TIM-1 AND TIM-2 AS REGULATORS OF T- CELL ACTIVATION

[TIM: T-CELL IMMUNOGLOBULIN AND MUCIN CONTAINING MOLECULE]

by

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The TIM proteins belong to a novel family of molecules contained within a single gene locus positioned on mouse chromosome 11B1.1 and human chromosome 5q33.2- a locus that been repeatedly linked with susceptibility to atopy and autoimmune diseases. Early evidences from genetic, epidemiologic and immune functional studies have also implicated these proteins in the regulation of immune responses associated with asthma and autoimmunity. However, at the time of commencing these studies there was a paucity of information regarding the underlying molecular basis of TIM function that directed these different effector responses. Based on existing information on putative signaling motifs contained within the TIM protein sequences and early evidence of TIM-2 tyrosine posphorylation we proposed that TIM-1 and TIM-2 could transduce intra-cellular biochemical signals in response to ligation of the receptor, by coupling to phosphotyrosine dependent signaling mechanisms in order to regulate T cell activation.

Employing a combination of biochemical, pharmacological and genetic approaches, our studies establish TIM-1 and TIM-2 proteins as -signal transducing- cell surface receptors. We show that, TIM-2 functions as a negative regulator of T cell activation by inhibiting NFAT/AP-1 dependent transcription. In contrast, TIM-1 can provide a co-stimulatory signal for T cell activation, and augment cytokine and NFAT/AP-1 dependent transcription. TIM-1-mediated signal transduction requires the

cytoplasmic tail and the conserved tyrosine 276 contained within the tail. Furthermore, TIM-1 can amplify classical TCR signaling cascades for NFAT/AP-1 dependent transcription and this occus in a manner that requires the TCR and contribution of most of the key signaling components elicited by the TCR. TIM-1 can also trigger distinct pathways that involve Lck-dependent tyrosine phosphorylation followed by recruitment and activation of p85-PI3K for up-regulation of surface markers associated with T cell activation. In this context TIM-1 requires Akt to enhance NFAT/AP-1 dependent transcription.

Eventually, elucidation of the biochemical signals underlying the mechanistic function of a family of molecules significantly involved in the regulation of T helper cell responses would present targets for therapeutic modulation of Th1 and Th2 -type immunity in health and in immune-mediated disease.

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PREFACE

I am filled with a sense of 'wonderment' as I begin to write this preface. I have listened to other graduate students giving their defense talks over the course of these few years and thought " Am I really going to get up there and deliver THAT one day?!!" And now I have finally reached this point where I write the preface of my own thesis. …….Wow!

My experience of graduate school is unique in that I joined a laboratory (lab) that was just starting up. My advisor Dr. Larry Kane had just come to Pittsburgh in July of 2003 and was setting up his own lab for the first time. I started my rotation in his lab two months later based on outstanding recommendations from some of the faculty. I have often wondered what made me rotate in and join a lab, so new! And I have come to the conclusion that it was partly the challenge that accompanies uncharted territory and a somewhat unexplainable 'fascination' for the unknown that enticed me to 'take the plunge'! But even these reasons would not have been sufficient were it not true that I was completely bowled over by Larry's tremendous scientific prowess and excelling traits, which couldn't but lead to success.

Four years later in the lab and I am not sorry for having chosen the path I took. I have grown tremendously during this period. I have observed first-hand what it takes to set up and establish a working lab and an academic career…and it is TOUGH! Should I ever want to do the same in future, my graduate experience and training will come in handy and it is invaluable.
I am grateful to Larry for all that I have learned during this period while in his lab and from him, academically related and otherwise.

I have also had the unique privilege of virtually starting a project from ground up, having started working on the TIM project 2-3 months after Larry began it. It was an exciting project to work on given the paucity of information about this novel gene family, but at times was also confusing, for much the same reason. Almost two years after I started working on TIM-1, and a lot of hard work later we published my first premier-author manuscript in 2005. Subsequent work on TIM-2 was slightly easier and much faster since we followed a similar manner of investigation and we knew where the pitfalls lay. And then I delved a little deeper into exploring the mechanisms of TIM-1 function.

I attribute a huge portion of my academic and all-round growth to all the faculty (primary and secondary), staff and fellow students primarily of the Department of Immunology, but also other department members with whom I have come in contact. And I sincerely mean this! I believe an optimum environment is required for optimum growth and I received this through all these people. Truly, I am proud to have been a student of this department. In addition, I cannot leave out any of my many friends who have sustained me through this period and made it so enjoyable.

I am especially grateful to all my committee members: Drs. Olja Finn, Anuradha Ray, Tom Smithgall and Binfeng Lu. They have given me expert direction and supported all my endeavors. It was very reassuring to me to have such sterling scientists as members of my committee. I have met with them at committee meetings and approached them at other times to seek their advice, and they have always kept an open door for me.

To my parents and my family (including both my very dear brothers) I am at a loss for words! I have received a continual supply of unconditional and endless love, support and guidance from my parents and brothers, so willingly given. I am deeply grateful to my parents, beyond expression. I know that I would not have reached this far in my graduate career were it not for their loving mentorship. In truth, I consider them my mentors. So I dedicate my thesis and all the knowledge I obtained therein, to my parents.
Finally, I am thankful for my belief in God, and I thank God for EVERYTHING!

I would like to leave you with two thoughts:

"Keep on seeking the truth till Truth finds you"
Dr. L.J. de Souza

"Truth is truth to the end of reckoning"
Shakespeare (Measure for Measure, V - 1)

1. CHAPTER 1: INTRODUCTION

The contents of this chapter have been modified from a published review (de Souza AJ and Kane LP (2006) *Immunological Research* 36:147-155). This chapter includes much additional information that was not incorporated in the published review.

1.1. IDENTIFICATION OF *TAPR* AND THE TIM GENE FAMILY

Among other loci, human chromosome 5q23-35 and the syntenic region in mice (11B1.1) has been repeatedly linked to asthma susceptibility and autoimmunity. This locus contains several immune- regulatory genes including interleukin 9 (IL-9), IL-12p40, the β- adrenergic receptor, CD14, Itk and the IL-4 gene cluster that, among other genes, includes the genes encoding IL-4, IL-5, IL-13 and GM-CSF. The syntenic region in mice (11B1.1) has also been implicated in type-1-diabetes (Idd4), and experimental autoimmune encephalomyelitis (EAE) (Eae6). Through genome-wide scans, human chromosome 5q23-35 has been linked with a predisposition to rheumatoid arthritis, autoimmune thyroid disease, type 1 diabetes, Crohn's disease, asthma and allergic disease [1-4].

In-order to analyze this region for new asthma-susceptibility genes, McIntire et al used congenic mice produced on a BALB/c background, each with a discrete genomic interval inherited from chromosome 11 of the DBA/2 mouse and syntenic with human 5q23-35. Several such lines were screened for the suppression of asthma susceptibility. One of the strains generated termed C.D2 Es-Hba (HBA), contained a distinct genetic interval that converted a BALB/c Th2 biased cytokine phenotype of enhanced IL-4, IL-13 production and airway hyper reactivity (AHR) to a DBA/2 phenotype of low Th2 cytokine levels, high IFNγ levels and low AHR. Subsequent genome linkage analyses indicated that genetic variation in a single locus (designated as *Tapr* for T cell and Airway Phenotype Regulator) regulated Th2 cytokine production and AHR in the HBA mice. *Tapr* was genetically separable from the nearby IL-12p40 and IL-4 cytokine gene clusters, and contained the **T** cell **I**mmunoglobulin and **M**ucin domain containing (TIM) gene family [5]. Analysis of individual TIM genes revealed polymorphisms in the coding sequences of TIM-1 and TIM-3 when comparing BALB/c and DBA/2 [5]. These findings in mice are consistent with epidemiological studies in humans that have linked polymorphisms in TIM-1 with atopy and asthma [6].

1.2. STRUCTURE OF THE TIM PROTEINS

The TIM gene family includes 8 genes present on mouse chromosome 11B1.1(encoding TIM-1 to TIM-4 and putative proteins TIM-5 to TIM-8) and 3 genes on human chromosome 5q33.2 (encoding TIM-1, TIM-3 and TIM-4) with no intervening genes in

either species but with a few TIM-like pseudogenes present [5]. The three human TIM proteins TIM-1, TIM-3, TIM-4 are most similar to mouse TIM-1, TIM-3 and TIM-4 respectively [5]. One other known murine TIM protein TIM-2 appears not to be conserved in primates. Yet since mouse TIM-2 shares much homology (64%) with mouse TIM-1, it has also been considered as an orthologue of human TIM-1 [7]. The TIM proteins are type-I cell-surface glycoproteins with common structural motifs, including a signal peptide, an extra-cellular IgV domain and mucin-like domain, a trans-membrane helix and an intracellular cytoplasmic tail. The mucin domain is rich in threonine, serine and proline residues and is predicted to be heavily O-glycosylated. While TIM-1 and TIM-2 contain 2 putative sites for N-linked glycosylation, TIM-3 contains 4 sites for N-linked glycosylation [5,8].

The cytoplasmic tail is the most heavily conserved domain in mouse and human orthologues [8]. Excluding TIM-4 whose short tail is lacking in any tyrosines, the other cloned TIM proteins contain a conserved intracellular tyrosine phosphorylation motif that may transduce signals in response to receptor-ligand interaction [5,7]. Mouse and human TIM-1 have very similar phosphorylation motifs, RAEDNIYIVED and QAEDNIYIENS respectively [5,7,8]. Excluding TIM-4, each of the TIM's also contains additional tyrosines that might also participate in signal transduction. TIM-4, on the other hand, may function as a decoy receptor or may associate with another binding partner that could possibly transduce signals [9].

While the TIM's belong to the Ig family of receptors, they share a striking difference in the conformation of the IgV domain. Unlike related Ig receptors, the TIM's are unique in containing a very high number of invariant cysteines (six) in a single Ig

domain. Two of the cysteines form the characteristic intra-disulphide bond connecting the B and F strands that is common to all Ig superfamily members. The 'extra' four cysteines contained in the CC′ loop are engaged in forming two intra-disulphide bonds and assume a distinctive folded conformation onto the GFC beta sheet. This is a distinguishing structural feature of the TIM's. In TIM-2 there is even greater divergence, with the CC′ loop structurally attached to the FG loop forming a protruding CC′FG epitope, which partially covers the GFC beta sheet. Related Ig receptors instead show the GFC beta sheet as a flat surface mediating ligand binding. Another piece of revealing information obtained from the crystal structure, pictured TIM-1's capability for homophilic interaction with neighboring cells but not for dimerization on the same cell. The interaction is mediated through the BED surface in the IgV domain, which lies opposite the GFC beta sheet. The polymorphic O-glycans in the mucin domain also contribute to this interaction. Paradoxically, TIM-2 molecules on the cell surface can only homodimerize with each other, thereby burying the domain surface that would otherwise be engaged in TIM-2 homophilic interaction or interaction with TIM-1 [10]. Collectively, these molecules have different ligand binding modes that might have a pivotal influence on TIM function

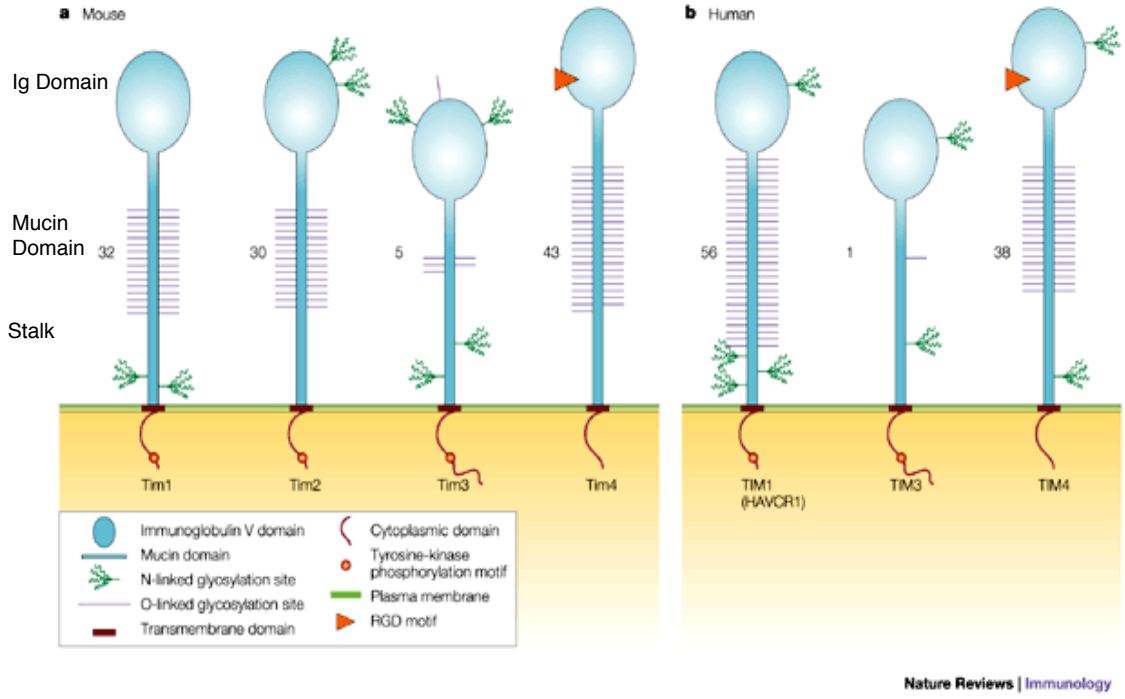


Figure 1: Diagrammatic Representation of TIM family members [7]

1.3. TIM MOLECULES HAVE A VARIED EXPRESSION

1.3.1. TIM-1 expression

TIM-1 was originally discovered as the cellular receptor for hepatitis A virus (HAVcr) in African green monkey kidney cells [11] and the human homolog, which shares 79% homology with its simian counterpart, functions similarly [12]. Both the Ig and the mucin

domains are essential for successful viral entry [13,14]. It was later described as kidney injury molecule-1 (KIM-1) in rats and humans with 43.8% shared identity[15]. KIM-1 is expressed at low levels in normal kidney but its expression is dramatically upregulated after renal injury, particularly renal ischemia-reperfusion injury and localized to proliferating and regenerating proximal tubule epithelial cells in damaged regions [15]. There exist two splice variants (a and b) of human KIM-1 that differ in their cyto-plasmic domain and tissue distribution. KIM-1a in humans is the major form in the liver and is the homolog of KIM-1 in rats. This variant has a truncated cytoplasmic tail lacking the tyrosine phosphorylation motif. KIM-1b, which retains the conserved tyrosine in the tail, is present predominantly in the kidney and was cloned as the homolog of HAVcr-1 in monkeys. KIM-1b is constitutively shed into the extracellular milieu as a soluble ectodomain, by metalloproteinases [16]. This earlier study also postulated TIM's function as a kidney epithelial cell adhesion molecule due to its similarity with MAdCAM-1 structure.

Recently TIM-1 expression was detected by quantitative RT-PCR from BALB/c mice to be in high abundance in lymph node and kidney and in relatively low abundance in spleen, lung and thymus while being undetectable in heart or liver of these mice [17]. Monoclonal antibody to murine TIM-1 detected low surface expression on unactivated CD4+ T cells, CD19+ B cells and CD11c+ bone marrow derived dendritic cells, but not on CD8+ T cells, from BALB/c spleens. In contrast, TIM-1 expression greatly increases on activated CD4+ T cells within 24 hour of activation and increases further by 48 hours. Its expression decreases on Th1 cells during the process of differentiation but is sustained on Th2 cells [17].

1.3.2. TIM-2 Expression

TIM-2 message is undetectable in resting murine T cells but is moderately expressed on activated T cells [18]. This expression dramatically increased in terminally differentiated Th2 cells, while being undetectable in Th1 T cells [18]. High surface expression levels of TIM-2 were also observed on B cells using a TIM-2 antibody targeted to the Ig domain [19]. Immuno-flourescence and immuno- histochemistry studies indicated a preferential concentration of TIM-2 in germinal centre B cells over follicular B cells [19]. There was no TIM-2 detected in T cell zones [19], possibly because the T cells were un-polarized or were Th1 cells. Like TIM-1, murine TIM-2 is also expressed in extra-lymphoid tissues. Antiserum directed against the cytoplasmic tail, showed localized expression of TIM-2 in bile duct epithelial cells of the liver and tubular epithelial cells of the kidney [19].

1.3.3. TIM-3 Expression

TIM-3 in mice is almost exclusively present on terminally differentiated Th1 cells [8]. Murine TIM-3 expression is significantly up-regulated *in vitro* only after three rounds of Th1 re-stimulation and is maintained on most CD4 T cells through at least six rounds of stimulation [8,20].These expression kinetics reflect probable TIM-3 involvement in the effector function of Th1 cells rather than a contribution to Th1 differentiation. *In vivo,* Tim-3 was mostly observed on effector memory T cells with a $CD62L^{llow}$, $CD44^{high}$ characteristic phenotype [21]. TIM-3 message in the mouse was undetectable in naive T cells, B cells and dendritic cells, but was found at low levels in *in-vivo* derived CD11b+

cells [8]. Like TIM-1, TIM-3 can exist in two forms, namely membrane-bound full-length and a secreted form composed of the IgV domain fused to the intracellular tail [22]. In contrast to TIM-1 however, the soluble version is not enzymatically cleaved but is rather an alternatively spliced variant [16,22]. Assessment of TIM-3 mRNA and protein levels *in-vivo* during the development of the Th1 dependent disease experimental autoimmune encephalomyelitis (EAE), revealed maximal expression at day seven in the lymph node just before the onset of disease and at day 10 in the brain at the onset of disease, with a return to basal levels as the disease progressed [8]. TIM-3 expression is also triggered on lymphocytes that have been adoptively transferred into allogeneic hosts, with a peak on day 3 after transfer, in a mouse model of graft versus host disease (GVHD) [20]. Human TIM-3 is 302 amino acids in length and its message had been detected in Th1 cells as well as CD8+ Tc1 cells [23].

1.3.4. TIM-4 Expression

TIM-4 mRNA can be detected in the stromal cells (or cells tightly associated with the stroma) of the spleen, lymph nodes and payer's patches [24]. Its expression is tightly linked with that of lymphotoxin (LT), which is important for lymphoid organogenesis and is a cytokine marker of Th1 cells. An analysis of splenic gene expression profile through cDNA micro arrays revealed a marked decrease in Tim-4 expression with LT deficiency (LTα, LTβ, LTR knockout mice). Similar results were obtained in lymph nodes and Payer's patches. Using *in situ* hybridization, the expression pattern of TIM-4 was localized to white pulp of the spleen, the sub capsular sinus and the para-cortical

areas of the lymph node and in lymphoid follicles of Peyer's patches [24]. A recent study using quantitative RT-PCR has extended this expression profile to antigen presenting cells such as macrophages, mature dendritic cells of the lymphoid lineage and, to a lesser extent, B cells [25]. While TIM-4 transcripts were detected in the spleen and lymph node, and at low levels in the lung, liver and thymus, they were undetectable in resting or differentiated Th1 or Th2 cells [25].

Collectively, the expression profiles of the TIM molecules reflect a diverse distribution that associated with varied functionalities could nevertheless, be specific to a differentiated cell type or organ.

1.4. MULTIPLE LIGANDS FOR THE TIM'S

The TIM molecules are comparable in their extra-cellular domain structure to several glycoprotein receptors, including the cell-adhesion molecule MAdCAM.1. Variations in either Ig or mucin domain may regulate receptor-ligand interaction, as shown for MAdCAM.1. While the Ig domain of this molecule binds the integrin $\alpha_4\beta_7$, the glycosylated mucin domain is required for interaction with the CD62L (L-selectin) [26]. Likewise, it is possible that each of the TIM molecules may also have more than one ligand. Moreover, their sticky glycosylated mucin domain may favor homotypic interactions as well as heterotypic interactions with other molecules, including other TIM proteins.

1.4.1. TIM-4 and HAV interact with TIM-1

Through flow cytometry, a soluble TIM-4-Ig fusion protein (consisting of the extracellular IgV and mucin domains of TIM-4 fused to an IgG1 Fc tail), revealed expression of a TIM-4 ligand - apparently TIM-1 - on a subset of inactivated B cells and on most activated B and T cells and there was a higher percentage of Th2 effector cells interacting with TIM-4, than Th1 cells [25]. The TIM-4 interaction was specific to TIM-1, as opposed to other TIM molecules, since TIM-4-Ig bound only TIM-1 transfectants and not TIM-4 or TIM-3 and no obvious homotypic interaction was detected [25]. Binding of TIM-4-Ig directly correlated with TIM-1 expression on activated primary murine T cells. This interaction is only partially blocked with an antibody targeted to the Ig domain of TIM-1, indicating that a part of the interaction between TIM-1 and TIM-4 involves the TIM-1 mucin domain. This feature resembles another natural ligand for human TIM-1, namely hepatitis A virus, which interacts with both the Ig and the mucin domains of TIM-1 for successful viral entry [13,14]. Although TIM-1 and TIM-2 share a great degree of homology, they do not also share a similar ligand in TIM-4 [25]. Recent studies have revealed the presence of another TIM-1 ligand, as yet unidentified, on unstimulated human Jurkat T cells and on murine T cells upon activation [27,28].

Interestingly, structural data obtained through X-ray crystallography indicates that TIM-1 can interact homophilically on neighboring cells in a manner that requires the Ig domain and is fortified by differential glycosylations of the mucin domain. TIM-1 homophilic interaction is conserved in both mouse and human and therefore suggests an important biological function. Considering that the participating mucin region is

polymorphic in different mouse and human strains this suggests an accompanying polymorphism in attached O-linked glycans, which in turn would affect TIM-1 homophilic interactions as well as the surface epitopes presented to potential ligands. Collectively, these findings support the notion that differential TIM molecule glycosylation in assorted cell types and under different conditions may specify the TIM molecule- ligand interaction, thereby determining functional outcomes.

1.4.2. TIM-2 and its ligands: Semaphorin 4a and H-Ferritin

In an elegant study to identify TIM-2 ligands, Chen TT et al showed that cell-free supernatants from resting and activated macrophage cell lines induced NFAT mediated LacZ expression in the BWZ.36 T cell line transfected with a TIM-2/ CD3ζ chimera. These studies thus indicated that macrophage cell lines produce a soluble ligand for TIM-2. Subsequent expression cloning experiments revealed that mouse H-ferritin but not L-ferritin served as a TIM-2 ligand. This interaction is specific for TIM-2 since recombinant H-ferritin does not bind detectably to TIM-1 or TIM-3 [19]. Ferritin is a major tissue iron-storage protein [29] composed of 24 sub-units consisting of acid/H chains and basic/L chains in a variable ratio [30]. A TIM-2-Ig fusion protein had also identified a TIM-2 ligand on a subset of activated B cells, macrophages, and on all activated CD11c+ dendritic cells, while being undetectable on T cells [18]. Identification of this ligand is still awaited and it could be similar or not to H-ferritin. Another independent study had previously demonstrated Semaphorin 4a present on dendritic cells and activated B cells, as a TIM-2 ligand [31]. These data indicate the presence of

multiple ligands for TIM-2 that may interact at the same or different regions of the molecule, with differing affinities, perhaps in competition with each other for binding to TIM-2.

TIM-2 shares high sequence homology with TIM-1. Yet paradoxically it interacts with different ligands. Further, TIM-2 cannot bind to TIM-1 and vice versa. Recent X-ray crystallographic studies of TIM-2, cited above, have begun to shed some light on the structural features of the molecule that underlie these observations. Unlike TIM-1, which engages in homophilic interaction, TIM-2 can dimerize on the cell surface. This and other conformational differences in their IgV domain discussed above, have predicted different ligand binding modes for the two molecules, which might explain the different immune functions observed for TIM-1 and TIM-2 [10].

1.4.3. Galectin-9 and TIM-3

Initial studies with a TIM-3-Ig fusion protein indicated presence of a TIM-3 ligand on both resting and activated effector-memory-regulatory CD4+T cells [20,22]. This was down regulated on Th1/Th2 clones and CD4+CD25- cells 48 hours after activation while it was sustained or even increased on CD4+CD25+ T regulatory cells [20]. A very low population of dendritic cells and macrophages also exhibited the ligand [20,22]. Subsequent studies have identified the 35kd protein Galectin-9 as a ligand for TIM-3 [21]. Galectin-9 is ubiquitously expressed in a variety of tissues, including lymph nodes and spleen, in both humans and mice (14,22). Galectin-9 was detected by TIM-3-Ig staining of the CD8+ mouse lymphoma cell line TK-1, and subsequently identified

through immuno-precipitation assays from surface biotinylated TK-1 cells followed by mass spectrometry [21]. TIM-3 binds both Galectin-9 and the long isoform Galectin 9L encoded in TK-1 cells. This interaction is specific for TIM-3 alone and not TIM-2 or TIM-4. The IgV domain of TIM-3 is sufficient to maintain the binding. While TIM-3 interacts best with Galectin-9, there do exist low-affinity interactions between TIM-3 and other members of the Galectin family; namely Galectin-4, Galectin-3, and Galectin-1. However, binding is completely abolished when sTIM-3-Ig is treated with the N-glycosidase PNGaseF. Moreover, the interaction is also eliminated when two conserved amino acids (R64 and R238) on Galectin-9, known to be important for carbohydrate recognition [32], are mutated [21]. Taken together, these data support the idea that the glycosylation in the IgV domain of TIM-3 is responsible for the interaction between TIM-3 and its natural ligand Galectin-9.

X-ray crystallographic studies of TIM-3 have predicted an additional ligand(s) that is still unknown. Common to the TIM family and distinguishing it from other Ig super family receptors, TIM-3 forms a unique surface in its IgV domain generated by two non- canonical disulphide bonds which place the CC' and FG loops in close proximity. The CC'-FG surface is predicted to serve as a ligand binding surface since mutagenesis of residues contained in this region led to abolishment of TIM-3-Ig binding. Moreover, this epitope is thought to bind ligands distinct from galectin-9 since the N- and O-glycans linked with the IgV domain which are essential for Galectin-9 binding, are located distantly from this epitope, and glycan-deficient TIM-3 tetramers bound a range of primary immune cells and cell lines [33].

1.5. IMMUNE REGULATION BY TIM-1

1.5.1. TIM-1 and T cell activation

Optimum T cell activation requires at least two signals. One signal is provided by encounter of TCR with antigen and MHC and subsequent TCR dependent signaling. The second signal is provided by co-stimulatory molecules following their interaction with specific ligands [34-36]. Several lines of evidence suggest that TIM-1 functions as a co-stimulatory molecule for T cell activation. Cross-linking of TIM-1 with anti-TIM1 antibody delivers a signal that greatly enhances proliferation of CD4+ T cells [17]. TIM-1 can augment T cell proliferation with antigen and antigen presenting cells (APC's) or with anti-TCR and anti-CD28 stimulation alone [17]. This suggests that TIM-1 may not require any other molecule on the surface of APC's for mediating its co-stimulatory function of increased proliferation. To determine whether cross-linking of TIM-1 with the antibody delivered a positive signal or if it blocked a negative interaction of TIM-1 with its ligand, monovalent Fab fragments of anti-TIM-1-mAb were included instead. Since monovalent Fab fragments were not able to replicate stimulatory effects of the intact antibody to TIM-1, it is unlikely that TIM-1 sends a negative signal that is blocked by the monoclonal antibody [17]. Similar effects of anti-TIM1 antibody were observed *in vivo*, with the exception of higher basal T cell proliferation levels upon *ex vivo* culture of T cells from mice treated with anti-TIM-1. This basal proliferation was also observed with the administration of TIM-1-Ig or TIM-4-Ig during an *in-vivo* Th1-biased immune response, indicating hyper-activation of immune cells [25]. Further analysis revealed that

the spontaneous hyperproliferation was entirely due to T cells interacting with TIM-4-Ig. It is possible that TIM-1-Ig might cluster TIM-4 on APC's, strengthening the interaction with TIM-1 thereby increasing the TIM-1 costimulatory signal. Interestingly, while sup-optimal *in vitro* T cell stimulation coupled with higher doses of TIM-4-Ig greatly enhanced proliferation, lower levels of TIM-4-Ig inhibited proliferation [25]. One probable explanatory mechanism is that at lower concentrations TIM-4 might bind infrequently to TIM-1 and perhaps engage a higher affinity ligand on T cells that transmits a negative signal. On the other hand TIM-1 itself or its binding partner might relay a negative signal at lower ligand density while this is reversed with higher TIM-4 levels [25]. In some support of this latter possibility, immobilized but not soluble human eTIM1 (TIM-1 extracellular domain) was able to inhibit T cell activation by arresting cell cycle at the G_0/G_1 phase through regulation of cell-cycle proteins [37].

The co-stimulatory effects of TIM-1 are strong enough to abrogate tolerance when anti-TIM-1 is administered during the induction phase of respiratory tolerance before and after exposure to intranasal antigen [17]. Consistent with TIM-1's role as a co-stimulatory molecule, enhanced IL-4 and IFN-γ cytokine production levels were demonstrated when anti-TIM1 or TIM-4-Ig was administrated *in vivo*, and these cytokines were further upregulated upon *in vitro* re-stimulation. Yet, *in vitro* reactivation of the cells treated with TIM-1-Ig, resulted in an inhibition of Th1 cytokine production and an enhancement of Th2 cytokines [17,25]. Further, within a solely *in vitro* system, anti-TIM1, in concert with TCR and CD28 stimulation, enhanced IL-4, but not IFN-γ levels in Th2 cells, and did not change IL-4 or IFN-γ levels in Th1 cells [17]. Either this is a selective effect of TIM-1 on Th2 cytokine production or lower TIM-1 expression

levels in Th1 cells may not be sufficient to exert an effect on Th1 cytokines. Yet, enforced expression of TIM-1 *in vitro* in un-polarized naïve murine T cells during primary stimulation, through retroviral transduction, increases the frequency of IL-4 producing cells but not IFN-γ [28]. Consistent with these findings, a similar bias towards the production of Th2 cytokines IL-4 and IL-10, was observed upon *in vitro* re-stimulation of mouse splenocytes that were previously treated *in vivo* with TIM-1-Ig [25].

