, including *cis* and *trans* binding. Clearer understanding of how Tim-1 mediates its functions will provide more specific targets for drug design and more specificity in modulation of the downstream signaling.

Figure Legends for Movies:

Movie 2-1: Tim-1 microclusters do not localize with peripheral ZAP-70 microclusters. Jurkat cells transiently transfected with Tim-1-GFP (green) and ZAP70-TagRFP (red) were allowed to settle on anti-TCR/CD28 coated coverslips. TIRF images were continuously taken on a Nikon A-1 microscope by Simon Watkins.

Movie 2-2: anti-CD3/CD28/Tim-1 stimulation mobilizes ZAP-70 microclusters. Jurkat cells transiently transfected with Tim-1-GFP (green) and ZAP70-TagRFP (red) were allowed to settle on anti-TCR/CD28/Tim1 (3B3) coated coverslips. TIRF images were continuously taken on a Nikon A-1 microscope by Simon Watkins.

Movie 2-3: WT Tim-1 moves away from the nascent IS after APC stimulation. Jurkat T cells transiently transfected with Tim-1-GFP (green) and ZAP70-TagRFP (red) were incubated with Raji cells incubated with 1 mg/mL SEE and stained with Cell Tracker Blue (blue). Mid-plane images were taken every 6.7 seconds over the course of 19 minutes in a heated chamber on an Olympus FluoView 1000 confocal microscope.

Movie 2-4: Imaging Tim-1 movement in response to latex beads. Jurkat T cells transiently co-transfected with Tim-1-GFP (green) and ZAP70-TagRFP (red) were incubated with anti-OKT3, anti-CD28 coated

latex beads. Mid-plane images were taken every 23 seconds over the course of 19 minutes in a heated chamber on an Olympus FluoView 1000 confocal microscope.

Movie 2-5: Tim-1^{QGQ} localizes to intracellular pools that can reside at the IS. Jurkat T cells transiently transfected with Tim-1^{QGQ}-GFP (green) and ZAP70-TagRFP (red) were incubated with Raji cells incubated with 1mg/mL SEE and stained with Cell Tracker Blue (blue). Mid-plane images were taken every 6.7 seconds over the course of 14 minutes in a heated chamber on an Olympus FluoView 1000 confocal microscope.

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