Finally, a recent study has used a distinct TIM-1 antibody, targeting a different TIM-1 epitope, during the *in vivo* development of a mouse model of asthma (airway hyperreactivity), and shown that introducing anti-TIM1 closer to challenge, rather than the priming phase actually inhibits asthma [38]. Thus, the functional effects of TIM-1 modulation *in vivo* may critically depend upon the timing of the treatment. Given the association of certain TIM-1 variants with asthma, and the inverse correlation between HAV exposure and the development of this disease, the interaction between HAV and different forms of TIM-1 may explain in part, differences in susceptibility to asthma [9]. However, this model has not yet been addressed directly.

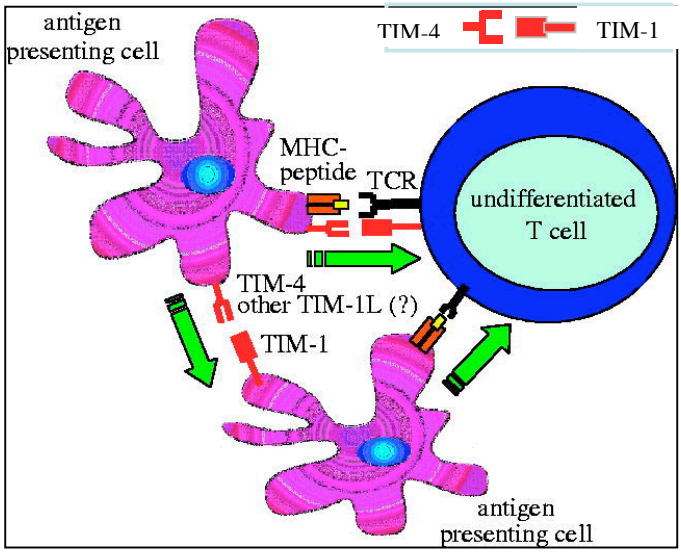


Figure 2: TIM-1 dependent costimulation of T cells.

(Adapted from Mariat et al [39])

TIM-1 is upregulated on T cell early after activation and interacts with its ligand expressed on the stimulating antigen-presenting cell (APC). TIM-1/TIM-1L interaction provides a positive signal that costimulates T cell activation. Alternatively, TIM-1 expressed on APC might directly deliver a positive signal to the APC, which in turn amplifies T cell activation.

1.5.2. TIM-1 and Th2 immune regulation

Several lines of evidence suggest a link between TIM-1 and regulation of Th2 effector responses. The genetic interval of human chromosome 5q33.2 has been repeatedly linked in the past to asthma and allergic susceptibility, as has the syntenic region on mouse chromosome 11B1.1[1-4]. The TIM gene family was positionally cloned in this region using a BALB/c-based congenic mouse model containing a distinct gene segment –apparently TIM gene family - from DBA/2 chromosome 11 and whose phenotype was associated with differences in airway hyperreactivity and Th2 responsiveness [5]. This

study provided the initial link with the TIM gene locus to asthma in mice. It also showed that TIM-1 message is upregulated very early during a primary immune response at a time when T cell activation and differentiation can be influenced. Higher surface expression of TIM-1 is also preferentially sustained on differentiated Th2 cells while it is lowered on Th1 cells, correlating with TIM-1's potential role in Th2 immunity [17]. In contrast, TIM-3 is expressed only on terminally differentiated Th1 cells [8]. Further, TIM-1 message was found at higher levels in human Th2 lines derived from patients with multiple sclerosis as compared to TIM-3 transcripts that were more prominent in Th1 lines [23]. An additional piece of evidence demonstrated that ectopic expression of TIM-1 through retroviral transduction in naïve CD4 T cells form C57BL/6 mice, resulted in greater frequency of IL-4 producing cells, but not IFN-γ upon primary activation [40]. This suggests that TIM-1 may influence Th2 differentiation. Importantly, there exists a remarkable degree of polymorphism in both TIM-1 and TIM-3 molecules between different strains of mice and this is also observed in human populations. Human TIM-1 also functions as the receptor for hepatitis A virus (HAV) [11]. Prior investigations have denoted an inverse correlation existing between HAV infection and development of asthma and atopic diseases [41,42]. Functioning as the HAV receptor, TIM-1 is thus implicated in an important role regulating Th2 immune responses. Subsequent epidemiological studies revealed that the inverse correlation was maintained only in HAV seropositive individuals expressing distinct TIM-1 polymorphic variants, one such variant being 157insMTTVP, which includes a six amino acid insertion in the mucin region and two additional single amino acid changes [6]. HAV-negative individuals did not exhibit any such correlation. One way, by which HAV might confer protection

against atopy, is by modulating T helper differentiation through its interaction with TIM-1[5,6]. A similar mechanism has also been observed for other viruses, such as the measles virus, which binds to its receptor SLAM on CD4 T cells thereby inhibiting Th1 differentiation[43]. SLAM is expressed on T and B cells after activation. Binding to measles virus interferes with its normal function in T cells. Further, it has been observed that mutations in viral receptors frequently alter binding and efficiency of viral infection as is the case for individuals bearing mutant alleles of the chemokine receptor gene CCR-5 are resistant to HIV-1 infection [44,45]. Finally, these studies are supported by several additional groups independently linking atopy with chromosome 5q33.2 at markers very close to TIM-1 or within the TIM-1 gene [46-48]. One study however, had contrasting data showing no association between atopy and TIM-1 in a Japanese population, perhaps due to a lack of HAV exposure [49]. Collectively, these studies strongly imply an important role for TIM-1 in regulating Th2 effector responses.

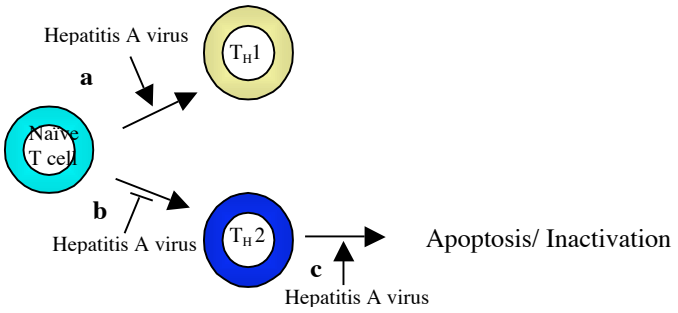


Figure 3: Possible mechanisms by which hepatitis A virus might reduce the development of atopic diseases. (Adapted from Kuchroo,VK et al [7]

a | Hepatitis A virus might productively infect T helper 2 (TH2) cells preferentially and eliminate them. **b** | Hepatitis A virus might bind to T-cell immunoglobulin mucin 1 (TIM1) and block the development of TH2 cells. **c** | Hepatitis A virus might bind to polymorphic variants of TIM1 on naive T cells and enhance the development of TH1 cells.

1.6. IMMUNE REGULATION BY TIM-2.

Emerging evidence suggests that TIM-2 may play an immune regulatory role. Administration of TIM-2-Ig *in vivo* coupled with immunizing antigen during the induction of EAE, resulted in a pronounced increase of basal splenocyte proliferation in the absence of *in-vitro* re-stimulation. This indicates a TIM-2-Ig mediated hyperactivation of splenic cells [18]. This hyper-proliferation is dependent on T cells from TIM-2-Ig treated mice but requires an interaction with antigen presenting cells such as dendritic cells and B cells. While the source of the T cell is dependent on TIM-2-Ig treatment, this is not the case for the antigen -presenting cell. Induction of EAE results in a marked Th1 cytokine profile [50,51]. And is accompanied by a strongly skewed Th1 immune response. Yet treatment with TIM-2-Ig during the induction phase completely reverses this phenotype with little to no production of the hallmark Th1 cytokine IFN-γ

upon *in vitro* re-stimulation [18]. Conversely, TIM-2-Ig induces a strong Th2 cytokine profile with high levels of IL-4 and IL-10. This suggests that the interaction between TIM-2 and its ligand on antigen presenting cells may function to inhibit T cell proliferation and Th2 immune responses. Introduction of TIM-2-Ig might block this interaction, consequently reversing the inhibition. Indeed, TIM-2-Ig is able to significantly lower the strength of EAE by delaying its progression and reducing the clinical scores in the brain and spinal cord [18], implying that TIM-2-Ig can mediate expansion of a Th2 immune response within a Th1 immune setting, since regulation and recovery from EAE is dependent on Th2 cytokine production [50,51]. Supporting the notion that interaction of TIM-2 with its ligand might down modulate Th2 effector responses, TIM-2 deficient mice show an exaggerated Th2 phenotype with high production of the prototypic cytokines IL-4, IL-5, IL-6, IL-10 and IL-13, but no corresponding decrease in IFN-γ levels which were either unaffected or raised. These observations were detected in a Th2 biased lung inflammation model of atopic airway response as well as in Th-1 dependent model, with T cells responsible for the dysregulated phenotype [52].

In contrast to the studies discussed above, another study by Kumanogoh et al, has shown enhancement of T cell activation and production of Th1/Th2 cytokines upon TIM-2 cross-linking with one of its ligands, namely semaphorin-4a [31]. This effect was observed both *in vivo* and *in vitro* using a soluble Sema 4a-Ig fusion protein and was blocked with anti-sema4a antibody. Enhancement was obtained only upon administration of Sema4-Ig at the same time as immunization and not even a day later, suggesting that it may influence very early T cell responses. In lymphoid tissues, Semaphorin 4a is

expressed on dendritic cells and activated B cells [31]. It is also detected on activated un-polarized and Th2 polarized T cells maximally at 24 hours with subsequent decrease in expression, while a sustained expression on Th1 cells is maintained even after three rounds of polarization [53]. The studies performed by Kumanogoh et al in connection with TIM-2, have for the most part used unpolarized T cells [31]. TIM-2 has a very low expression on resting and activated T cells. Its expression is significantly up-regulated only in the late stages of Th2 cell differentiation [18]. Therefore, it is possible that Semaphorin-4a might interact with a ligand other than TIM-2 that is either well expressed on the cell surface or with higher affinity, to mediate its co-stimulatory function early in an immune response.

Interestingly, recent data have identified the more acidic chain of ferritin (H-ferritin), as a ligand for TIM-2 [19]. While extra-cellular ferritin is present in most body fluids at low levels (Ruggeri G), the plasma levels are elevated in inflammatory states and liver disease [54]. Enhanced levels of H-ferritin are also associated with different cancers although certain melanomas may secrete both H and L- ferritin [55-60]. A number of immunological functions have been attributed to extra-cellular ferritins including regulation of myelopoiesis [61], regulation of lymphocyte migration [62], and as an immunosuppressive agent [63]. An increasing body of evidence has indicated that H-ferritin but not L-ferritin can suppress anti-CD3 stimulated T cell proliferation[60,64-66], through increased production of IL-10 [60] from regulatory T cells [67,68] and monoclonal antibodies against H-ferritin or against 1L-10 can reverse these suppressive effects [60]. The up-regulated IL-10 is associated with small decreases in IL-2 and IL-4 Th2 cytokine production, and an increase in IFN-γ Th1 cytokine production [60].

Increased production of IL-10 occurs in part, through effects of H-ferritin on antigen presenting cells to induce greater dendritic cell expression of CD86 and B7-H1, which are ligands for CTLA-4 and PD-1 respectively [67]. It is likely that H-ferritin may function through its receptor TIM-2 to exert these immunosuppressive effects, but this connection has yet to be established. Notably, while H-ferritin may have immunosuppressive effects, it does not also promote apoptosis of the cell [69]. Conversely, H-ferritin up regulated by NF-κB, suppresses apoptosis induced by stimuli such as TNFα. [69]. It mediates the inhibition by preventing the induction of reactive oxygen species[69] that in turn are critical mediators of apoptosis [70,71], in a manner that is dependent upon iron sequestration [69].

The recent findings on TIM-2 so far, clearly indicate that there exists more than one ligand for this molecule. The overall functional outcome mediated by TIM-2 may be ultimately dictated by its expression pattern and the kinetics of this expression under different condition and consequent interaction with a tissue-specific ligand.

1.7. IMMUNE REGULATION BY TIM-3

The outcome of immune responses to different pathogens and auto-antigens are regulated by Th1 and Th2 pathways [72-75]. Th1 cells are characterized by their production of canonical cytokines IFN-γ, IL-2, TNF-α and lymphotoxin, which are associated with cell-mediated immune responses against intracellular pathogens [72-75]. One example of a qualitative and quantitative Th1 cell surface marker is TIM-3 due to its expression on terminally differentiated Th1, but not Th2 cells [8]. The functional role of TIM-3 is

beginning to be elucidated and accumulating data suggest that it negatively regulates Th1 immune responses.

Treatment of mice with anti-TIM3 antibody *in vivo* during the development of the Th1 dependent disease-EAE resulted in severe clinical disease with hyperacute inflammation and demyelinating lesions in the CNS [8]. The lesions were filled with activated macrophages probably induced by anti-TIM3, that were phagocytosing myelin fragments in the CNS. Antibody-mediated disruption of TIM-3's interaction with its physiological ligand enhanced basal splenocyte expansion *in vivo,* with macrophages being the principle proliferating cell population [8]. Immune hyperproliferation in anti-TIM-3 treated mice was an outcome of a synergistic and cognate interaction between TIM-3 expressing Th1 cells and non-T cells (B cells plus CD11b+ cells) [8]. Mechanistically, the antibody might cross-link TIM-3 on Th1 cells, inducing an amplification of inflammatory cytokine production that in turn activates macrophages. On the other hand, anti-TIM-3 could block an inhibitory interaction of TIM-3 with its natural ligand on T cells *in vivo,* thereby enhancing cytokine production. Activation of macrophages might be a secondary consequence of the disturbance.

Interference with endogenous TIM-3 interactions through fl-TIM3Ig or sTIM-3-Ig fusion proteins conducted in a parallel study, also led to *in vivo* basal splenic hyperproliferation [22]. Distinct from the effects of anti-Tim3 antibody, however, here T cells contributed primarily to the proliferative response, which was maximally observed with B cell interaction. TIM3-Ig induced a concomitant increase in Th1 cytokine production of IFNγ from T cells [22]. As with TIM2-Ig, the effects of TIM3-Ig could be

accounted for if the fusion protein were to disrupt the integrity of an inhibitory interaction between TIM-3 and its ligand.

The importance of this association was reinforced with the finding that TIM-3 deficient mice are resistant to high dose tolerance, and the fact that administration of anti-Tim3 antibody or flTIM3-Ig in NOD mice hastened the onset of diabetes [20,22]. Further, EAE-tolerized mice with (high doses of soluble antigen), treated with TIM3-Ig regained a large proliferative response and Th1 cytokine production of IL-2 and IFN-γ upon subsequent activation [22]. Since this response is in direct contrast to the normal phenotype of tolerized mice, it is indicative of an abrogation of tolerance induction. Underscoring TIM-1's role in down regulating Th1 responses, was the finding that T cells clones of patients suffering from multiple sclerosis had lower TIM-3 levels, exhibited a marked increase in IFN-γ production, and were also refractory to induction of tolerance. [76]. Consistent with these studies, administration of an anti-TIM3 antibody during the induction of pulmonary inflammation skewed a prototypical Th2 response to enhanced Th1 cytokine production [77].

Collectively, targeting the TIM3-TIM3 ligand pathway either by TIM3-Ig, or anti-TIM3 antibody, or through TIM3-deficient mice accelerated autoimmunity, thereby increasing the likelihood that TIM-3 triggers a negative signal in Th1 cells and/or amplifies pathways that dampen autoimmune responses [20,22].

Indefinite, donor-specific transplantation tolerance is induced in an islet allograft model in which the recipient is treated with DST (donor specific transfusion) together with anti-CD154 (CD40L) to achieve CD40-CD40L blockade [78,79] or in a model using CTLA4-Ig. Nevertheless, allograft rejection was rapidly precipitated when this treatment

was coupled with TIM3-Ig or was conducted in TIM-3 deficient Balb/c mice, denoting TIM-3 as a critical regulator of immunological tolerance [20]. All the same, interaction of TIM3 with its ligand on CD4+CD25+ regulatory cells-, key enforcers of allograft tolerance, was not a prerequisite for CD4+CD25+ to prevent allograft rejection [20]. In essence, TIM-3 was required for the generation of an enhanced immunosupressive regulatory cell population during tolerance induction, but not for its immunosuppressive effector function [20].

One probable molecular mechanism for TIM-3 mediated inhibition of Th1 immune responses is through cell death of this helper population. Importance for this mechanism was emphasized with the discovery of Galectin-9 as a TIM-3 ligand [21]. Galectin-9 is expressed in the immune system and its expression is downregulated on naïve CD4+T cells following activation, consistent with the expression kinetics observed with a TIM-3-Ig fusion protein [20,21]. Galectin-9 can induce cell death of thymocytes and peripheral CD4 or CD8 T cells and it selectively induces Th1 but not Th2 cell death [21,80,81]. This pattern of Galectin-9 mediated cell termination correlates well with TIM-3 expression on Th1 cells. A calcium-calpain-caspase-1 pathway has been linked to Galectin-9 mediated cell death [80]. Additionally, a highly conserved tyrosine present in the TIM-3 tail is phosphorylated by Itk and TIM-3 tyrosine phosphorylation is increased upon crosslinking with Galectin-9, suggesting a participation in the molecular events associated with Galectin-9 linked cell death [82]. In response to exogenously administered Galectin-9, Tim-3 deficient Th1 cells demonstrated a lower Ca2+ flux and lower cell death than wild type cells [21]. Since the effects of Galectin-9 were only partially abrogated, it suggests that only a portion of Galectin-9 induced cell death is

mediated through TIM-3 [21]. Cell death occurs by apoptotic and necrotic mechanisms and is preceded by T cell clustering reflecting probable crosstalk between adjacent cells. Further, an in-vivo administration of gal-9 during a Th1 immune response (EAE) significantly reduces the IFN-γ production from antigen specific Th1 cells, ameliorating EAE and these effects were reversed by galectin-9-siRNA. These data indicate that Gal-9 functions to control pro-inflammatory, IFN-γ secreting, Th1 effector cells during an autoimmune immune response in part through TIM-3 [21]. Galectin-9 can also be induced by IFN-γ or IL-1β [83,84] which in turn participates in a feed-back inhibitory mechanism to terminate Th1-mediated inflammation via Galectin-9.

1.8.FUTURE DIRECTION

The last few years have witnessed a significant increase in our understanding of the immunological roles of TIM family proteins. The field in now poised to further develop these observations into a more detailed understanding of the molecular mechanisms that underlie the functional effects of the TIM's on specific leukocytes sub-populations. Eventually, these proteins may represent desirable targets for pharmacological modulation of immune responses.

2. CHAPTER 2: STATEMENT OF THE PROBLEM

TIM-1 and TIM-2 belong to a novel gene family positionally cloned on mouse chromosome 11B1.1 analogous to human chromosome 5q33.2– a region that has been repeatedly linked to asthma, allergic and autoimmune diseases. The discovery of the TIM genes stemmed from genetic studies in congenic mice that linked the TIM locus to phenotypic consequences of differential Th2 responsiveness and airway hyperreactivity, a hallmark of human asthma. Thus a gene locus was linked to disease susceptibility and there exist distinct natural polymorphisms in TIM-1 and TIM-3 alleles that are not a result of alternative splicing. TIM-1 is expressed early upon T cell activation and could potentially regulate these events. Additionally, TIM-1 and TIM-3 are differentially expressed on Th1 and Th2 effector cells, respectively, and TIM-3 had been implicated in inhibition of Th1-dependent autoimmune responses. These early studies thus provided genetic and functional indications for the role of TIM's in allergic and autoimmune diseases

At the time of commencing these studies, there was an almost complete dearth of information regarding the molecular basis for TIM function. Excluding TIM-4, all the TIM's have conserved tyrosine kinase phosphorylation motifs, which are distinct between different family members, thus implying their potential role as - signal transducing - cell surface receptors. TIM-1 and TIM-2 share a high degree of sequence

homology with each other, and there was early indication from another group that TIM-2 could be tyrosine phosphorylated.

This led to the hypothesis that TIM-1 and TIM-2 could transduce intracellular biochemical signals in response to ligation of the receptor, by coupling to phosphotyrosine dependent signaling mechanisms in order to regulate T cell activation and helper immune responses. Consequently, the studies presented here were designed to achieve a greater understanding of the underlying biochemical signaling pathways that are triggered by TIM-1 and TIM-2, in an attempt to mechanistically explain the role of these proteins in health and disease. Elucidating the molecular mechanisms of a family of molecules that participate in T cell immunity, may lead to the identification of targets for therapeutic manipulation of immune responses in health and disease.

3.0 CHAPTER 3: TIM-1 PROVIDES A CO-STIMULATORY SIGNAL FOR T-CELL ACTIVATION

This chapter has been adapted from a published study (de Souza AJ et al (2005) *Proc Natl Acad Sci USA* 102:17113-17118). The chapter includes additional data that was not shown in the published paper.

3.1. INTRODUCTION

Many helper T cell-dependent immune responses skew toward one of two stereotyped profiles. Generally speaking, the T-helper 2 (Th2) type response has been selected for its ability to eliminate parasitic worm infections, while a Th1 response is more effective at dealing with viruses and intracellular bacteria [85,86]. A Th2 response is characterized by the generation of CD4+ T cells that make and secrete the cytokines IL-4, IL-5 and IL-13, which help drive a humoral immune response characterized by relatively high levels of antigen-specific IgE, as well as having effects on cell types such as eosinophils and mast cells. By contrast, a Th1 type of immune response is characterized by the production of high levels of interferon gamma, among other cytokines, that are important for cell-mediated immunity. Just as many protective immune responses can be categorized

according to the Th1/Th2 framework, so too can many harmful responses. For example, diseases like atopic dermatitis, allergic rhinitis and asthma are characterized by aberrant Th2 responses to certain otherwise "innocuous" antigens (allergens). Conversely, some autoimmune diseases, like type I diabetes and multiple sclerosis, are caused by inappropriate Th1 responses to self-antigens.

It is still not clear why some individuals respond to certain allergens with strongly polarized Th2 immune responses. Environmental factors likely play a role, but there is strong evidence from man and mouse that genetic factors also influence the outcome. A number of human linkage analyses have been performed, and the results implicate at least fifteen genetic loci in human atopy and asthma [40,87]. One human genetic locus that has received a great deal of attention is 5q23-35. The syntenic region of mouse chromosome 11 has also been implicated in the development of atopic asthma [5], by analysis of a series of congenic mice that were bred to be identical to the asthma-susceptible BALB/c strain, but for small loci inherited from the relatively asthma-resistant strain DBA/2. In one of these congenic lines, termed 'HBA,' suppression of asthma susceptibility was mapped to a genetic interval inherited from DBA/2, which the authors named *tapr* (for T cell and airway phenotype regulator). Further analysis implicated two genes in this locus, *tim1* and *tim3*, named for the fact that they encode proteins expressed on T cells and contain immunoglobulin and mucin domains. The TIM's are type I transmembrane proteins, with extracellular immunoglobulin and mucin domains, and intracellular domains that contain an average of forty amino acids, and which may be tyrosine phosphorylated [88].

In addition to the genetic linkage to asthma in mice, there are several lines of evidence suggesting that TIM-1 is involved in helper T cell differentiation. First, TIM-1 mRNA is upregulated by CD4+ T cells within seven hours of stimulation [5], a time when helper T cells are undergoing differentiation to become effector cells. Also, messages for TIM-1 were found at higher levels in Th2 lines derived from patients with multiple sclerosis (MS), compared to TIM-3 message, which was more abundant in Th1 lines [89]. In the same study, levels of TIM-1 message correlated positively with levels of message for the Th2-associated cytokine IL-10, and inversely with IFN-γ [89]. One hypothesis for how TIM-1 functions in T cells is that it acts as a co-stimulator to affect T cell activation, either quantitatively or qualitatively.

Recent studies have examined the expression and function of murine TIM-1 in some detail, using an anti-TIM-1 antibody [17] or a TIM-1-Ig fusion protein [25]. Consistent with the studies cited above, TIM-1 protein was found on activated T cells, with the highest levels seen on Th2 cells [17]. Furthermore, addition of the anti-TIM-1 antibody provided a co-stimulatory signal for T cell activation. The co-stimulatory function of TIM-1 may be induced by binding to TIM-4, which was shown to be a ligand for TIM-1 [25].

We report here that TIM-1 protein is expressed *in vivo* on the surface of T cells from lung draining lymph nodes after intranasal immunization. Furthermore, ectopic expression of TIM-1 increases the frequency of IL-4 producing cells when T cells are stimulated under non-polarizing conditions. We have also found that TIM-1 augments the activation of the IL-4 promoter in a Th2 T cell clone, a co-stimulatory effect that may occur through increased activation of the composite NFAT/AP-1 transcription factors.

Finally, we provide evidence that the cytoplasmic tail of TIM-1 is required for its co-stimulatory activity, through a mechanism that is dependent on tyrosine phosphorylation.

3.2. MATERIALS AND METHODS

3.2.1. DNA constructs

A cDNA clone containing the entire coding sequence of murine TIM-1 (from strain C57BL/6), originally isolated by the I.M.A.G.E. consortium, was purchased from Open Biosystems (Huntsville, AL). The open reading frame of TIM-1 (excluding the start codon and signal sequence) was amplified from this clone by PCR with the Expand polymerase (Roche; Indianapolis, IN) and forward and reverse primers containing Cla I and Eco R1 sites, respectively. The amplified product was ligated into a pCDEF3 expression plasmid containing a signal sequence from human CD8a followed by the coding sequence for the Flag epitope tag [90]. A Flag-TIM-1 construct lacking the cytoplasmic tail (delta-cyto TIM-1) was generated in an identical fashion, except that the reverse primer was designed around the boundary of the transmembrane and cytoplasmic domains, with a stop codon at the 5' end of the primer. The potential site of src-mediated tyrosine phosphorylation in the cytoplasmic tail of TIM-1 (Y276 in the BL/6 allele) was mutated (within the Flag-TIM-1 construct) to phenylalanine with the QuickChange site-directed mutagenesis kit from Stratagene. All DNA constructs were verified by automated DNA sequencing.

3.2.2. Antibodies and flow cytometry

Anti-Flag antibody (M2) was from Sigma (St. Louis, MO). Antibody to murine TIM-1, rat isotype control and anti-rat-FITC were from e-Biosciences (San Diego, CA). Hamster antibodies to murine CD3 (500A2) and CD28 (37.52) were obtained from BD-Pharmingen (San Jose, CA) or Caltag (Burlingame, CA). Anti-CD4-PE was from BD-Pharmingen. Other fluorophore-conjugated secondary and cytokine antibodies were from Caltag. TIM-1-Ig was described previously [25], and was obtained from V. Kuchroo (Harvard Medical School, Boston, MA). Mouse monoclonal anti-phospho-tyrosine 4G10 was obtained from Upstate, Inc. Flow cytometry was performed with a Becton Dickinson LSR II, using FACS Diva software, or with a FACS Calibur, using CellQuest software.

3.2.3. Induction of airway tolerance or inflammation

BALB/cByJ mice were immunized as described previously [91]. Briefly, mice were given three consecutive daily treatments of intranasal ovalbumin alone, to induce airway tolerance, or ovalbumin plus cholera toxin adjuvant, to induces Th2-mediated airway inflammation. Three or five days following the last intranasal treatment, lung-draining lymph nodes were harvested and the cells were stained for expression of CD4 and TIM-1. Analysis was performed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

3.2.4. Transient transfections and luciferase assays

Jurkat or D10 T cells were transfected by electroporation and luciferase assays were performed, both as described previously [92,93]. A rapidly dividing variant of the D10 T cell clone was obtained from M. Krummel (U.C.S.F.). Cells were re-stimulated every three to four weeks with chicken conalbumin (Sigma) and irradiated or mitomycin c-treated APC's (rbc-depleted splenocytes from B10.BR mice). During the intervening periods, cells were maintained in complete D10 medium (RPMI supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 mM 2-mercaptoethanol, 100 U/ml penicillin, 100 mg/ml streptomycin, and 25 U/ml rhIL-2 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Maurice Gately, Hoffmann-La Roche, Inc).

3.2.5. Generation of recombinant retrovirus and infection of primary T cells

The Flag-TIM-1 construct was sub-cloned into the MSCV2.2 retroviral vector, which contains an IRES and GFP open reading frame downstream of the multiple cloning site. To generate recombinant retrovirus, MSCV-TIM-1 (or empty vector) was transfected into the Phoenix packaging cell line by the calcium phosphate method, along with the ecotropic packaging vector, to increase the efficiency of virus production. Viral supernatant was harvested at 48 and 72 hours after transfection.

T cells were purified from spleen and lymph node of six-week-old C57BL/6 mice by negative selection, using the murine T cell purification kit from Miltenyi (Auburn,

CA). Purified T cells were stimulated in 24-well plates coated with rabbit-anti-hamster antibody (Sigma, St. Louis, MO) and hamster antibodies to CD3 and CD28, at a concentration of one million cells per well. Twenty-eight hours later T cells were subjected to two rounds of spin infection, at four hour intervals, with retroviral supernatant supplemented with IL-2 and polybrene. Cells were expanded one day after infection, re-stimulated the next day with anti-CD3 plus Brefeldin A (Sigma), and intracellular cytokine staining performed after overnight stimulation.

3.2.6. TIM-1 tyrosine phosphorylation

D10 or Jurkat cells (20 million) were transfected as described above with empty vector or Flag-TIM-1. NP-40 cell lysates were made the next day and IP'd with anti-Flag antibody M2 covalently coupled to agarose beads (Sigma). IP's were separated on SDS-PAGE gels and western blotted with 4G10 and goat-anti-mouse-HRP (Pierce). Blots were developed with SuperSignal Pico ECL substrate (Pierce) and digitally imaged on a Kodak Image Station 2000R, with accumulation automatically set to stop at 2000 gray levels. Blots were then stripped and re-probed with M2 and anti-mouse-HRP to visualize total TIM-1 present in the IP's. Digital images were exported as JPEG images and final figures composed in Canvas 8.

3.3. RESULTS

3.3.1. Expression of TIM-1 on CD4+ T cells *in vivo.* (This experiment was performed by Dr. Tim Oriss in Dr. A. Ray's Laboratory)

The available data suggest that TIM-1 expression is associated with development of Th2 T cell responses. We examined TIM-1 expression on T cells *in vivo* during a Th2 immune response initiated by intranasal immunization with ovalbumin (OVA) plus cholera toxin (CT) [91,94-96]. As shown in **Figure 4**, a significant proportion of T cells isolated from lung draining lymph nodes after OVA/CT immunization expressed TIM-1 five days (and also three days; data not shown) after treatment (right column). We also assessed the expression of TIM-1 on T cells after treatment with OVA alone, which induces an abortive course of T cell activation and partial Th2 effector development that results in tolerance. As shown in **Figure 4** (left column), this treatment also led to the expression of TIM-1 at days three and five, consistent with upregulation of TIM-1 expression early after activation, but before the induction of tolerance. No detectable TIM-1 staining was observed on naïve T cells (data not shown). Thus, intranasal immunization of T cells under inflammatory or tolerizing conditions leads to the expression of TIM-1. This is the first demonstration of TIM-1 expression on T cells isolated from a murine model of asthma, a Th2-mediated disease.

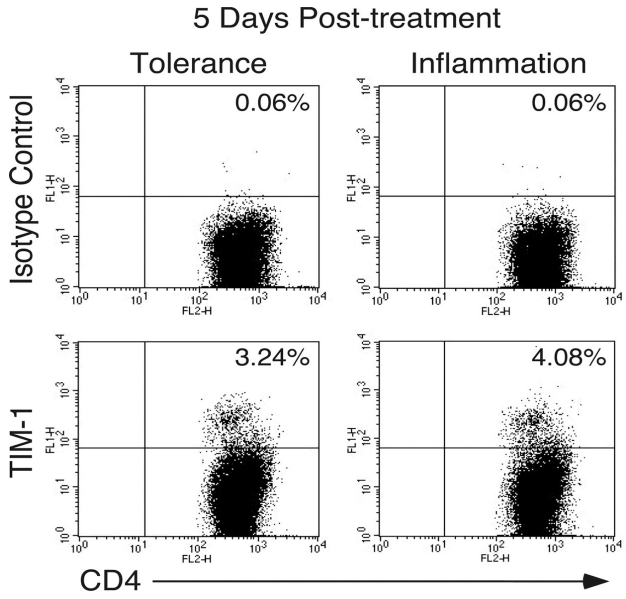


Figure 4: TIM-1 is expressed on lung-draining lymph node CD4 T cells under conditions of airway tolerance and inflammation. (This figure is adapted from Dr. Anuradha Ray's Laboratory)
Mice were immunized as described in *Materials and Methods*, with OVA alone to induce tolerance (*Left*) or with OVA plus cholera toxin to induce inflammation (*Right*). Five days after the last intranasal treatment, lung-draining lymph nodes were harvested, and cells were stained with antibodies to CD4 and TIM-1. Results are gated on CD4+ cells.

3.3.2. Generation of Flag-TIM-1

When we commenced these studies, an antibody to murine TIM-1 was not available, and therefore we generated a TIM-1 construct with an extracellular Flag epitope tag (**Figure 5**). A full-length TIM-1 clone (containing the BL/6 allele) was obtained from the I.M.A.G.E. consortium. The TIM-1 coding sequence downstream of the signal sequence was amplified and ligated in-frame to a human CD8a signal sequence and Flag epitope tag contained within the pCDEF3 mammalian expression vector [97]. The CD8-Flag-

TIM-1 sequence was also sub-cloned into the MSCV 2.2 retroviral expression vector, for generation of retrovirus to infect primary T cells.

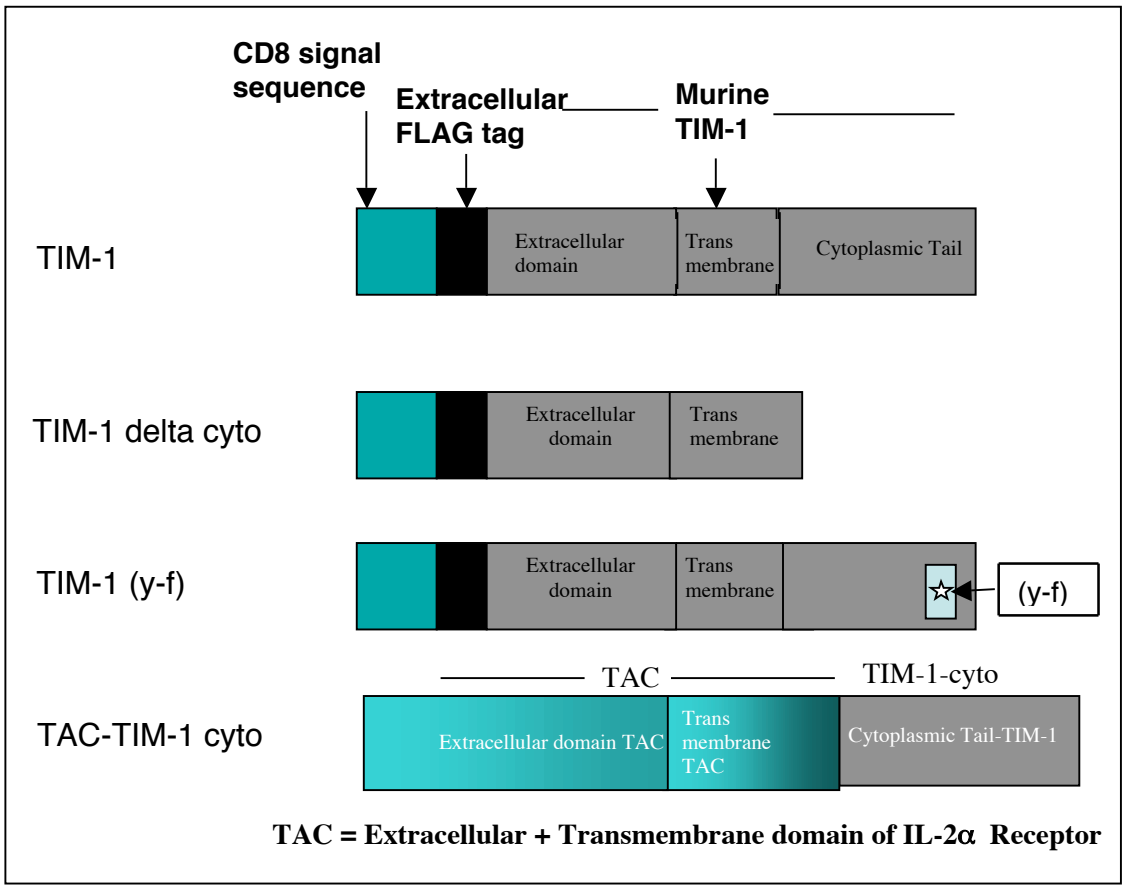


Figure 5: Flag-TIM-1 constructs used for the study

Flag-tagged murine TIM-1, TIM-1-delta cyto and TIM-1 (y-f) were generated as described in the text and in Material and Methods.

3.3.3. Effects of ectopic TIM-1 expression on helper T cell cytokine production

Expression of the TIM molecules appears to be tightly regulated [22,31]. We wanted to assess directly the effect of enforced TIM-1 expression during T cell activation and differentiation, so we employed a widely used retroviral system for expression of exogenous genes in primary T cells [98,99]. Purified T cells from C57 BL/6 mice were stimulated *in vitro* with antibodies to CD3 and CD28, under neutral conditions, then infected with retrovirus-containing supernatant from packaging cells transfected with either Flag-TIM-1 or an empty vector. Cells infected with control or TIM-1 retrovirus could be followed for expression of GFP, which is also encoded for by the retroviral vector. In addition, cells infected with TIM-1 retrovirus were stained with anti-Flag antibody and analyzed by flow cytometry (data not shown), to confirm expression of Flag-TIM-1. After retroviral infection, cells were rested and re-stimulated with anti-CD3/CD28 antibodies. Intracellular cytokine staining was performed after re-stimulation, to assess the number of cells producing IFN-γ and IL-4, the hallmark cytokines of Th1 and Th2 cells, respectively. As shown in **Figure 6**, cultures infected with TIM-1 retrovirus contained significantly more (about two-to-three fold) cells making IL-4, compared with cells infected with control retrovirus. By contrast, expression of TIM-1 had little-to-no effect on the number of cells producing IFN-γ in these same unpolarizing ultures. Somewhat unexpectedly, these results were observed in the absence of any active crosslinking of the Flag-TIM-1 protein, and addition of anti-Flag antibody had no further positive or negative effects (data not shown).

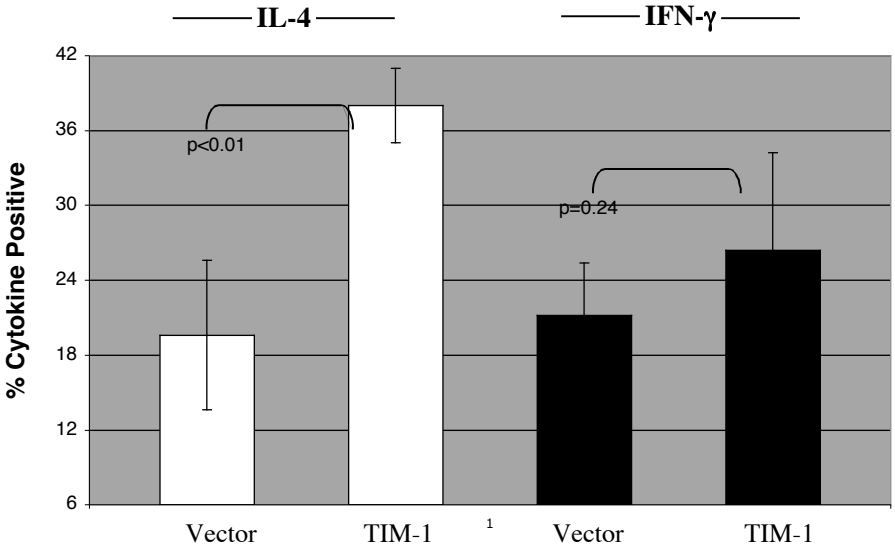


Figure 6: Co-stimulation of Th2 differentiation by TIM-1.

Purified T cells were stimulated *in vitro* under neutral conditions, then infected with control MSCV-GFP retrovirus ("vector") or virus encoding Flag-tagged murine TIM-1. Cells were rested and restimulated with anti-CD3 antibody, then stained for intracellular cytokines. Results are the percentage of cytokine-positive cells of the GFP+ population in each case and are the average ± SD of six samples from three experiments. *P* values were derived from Student's *t* test analyses.

3.3.4. Co-stimulation of inducible transcription by TIM-1

Because our results suggested that the development of IL-4 producing cells is enhanced in the presence of TIM-1, we wished to determine whether this effect was due, at least in part, to increased IL-4 transcription. We co-transfected an IL-4 promoter luciferase reporter into the D10 Th2 T cell clone [100], along with either an empty vector or Flag-TIM-1 expression plasmid. Flag-TIM-1 was expressed efficiently on the surface of transfected D10 cells (**Figure 8**), and cells expressing TIM-1 responded significantly better to a range of stimuli than cells transfected with empty vector (**Figure 7A**). This

conditions included stimulation through the TCR/CD3 complex, with or without CD28 co-stimulation, or ionomycin, all of which were enhanced in the presence of TIM-1. By contrast, PMA and ionomycin, which bypass the proximal tyrosine kinases and PLC-γ1, induced a level of stimulation that was not significantly affected by TIM-1 ($P = 0.15$). In addition, the basal level of IL-4 promoter transcriptional activity was not affected by the transfection of TIM-1.

Given the above results, we wanted to determine whether TIM-1 expression also affects transcription of IFN-γ. When an IFN-γ-luciferase reporter was transfected into Jurkat T cells, we noted that co-transfection of TIM-1 led to a significantly higher level of basal luciferase activity, i.e., in the absence of stimulation (**Figure 7B**). TIM-1 expression also augmented the stimulation of this reporter by signaling through the TCR. Therefore, at least when expressed in Jurkat T cells, TIM-1 can also augment transcription from the IFN-γ promoter.

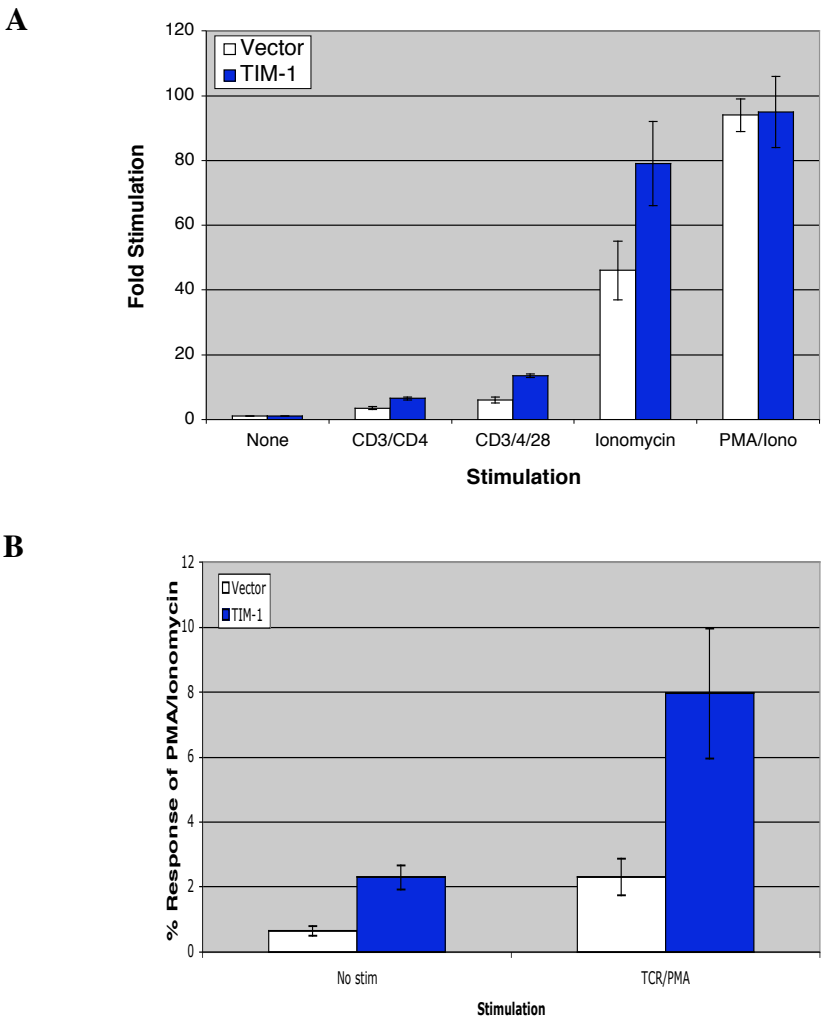


Figure 7: Costimulation of cytokine transcription by TIM-1.

(*A*) D10 T cells were transfected with an IL-4 promoter luciferase reporter and either an empty vector or Flag-TIM-1. The next day, cells were stimulated as indicated (PMA, phorbol 12-myristate 13-acetate) and analyzed for luciferase activity. Results are presented as relative light units (mean ± SD) of four samples, from two separate experiments. (*B*) Jurkat T cells were transfected with a murine IFN- promoter luciferase construct, along with either an empty vector or Flag-TIM-1 plasmid. Cells were stimulated as indicated (TCR, T cell receptor) and luciferase activity was determined. Results are the mean response, normalized to PMA/ionomycin for duplicate points of a single experiment, representative of the three performed.

Cytokine transcription is controlled by a number of transcription factors [101]. Perhaps the best studied of these are the proteins of the NFAT family, four of which are expressed by lymphocytes [102]. Most NFAT family members are regulated by calcium, their entry into the nucleus occurring after calcineurin-mediated de-phosphorylation, which requires increases in intracellular calcium [102]. In many promoters, NFAT binds cooperatively to DNA with transcription factors of the AP-1 family [103]. The IL-4 promoter contains both *cis*-acting elements that can bind NFAT alone, as well as sites where NFAT binds cooperatively with AP-1 [101]. We co-transfected an NFAT/AP-1 luciferase reporter into D10 T cells, along with empty vector or the Flag-TIM-1 expression plasmid. As shown in **Figure 9A**, cells expressing TIM-1 and the NFAT/AP-1 reporter responded more robustly to stimulation through the TCR/CD3 complex, compared with cells transfected with empty vector. Consistent with the results shown in **Figure 7A**, no increase in basal NFAT/AP-1 reporter activity was observed in the TIM-1-transfected D10 cells.

We also assessed the ability of TIM-1 to co-stimulate NFAT/AP-1-dependent transcription in Jurkat T cells. Flag-TIM-1 could be detected on Jurkat T cells after transient transfection (**Figure 8**). When luciferase activity was determined (**Figure 9B**), we noted that TIM-1 (black bars) significantly increased the basal activity of the NFAT/AP-1 reporter, in contrast to what was seen in D10 cells (**Figure 9A**). Consistent with the IL-4 and NFAT/AP-1 results obtained with D10 cells, TIM-1 could also co-stimulate transcription induced through the TCR and CD28. Treatment with anti-Flag antibody, however, neither enhanced nor inhibited the basal NFAT/AP-1 activity induced by TIM-1. As a positive control for the anti-Flag treatment, we transfected Jurkat cells

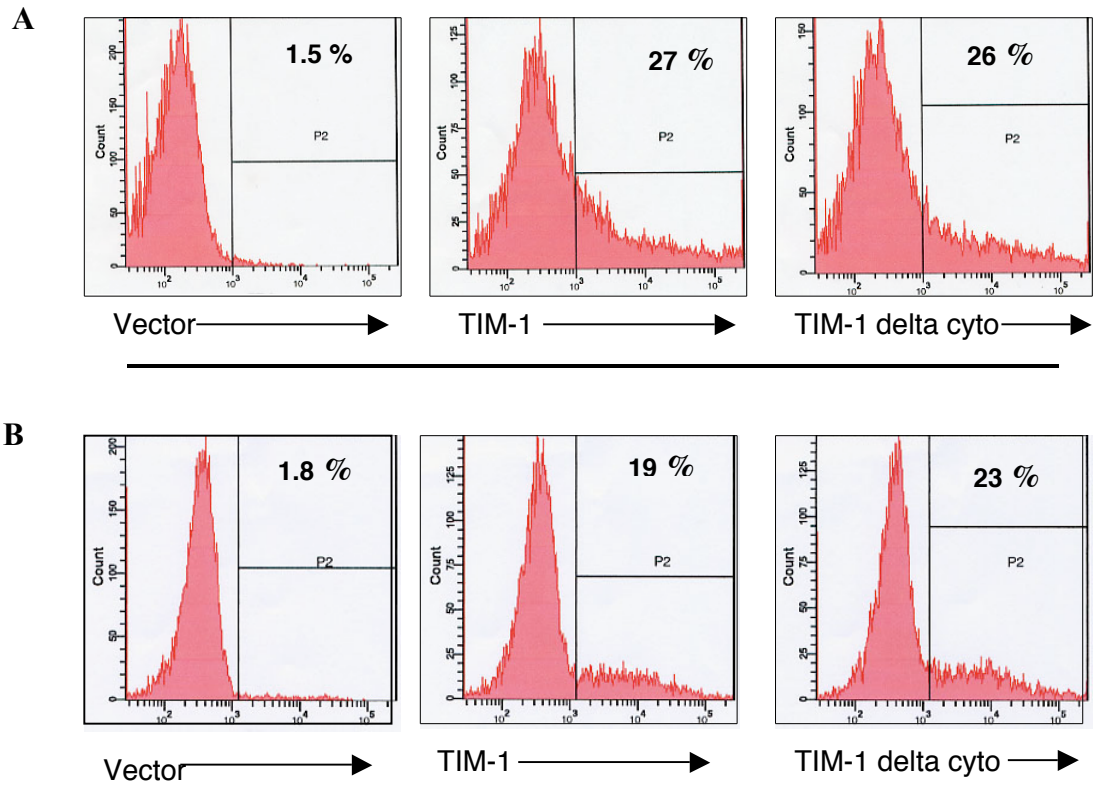


Figure 8: Expression of the Flag-TIM-1 constructs on (A) Jurkat and (B) D10 T cells.

Jurkat (A) or D10 (B) T cells were transfected with empty vector or Flag-TIM-1 or Flag-TIM-1-delta cyto. Eighteen hours later, cells were stained with anti-Flag-Antiody M2, and PE-conjugated secondary antibody.

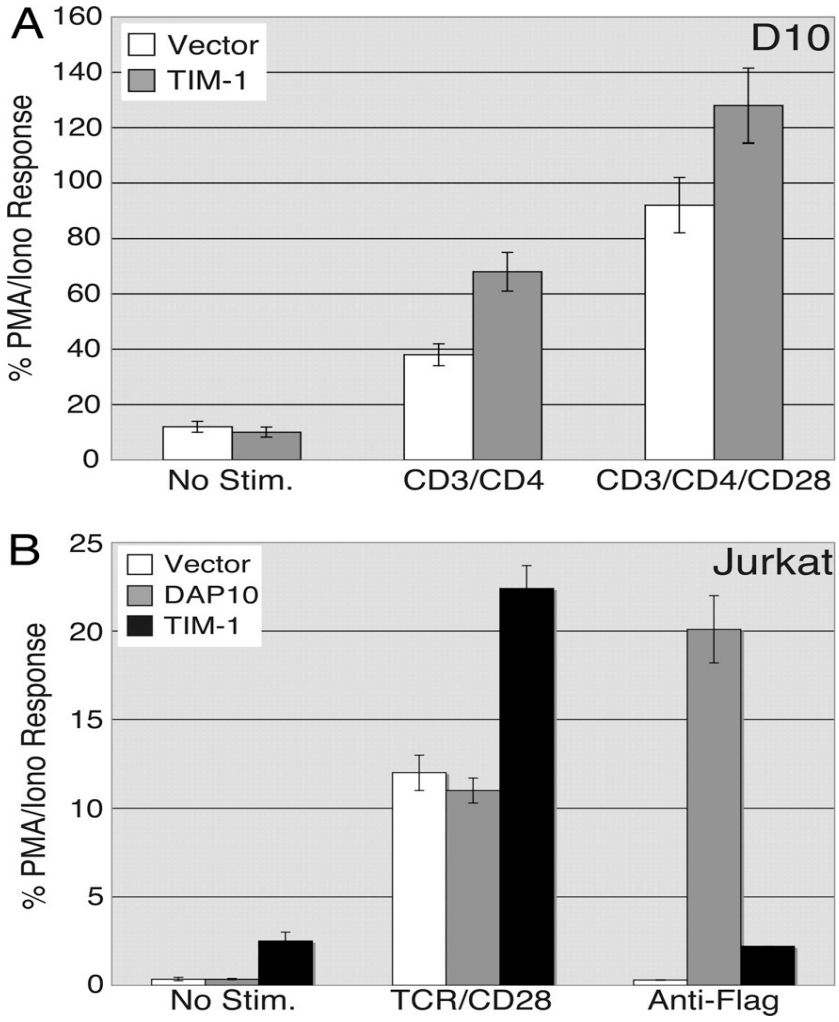
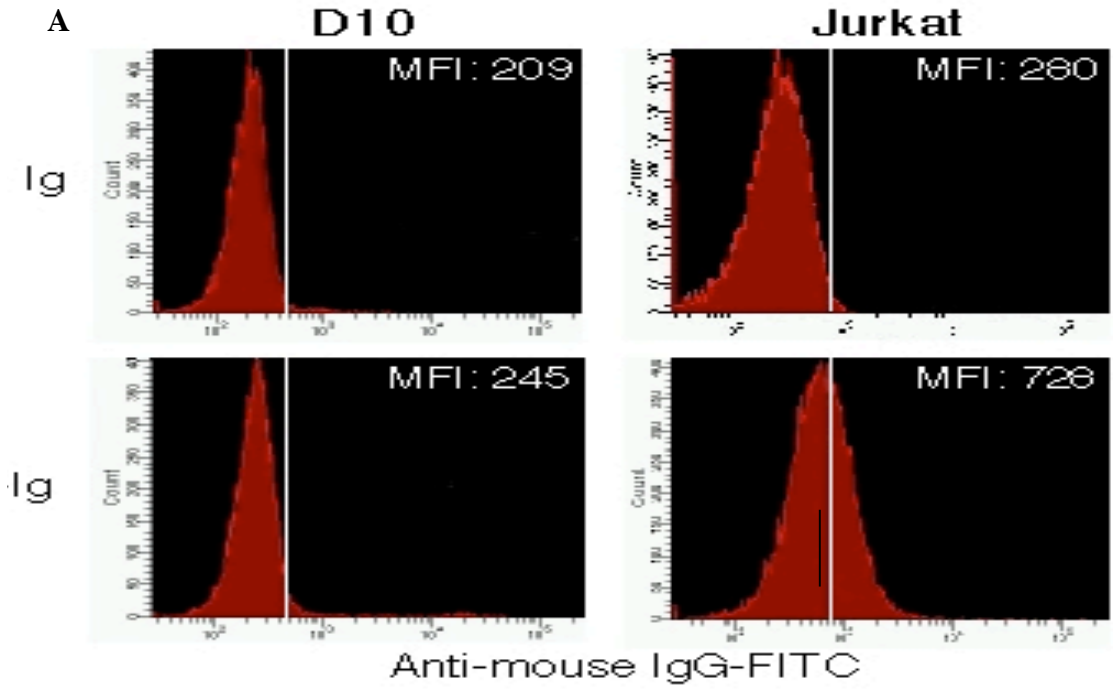


Figure 9. TIM-1 costimulates NFAT/AP-1-dependent transcription.

D10 (*A*) or Jurkat (*B*) T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated plasmids. Cells were treated the next day with the indicated stimuli and analyzed for luciferase activity, which is expressed as the percentage (mean ± SD) of the response with PMA/ionomycin from triplicate points of a single experiment. (*A*) NFAT/AP-1 activity in D10 T cells transfected with empty vector or Flag-TIM-1. Data displayed are representative of three experiments that were performed. (*B*) NFAT/AP-1 activity in Jurkat cells transfected with empty vector, Flag-DAP10, or Flag-TIM-1. Data displayed are the means of triplicate points from a single experiment, representative of >10 experiments.

with Flag-tagged DAP10, a component of some activating receptors in NK cells [90]. Consistent with previous results (data not shown), crosslinking of this construct could activate transcription from an NFAT/AP-1 reporter, but unlike TIM-1, DAP10 expression alone did not lead to increases in basal or TCR-stimulated NFAT/AP-1 reporter activity (**Figure 9B, gray bars**). It is possible that the effects of TIM-1 we observe may have resulted in part from interaction with a ligand distinct. Indeed, we have obtained evidence for a TIM-1 ligand on Jurkat, but not D10, T cells (**Figure 10A**). Thus far, however, we have only observed a partial decrease in basal levels of NFAT/AP-1 reporter activity when using TIM-1-Ig to block interactions with this putative ligand (**Figure 10B**).

Additional luciferase reporter experiments showed that TIM-1 expression could also co-stimulate induction of an AP-1 reporter (**Figure 11A**) independently. However, in contrast to the co-stimulatory molecule CD28, under no conditions did we observe any effect of TIM-1 on an NF-κB reporter **(Figure 11B**). Thus, TIM-1 appears to have a unique co-stimulatory profile, augmenting NFAT and AP-1, but not NF-κB, signaling.



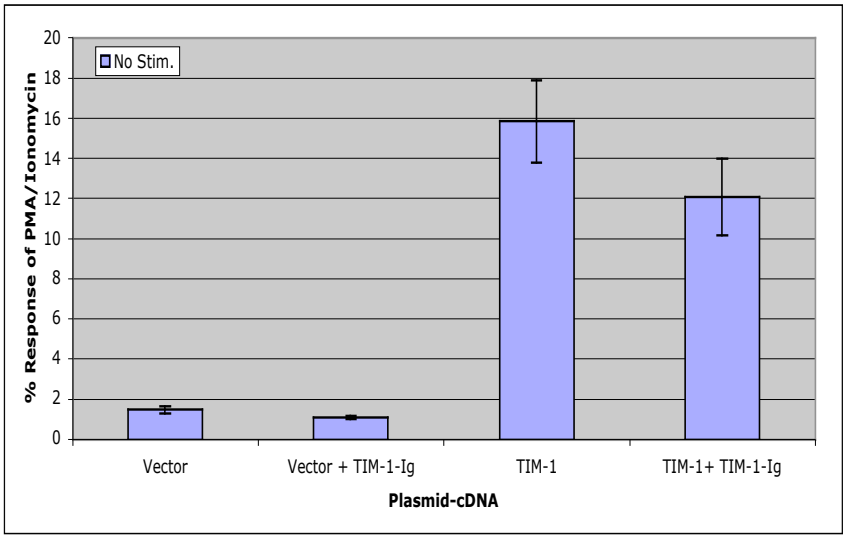


Figure 10. Use of a TIM-1-Ig protein for ligand detection and it's effects on NFAT/AP-1 reporter activity.

(*A*) Jurkat and D10 cells were stained with mouse IgG2A or TIM-1-Ig, followed by anti-mouse IgG secondary antibody conjugated to FITC. (*B*) Jurkat cells were transfected with NFAT/AP-1-luciferase and either empty vector or Flag-TIM-1, followed by overnight culture with or without TIM-1-Ig. The next day, cells were stimulated as indicated and luciferase activity was determined.

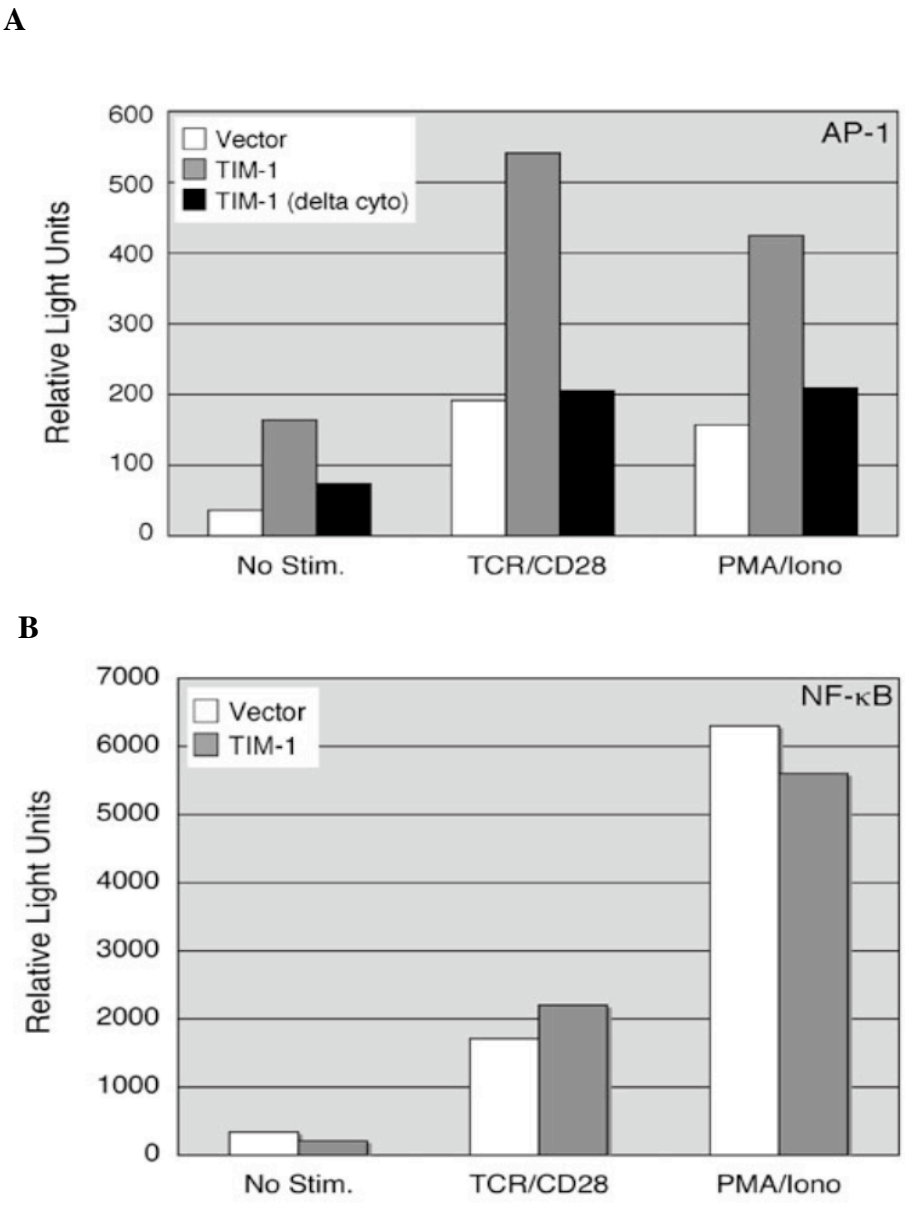


Figure 11: Effect of TIM-1 expression on AP-1 and NF-κB transcriptional reporters.

Jurkat cells were transfected with an AP-1 (*A*) or NF- κB (*B*) transcriptional reporter, along with empty vector or Flag-TIM-1. The next day, cells were stimulated for 6 h as indicated, and luciferase activity was determined. Results are presented as the mean relative light units obtained from duplicate samples of a single experiment, representative of at least three that were performed with each reporter. In *A*, a transfection was also performed with the Δ-cyto form of TIM-1, which behaves as shown for the NFAT/AP-1 reporter (Figure 6).

3.3.5. Role of the TIM-1 cytoplasmic tail in co-stimulation

We next wanted to determine whether the co-stimulatory signal provided by TIM-1 requires its cytoplasmic tail, analogous to other co-stimulatory molecules like CD28. We therefore generated a Flag-TIM-1 construct lacking the cytoplasmic tail, and compared its ability to co-stimulate NFAT-dependent transcription with full-length Flag-TIM-1. As shown in **Figure 8**, delta-cyto Flag-TIM-1 was expressed at the surface of transfected D10 and Jurkat T cells at levels roughly equivalent to the full-length construct. When the constructs were co-transfected into D10 cells with an NFAT/AP-1 luciferase reporter (**Figure 12A**), delta-cyto TIM-1 displayed an almost complete loss of co-stimulatory activity. Similarly, the delta cyto TIM-1 construct displayed almost no co-stimulatory activity when transfected into Jurkat T cells (**Figure 12B**). However, for reasons that are still unclear, expression of truncated TIM-1 in Jurkat cells still led to increased levels of basal reporter activity, i.e. in the absence of additional stimulation.

In-order to better understand the contribution of the cytoplasmic tail to TIM-1 signaling, a chimeric molecule was generated consisting of the extracellular and transmembrane domains of the IL-2α receptor (Tac) fused to the cytoplasmic tail of murine TIM-1. a chimeric molecule was generated consisting of the extracellular and transmembrane domains of the IL-2α receptor (Tac) fused to the cytoplasmic tail of murine TIM-1 (Tac-TIM1-cyto) (**Figure 12**). As observed with TIM-1 delta cyto, the Tac-TIM1-cyto chimera increased reporter activity, when cross-linked with anti-Tac antibody, in the absence of TCR/CD28 antibodies (**Figure 13A**). Under stimulation with TCR and CD28, Tac-TIM-1-cyto was also able to co-stimulate induction of the reporter as observed with

full length TIM-1 (**Figure 13B**). Similar observations were detected with transfection of an Elk-1 reporter instead (**Figure 12C**). Elk-1 functions to up-regulate the FOS element required for the AP-1 transcription factor (Fos/Jun). Taken together, the co-stimulatory activity of TIM-1 requires its cytoplasmic tail.

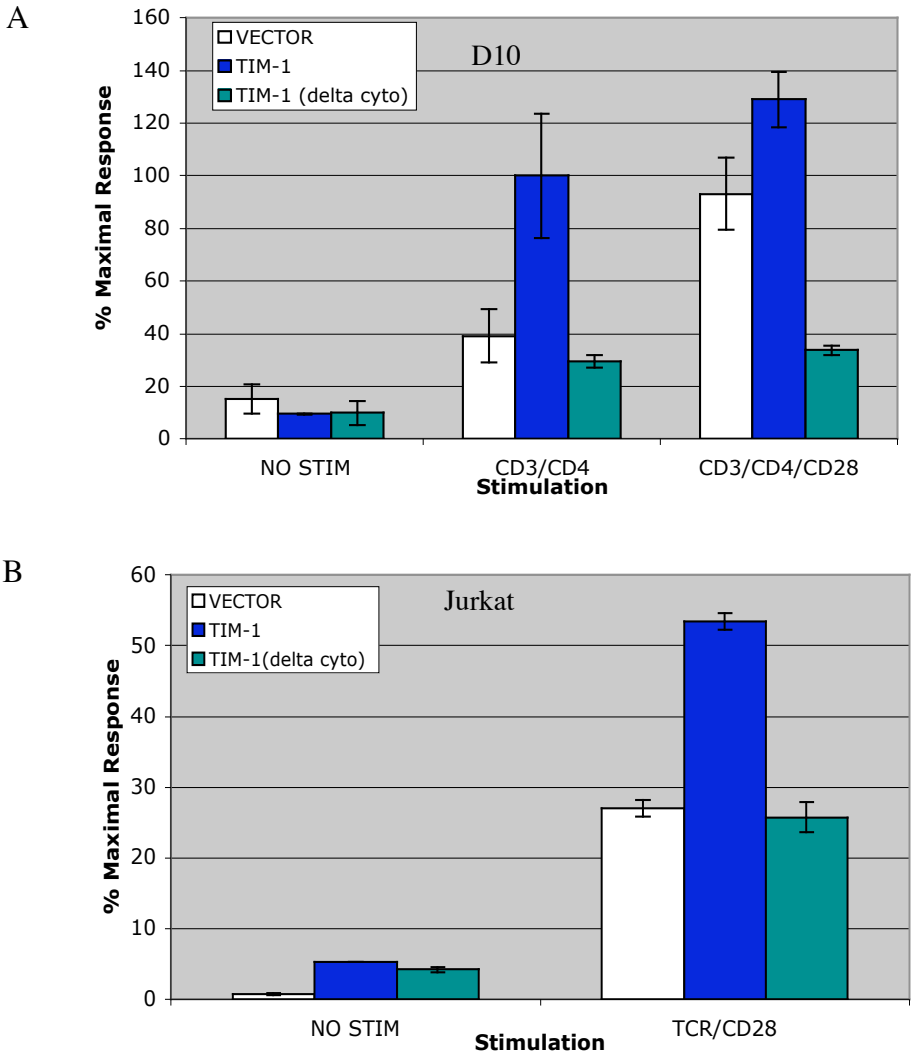


Figure 12: The TIM-1 cytoplasmic tail is required for costimulation of transcription.

D10 or Jurkat T cells were transfected with the NFAT/AP-1 luciferase reporter and either the empty vector, Flag-TIM-1, or the truncated Flag-TIM-1. (*A*) Luciferase activity of unstimulated or anti-CD3/CD4-stimulated D10 cells transfected with the indicated constructs. Results shown are the mean of duplicate points from a single experiment, representative of four that were performed. (*B*) Luciferase activity in Jurkat T cells transfected with the same constructs as in *A*. Luciferase activity is expressed as the percentage (mean ± SD) of the response with PMA/ionomycin from triplicate points of a single experiment, representative of eight that were performed.

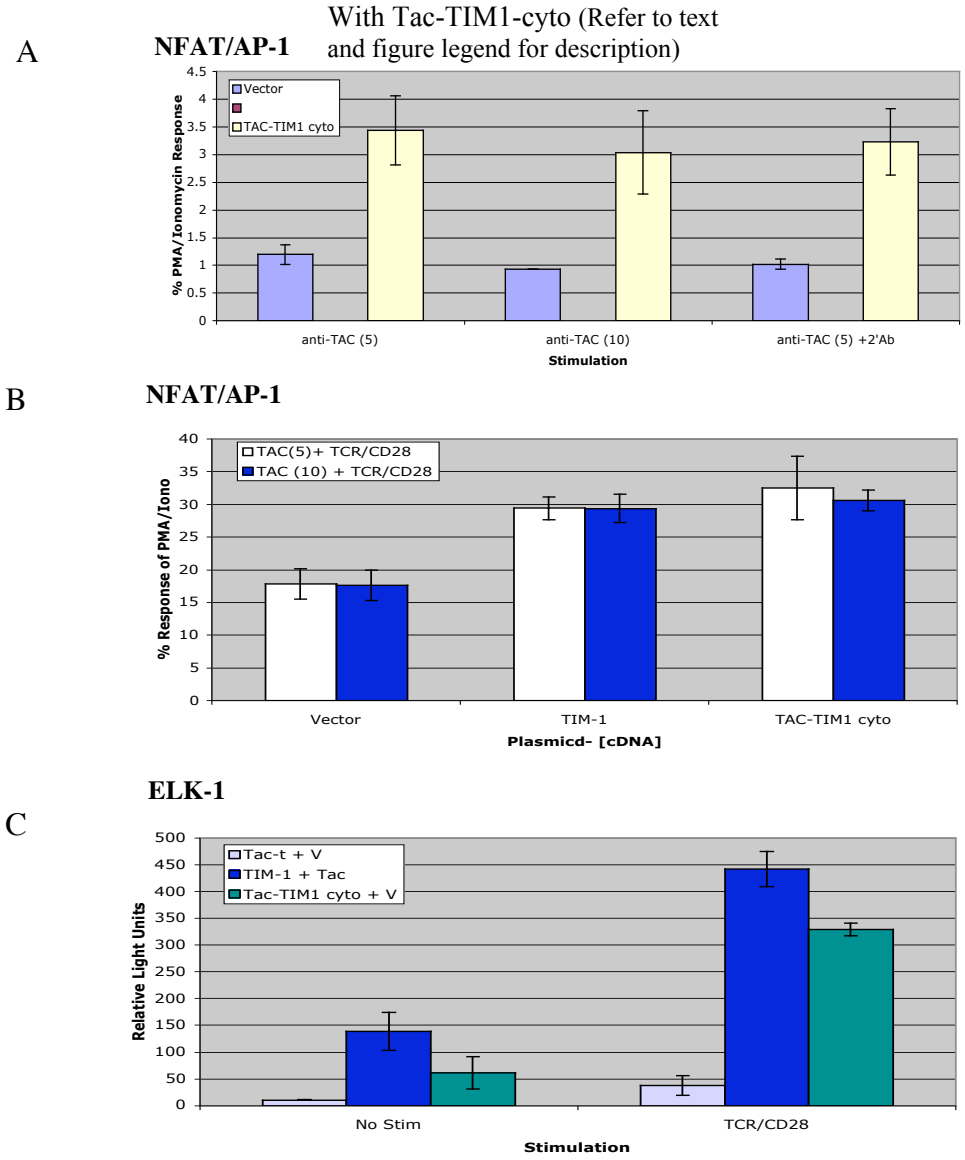


Figure 13: Role of TIM-1 cytoplasmic tail in the absence of extra-cellular and transmembrane domains, for NFAT/AP-1 induction.

Jurkat T cells were transfected with the NFAT/AP-1 luciferase (A & B) or Elk-1 (C) reporter and either the empty vector, Flag-TIM-1, or the Tac-TIM-1cyto. **[Tac-TIM-1-cyto is a chimera consisting of the extracellular and transmembrane domains of IL2α repector (Tac) fused to the cytoplasmic tail of Tim-1**. Elk-1 induces AP-1 activation by upregulating the Fos element of AP-1.] The next day cells were left unstimulated or were stimulated with anti-TCR/CD28 or anti-Tac (5or 10 ug/ml), and luciferase assay was performed. Results shown are the mean of duplicate or triplicate points from a single experiment done two to four times in each case.

3.3.6. Tyrosine phosphorylation of TIM-1

Inducible tyrosine phosphorylation is critical for the initiation of TCR/CD28-mediated signal transduction and T cell activation, so we were interested in determining whether TIM-1 could be phosphorylated on tyrosine. Flag-TIM-1 was transfected into Jurkat or D10 T cells, which were divided the next day and either left un-stimulated or treated with pervanadate to pharmacologically induce tyrosine phosphorylation. After lysis of cells, Flag-TIM-1 was IP'd and analyzed for tyrosine phosphorylation by western blotting with the antibody 4G10. As shown in **Figure 14A**, TIM-1 expressed in both un-stimulated Jurkat or D10 cells displayed a low level of basal tyrosine phosphorylation, which was greatly increased after treatment of cells with pervanadate. These results demonstrate that TIM-1 can be tyrosine phosphorylated when expressed in T cells.

The cytoplasmic tail of TIM-1 contains two tyrosines, one of which (Y276 in the BL/6 allele) conforms well to a consensus site for src family tyrosine kinase phosphorylation [88]. The other tyrosine is only two residues removed from the transmembrane domain, and is a poor candidate for phosphorylation. To test the functional relevance of TIM-1 tyrosine phosphorylation, tyrosine 276 in TIM-1 (with a Flag tag) was mutated to phenylalanine. As with the form of TIM-1 lacking the cytoplasmic domain (**Figure 8**), TIM-1 (Y-F) is expressed at equivalent levels on the surface of transfected T cells, compared to wild-type Flag-TIM-1 (data not shown). Also, like the delta-cyto form of TIM-1 (**Figure 12**), TIM-1 (Y-F) is deficient in co-stimulation of NFAT transcriptional responses (**Figure 14B and C**). These data demonstrate that

tyrosine 276 in the cytoplasmic tail of TIM-1 is required for co-stimulation of NFAT/AP-1-dependent transcription by this molecule.

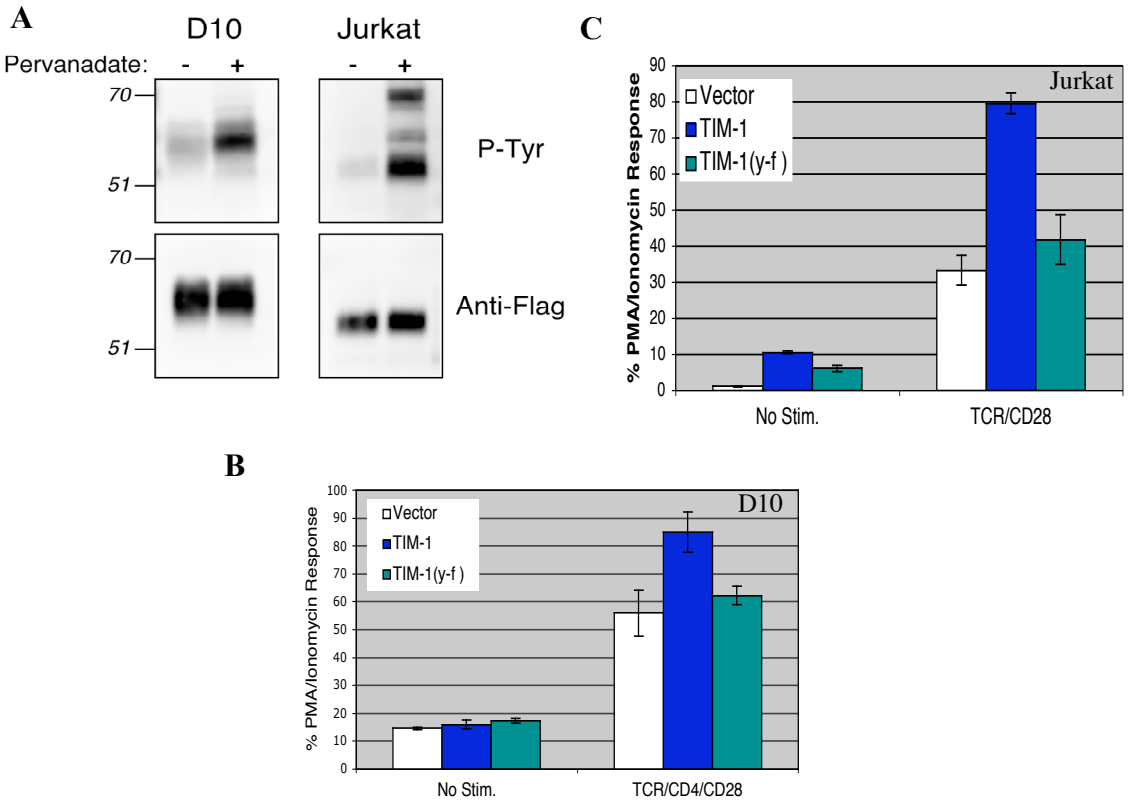


Figure 14: TIM-1 tyrosine phosphorylation and requirement for tyrosine-276 in costimulation of NFAT.

(*A*) Jurkat or D10 T cells were transfected with Flag-TIM-1. The next day, transfected cells were divided and left unstimulated or stimulated with pervanadate. Immunoprecipitations were performed with anti-Flag mAb, then the precipitates were separated by SDS/PAGE and Western-blotted. Blots were probed with anti-Flag (*Lower*), then stripped and reprobed with anti-phosphotyrosine antibody 4G10 (*Upper*). D10 (*B*) or Jurkat (*C*) cells were transfected with NFAT/AP-1-luciferase plus empty vector, wild type Flag-TIM-1, or Flag-TIM-1 (Y-F). Luciferase activity is expressed as the percentage of the PMA/ionomycin response from triplicate samples (mean ± SD) of a single experiment, representative of five that were performed.

3.4. DISCUSSION

Here we have provided direct evidence that TIM-1 plays a role in T cell activation and differentiation. Thus, we have demonstrated that TIM-1 is expressed on T cells of the lung-draining lymph nodes after intranasal immunization. Furthermore, ectopic retroviral expression of TIM-1 in developing effector T cells increases the number of IL-4 producing cells. This finding is consistent with our observation that expression of TIM-1 in a Th2 T cell clone provides a co-stimulatory signal for increased transcription from the IL-4 promoter. TIM-1 expression can also augment signaling to NFAT and AP-1, critical transcription factors for Th2 development, and IL-4 production. Finally, we have obtained evidence that the co-stimulatory function of TIM-1 requires a tyrosine within its cytoplasmic tail, which likely couples the molecule to intracellular signal transduction pathways. We conclude that TIM-1 co-stimulatory function is mediated at least in part through effects on inducible transcription factors.

We have observed that TIM-1 is expressed on CD4+ effector T cells after immunization protocols that result either in inflammation or tolerance (**Figure 4**). Because the induction of tolerance in this model (and others) occurs after some level of T cell activation and effector differentiation ([91] and T.B.O. and A.R., unpublished data), TIM-1 expression most likely occurs before anergy is established. Consistent with a defect downstream of IL-4 receptor signal transduction [91], even the level of IL-4 produced in the presence of TIM-1 is apparently insufficient to overcome the tolerizing block. Intriguingly, using a similar model, Umetsu et al. showed recently that pre-treatment of animals with a TIM-1 monoclonal antibody can inhibit tolerance induction

[17]. It may be that the TIM-1-expressing cells that we observe after tolerance induction have resulted from bystander activation, rather than being antigen-specific, although further analysis is required to determine whether this is the case.

The effects of TIM-1 on IL-4 production and transcription would appear, at first glance, to be rather modest. However, co-stimulatory receptors generally do not function in an "all-or-none" fashion. Rather, they modify the initial stimulation conditions such that more cells can surpass the threshold for entry into the cell cycle and differentiation. Absence of a particular co-stimulatory molecule would therefore be predicted to have modest-to-severe effects on the ability to develop an immune response, depending on the initial conditions. For example, absence of CD28 expression has varying effects on the ability to generate an immune response, depending upon the strength of the signals received through the TCR. Th2 immune responses appear to be particularly sensitive to the initial "strength" of stimulation, as previous studies have indicated that full differentiation to the Th2 lineage requires additional rounds of cell division, compared with development of Th1 effector T cells [104,105]. Also, a number of co-stimulatory and signaling molecules are preferentially required for the generation of Th2, compared to Th1, responses [106-108]. It therefore appears that the requirement for further rounds of cell division in their development has made Th2 cells more dependent upon co-stimulation and sustained signaling. This could be the result of less selection pressure to generate a rapid response, since Th2 responses are tailored toward fighting relatively slow-growing extracellular pathogens such as parasitic worms.

The increased number of IL-4-producing cells after TIM-1 expression (**Figure 6**) is consistent with our findings that transcription from the IL-4 promoter and isolated

NFAT/AP-1 elements are also augmented by TIM-1. However, the effects of TIM-1 on inducible transcription cannot completely explain the preferential development of IL-4-producing cells, because we have also observed that ectopic expression of TIM-1 can augment transcription from an IFN-γ luciferase promoter reporter (**Figure 7B**). TIM-1 may therefore have a preferential effect on the survival and/or proliferation of Th2 cells, the latter being consistent with the recent finding that TIM-1 ligation can augment TCR-driven proliferation [17]. Further investigation will be necessary to determine whether this is the case.

Our functional results are largely in agreement with recent studies that examined TIM-1 function in murine T cell activation [17,25]. Using a monoclonal anti-TIM-1 antibody, Umetsu et al. provided compelling evidence for a co-stimulatory role for TIM-1 in T cell activation and differentiation [17]. In an accompanying report, Meyers et al. showed that TIM-1 co-stimulation of T cell activation can occur as a consequence of binding to the TIM family protein TIM-4 on antigen-presenting cells [25]. However, for reasons that are still unclear, we have not observed any effect, either positive or negative, of crosslinking the Flag-TIM-1 construct in our transcriptional reporter experiments. Our experiments were carried out with purified T cells and T cell lines, making it unlikely that TIM-4 was present during our analyses, based on the finding of Meyers et al. that TIM-4 is expressed on neither naïve nor activated T cells [25]. The effects of TIM-1 that we have reported here may have resulted in part from interaction with a ligand distinct from TIM-4. Indeed, we have obtained evidence for a TIM-1 ligand on Jurkat, but not D10, T cells (**Figure 10A**). Thus far, however, we have only observed a partial decrease in basal

levels of NFAT/AP-1 reporter activity when using TIM-1-Ig to block interactions with this putative ligand (**Figure 10B**).

It is also possible that TIM-1 may homo-dimerize (at least in our experiments with ectopic expression) in a fashion regulated by its heavily glycosylated mucin domain, perhaps in a manner similar to CD45[109]. This is a particularly intriguing model in light of the fact that polymorphisms in both murine and human TIM-1 associated with relative asthma susceptibility are found in the mucin domain. We do not believe that the Flag epitope tag has an influence on the activity of TIM-1 in our system, since we have confirmed that expression of a construct lacking the Flag epitope tag can also co-stimulate anti-TCR-induced NFAT activity (data not shown). Also, expression of a Flag-tagged version of the NK-activating DAP10 molecule does not lead to constitutive NFAT activity, but rather requires anti-Flag crosslinking (**Figure 9A**). Differential glycosylation of TIM-1 may occur in the two main cell types that we have employed in these studies - the Jurkat and D10 T cell lines. Thus, when we examined TIM-1 phosphorylation (**Figure 14A**), we noted that TIM-1 runs at different apparent molecular weights when expressed in Jurkat or D10 cells. The role of glycosylation in TIM-1 function warrants further study.

Virtually nothing is known about how TIM family proteins connect to intracellular signaling pathways. Consistent with our findings (**Figure 14A**), a previous report showed that TIM-2 can be tyrosine phosphorylated after interaction with a putative ligand, when expressed in a fibroblast cell line [31]. The tyrosine at residue 276 in TIM-1 appears to be an ideal site for phosphorylation by a src family kinase. Many of the residues surrounding this tyrosine are conserved between rodent and primate orthologues

of TIM-1, consistent with both the likely importance of this motif and our data demonstrating a function for this tyrosine in the co-stimulation of NFAT. We are currently attempting to determine if this tyrosine is also required for TIM-1 function in primary T cells and, if so, whether it mediates interactions with one or more SH2-domain-containing proteins.

In summary, we have demonstrated in this report that TIM-1 can provide a co-stimulatory signal that influences effector T cell differentiation and TCR-dependent activation of the IL-4 promoter and NFAT/AP-1 transcription factors. It will be of interest to determine how TIM-1 co-stimulatory signals intersect with those originating at the TCR/CD3 complex and the wider consequences of this co-stimulatory activity for T cell function.

4.0. CHAPTER 4: INHIBITION OF T-CELL ACTIVATION BY TIM-2

This work has been adapted from a published study where- de Souza AJ- is co-first author. (Knickelbein JA, de Souza AJ et al (2006) *Cutting edge: Journal of Immunology* 177:4966-4970). This work was done in collaboration with a rotation student (Jared Knickelbein).

4.1. INTRODUCTION

Transmembrane proteins of the T cell immunoglobulin and mucin domain (TIM) family have recently been implicated in the regulation of T cell activation and differentiation [7,39]. Thus far, four of these proteins (TIM-1 through TIM-4) have been studied in mice. These genes are generally well conserved in humans, although the human genome does not contain a gene encoding TIM-2. Given its close sequence homology to TIM-1, it has been postulated that murine TIM-2 may share some of the functions carried out by human TIM-1 [9]. Several reports now suggest that TIM-1 can function to augment TCR-dependent T cell activation [17,25,28]. The activity of TIM-1 may be regulated *in vivo* by binding to another TIM protein - TIM-4 - which is preferentially expressed by APC's [25].

TIM-2 has also been reported to increase the efficiency of T cell activation through the TCR, possibly through binding to one of its ligands - the semaphorin Sema4A [31]. A recent study has identified another ligand for TIM-2, the heavy chain of ferritin [19]. It is not yet clear how this ligand for TIM-2 might regulate its function. Another group has shown that TIM-2 is preferentially expressed by Th2 T cells and serves as a negative regulator of this cell type [18], in contrast to the initial description of TIM-2 as a positive regulator of T cell activation [31]. Since we and others recently demonstrated that TIM-1 can provide a co-stimulatory signal, we wanted to determine whether TIM-2 behaves in a similar fashion. Also, as murine models are being used to address the function of TIM's, including TIM-1 [17,25,38], it is important to know whether mouse TIM-1 and TIM-2 are redundant, or whether they have distinct functions. Thus, we have transiently expressed TIM-2 in human and murine T cell lines, and probed its effects on downstream signaling pathways with well-characterized transcriptional reporter assays.

4.2. MATERIALS AND METHODS

4.2.1. Cell Lines, antibodies and reagents

Experiments were performed with the Jurkat human T cell leukemia cell line or a fast-growing derivative of the murine D10 T cell clone [93]. Antibodies for stimulation of Jurkat and D10 T cells were as described previously [28]. PMA and ionomycin were

obtained from EMD Biosciences (San Diego, CA) and used at 25 ng/ml and 1 mM, respectively. Anti-Flag antibody M2 and carbachol were obtained from Sigma (St. Louis, MO); carbachol was used at a final concentration of 500 mM. Anti-Flag staining was coupled with anti-mouse-PE (Caltag) for flow cytometry.

4.2.2. DNA Constructs

A full-length TIM-2 cDNA (clone # 4158605) generated by the I.M.A.G.E. consortium was purchased from Open Biosystems (Huntsville, AL). The open reading frame (excluding the signal sequence) was PCR-amplified from this plasmid and cloned in-frame with the human CD8 signal sequence and a Flag epitope tag, as described previously [28]. A TIM-2 fusion protein lacking a cytoplasmic tail was generated by PCR, using the same forward primer as for the full-length construct, and a reverse primer designed to terminate the protein two residues after the presumptive transmembrane domain [5]. Constructs were verified by automated sequencing.

Transfection efficiency was monitored with pMax-GFP from Amaxa (Gaithersburg, MD). Constitutively active Ras (V12) and calcineurin (regulatory domain-deleted) were from Dr. A. Weiss. Full-length (un-tagged) murine TIM-1 (from the BL/6 strain) was generated by PCR amplification of the full ORF, verified by automated sequencing and cloned into pCDEF3.

4.2.3. Transient Transfections, Stimulations and Luciferase Assays

Jurkat and D10 cells were transfected by electroporation, then stimulated the next day, followed by determination of luciferase activity, all as described previously [93,98]

4.2.4. ERK phosphorylation

Jurkat T cells were transfected with empty vector or Flag-TIM-2, plus pMax-GFP. The next day, cells were stimulated with anti-TCR and -CD28 antibodies. Cells were then fixed (1.5% PFA), permeablized (MeOH) and stained with a PE-conjugated antibody to phospho-ERK (BD Biosciences; San Jose, CA). PE staining within the GFP positive population was determined on a BD LSR II flow cytometer.

4.2.5. Calcium Mobilization

Jurkat T cells were transfected with empty vector or Flag-TIM-2. The next day, cells were loaded with Calcium Green AM ester (Invitrogen; Carlsbad, CA) and stained with an anti-Flag antibody. Intracellular calcium was monitored in the FITC channel before and after addition of anti-TCR/CD28 antibodies. Samples were maintained at 37 degrees for the duration of analysis.

4.3. RESULTS

4.3.1. Inhibition of Inducible transcription by TIM-2

In order to easily track expression and possibly manipulate dimerization of TIM-2, we constructed a version of the molecule that contains an extracellular Flag tag. This construct is expressed at the surface of transfected Jurkat and D10 T cells, as shown in **Figure 15**. Our previous studies had shown that ectopic expression of TIM-1 in these cell lines leads to increased basal activation of an NFAT/AP-1 reporter, as well as augmented TCR/CD28-stimulated NFAT/AP-1 activity [28]. As shown in **Figure 16**, we observed that ectopic expression of Flag-TIM-2 in Jurkat (panel A) or D10 (panel B) T cells did not result in an increase in NFAT/AP-1-dependent transcription, but rather inhibited activation of this reporter, whether stimulation was in the form of TCR/CD3 plus CD28 antibodies or the pharmacological stimuli PMA plus ionomycin. The inhibitory activity of TIM-2 appears to be mediated mainly by its cytoplasmic tail, since expression of a Flag-TIM-2 construct lacking the cytoplasmic tail led to little if any inhibition of NFAT/AP-1 reporter activity (**Figure 16C**), although this construct is expressed at the cell surface equivalently to full-length TIM-2 (data not shown).

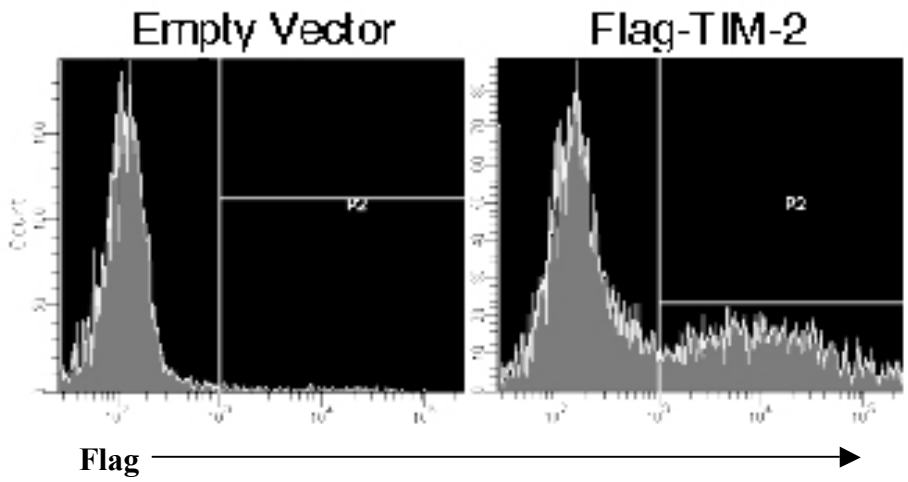


Figure 15: Expression of flag-TIM-2 on the surface of transfected T cells.

Jurkat T cells were transfected with empty vector or Flag-TIM-2. Eighteen hours later, cells were stained with anti-Flag Antibody M2 and PE-conjugated secondary Antibody.

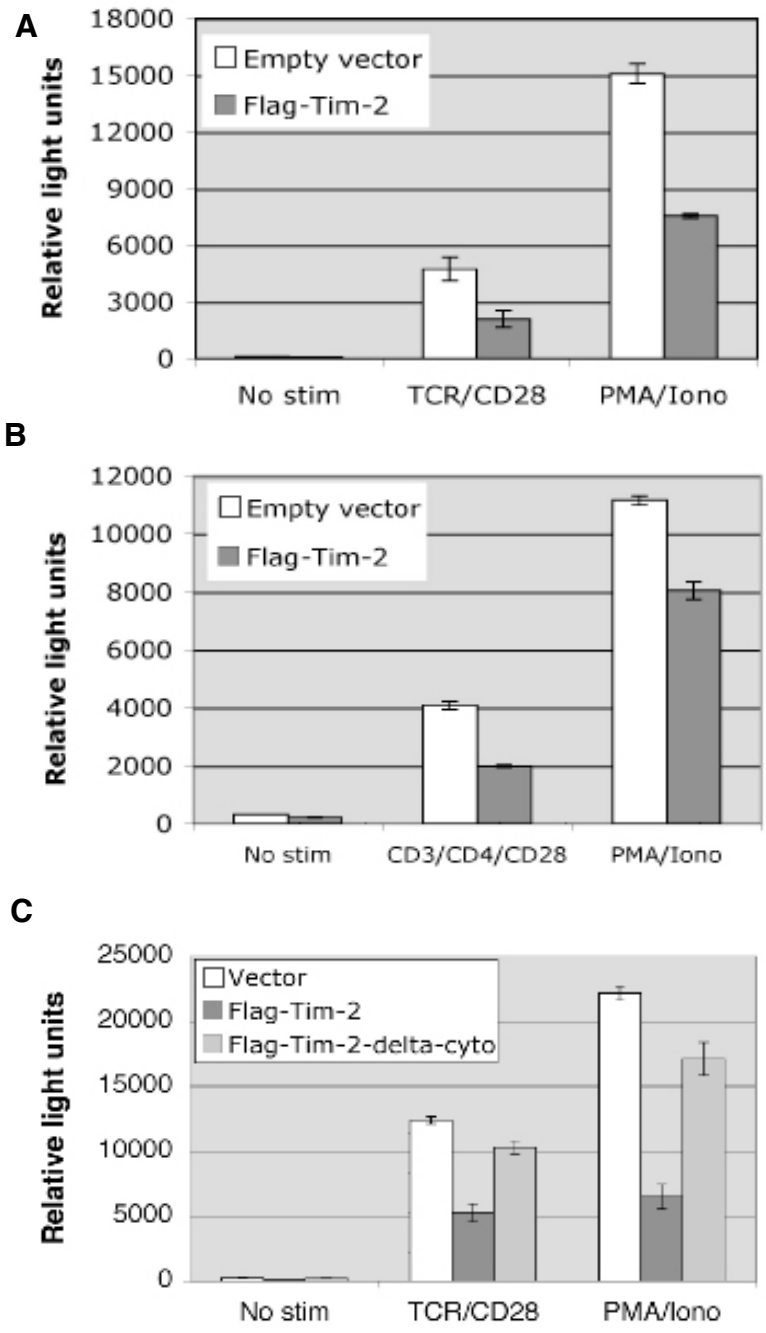


Figure 16: Suppression of NFAT/AP-1 Activation by TIM-2.

(A), Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and either empty vector or Flag-TIM-2. The next day, cells were treated with the indicated stimuli for 6 h, followed by determination of luciferase activity. Results are the average (± SEM) of triplicate determinations from a single experiment, representative of over ten that were performed. (B), D10 T cells were transfected and stimulated as shown in B. Results are representative of four experiments. (C), The cytoplasmic tail of TIM-2 is required for optimal inhibition of NFAT/AP-1. Jurkat T cells were transfected as shown in A, with the indicated constructs. Results are representative of six that were performed.

Since we observed inhibition of antibody-induced reporter activity in D10 cells, we also investigated whether stimulation of these cells with antigen/APC's is affected by TIM-2. Indeed, we did observe significant inhibition of antigen-dependent NFAT/AP-1 responses when Flag-TIM-2 was expressed in D10 cells (**Figure 17**).

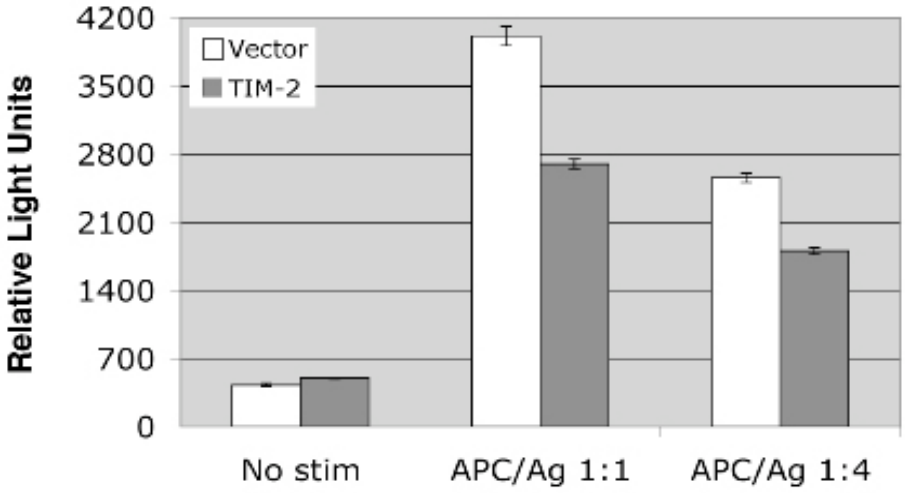


Figure 17. Inhibition of NFAT/AP-1 by TIM-2 in D10 cells stimulated with Ag/APC.
D10 T cells were transfected with NFAT/AP-1-luciferase and either empty vector or TIM-2. Cells were stimulated for 8 h with CH27 B cells, with or without conalbumin, either in an equal proportion to or at one quarter the number of D10 cells.

4.3.2. Scope of Inhibition by TIM-2

To further explore how general TIM-2 inhibition of signaling might be, we transfected Flag-TIM-2 into the Jurkat derivative J.HM-1, which expresses the human type 1 muscarinic receptor [110]. Stimulation of this cell line with the agonist carbachol results in G-protein-dependent activation of NFAT/AP-1, downstream of PLC-β. As shown in **Figure 18A**, expression of Flag-TIM-2 significantly impaired the ability of carbachol to

stimulate the activity of a co-expressed NFAT/AP-1 luciferase reporter, in addition to its inhibition of the effects of TCR/CD28 cross-linking and PMA/Iono. This result suggests that the inhibitory effect of TIM-2 occurs downstream of the proximal tyrosine kinases and adaptor proteins that result in PLC-γ1 activation. Inhibition by TIM-2 may therefore occur at the level of PLC itself (although carbachol stimulation requires PLC-β), or further downstream. We recently reported that transfection of TIM-1 into Jurkat T cells leads to a significant increase in basal levels of NFAT/AP-1 reporter activity [28]. As shown in **Figure 18B**, co-expression of Flag-TIM-2 with an un-tagged form of TIM-1 resulted in suppression of TIM-1-mediated NFAT/AP-1 activation, to a similar degree as its inhibition of signals from the TCR, PMA/Iono or carbachol. Importantly, expression of TIM-1 was not affected by co-transfection of Flag-TIM-2 (data not shown).

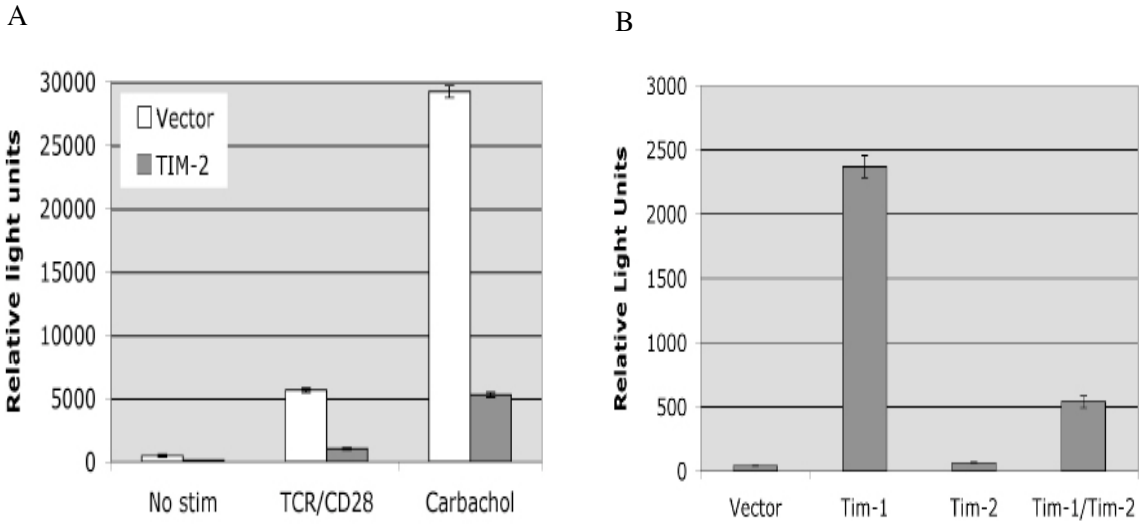


Figure 18. Scope of NFAT inhibition by TIM-2.

(A) J-HM1 Jurkat cells, were transfected with NFAT/AP-1-luciferase plus empty vector or Flag-TIM-2. The next day cells were stimulated as indicated and assayed for luciferase activity. (*B*) Jurkat T cells were transfected with NFAT/AP-1-luciferase and the indicated plasmids. Results are the average (± SEM) of triplicate determinations from a single experiment, representative of two that were performed in each case.

4.3.3. Effect of TIM-2 on T cell viability

We were concerned that the inhibition of T cell activation by TIM-2 was the result of a general effect on cell viability. Therefore, we co-transfected increasing amounts of Flag-TIM-2, along with a constitutively expressed GFP reporter. Cells were analyzed for GFP expression by flow cytometry the next day. Expression of the GFP marker remained relatively constant, regardless of the amount of co-transfected Flag-TIM-2 (**Figure 19**). Furthermore, the percentage of cells expressing the co-transfected GFP marker with either empty vector or Flag-TIM-2 did not diverge over the same time period in which luciferase activity was measured (i.e. 24 hours after transfection), or even up to 48 hours after transfection (data not shown). As an additional assay for possible effects on cell viability, we determined whether the expression of TIM-2 resulted in increased numbers of apoptotic cells. Cells were stained the day after transfection with Annexin V, which binds exposed phosphatidylserine, an early marker of apoptosis. The day after transfection with empty vector or Flag-TIM-2, there was no difference in the proportion of Annexin V positive cells (data not shown). We conclude that TIM-2 inhibits signaling pathways that lead to NFAT/AP-1-dependent transcription, but not general cell viability, at least in these relatively short-term assays.

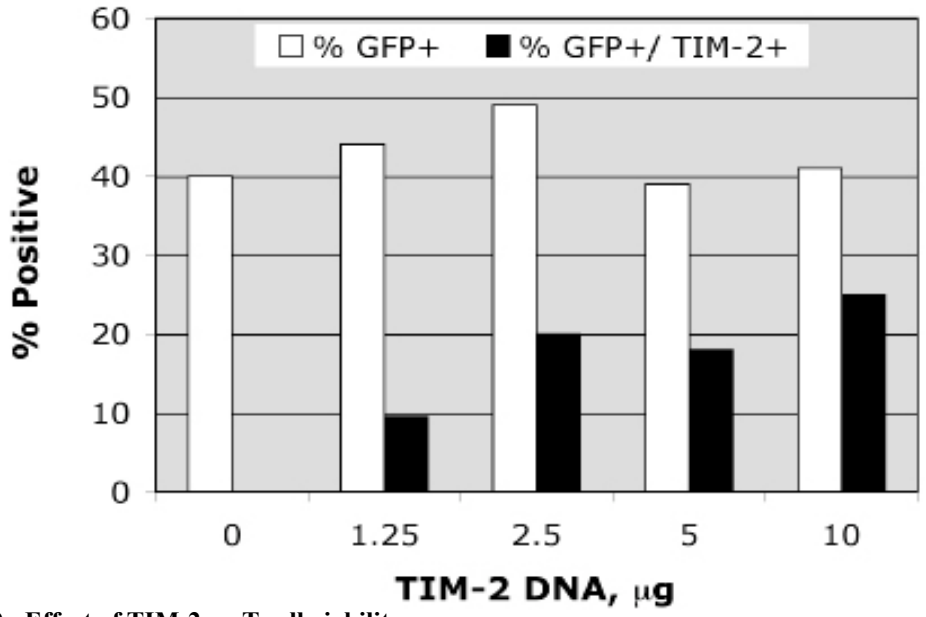


Figure 19: Effect of TIM-2 on T cell viability

TIM-2 expression does not cause nonspecific death of transfected cells. Jurkat T cells were transfected with pMax-GFP and the indicated amounts of Flag-TIM-2. Total amounts of DNA were equalized with empty vector (pCDEF3). Cells were analyzed after 24 h for expression of GFP (white bars) and Flag-TIM-2 (black bars).

4.3.4. Effect of TIM-2 on Calcium dependent and Ras-MAP kinase dependent pathways

To determine whether we could bypass the block imposed by TIM-2, we expressed active forms of signaling molecules located further downstream from where PMA and ionomycin act. First, we expressed an oncogenic, constitutively active, form of Ras (Ras V12). As shown in **Figure 20A**, expression of Ras V12 reversed the TIM-2 inhibition of TCR/CD28-induced NFAT/AP-1 reporter activity (gray bars). Ras V12 could also synergize with ionomycin to activate this reporter [111], and this response was not

inhibited by co-transfection of TIM-2 (black bars). Conversely, expression of a constitutively active form of calcineurin (**Figure 20B**) allows for much greater NFAT/AP-1 activation with PMA alone, which was also not inhibited by TIM-2 expression. TIM-2 inhibition of CD3/CD28-stimulated NFAT/AP-1 was also reversed by active calcineurin (data not shown). A curious finding in these experiments was *increased* NFAT/AP-1 activation (about 40%) when TIM-2 was co-expressed with activated Ras or calcineurin, compared with either of these signaling proteins alone. We have not yet explored this observation further, given the admittedly artificial nature of the experiment and the consistent inhibition of NFAT/AP-1 by TIM-2 under most other conditions.

Given the ability of TIM-2 to inhibit activation of an NFAT/AP-1 reporter, we wanted to know whether TIM-2 expression affects calcium mobilization downstream of the TCR. Jurkat T cells were transfected with empty vector or TIM-2 and analyzed by flow cytometry for intracellular calcium concentration before and after stimulation. As shown in **Figure 21A**, cells transfected with TIM-2 responded with a lower overall level of calcium mobilization after addition of anti-TCR/CD28 antibodies, and also maintained cytoplasmic free calcium above basal levels for a shorter amount of time. Activation of an NFAT/AP-1 reporter requires not only calcium mobilization but also Ras/MAP kinase activation [111], so we also examined the effect of TIM-2 on activation of the MAP kinase ERK. As shown in **Figure 21B**, induction of ERK phosphorylation by anti-TCR/CD28 antibodies was also impaired by expression of TIM-2. Thus, both the calcium and Ras/MAP kinase pathways appear to be inhibited by TIM-2.

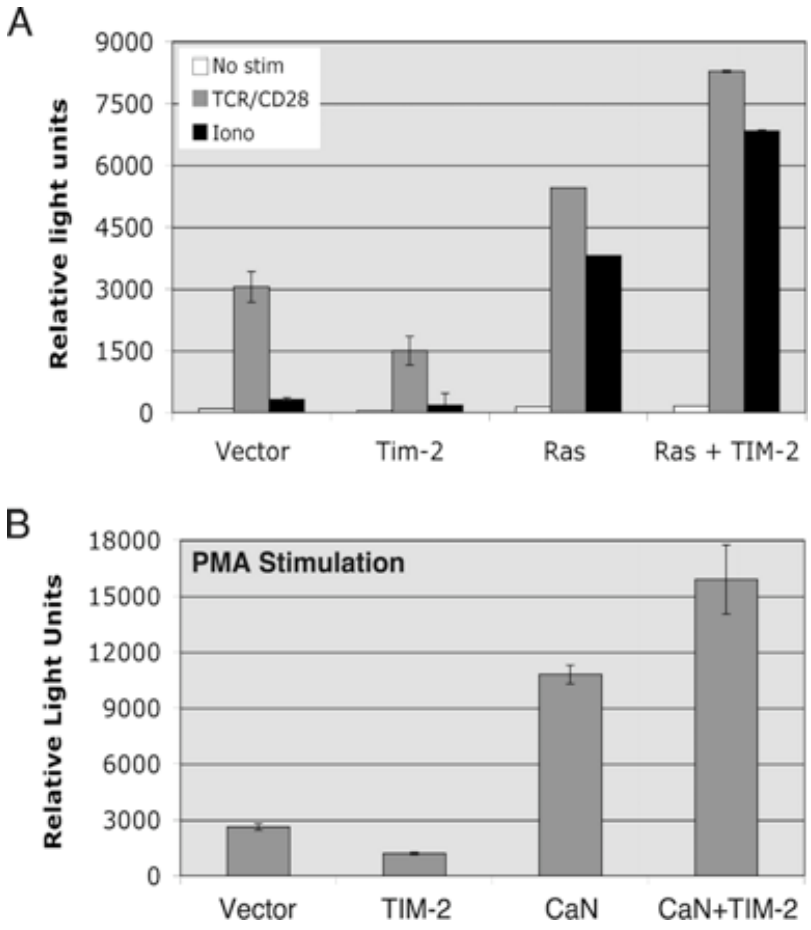


Figure 20: Constitutively active Ras or calcineurin can bypass TIM-2-mediated suppression of NFAT/AP-1 reporter activity.

Jurkat (*A*) or D10 (*B*) T cells were transfected with NFAT/AP-1-luciferase and the specified plasmids, then stimulated the next day as indicated. "Ras" denotes the oncogenic V12 form of Ras, whereas "CaN" denotes a truncated, active, form of calcineurin. Results shown are the average (± SD) of triplicate samples from a single experiment, representative of two experiments in each case.

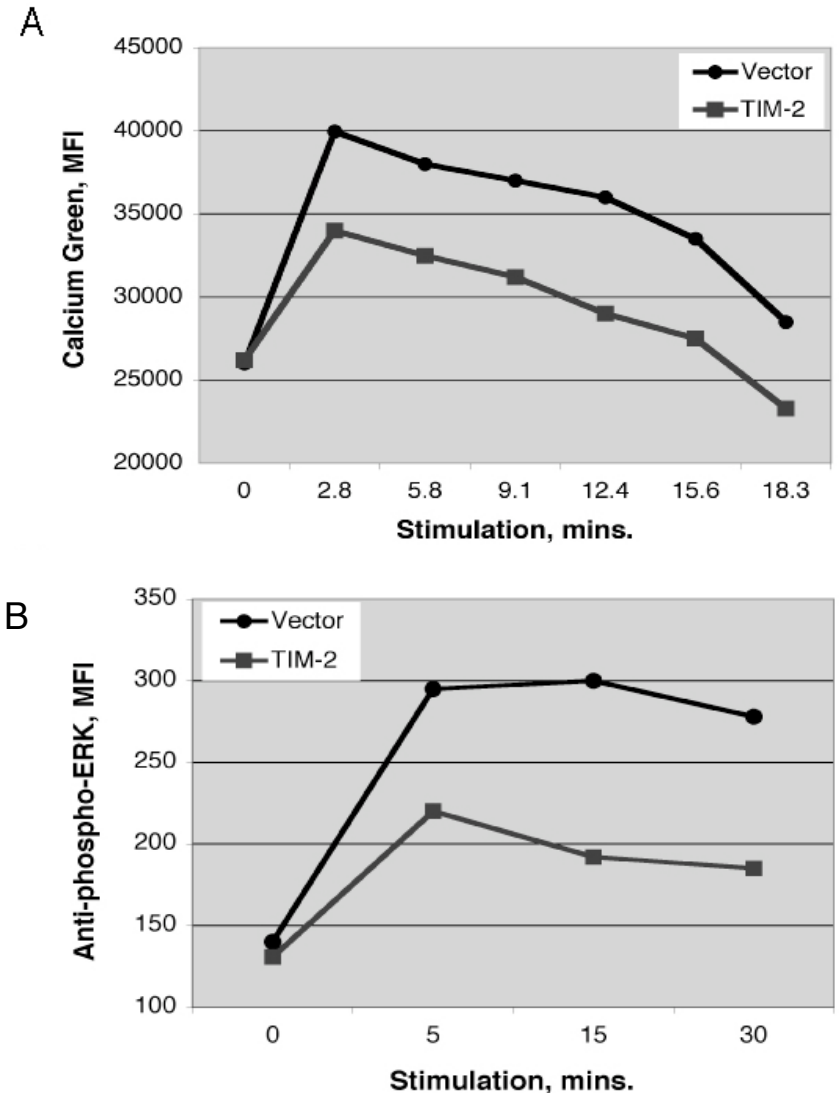


Figure 21. TCR-induced activation of ERK and calcium mobilization are impaired in TIM-2-expressing cells.

A, Jurkat T cells were transfected with empty vector or Flag-TIM-2. Calcium mobilization was assessed the next day, as described in *Materials and Methods*. Results are presented as the mean fluorescence intensity (MFI) of all cells (vector) or Flag-positive cells (TIM-2), and are representative of two experiments. *B*, Jurkat T cells were transfected with empty vector or Flag-TIM-2, plus pMax-GFP, then stimulated the next day with anti-TCR/CD28 Abs. Intracellular staining was performed to analyze ERK phosphorylation and is represented as the PE channel MFI within the GFP+ gate. Results are representative of two experiments.

Since we observed inhibition of MAP kinase activation in the presence of TIM-2, we also examined its effects on a pure AP-1 reporter. As shown in **Figure 22A**, expression of TIM-2 with an AP-1 reporter resulted in significant impairment of its activation by TCR/CD28 or PMA, similar to the results with the NFAT/AP-1 reporter. We wanted to determine if we could bypass this block by co-transfection of activated signaling molecules, as shown above for NFAT/AP-1 (**Figure 20**). We first employed a truncated, constitutively active, form of the MAP kinase kinase kinase MEKK1, which is known to activate AP-1-dependent transcription through the stress-activated protein kinases JNK and p38 [112,113]. Expression of activated MEKK1 resulted in potent activation of the AP-1 reporter activity, which was completely insensitive to co-expression of TIM-2 (**Figure 22B**). We next moved further upstream in the AP-1 pathway, transfecting this time a partially activated allele of PKC θ('PKC θ A/E') to activate the reporter [114]. Thus, as shown in **Figure 22C**, PKC θ A/E expression led to robust AP-1 activation. When TIM-2 was also expressed, it led to a modest (about 20%), although significant ($p<0.01$) decrease in the PKC θ-mediated AP-1 activity, which was not due to an effect on levels of PKC θ protein (data not shown).

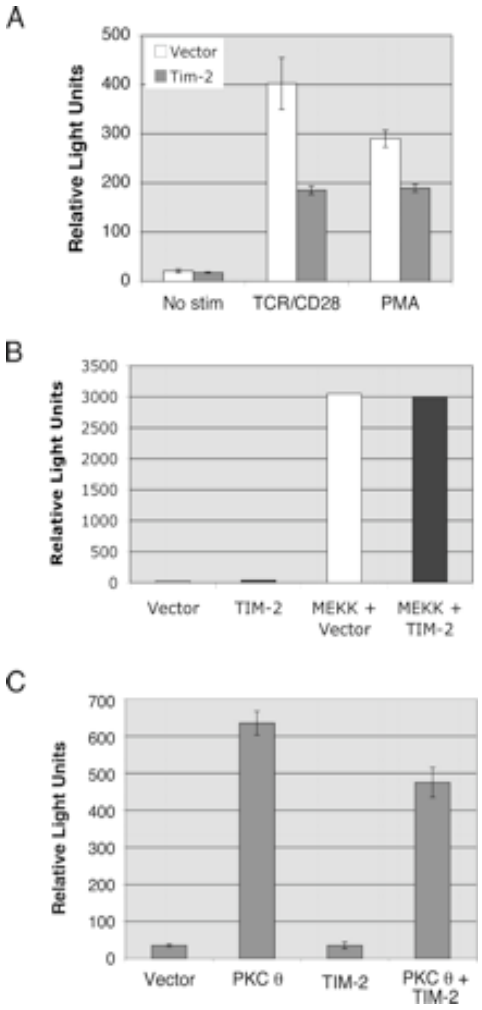


Figure 22. TIM-2 blockade of AP-1 reporter activity and reversal by expression of active signaling intermediates.

(A) Jurkat T cells were transfected with an AP-1 reporter plus empty vector or TIM-2. Cells were stimulated as indicated the next day. Results are the average light units (± SD) from triplicate determinations of a single experiment, representative of four. *B* and *C*, Jurkat T cells were transfected as above, with or without activated alleles of MEKK1 (*B*) or PKC (*C*). Luciferase activity in *B* and *C* was measured 24 h later, without any additional stimulation. Results shown are the average of duplicate points from a single experiment, representative of two performed (*B*) or the average triplicate points (± SD) from a single experiment, representative of four.

4.4. DISCUSSION

T cell Ig and mucin domain protein 2 (TIM-2) has been shown to regulate T cell activation in vitro and T cell-mediated disease in vivo. However, it is still not clear whether TIM-2 acts mainly to augment T cell function or to inhibit it. We have directly examined the function of TIM-2 in murine and human T cell lines. Our results indicate that expression of TIM-2 significantly impairs the induction of NFAT and AP-1 transcriptional reporters by not only TCR ligation but also by the pharmacological stimuli PMA and ionomycin. Our data suggest that TIM-2 acts to inhibit T cell activation by interfering with the TCR signaling cascade just downstream of PLC activation, but upstream of the NFAT and AP-1 transcription factors. Our results are consistent with the phenotype of TIM-2 knockout mice, which, in a murine model of asthma, display increased inflammation and Th2 cytokine production, apparently due to an increased sensitivity of TIM-2-deficient T cells to activation [115]. The fact that either Ras or calcineurin could rescue NFAT induction is difficult to reconcile with the fact that stimulation with PMA and ionomycin was compromised by TIM-2. However, the PKC/Ras and calcium signaling modules do not function in a completely separate manner. Intriguingly, recent evidence points to a role for PKC θ in the regulation of PLC-γ1 [116,117]. Also, classical PKC isoforms (like α andβ) are known to require calcium as a co-factor for optimal activation, in addition to diacylglycerol.

We note that transient expression of TIM-2 was itself sufficient to inhibit T cell activation. We did not observe any augmentation or reversal of the inhibitory effect by actively crosslinking Flag-TIM-2 with anti-Flag antibody (data not shown). Furthermore,

we could not detect the presence of any putative TIM-2 ligands on either the Jurkat or D10 T cells used in our experiments, as neither cell type exhibited positive staining with TIM-2-Ig (data not shown). Given its recent identification as a ligand for TIM-2 [19], we considered the possibility that ferritin contained in the FCS in our culture medium was modulating TIM-2 function. We conducted several experiments in the presence of 0.5% serum (including the post-transfection overnight culture), rather than the usual 5%, but still observed the same degree of inhibition of NFAT/AP-1 by TIM-2 (data not shown). This is reminiscent of our previous findings on TIM-1, ectopic expression of which provides a co-stimulatory signal in the absence of active crosslinking [28]. The apparent ligand-independent function of TIM-2 in our system could be explained by its homo-dimerization under conditions of transient expression, possibly through its heavily glycosylated mucin domain. CD45, which is heavily glycosylated and expressed at high levels on leukocytes, has been reported to homo-dimerize in a manner dependent on the level of glycosylation [109]. Nonetheless, ligand-independent signaling is not a common phenomenon of transiently expressed transmembrane proteins since CD28 (L.P.K., unpublished data) and DAP10 [28], for example, still require active cross-linking in order to transmit signals when transiently expressed.

We are currently attempting to understand better the role of dimerization in the regulation of TIM-2 function, as well as the precise biochemical mechanism by which it inhibits the signaling pathways that activate NFAT/AP-1-dependent transcription. Such knowledge should contribute to our understanding of how molecules of the TIM family regulate immune responses.

5. CHAPTER 5: TIM-1 MEDIATED T CELL ACTIVATION REQUIRES RECRUITMENT AND ACTIVATION OF PI3 KINASE

This chapter has been submitted for publication

5.1. INTRODUCTION

Preceding evidences so far have designated the TIM gene family as a set of costimulatory molecules functioning in differential capacity to positively or negatively modulate T cell activation and effector function. This relatively novel family consists of eight genes in the mouse and three in human [5,7,9]. The TIM proteins are cell surface receptors that may also be secreted and belong to the Ig superfamily sharing comparable structural features: an extracellular Ig-like domain, followed by a polymorphic mucin-like domain, transmembrane domain and cytoplasmic tail of variable length [5,7,9].

Of these family members TIM-1 has been implicated in the immune regulation of asthma and atopic diseases [9,118]. More recent studies have shown that ligation of TIM-1 with an agonistic TIM-1 antibody or with higher doses of its ligand TIM-4 can co-stimulate proliferation of T cells as well cytokine production *in vivo* and *in vitro* and

abrogate tolerance in a Th2 model of respiratory tolerance, collectively suggesting that it functions as a positive immune regulator[17,25]. Our own prior work also showed that murine Tim-1 can transduce a costimulatory signal for activation and cytokine production of T cells [28], and this is consistent with a recent report that human TIM-1 can associate with the TCR-CD3 complex to upregulate T cell activation signals [119]. Nevertheless, depending on the affinity and targeted epitope of the cross-linking antibody, coupled with the timing of its application, opposing conclusions have been reached whether TIM-1 positively or negatively regulates T cell activation and disease outcomes [37,38,120,121]. Thus, a greater understanding of the biochemical signals mediated by TIM-1 is required to mechanistically explain its role in immune activation.

The TCR/CD3 complex and costimulatory molecules interact with key intracellular signaling proteins that determine which downstream effector pathways are triggered. Of these, the phosphotidylinositol 3-kinases (PI3K) have been frequently implicated in costimulatory signaling. They are enzymes that regulate diverse biological processes including cell growth, proliferation, cytoskeletal organization, apoptosis and vesicular trafficking [122-126] consequently playing important roles in lymphocyte development, activation, and effector function. Of the three PI3K classes (Class I, Class II and Class III), Class IA proteins are linked with tyrosine kinase dependent signaling by lymphocyte antigen receptors, costimulatory receptors and cytokine receptors [126]. Class IB is associated with G-protein coupled receptors such as the chemokine receptors [126]. These proteins exist as heterodimers of a regulatory subunit and a catalytic subunit. The Class IA adaptor subunits p85α, p85βα and p85γ arise from the *Pik3r1*, *Pik3r2* and *Pik3r3* genes. *Pik3r1* generates three splice variants p85α, p55α and p50α.

The catalytic subunit comprises of p110α, p110β and p110δ [126,127]. While p110δ is predominant and mainly restricted to leucocytes, each p110 isoform can associate with any of the regulatory subunits suggesting some functional redundancy and compensatory activity [126,127].

Phosphotidylinositol 3-kinases operate as membrane associated lipid kinases and their chief property is to phosphorylate inositols at their 3' position to generate phosphotidylinositol (PI), phosphotidylinositol-3,4-bisphosphate (PIP2), and phosphotidylinositol 3,4,5 trisphosphate (PIP3) lipid second messengers [122,123,128,129]. These catalytic products are stationed at the inner leaflet of the plasma membrane where their major function appears to be to recruit signaling molecules with pleckstrin homology domains that can bind to D3 lipids. Recruitment to the membrane assists their activation and ensuing initiation of signaling pathways. Additionally, the p85 adaptor subunit contains several other interesting protein domains including SH2, SH3, proline rich domains, and a BCR-homology region [130,131]. These domains facilitate protein-protein interactions that may be able to mediate signal transduction events independently of p110 [132,133].

To date, there is little known about the molecular basis for TIM-1 co-stimulation. We have previously showed that TIM-1 could be inducibly tyrosine phosphorylated. Furthermore, TIM-1 expressed in human and murine T cells can transduce a signal thereby activating IL-4 and IL-2 dependent transcription in part, through NFAT/AP-1 transcription factors, in a manner that requires a conserved tyrosine 276 in its tail [28]. In this present study, we show that TIM-1 tyrosine phosphorylation requires the src family kinase Lck, which is also critical for TCR- mediated signal transduction. Lck-induced

TIM-1 phosphorylation is essential for subsequent activation of NFAT and AP-1 dependent transcription. Following phosphorylation, TIM-1 recruits the PI3K adaptors p85α/β. We also provide evidence that PI3K activity is important for TIM-1 function. Finally, we show that TIM-1 can enhance activation of primary murine T cells in a manner that requires p85 expression and PI3K activity.

5.2. MATERIALS AND METHODS

5.2.1. DNA Constructs.

Murine TIM-1 (from strain C57BL/6) was tagged with extracellular Flag and ligated into pCDEF3 expression plasmid, as described previously [28]. Similarly, generation of a flag-TIM-1 construct lacking the cytoplasmic tail (TIM-1 Δ cyto), and flag-TIM-1 (Y-F) where tyrosine 276 was mutated to phenylalanine has also been described elsewhere [28].

5.2.2. Cell Lines and Mice.

The Jurkat cell lines used were Jurkat- derived Lck deficient variant JCam1.6; Lck-reconstituted JCam1/Lck; [134,135] and Jurkat cell line (ATCC). D10 cells were obtained from ATCC. CHO cells stably transfected with HA-tagged TIM-4 (CHO-TIM4) or control vector (CHO-mock) were a gift from Vijay Kuchroo and generated as previously described [25]. Spleens from *Pik3r2*^{n} *(*p85 beta null) mice and from p85 T cell

deficient mice [*Pik3r1*^{f} mice bearing floxed alleles of *Pik3r1* (p85α, p55α, p50α) were bred with *Pik3r2*^{n} mice and then crossed with Lck-Cre transgenic mice] were a generous gift from David Fruman, and were generated as described in Refs. [136-139]. C57BL/6 mice were purchased from Jackson Laboratories. D10, Jurkat mutants and primary murine T cells were maintained in RPMI 1640 medium supplemented with 10% bovine growth serum, and 5% each of penicillin, streptomycin, L-glutamine, Sodium pyruvate, Non-essential amino acids, 1M HEPES buffer (pH 7.4), and beta-mercaptoethanol. Media for D10 cells was additionally supplemented with recombinant human IL-2 at 1:2000. Jurkat cells were maintained in RPMI 1640 medium supplemented with 5% bovine growth serum, and 5% each of penicillin, streptomycin and L-glutamine. CHO cells were maintained in RPMI 1640 / DMEM medium (1:1) supplemented with 1.5mg/ml of G418, 10% bovine growth serum and 5% each of penicillin, streptomycin and L-glutamine.

5.2.3. Antibodies and reagents.

Clonotypic antibody to the Jurkat TCR (C305) was from A.Weiss (University of California, San Francisco). Anti-human CD28, hamster anti-mouse CD3 and hamster anti-mouse CD28 were from Caltag (Burlingame, CA). Anti-PI3Kinase p85 (Rabbit anti-serum) and anti-phosphotyrosine 4G10 was from Upstate/Chemicon (Temecula, CA). Biotin-anti-mouse CD28 (37.51), Biotin-anti-mouse CD3ε (145–2C11) and FITC-conjugated anti-mouse CD69 were from BD Pharmingen (San Jose, CA). Biotin-anti-mouse CD4 (L3T4) was from eBioSciences (San Diego). Streptavidin was from Zymed

(San Francisco, CA). Rabbit anti-Syrian hamster IgG and APC-conjugated donkey anti-rat IgG was from Jackson Immunoresearch (West Grove, PA). Akti 1/2 (Akti1 and Akti inhibitor), phorbol myristate acetate (PMA), PP2 and Ly294002 were from EMD Biosciences (La Jolla, CA). Anti-flag (M2), β-actin mAb's and ionomycin was from Sigma (St. Louis, MO). Rat anti-mouse TIM-1 (3B3) was a generous gift from Dr. Rosemarie DeKryuff. Anti-phospho Akt (S473) rabbit mAb was from Biosource/ Invitrogen (Carlsbad, CA).

5.2.4. Transient Transfections and Luciferase Assay.

Jurkat / D10 / J.Cam1.6 or J.Cam1-Lck cells, were transfected by electroporation using a gene pulser (Bio-Rad Laboratories, Hercules, CA), at a setting of 250-260V and 960μF, in cuvettes containing 1-2 × 10^7 cells with 0.4 ml serum free media. The amount and type of DNA plasmids transfected for an experiment are as described in the respective figure legends. Following transfection, the cells were cultured in 10ml-supplemented medium for 16-20 hours, and then stimulated for six hours in 96 U-bottom well plates. Reporter assays were performed as described previously [92]. Luciferase activity was determined with an Orion luminometer (Zylux, Oak Ridge, TN).

5.2.5. SH2 domain arrays.

A thirteen amino-acid peptide of TIM-1 cytoplasmic tail was synthesized and this sequence included the tyrosine 276 with six amino acids of the tail sequence on either

side of the tyrosine (Peptide Synthesis facility, Biotechnology Center, Molecular Medicine Institute, University of Pittsburgh). The peptide was biotinylated on the N-terminus and the tyrosine was phosphorylated. This was then subjected to HPLC purification; the end product was greater than 90% purity, as determined by mass spectrometry. The purified peptide was incubated with the SH2 domain array as per the manufacturer's instructions, and subsequent processing steps were also performed exactly as mentioned in the kit (Panomics). Processed arrays were imaged on a Kodak Image Station 2000R, with accumulation to 2,000 grey levels. When employed as a competitive inhibitor, phenylphosphate was added both after blocking and during peptide incubation, at 100 mM. Quantitation was performed by averaging the intensity values for the duplicate p85α and p85β spots, and dividing by the average of all control spots. These values were then compared for experiments performed with or without phenylphosphate to derive a percent inhibition.

5.2.6. TIM-1 tyrosine phosphoryation and endogenous p85 association.

Jurkat, or Lck mutants JCam.1.6 or JCam.Lck cells (2×10^7) were transfected with flag-tagged TIM-1 or flag tagged mutant TIM-1 constructs, stimulated with pervanadate (1:100) [140], and/ or coupled with PP2 (10μM). Non-idet P40 lysates were immuno-precipitated with M2-conjugated agarose beads (Sigma), and separated by SDS-PAGE followed by Western blotting onto a PVDF membrane [92]. Blots were probed with M2 and horseradish peroxidase (HRP) –conjugated goat anti-mouse IgG (Pierce) or with anti-p85 pan antibody and HRP-conjugated Protein A (Pierce). They were developed with

Super Signal Pico ECL Substrate (Pierce) and digitally imaged on a Kodak Image Station 2000R, with accumulation set to stop at 2,000 gray levels. For detection of tyrosine phosphorylation, blots were then stripped and re-probed with 4G10 and HRP-conjugated anti-mouse IgG.

5.2.7. Analysis of T cell activation markers.

CD4 T cells were purified from C57BL/6 mice or from p85 beta k.o. or p85 double k.o. mice by negative selection using a kit from Miltenyi Biotec (Auburn, CA). The cells were stimulated in 24 well plates previously coated with rabbit anti-Syrian hamster IgG secondary antibody (10μg/ml overnight at 4^{o}C) followed by primary antibodies (2-3 hours at 37^{o}C) of hamster anti-mouse CD3 (0.02–1.0 μg/ml) and hamster anti-mouse CD28 (0.5 μg/ml). Additional inputs of anti-TIM1 antibody and/ or inhibitors were included as indicated in the experiment. T cells were harvested at day one and day three post-stimulation and examined for CD69 and CD25 surface expression by flow cytometry on a BD LSR II, using PE-conjugated anti-mouse CD25 and FITC- conjugated anti-mouse CD69 antibodies respectively. TIM-1 expression was determined with anti-TIM1 (3B3) followed by APC-conjugated anti-rat IgG antibody.

5.2.8. IL-2 ELISA

Purified splenic CD4+ T cells from p85β or p85α/β knockout mice were stimulated as indicated at one million per well in a 24 well plate. Supernatants were assayed for murine

IL-2 usng an OptEIA kit from B-D Biosciences, according to the manufacturer's instructions.

5.3. RESULTS

5.3.1. Regulation of TIM-1 tyrosine phosphorylation

We showed previously that TIM-1 can be inducibly phosphorylated on tyrosine [28] and that tyrosine 276 in the cytoplasmic tail is functionally required for TIM-1 mediated co-stimulation of NFAT/AP-1 dependent transcription in T cells [28]. This tyrosine is conserved between different species [5] and is contained in a motif favorable for recognition by src-family tyrosine kinases as indicated by the charged residues glutamic acid and aspartic acid lying upstream of tyrosine 276 [88]. In order to ascertain whether src kinases were indeed required for TIM-1 phosphorylation, we examined tyrosine phosporylation in the presence of a potent inhibitor of src kinases, namely PP2 [141]. Jurkat cells were transfected with epitope tagged TIM-1, and stimulated with pervanadate, in the presence or absence of PP2, followed by immuno-precipitation (IP) of TIM-1 and Western blots were probed with phosphotyrosine antibody. As shown in **Figure 23A**, potent tyrosine phosphorylation was induced by treatment with pervanadate. Coupling of PP2 treatment along with stimulation by pervanadate led to a significant decrease in the phosphorylation intensity which was similar to basal levels. This indicates a requirement of src kinases for TIM-1 tyrosine phosphorylation, even under conditions

where potent tyrosine kinase activation is induced, i.e. by pervanadate. Since Lck is a prominent src family tyrosine kinase essential for TCR-mediated T cell activation, we used an Lck – deficient mutant variant of Jurkat cells called JCam.1 [135] to probe the role of Lck in TIM-1 phosphorylation and function. The data shown in **Figure 23B** demonstrate that the ability of TIM-1 to become tyrosine phosphorylated in J.Cam-1 cells is severely compromised. In contrast, JCam.1 cells that have been stably reconstituted with Lck (JCam1-Lck) now showed a remarkable recovery of inducible TIM-1 phosphorylation similar to what was observed in wild-type cells (**Figure 23A)**.

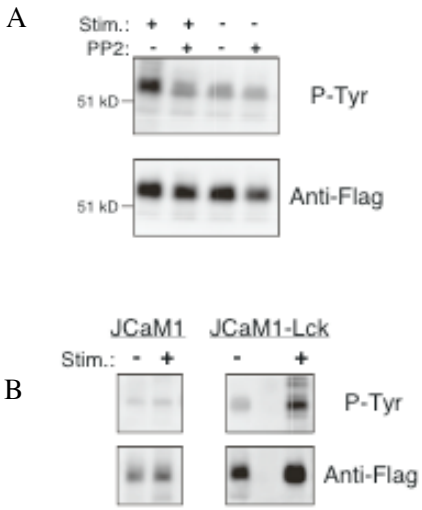


Figure 23: Role of Lck in TIM-1 tyrosine phosphorylation.

(A) Jurkat T cells expressing Flag-Tim-1 were stimulated as indicated, with or without the Src kinase inhibitor PP2. Anti-Flag IP's were separated by SDS-PAGE and western blotted with anti-Flag (lower panel), then stripped and re-probed with anti-phosphotyrosine antibody 4G10 (upper panel). (B) Lck-deficient JCaM1 or Lck-reconstituted JCaM1 cells expressing Flag-Tim-1 were stimulated and analyzed as in part a. Each experiment is representative of two (a) or three (b) that were performed.

In order to establish the functional relevance for lck-dependent TIM-1 tyrosine phosphorylation we examined TIM-1 mediated NFAT/AP-1 activation in these Lck mutant Jurkat cells lines. Consistent with our previously published data with parental Jurkats [28], TIM-1 expression in lCk-reconstituted JCaM1 cells resulted in enhanced transcription of an NFAT/AP-1 luciferase reporter (**Figure 24**). This enhancement was observed both basally as well as in conjunction with stimulation through the TCR. In contrast, Lck deficient JCam.1 cells displayed little if any reporter activity either with or without stimulation, despite the fact that Flaf-TIM-1 expression was equivalent on both cell lines (data not shown). These data suggest that TIM-1 enhances T cell activation, at least in part, by coupling to phosphotyrosine-dependent signaling pathways.

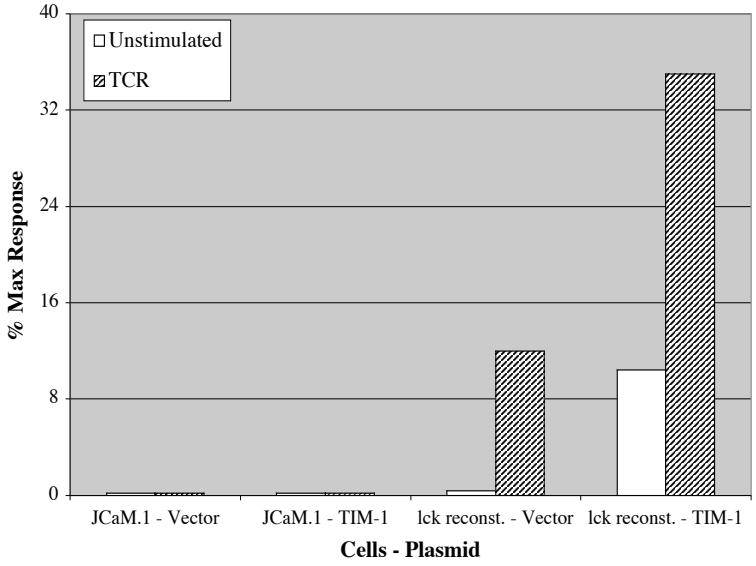


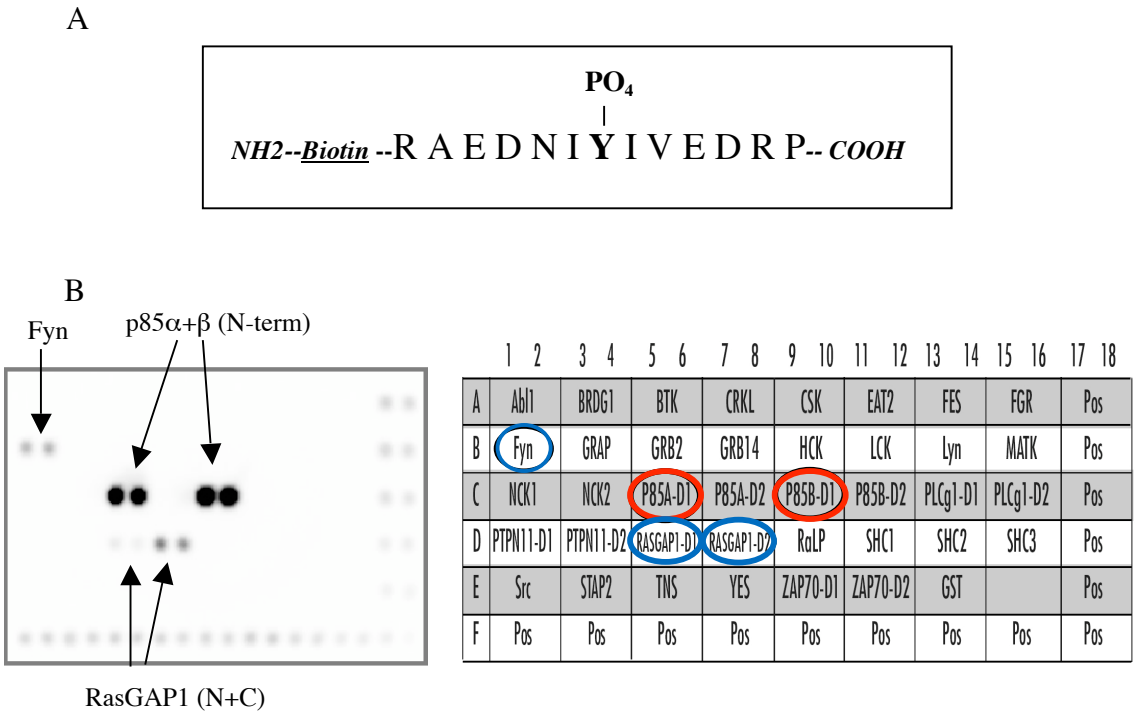
Figure 24: Role of Lck in TIM-1 function.

JCaM1 or JCaM1-Lck cells were transfected with an NFAT/AP-1 luciferase reporter and either empty vector or Flag-Tim-1. Cells were stimulated the next day and luciferase activity was determined. This experiment is representative of three that were performed.

5.3.2. Interaction of TIM-1 with p85 subunits of PI3K

One mechanism by which phosphotyrosine-dependent signaling complexes are formed is through phosphotyrosine-SH2 domain interactions. In order to identify SH2 domain - containing signaling proteins that might specifically bind to phosphotyrosine 276 of the TIM-1 tail, a solid phase screen was performed. The TranSignal^{TM} SH2 domain array from Panomics contains 38 SH2 domains of various signaling proteins, folded in their native conformation and immobilized in duplicates on the array membrane. The tyrosine phosphorylated peptide or protein of interest is incubated with the membrane. Protein interactions take place on the membrane surface and are visualized by HRP-based chemiluminescence detection where the signal strength correlates directly with the strength of protein- protein interaction. Accordingly as shown in **Figure 25A**, a thirteen amino-acid peptide was artificially synthesized that contained the tyrosine 276 of TIM-1 tail, with six amino acids on either side. The peptide was tyrosine phosphorylated and biotinylated at one end and incubated with the membrane. The array membrane was processed like a normal protein blot, and protein interactions were detected by Streptavidin-conjugated HRP combined with chemiluminescence imaging system. As shown in **Figure 25B**, two prominent spots were consistently obtained. These corresponded to the N-terminal SH2 domain of p85 alpha and p85 beta. Notably, this association is distinct from other costimulatory molecules such as CD28, CTLA-4 and ICOS that interact with the C-terminal SH2 domain of p85 via consensus YXXM motifs [142-147]. Other spots that were not consistent and of a much lower signal intensity corresponded to SH2 domains from RAS-GAP and the tyrosine kinase Fyn. We next

performed the experiment in the presence of a competitive inhibitor – phenylphosphate – to confirm that the binding observed was dependent on phosphotyrosine. As shown in **Figure 26B**, a much weaker signal was obtained when the SH2 arrray was incubated with phosphorylated TIM-1 peptide in the presence of phenylphosphate. By comparing the p85 signal with the positive control spots along the right and bottom edges, we determined that binding of the peptide to the p85α and p85β N-terminal SH2 domains was inhibited by 99% and 95%, respectively.



	1 2	3 4	5 6	7 8	9 10	11 12	13 14	15 16	17 18
A	Abl1	BRDG1	BTK	CRKL	CSK	EAT2	FES	FGR	Pos
B	Fyn	GRAP	GRB2	GRB14	HCK	LCK	Lyn	MATK	Pos
C	NCK1	NCK2	P85A-D1	P85A-D2	P85B-D1	P85B-D2	PLCg1-D1	PLCg1-D2	Pos
D	PTPN11-D1	PTPN11-D2	RASGAP1-D1	RASGAP1-D2	RaLP	SHC1	SHC2	SHC3	Pos
E	Src	STAP2	TNS	YES	ZAP70-D1	ZAP70-D2	GST		Pos
F	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Figure 25: Specificity of SH2 domain binding to phosphorylated Y276 in the TIM-1 cytoplasmic tail. (a) Sequence of the peptide, and modifications, used to determine the SH2 specificity of phopsho-Y276. (b) Results of the SH2 domain array screen, using the peptide illustrated in part a. SH2 domains are present as duplicate spots. The dashed box indicates the location of negative control spots; the bottom and right edge contain positive control spots. These results are representative of those obtained in three separate experiments, using two different lots of membrane.

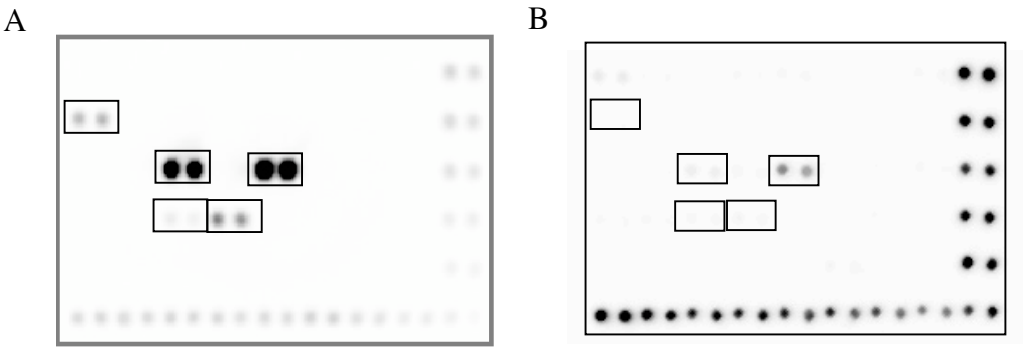


Figure 26: TIM-1 requires phosphorylated Y276 for binding to p85.

Competitive inhibition of TIM-1 peptide binding, in the presence of the phosphotyrosine analogue phenylphosphate (Part A is reproduced from figure 25B, Part B is performed in the presence of phenylhosphate) The blot shown is representative of two experiments.

To validate the TIM-1-p85 association with full-length proteins, the interaction was also established in T cells by conventional immuno-precipitations. Accordingly, Flag-TIM-1 transfected Jurkat cells were stimulated with pervanadate followed by anti-flag immunoprecipitation and western blotted with an anti-pan p85 antibody. While there was a reproducibly strong interaction of TIM-1 with p85 under conditions of pervanadate stimulation, a weaker and less consistent association was also detected under resting conditions, suggesting some low levels of basal TIM-1 phosphorylation (**Figure 27**). Consistent with the notion that the protein interaction is dependent on tyrosine 276 of TIM-1 tail, there was an almost complete loss of inducible p85 binding to the mutant forms of TIM-1 either lacking the cytoplasmic tail or with the Y276 mutated to phenylalanine (**Figure 28A**). Again in agreement with data discussed above, this interaction was also significantly diminished in cells treated with PP2 (**Figure 28B**).

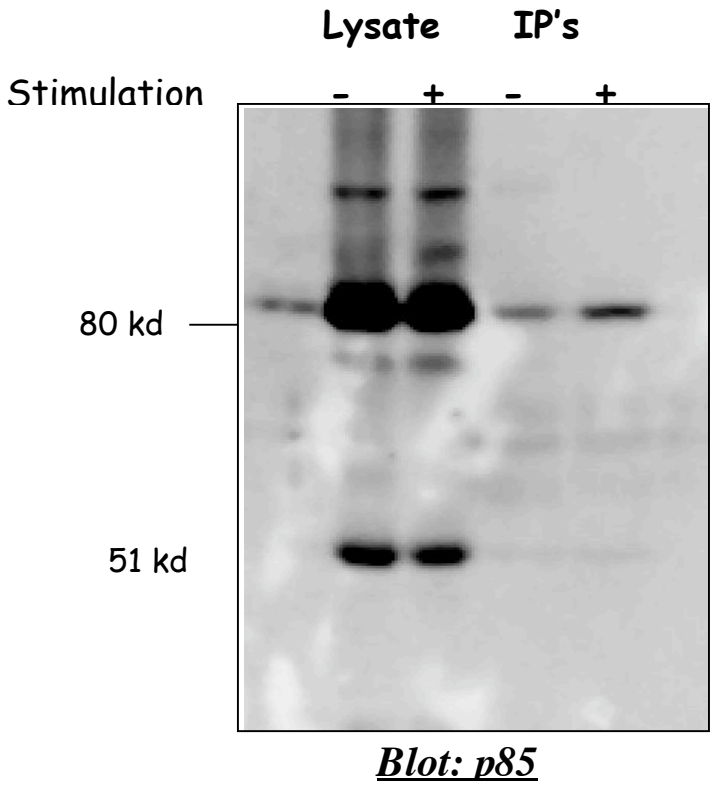


Figure 27: Recruitment of PI3 kinase by TIM-1.

Jurkat T cells expressing Flag-Tim-1 were stimulated as indicated, lysed and IP'd with anti-Flag. Whole cell lysates and IP's were separated by SDS-PAGE and western blotted with a polyclonal antiserum to p85a/b. Results are representative of five performed.

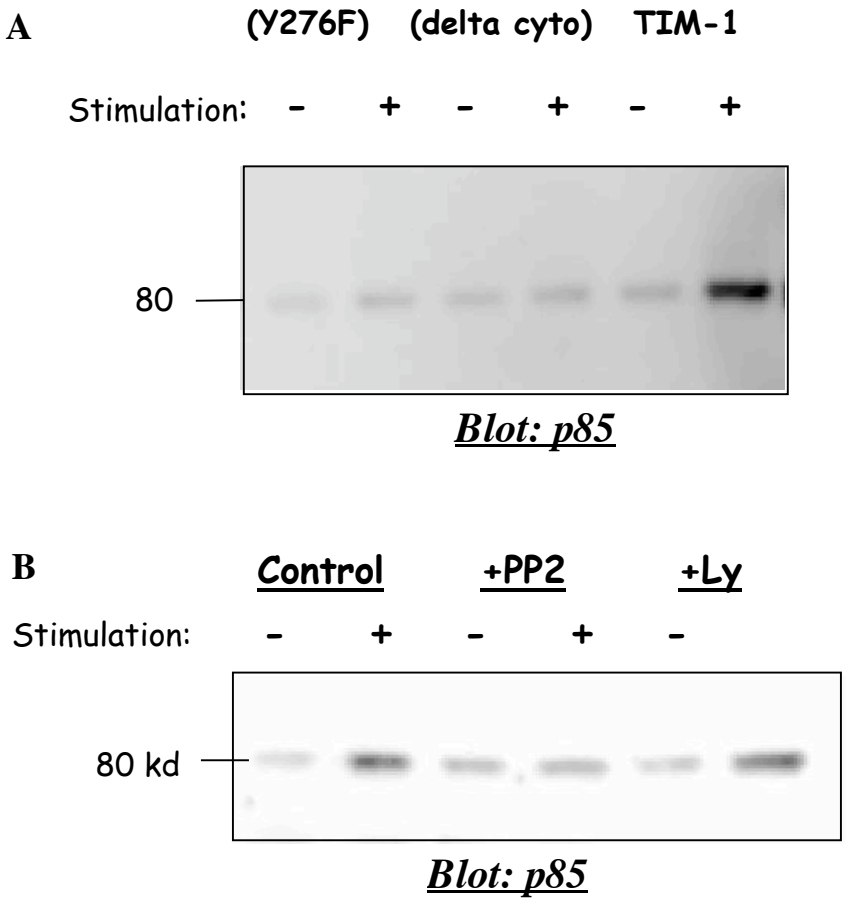


Figure 28: Recruitment of PI3 kinase requires Y276 of TIM-1.

(A) Jurkat T cells expressing the indicated Tim-1 constructs were stimulated and analyzed for p85 binding as described earlier. Results are representative of three performed. (B) Jurkat T cells expressing TIM-1 construct were stimulated as indicated and analyzed for p85 binding as described earlier.

A major target for PI3K-derived lipids is the serine/threonine kinase Akt, activation of which is absolutely dependent upon PI3K. We therefore examined whether Tim-1 ligation results in activation of Akt, as assayed by its phosphorylation at serine 473. As shown in **Figure 29**, we were able to observe inducible Akt activation, in both primary T

cell blasts and the Th2 T cell clone D10, after stimulation (of untransfected cells) with anitobies to CD3 and CD28 or TIM-1. Akt activation by either CD3/CD28 or TIM-1 was completely inhibited by pre-treatment with the PI3K inhibitor LY294002 (data not shown). These results demonstrate that TIM-1 can interact with p85 and activate PI3K signaling, when expressed in T cells, in a phosphotyrosine-dependent manner.

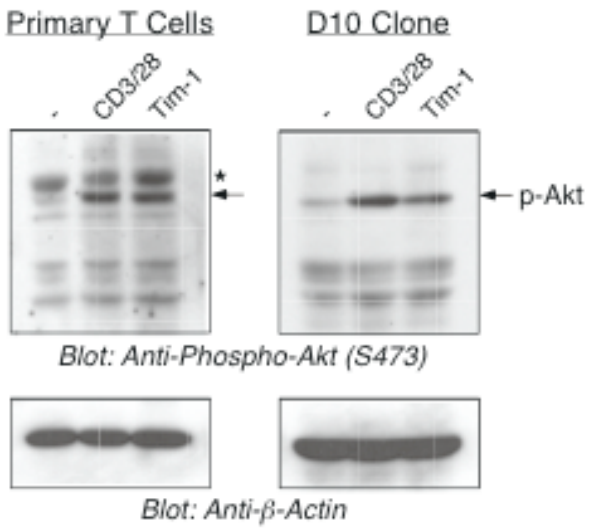


Figure 29: Activation of PI3 kinase by TIM-1.

T cell blasts were stimulated *in vitro* as indicated, with CD3, CD28 and TIM-1 antibodies. Lysates were separated by SDS-PAGE and western blotted with a rabbit mAb to phospho-Akt (S473; upper panels). Blots were stripped and re-probed with a mouse mAb to β-actin (lower panels), to control for protein loading. The arrows indicate the location of phospho-Akt, while the star indicates a non-specific band, which is only observed in primary T cells. Results are representative of three experiments that were performed.

5.3.3. Role of p85 and PI3K in TIM-1 Function

p85 functions as the regulatory sub-unit of phosphotidylinositol 3-kinase (PI3K), and as such regulates localization and activation of the associated catalytic p110 proteins. In

order to determine whether the catalytic property of PI3K was required for TIM-1 mediated co-stimulation of downstream NFAT/AP-1 dependent transcription, D10 cells (which possess a normally regulated PI3K pathway) were co-transfected with an NFAT/AP-1 luciferase reporter, along with either vector or TIM-1. These cells were then treated with a range of stimuli in the presence or absence of Ly294002, an inhibitor of PI3K activity [148]. As shown in **Figure 30A**, stimulation through CD3 and CD28 induced an enhanced activation of the reporter in TIM-1 transfected cells, compared with cells transfected with empty vector. However, in the presence of Ly294002, the effect mediated by TIM-1 was markedly reduced. These data suggest that one way by which TIM-1 co-stimulates T cell activation is through the catalytic property of PI3K. Therefore, we examined the role of a known downstream target of PI3K - the serine/ threonine kinase Akt/PKB [149], which as shown above is activated by TIM-1. A similar reporter assay was performed as shown above, with one exception. In addition to stimulation through CD3/CD28, an inhibitor of Akt (Akti) was used. **Figure 30B** reveals a clear requirement for Akt in TIM-1 function, since TIM-1 mediated activation of NFAT/AP-1 is inhibited to a level comparable to that obtained with vector-transfected cells stimulated through the TCR and CD28 alone. These data suggest that TIM-1 exerts an effect on NFAT at least in part, through activation of Akt. One possible mechanism is through subsequent inactivation of GSK-3[150], thereby increasing the residency of activated NFAT in the nucleus, in turn up regulating the production of IL-2.

We attempted to more definitively determine the role of p85 in TIM-1 function by siRNA- mediated knock-down of p85 in D10 T cells. We were able to achieve at best approximately 60% decrease of the protein (**Figure 31A),** likely due in part to the

necessity toeliminate expression of both p85α and p85β; this resulted in a partial inhibition of TIM-1 function, as determined with the NFAT/AP-1 reporter assay (**Figure 31B**). Taken together, the data in **Figure 30 & 31** indicate that TIM-1 exerts an effect on NFAT/AP-1 at least in part thought the PI3K/Akt pathway.

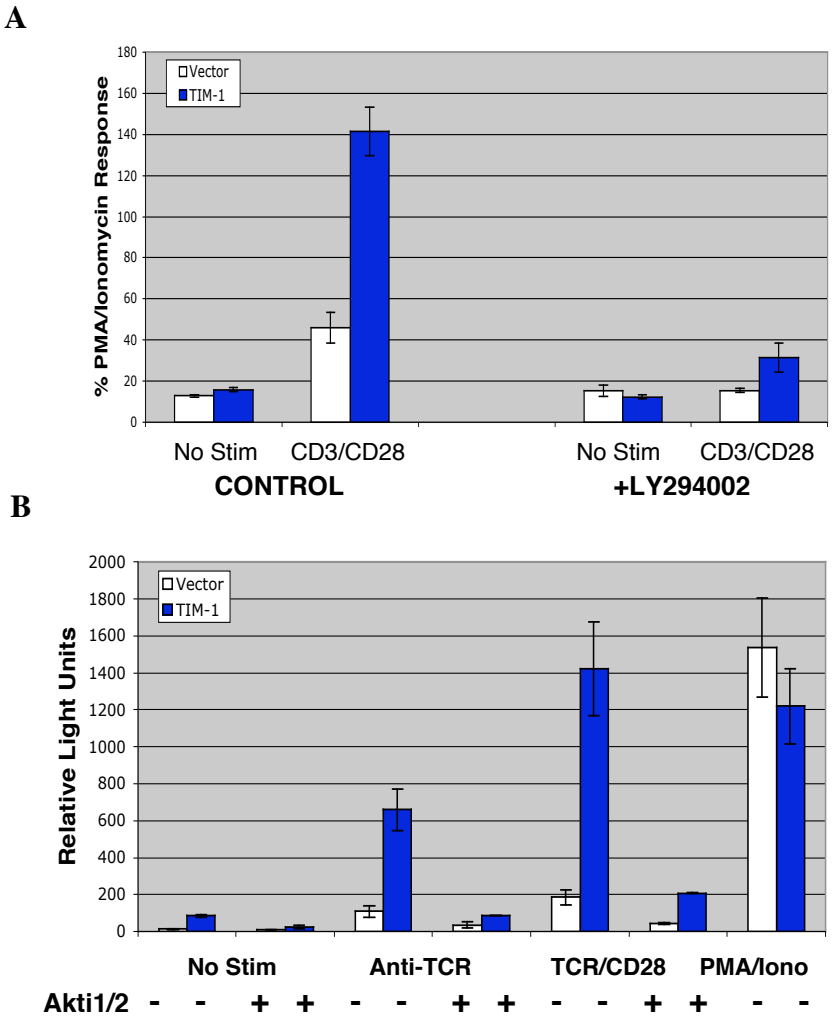
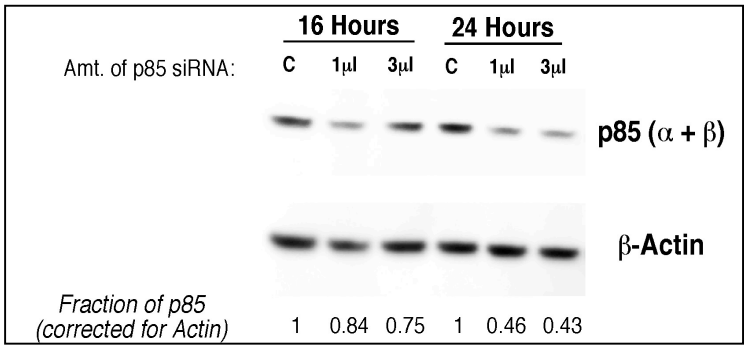


Figure 30: Role of the PI3K pathway in TIM-1 activation of NFAT/AP-1.

T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. (A) Cells were stimulated in the presence or absence of the PI3K inhibitor LY294002. (B) Cells were stimulated in the presence or absence of the Akt inhibitor Akt*i* 1/2. Results shown in each part are representative of three experiments.

A



B

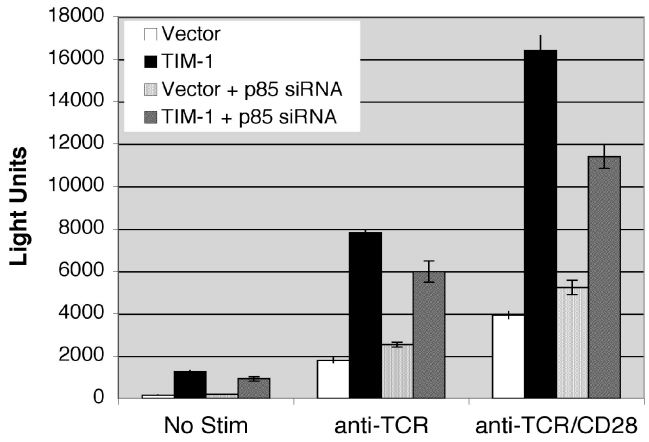


Figure 31: p85 siRNA inhibits optimal TIM-1- mediated activation of NFAT/AP-1.

T cells were transfected with an NFAT/AP-1reporter, plus either empty vector or Flag-TIM-1, and the indicated amounts of siRNA SmartPool oligos (Dharmacon) specific for p85α and p85β. Twenty-four hours after transfection, cells were stimulated for six hours, followed by determination of luciferase activity. Error bars indicate standard deviation for triplicate points in a single experiment. Results shown in each part are representative of three experiments.

One of the earliest activation markers expressed by T cells is the cell surface glycoprotein and C-type lectin domain-containing molecule, CD69. This molecule is expressed within one or two hours following TCR cross-linking and persists for at least three days [151,152]. In order to assess the effect of TIM-1 on CD69 up regulation, purified CD4 T cells from C57BL/6 mice were activated for 24 hours with an increasing

range of plate-bound anti-CD3 concentrations coupled with a fixed amount of anti-CD28, in the presence or absence of an agonistic TIM-1 antibody (3B3). Ligation of TIM-1 considerably enhanced the upregulation of CD69 expression (**Figure 32 A**) with the greatest effect observed at lower concentrations of anti-CD3. CD69 expression increased proportionally with increasing anti-CD3 concentration in TIM-1 treated cells through a range of concentration, eventually leveling off. Conversely, a mounting titration of anti-TIM-1 up to a point also corresponded with stronger co-stimulation (data not shown). TIM-1 also enhanced CD69 surface expression in the Jurkat T cell line in a manner that required Y276 of TIM-1 tail (**Figure 33B**). Intriguingly, some increase in CD69 expression was observed for the primary T cells treated with anti-TIM-1 alone. In light of the data discussed above, we were also interested in determining whether PI3K catalytic activity is required for TIM-1 mediated CD69 up-regulation. For that reason, this experiment was performed with Ly294002. As shown in **Figure 32A**, there was an apparent reduction in CD69 expression for the cells treated with TIM-1 in the presence of Ly294002, compared with anti-TIM-1 treated cells in absence of the inhibitor. Supporting this data, **Figure 32B** shows an increase of 15-20% in CD69 positive T cells treated with anti-TIM-1 at different concentrations of anti-CD3, over cells untreated with this antibody and this increase was significantly dampened with Ly294002, but was still higher than TIM-1 untreated cells with Ly294002. Of interest, CD69 increases induced by TIM-1 alone, also required PI3K activity to some extent. To address whether TIM-1 ligation with its natural ligand TIM-4 had similar effects, CHO cells stably transfected with TIM-4 were incubated with CD4 T cells for 24 hours and assessed for CD69 expression. Consistent with the results of antibody cross-linking described above, TIM-1

ligation by TIM-4 also induced an increase in CD69 expression, and this was inhibited with Ly294002 (**Figure 33A**). Taken together these data indicate that TIM-1 co-stimulates surface expression of the early activation marker CD69 on primary T cells in a manner that requires PI3K.

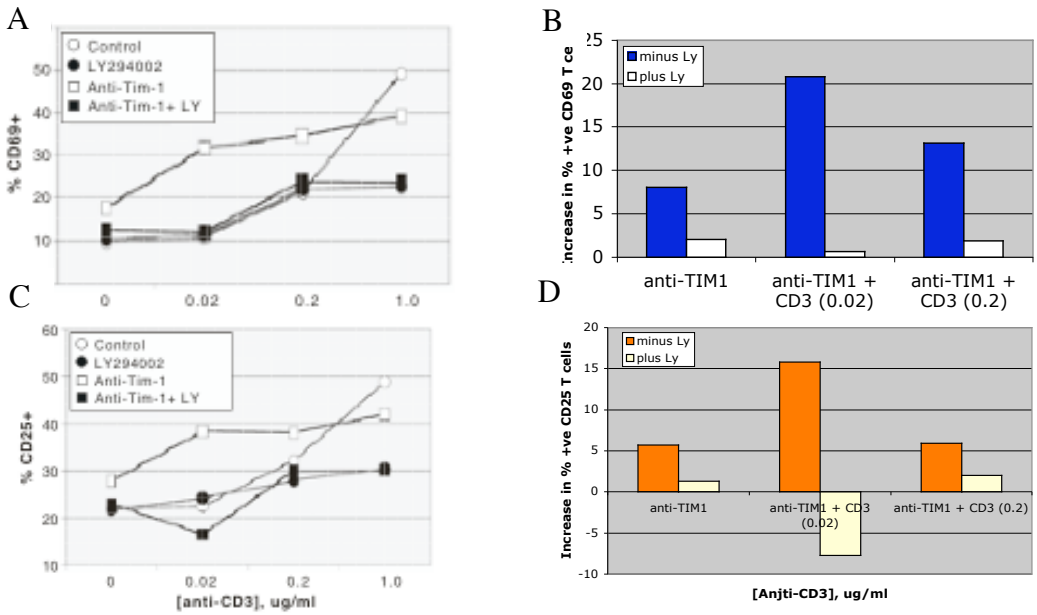


Figure 32: PI3K activity is required for upregulation of early activation markers by endogenous Tim-1 on primary T cells.

Purified CD4+ T cells from C57 BL/6 spleen and lymph node were stimulated overnight with the indicated concentrations of anti-CD3 antibody (and a fixed concentration of anti-CD28 antibody), with or without anti-Tim-1 antibody, in the presence or absence of PI3K inhibitor LY294002. Cells were stained with fluorescently labeled antibodies to CD69 (A) or CD25 (C) and analyzed by flow cytometry. (B) and (D) represent the increase in percent positive CD69 and CD25 T cells with anti-Tim-1 respectively. Results in each panel are representative of three experiments that were performed.

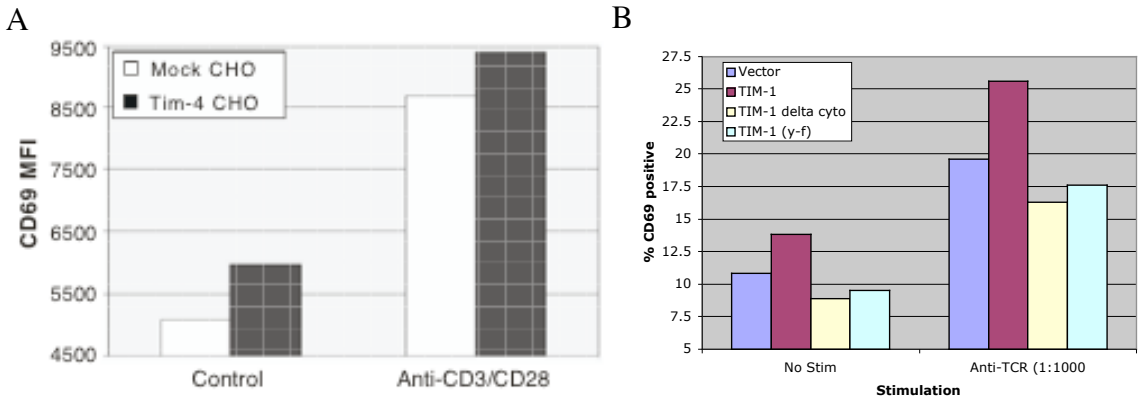


Figure 33: Upregulation of CD69 in primary T cells by the TIM-1 ligand TIM-4 as well as by overexpressing TIM-1 in Jurkat cells.

a) CD4+ T cells were stimulated with control CHO cells ('mock') or CHO cells expressing the TIM-1 ligand TIM-4, either alone or with anti-CD3/CD28 antibodies, then analyzed by flow cytometry for CD69 expression. b) Jurkat T cells were transfected with either TIM-1 or TIM-1-delta cyto or TIM-1 (y-f) constructs, and stimulated with anti-TCR antibody. 24 hours after stimulation, the cells were harvested and analyzed by flow cytomtery for CD69 expression. Results (in a & b) are representative of the experiment performed twice.

Another inducible cell surface marker essential for T cell commitment to activation, and thus representative of activated T cells is the high affinity IL-2 receptor alpha chain (CD25). To evaluate whether TIM-1 selectively enhanced expression of the early activation antigen CD69, or if this phenomenon was a more general consequence of its effects on T cell activation, we have also examined CD25 up-regulation (**Fig 32C & 32D**), which arises after CD69. As observed with CD69, TIM-1 cross-linking also co-stimulated CD25 surface expression and this was more evident with lower amounts of anti-CD3. Again, as with CD69, TIM-1 ligation alone led to some up-regulation of CD25 expression. Treatment with Ly294002 significantly inhibited TIM-1-mediated CD25 up-regulation, both by itself and in conjunction with CD3/CD28 signals. Maximal inhibition

was detected at lower anti-CD3 concentration that is normally most effective for TIM-1 function. Thus, these data suggest that TIM-1 induces expression of the activation markers CD25 and CD69 in a PI3K- dependent manner.

In order to determine more conclusively the relevance of p85 interaction for endogenous TIM-1 function, a genetic model of p85 deficient mice were used. These mice were generated by crossing *Pik3r1*^{f} mice, which have floxed alleles of *Pik3r1* (encoding p85α, p50α and p55α) [137], with *Pik3r2*^{n} strain deficient in the germ line for *Pik3r2* (encoding p85 beta) [138]. The resulting mice were then bred to Lck-Cre transgenic mice [139] yielding mice with T cells specifically ablated of PI3K regulatory sub-units from the double negative stage of thymocyte development forward [136]. T cells from p85 beta null mice develop normally and possess no detectable defects in PI3K signaling or any compensatory changes in PI3K isoform expression, so these mice served as controls [136,153]. p85 double knockout mice also possess T cells with no gross developmental problems but have selective defects in TCR dependent signaling and cytokine production [136]. Also, importantly, expression of Tim-1 is not impaired on T cells from the p85a/b knockout mice, with expression in the resting (low levels) and activated state (higher levels) indistinguishable from p85b or wild-type mice **(Figure 35).**

To confirm and extend the inhibitor studies performed with T cells from C57BL/6 mice, we examined TIM-1 effects on surface expression of CD69 and CD25 in CD4+ T cells from these p85 mutant mice. Representative flow cytometry plots for results obtained with CD69 are shown in **Figure 34**. Thus, while stimulation with anti-CD3/CD28 alone was relatively unaffected by the complete lack of p85 (compare part b

with a) in this experiment, the ability of TIM-1 to co-stimulate CD69 upregulation was severely impaired. Because we an others have observed both stimulatory and co-stimulatory effects of TIM-1 ligation, we wanted to determine whether both or either of these activities would be affected by p85 deficiency.

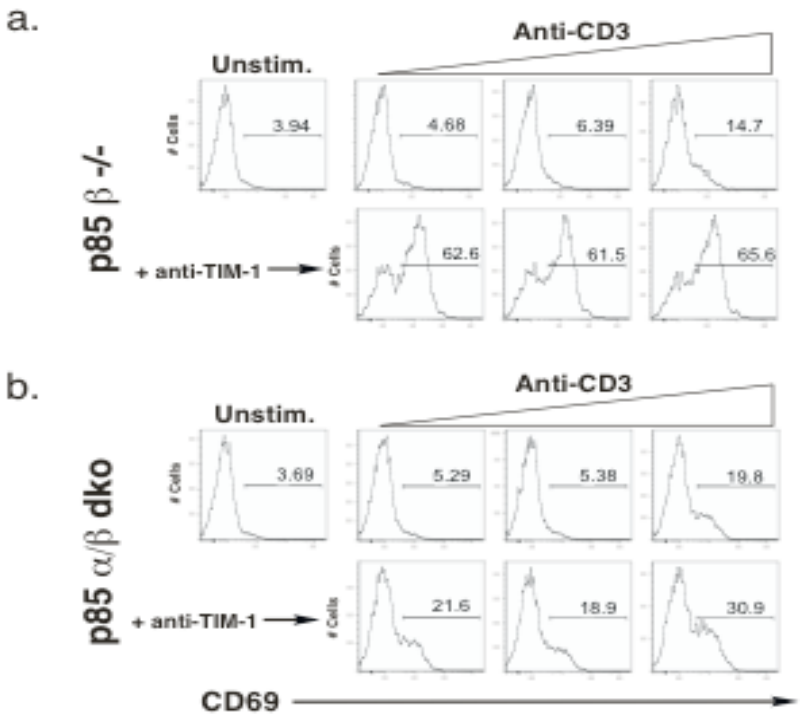


Figure 34: p85 is required for TIM-1-mediated co-stimulation of CD69 expression.
Splenocytes from p85β (a) or p85α/β (b) deficient mice were stimulated overnight with various concentrations anti-CD3 antibody (0.05, 0.2 and 1 ug/ml), along with a fixed concentration of anti-CD28 (1 ug/ml). Anti-TIM-1 antibody was also added to some cultures, at a concentration of 8 mg/ml. Cells were stained the next day for CD4 and CD69 and analyzed by flow cytometry. Results shown were gated on CD4+ cells

We therefore examined effects of agonistic TIM-1 antibody on upregulation of both CD69 (**Figure 36 a, c, e**) and CD25 (**Figure 36 b,d,f**). This was carried out in either the presence of CD3/CD28 engagement (**Figure 36 a-d**) or without any additional stimuli

(**Figure 36 e and f**). As can clearly be seen from these data, the absence of p85 expression, in the p85a/b double knockout (dko) T cells, had a profound effect on the ability of TIM-1 to induce increases in CD69 and CD25, either by itself or in conjunction with CD3/CD28 signaling. Previous studies with p85-deficient mice had demonstrated that the proliferation defect was largely corrected with the addition of exogenous IL-2 [136]. Although activation markers were assessed relatively early in the course of T cell activation, we wanted to ensure that proliferation differences did not underlie the effects we were observing. Thus, as expected, addition of IL-2 to the cultures had no effect on the results (data not shown).

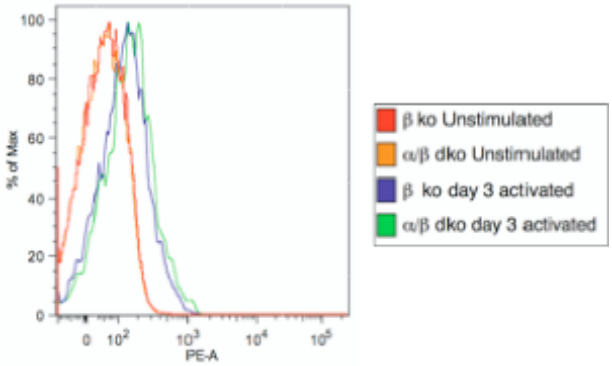


Figure 35: Expression of TIM-1 on p85 deficient T cells.

Splenic CD4+ T cells from p85β or p85α/β deficient mice were stained with anti-TIM-1 mAb and PE-conjugated secondary antibody, immediately after purification ("unstimulated") or after three days of *in vitro* stimulation with anti-CD3/CD28 antibodies, then analyzed by flow cytometry.

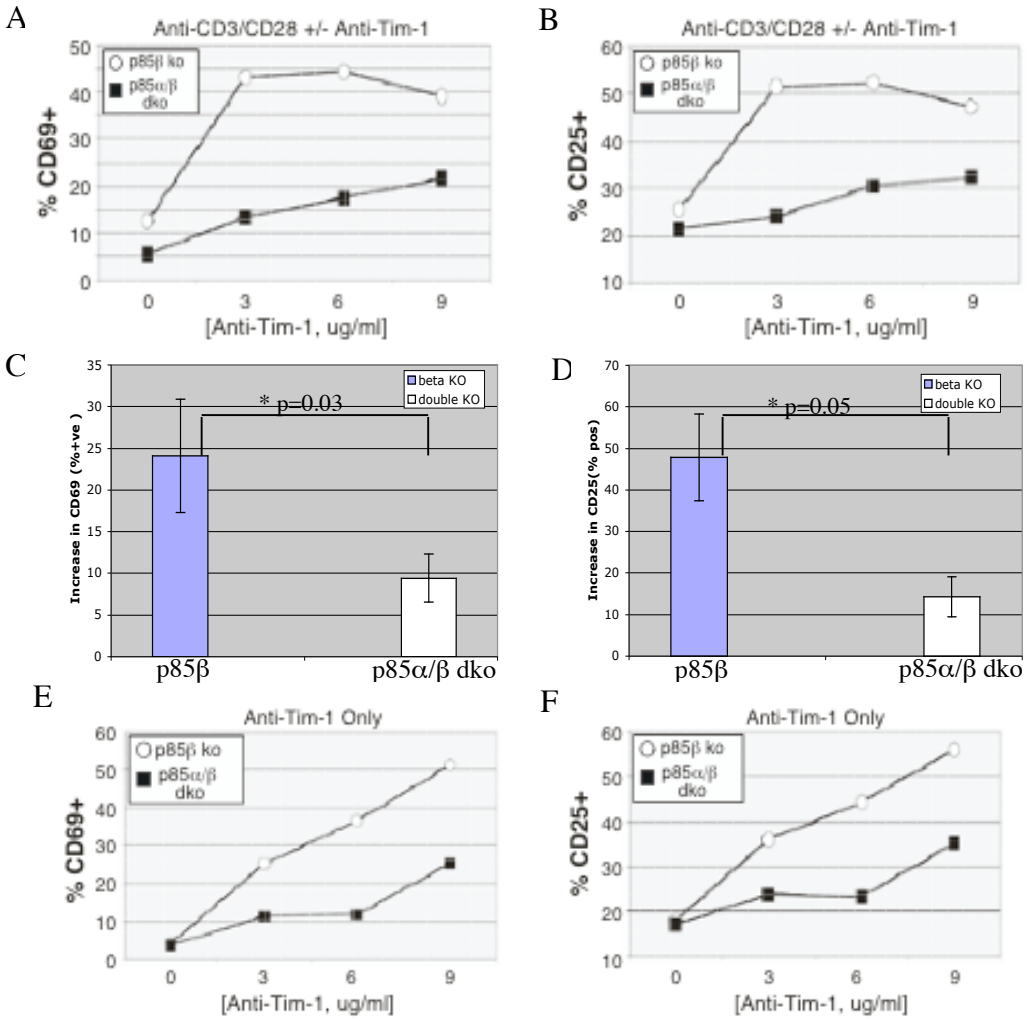


Figure 36: p85 is required for TIM-1-mediated CD69 and CD25 upregulation.

Purified CD4+ T cells from p85β or p85α/β deficient mice were stimulated *in vitro* with anti-CD3/CD28 antibodies and the indicated concentrations of TIM-1 antibody (A-D) or with anti-TIM-1 antibody alone (E and F). Cells were harvested and stained for CD69 (A, C, E) or CD25 (B, D, F) expression, and analyzed by flow cytometry. Results in A-B and E-F are single experiments, representative of three, or two, separate experiments, respectively. Panels C-D show effects of TIM-1 co-stimulation (+/- S.D.) averaged over three experiments, with *p* values indicated.

Finally, we examined cytokine production by the mutant p85 T cells and found that TIM-1 induction of IL-2 secretion was impaired in the p85 double knockout cells,

compared with T cells lacking p85β alone (**Figure 37**). In contrast to the more potent stimulatory effects of TIM-1 antibody on CD69 and CD25 induction, TIM-1 crosslinking by itself had a small effect on IL-2 secretion (note difference in scales between panels A and B), which was partially impaired in the p85 α/β deficient T cells **(Figure 37**). However, in the presence of anti-CD3/CD28 stimulation, Tim-1 ligation significantly enhanced the production of IL-2, an effect that is severely impaired in the p85 α/β deficient T cells (**Figure 37B**).

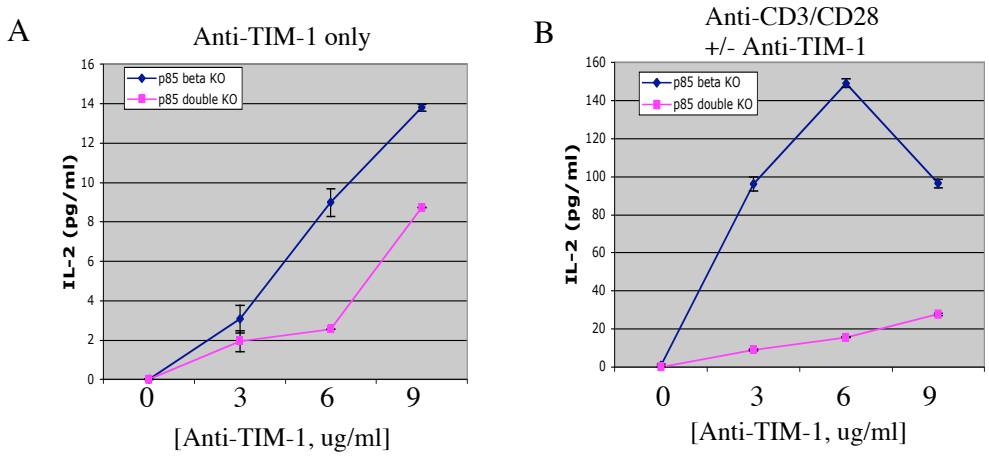


Figure 37: p85 is required for TIM-1-mediated IL-2 production

Purified CD4+ T cells from p85β or p85α/β deficient mice were stimulated in vitro with anti-CD3/CD28 antibodies and the indicated concentration of TIM-1 antibody (B) or with anti-TIM-1 alone (A). Supernatants wree harvested after 24 hours and analyzed by ELISA for IL-2. Error bars (obscured by the marker in most cases) indicate standard deviation for triplicate points withing a single experiment, which is representative of three that were performed.

5.4. DISCUSSION

Recent studies have demonstrated that the transmembrane protein TIM-1 can influence the course of an immune response through effects on T cell activation. However, the biochemical signaling pathways underlying these effects of TIM-1 ligation are not understood. Employing a combination of biochemical, pharmacological and genetic approaches, we delineate a signaling pathway, starting with Src kinase-mediated phosphorylation of TIM-1, to recruitment and activation of PI3K and Akt, which are required for induction of NFAT/AP-1-dependent transcription and up-regulation of surface activation markers and cytokines that are important for T cell activation and function.

The data we present here reveal a requirement for the src family kinase Lck for TIM-1 tyrosine phosphorylation, and subsequent NFAT/AP-1- dependent transcription. Our current model is that Lck first phosphorylates the cytoplasmic tail of TIM-1 and this allows subsequent recruitment of signaling proteins to Y276, a residue that is required for TIM-1 function in T cell lines. It should be noted that our data are also consistent with a possible role for the related tyrosine kinase Fyn in the regulation of TIM-1 function. Thus, the JCaM cell line, while lacking expression of Lck, also contains little if any Fyn protein [154]. In addition, the kinase inhibitor PP2, while relatively specific for Src family kinases, cannot distinguish between possible functions for Lck or Fyn. Finally, in some SH2 domain array experiments, we noted weak binding of the TIM-1 phospho-

Y276 peptide to the SH2 domain of Fyn. Further investigation is warranted to address whether Fyn contributes to Tim-1 function.

The protein array is a relatively novel technique for detection of protein interactions that has been used with considerable success by other groups ([155-157]. It is a slight variation from antibodies bring blotted onto membranes, and detected with fluorescent dyes or markers. The SH2 domain array from Panomics has protein domains, which in this case are SH2 domains, arranged in a predefined fashion and arrayed into membranes. The advantage of such a technique is that it allows for detection of interacting proteins by testing a large number of probable candidates simultaneously, using very little starting material. Using this unbiased approach, we have found that phosphorylation of Y276 leads to recruitment of the p85 subunit of PI3K, and we further confirm this interaction with endogenous p85 in T cells expressing TIM-1. Interestingly, TIM-1 interaction with p85 is a common feature shared by other costimulatory molecules such as CD28, ICOS, and CTLA-4. However, in contrast to these molecules, which preferentially bind the carboxy terminal SH2 domain of p85, TIM-1 binds the amino terminal SH2 domain as revealed by the SH2 domain array. Another difference is that p85 binds to these other co-stimulators via a consensus YXXM motif, while it binds to TIM-1 at a non-canonical sequence (YIVE). Importantly, the amino-terminal SH2 domain has a ten-fold lower avidity for the YXXM motif than does the carboxy terminal SH2 domain [142] which may explain its preferential interaction with TIM-1. These data suggest that TIM-1 might signal differently from other co-stimulators such as CD28. Indeed, we showed previously that TIM-1, in contrast to CD28 does not activate NF-κB dependent transcription in T cells [28] .

The data here show that ligation of TIM-1 augments surface expression of representative T cell activation markers CD69 and CD25 on primary murine T cells. CD69 is one of the earliest activation markers expressed within 1-2 hours following TCR ligation, perhaps indicating that TIM-1 can modulate very early events in T cell activation which is in keeping with previously described kinetics of early TIM-1 mRNA expression and function following activation [5,28]. The timing of TIM-1 ligation *in vivo* may therefore be critical for its function in T cell activation. CD69 itself has a functional involvement in T cell activation, since in conjunction with TCR ligation or PMA it enhances AP-1 dependent transcription, Ca2+ mobilization, IL-2 and IFNγ cytokine production, proliferation and CD25 surface expression [158]. Thus, perhaps one feed-forward mechanism of sustaining TIM-1 co-stimulation is indirectly through CD69 surface enhancement.

We provide evidence that TIM-1 can regulate the surface expression of CD25 in addition to CD69 for T cell activation. One cannot exclude the possibility that it may also modulate the generation or function of CD4+CD25+ regulatory T cells that express Foxp3 (Forkhead box P3). Indeed, anti-TIM1 antibodies, RMT1-10 and 3B3, differing by 17-fold in binding affinity to the same or closed related TIM-1 epitope in the IgV domain exert opposite effects on T cell function [120]. TIM-1 ligation by RMT1-10 apparently induces inhibitory consequences, whereas cross-linking with higher affinity 3B3 potentiates T cell activation. Given the evidence provided here and elsewhere that 3B3-ligated TIM-1 costimulates T cell activation, TIM-1's contribution to promoting T regulatory cell development rather than its suppression seems unlikely. This is corroborated by the fact that FOXP3 functions as an NFAT repressor [159], while our

studies denote that TIM-1 induces potent NFAT activation [28]. Nevertheless, these possibilities call for further investigation.

Consistent with its recruitment of p85, we also show that TIM-1 requires PI3K activity to mediate its co-stimulatory role in primary murine T cell function since Ly294002 inhibited TIM-1 function. While being a potent inhibitor of Class 1A PI3K, which includes the p85 regulatory sub-units, Ly can also inhibit other PI3K classes such as Class 1B PI3K as well as the mammalian target of rapamycin (mTOR)[160-162], both required for T cell proliferation. Each of the five regulatory sub-unit isoforms of Class 1A PI3K (p85α, p55α, p50α, p85β, p55γ) can interact with each p110 species to form a functional PI3K enzyme that is activated by tyrosine kinases for immune signaling. Previous studies have used mice lacking in p85α, or p85α, p55α, p50α, or p85β which show normal or enhanced T cell proliferation [153,163-165] and the germ line p85αβ deletion is embryonic lethal. In this study we have employed a recently described genetic model, which is based on complete deletion of the regulatory sub-units of PI3K (encoded by the *Pik3r1* and *Pik3r2* genes) selectively in T cells [136]. This is accompanied by a significant reduction in levels of the catalytic p110 sub-unit isoform (which is normally stabilized by p85 binding), resulting in abrogation of Class IA, but not Class IB, PI3K activity in T cells. Consistent with previous data [136], CD25 and CD69 upregulation triggered by TCR stimulation was only mildly affected in the p85β and double k.o. T cells. However, the ability of TIM-1 to co-stimulate CD25 and CD69 was severely impaired in the double k.o. T cells, when compared with p85β- deficient T cells. Again, consistent with previous findings [136], p85β- deficient T cells behaved identically to wild-type T cells with regard to CD25 and CD69 induction (data not shown). Of interest,

TIM-1 could also induce some activation marker expression by itself and this was also substantially impaired in p85 double k.o. T cells.

T cell activation induced by TIM-1 alone was observed in our earlier studies performed in human Jurkat cells and is also in agreement with other studies where hyperproliferation of T cells was observed in mice treated with soluble mTIM-1 molecules, as well as increased basal signaling observed in primary human cells treated with anti-TIM1 antibody [25,28,119]. A recent study has shown that TIM-1 can associate with the TCR-CD3 complex in the basal state and co-localization is fortified by TIM-1 cross-linking or anti-CD3 ligation [119]. Further, crystal structure analysis of the TIM's have revealed the basis for homophilic interaction of TIM-1 at the intercellular junctions on neighboring cells, and this interaction is conserved between mouse and humans implying an important biological function [10]. TIM-1 homophilic interaction coupled with its association with the TCR, could lead to an aggregation of molecules, the stability of which may facilitate TIM-1phosphorylation along with phosphorylation of signaling mediators, in turn triggering downstream effector pathways. The pathways initiated could be both TCR dependent and p85 dependent. Ligation of TIM-1 when coupled with TCR stimulation would enhance distinct TIM-1 signaling mechanisms mediated in part by p85. The quality of the signaling outcome might vary proportionately with the stability and perhaps more importantly the composition of the molecular clustering that is ultimately induced by an epitope specific anti-TIM1 antibody or ligand. Raising the dose of the agonistic TIM-1 antibody would engage additional TIM-1 molecules on the surface that would form a cluster with the TCR-CD3 complex. Indeed, we show that an increasing titration of 3B3 alone is accompanied by an increasing surface expression of CD69.

While there is a significant dampening of this response in T cells from p85 double knockout mice, treatment with higher doses of 3B3 can induce some basal CD69 augmentation. Though these T cells lack p85 regulatory sub-units, proximal TCR induced signaling events seem relatively unaffected since ZAP-70 phosphorylation was intact in these cells ([136]. Given that TIM-1 can also induce ZAP-70 phosphorylation through its interaction with TCR [119], this suggests that the residual effects of TIM-1 observed in these p85 deficient mice are TCR-dependent.

The data presented here support TIM-1's function as a positive costimulatory molecule. Recent structural studies predict different ligand binding modes [10,33]. Indeed thus far, there exist more than one ligand for each of the TIM's, and presence of additional ligands are predicted [7,19,21,25]. It is also possible that at any given time, more than one ligand might interact with TIM-1 concurrently on different faces of the molecule. Furthermore, homophilic interaction between individual TIM-1 molecules are mediated through their Ig domain but these interaction are also affected by glycosylations of the mucin domain [10], the sequence of which is polymorphic. Anti-TIM-1 antibodies targeting different epitopes of the TIM-1 exracellular domain have also been shown to initiate either positive or negative effects on T cell activation [38,120,121]. Thus, the cis- and trans interaction of TIM-1 with its binding partners regulated in part by the timing of their expression in immune development and TIM-1's inherent polymorphisms, would modulate the subsequent biochemical signaling pathways that are triggered.

While our study indicates that PI3K plays an important role in mediating TIM-1 costimulatory signaling, this does not exclude the possibility that there may be additional molecules that can also bind to TIM-1 with different functional effects. Based on data

presented here, we suggest that ligation of TIM-1, when coupled with TCR stimulation, enhance downstream signaling pathways that induce T cell activation at least in part through PI3K and its downstream effector Akt. Future studies will focus on the mechanisms by which TIM-1 mediated PI3K activation affects T cell activation.

6. CHAPTER 6: TIM-1 REQUIRES TCR SIGNALING COMPONENTS TO ENHANCE NFAT/AP-1 DEPENDENT TRANSCRIPTION.

6.1. INTRODUCTION

Our findings presented earlier in chapter three provided evidence that TIM-1 can function as a co-stimulatory molecule for T cell activation and can transduce signals to augment NFAT/AP-1 dependent transcription. These findings were supported by another group who showed that ligation of TIM-1 with an agonistic anti-TIM-1 antibody coupled with stimulation through the TCR could significantly co-stimulate T cell proliferation, enhance cytokine production and even abrogate tolerance in a Th2 –driven model of respiratory tolerance [17]. At about the same time, work from a third group indicated that TIM-4 can serve as a natural ligand for TIM-1 and administration of higher amounts of TIM-4-Ig chimera with sub-optimal levels of TCR stimulation could also enhance T cell proliferation and cytokine production [25].

In chapter three we establish TIM-1 as a signal transducing cell surface receptor. A general immunological paradigm for co-stimulatory molecules is that they are unable to bring about T cell proliferation and immune effector function in the absence of TCR ligation by antigenic peptide in context with MHC or by anti-TCR antibody [166].

Conversely, TCR ligation in the absence of a second signal generated through co-stimulatory signaling leads to anergy [167]. Co-stimulatory molecules amplify and sustain TCR signals so that a critical threshold can be crossed for activation of the T cell [168-170]. Some important questions that stem from this discussion are whether co-stimulatory signaling pathways qualitative in nature and trigger pathways distinct from TCR stimulation, or do serve to quantitatively increase TCR signals or both? In the case of TIM-1, chapter five delineates a signaling mechanism whereby TIM-1 can recruit and activate p85-PI3K-mediated signaling pathways in a phospho-tyrosine dependent manner. Do TIM-1-dependent signals intersect with those originating from the TCR as well, and how is the synergy brought about? This present study therefore begins to address these important issues in order to elucidate the nature of signals mediated by TIM-1 in relation to TCR signaling.

An overview of TCR signaling is depicted schematically below (**Figure 38**) Briefly, T cell- dependent immune responses are thought to begin with an encounter between antigenic peptide expressed by MHC on an antigen presenting cell (APC) and the T cell receptor (TCR) on a naïve T cell. This event triggers an entire cascade of signaling events culminating with activation of the T cell. Following engagement of the TCR with antigen, the src family tyrosine kinases Lck and Fyn associated with the co-receptor CD4 get activated [135,171]. This leads to the phosphorylation of tyrosines contained in the ITAM (immunoreceptor tyrosine based activation motif) motifs in the TCR ζ- chain [171]. The SYK family protein tyrosine kinase (PTK) ZAP-70 is thereby recruited to phosphorylated ITAMs and itself get phospho/rylated and activated by Lck and other Src family PTK's such as Fyn [172-174].

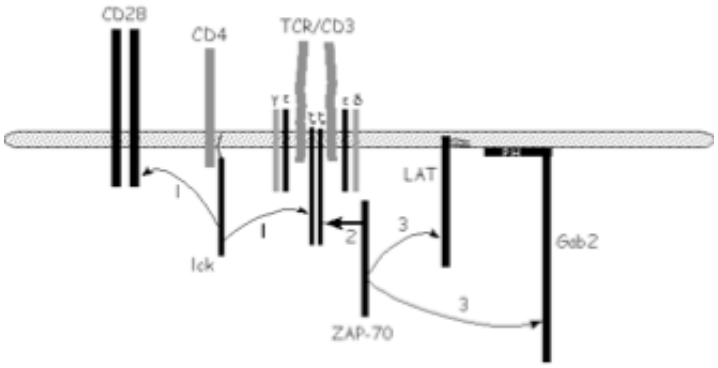


Figure 38: Schematic model of proximal TCR signaling.

(Adapted from Kane LP et al, [175])

Molecules implicated in PI-3 kinase activation are represented in black. Phosphorylation events are designated by thin curved arrows and inducible protein–protein associations with thick straight arrows. The order of these early events is indicated by the numbered arrows.

Activated ZAP-70 phosphorylates and activates several downstream substrates. Of special significance amongst these is the adaptor protein LAT [176] (**Figure 39**). LAT phosphorylation provides binding sites for several important SH2 domain-containing signaling proteins including Grb2, PLCγ1, Itk, p85 sub-unit of PI3K, and the adaptor GADS [176-179]. The adaptor molecule SLP-76, can therby associate with LAT through GADS following TCR ligation[177-180]. Since LAT is stationed in lipid rafts at the cell membrane [181-183], one functional model for LAT-GADS-SLP-76 multi-molecular scaffold is to recruit signaling molecules to lipid rafts, a site of active TCR signaling [184]. Activated SLP-76 can itself interact with Vav, Nck, Itk and/or other Tec kinases [185-188]. Evidence indicates that SLP76 also constitutively associates with the SH3 domain of PLCγ1 [189], and that formation of a multimolecular complex between LAT,

GADS, SLP76 and PLCγ1 is required for optimal PLCγ1 activation. PLCγ1 is phosphorylated by Itk and other Tec family members and by ZAP-70 and thus activated [187,190,191]. Activation of PLCγ1 results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 production leads to increases of cytosolic free Ca2+ [Ca2+]i, whereas DAG can activate both protein kinase C (PKC) and Ras guanyl nucleotide-releasing protein (RasGRP) [192]. PKCθ is activated by DAG/Vav/Rac mediated events [193-197]. Phosphorylated LAT also recruits the SH2 domain of GRB2 to lipid rafts, and therefore, the GRB2-associated RasGEF, SOS, thereby providing an additional possible mechanism of Ras activation through LAT [198-200]. These events collectively results in the downstream activation of MAP kinases and Calcium dependent pathways, in turn inducing transcription factors that direct gene transcription.

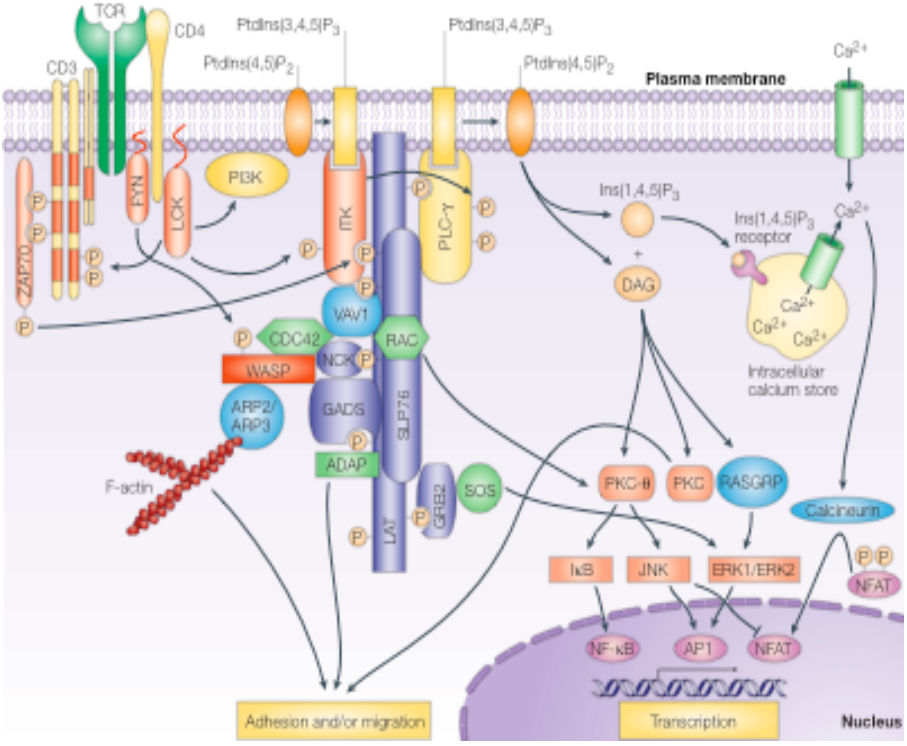


Figure 39: Overview of TCR Signaling

(Adapted from Schwartzberg PL et al [201] and Koretzky G et al [184]) See text for description

6.2. MATERIALS AND METHODS

6.2.1. Cell lines, antibodies and reagents

Experiments were performed with the Jurkat human T cell leukemia cell line, or with different variants of parental Jurkat: J.Cam.1 (Lck-deficient); Lck-reconstituted J.Cam.1; p116 (ZAP-70 deficient); JRT3-T3.5 (TCR-β deficient) and J14 (SLP-76 deficient). The cells were maintained as described previously (Refer chapter five).

Clonotypic antibody to the Jurkat TCR (C305) was from A.Weiss (University of California, San Francisco). Anti-human CD28 was from Caltag (Burlingame, CA). APC-conjugated donkey anti-rat IgG was from Jackson Immunoresearch (West Grove, PA). Phorbol myristate acetate (PMA) was from EMD Biosciences (La Jolla, CA). Anti-flag 77(M2) and Ionomycin was from Sigma (St. Louis, MO).

6.2.2. Transient transfections and luciferase assay

Jurkat / JRT3.T3.5 / J.Cam1.6/ J.Cam1-Lck / p116 / J14 cells, were transfected by electroporation using a gene pulser (Bio-Rad Laboratories, Hercules, CA), at a setting of 250-260V and 960μF, in cuvettes containing 1-2 × 10^7 cells with 0.4 ml serum free media. The amount and type of DNA plasmids transfected for an experiment are as described in the respective figure legends. Following transfection, the cells were cultured in 10ml-supplemented medium for 16-20 hours, and then stimulated for six hours in 96 U-bottom well plates. Reporter assays were performed as described previously [92]. Luciferase activity was determined with an Orion luminometer (Zylux, Oak Ridge, TN).

6.2.3. Calcium mobilization

Jurkat T cells were transfected with empty vector or Flag-TIM-1. The next day, cells were loaded with Calcium Green AM ester (Invitrogen; Carlsbad, CA) and stained with an anti-Flag antibody. Intracellular calcium was monitored in the FITC channel before

and after addition of anti-TCR/CD28 antibodies. Samples were maintained at 37^{o}C for the duration of analysis.

6.2.4. Detection of protein phosphorylation

Jurkat T cells were transfected with empty vector or Flag-TIM-1, plus pMax-GFP. The next day, cells were stimulated with anti-TCR and -CD28 antibodies. Cells were then fixed (1.5% PFA), permeablized (MeOH) and stained with a PE-conjugated antibody to phospho-ERK or phospho-JNK or APC-conjugated phospho-PLCγ1 (BD Biosciences; San Jose, CA). PE and APC staining within the GFP positive population was determined on a BD LSR II flow cytometer.

6.3. RESULTS

6.3.1. Requirement of TCR for TIM-1 co-stimulatory function

We have previously shown that TIM-1 can enhance activation of NFAT/AP-1 dependent transcription when expressed in T cells [40]. In Jurkat cells, some enhancement is detected at resting levels, and this is increased along with TCR stimulation. A general immunological paradigm for co-stimlulatory receptors is that they are unable to initiate T cell proliferation and effector function independently of TCR ligation by antigenic peptide or antibody [202]. Yet, surface expression of TIM-1 alone can induce basal

activation of the reporter. Therefore, in order to assess the extent of TCR involvement for TIM-1 co-stimulatory function, we employed mutant Jurkat cells JRT3-T3.5 (TCRβ -ve). These cells lack protein synthesis of the beta strand of the TCR heterodimer and therefore cannot assemble the TCR-CD3 membrane complex and so are essentially devoid of TCR surface expression [203]. The complete abrogation of NFAT/AP-1 activation observed in vector-transfected mutant cells when stimulated through the TCR is indicative of a non-functional TCR in JRT3-T3.5 cells. However, co-transfection of TIM-1 with NFAT/AP-1 luciferase reporter in these mutant cells, showed partial activation of the reporter with TIM-1 alone, and this was increased four fold by TIM-1 when coupled with TCR/CD28 stimulation (**Figure 40**). This data thus suggests that TIM-1 can trigger signaling pathways independent of TCR ligation. Nevertheless, expression of TIM-1 in cells that are reconstituted with TCR beta chain – a competent TCR- now reveal a profound increase in comparison, of NFAT/AP-1 induction when stimulated through TCR and CD28. This is several fold higher (>10) than TIM-1's effect in TCRβ-ve cells and approximately four fold higher than the level observed in vector transfected TCR reconstituted cells. There is also a concomitant rise in NFAT/AP-1 activation with TIM-1 alone in the reconstituted cells. Therefore, these findings indicate that optimal TIM-1-mediated signal transduction is dependent on TCR ligation. However, TIM-1 may also initiate distinct pathways that are likely to be amplified by TCR activation.

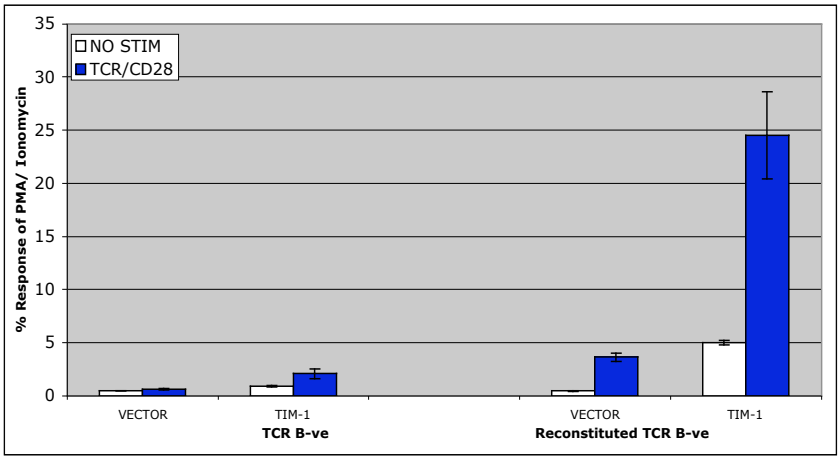


Figure 40: Requirement of TCR for TIM-1-mediated NFAT/AP-1 activation.

JRT3-T3.5 (TCR β -ve) and Reconstituted JRT3-T3.5 Jurkat variants were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. Results are representative of the average of triplicates from a single experiment performed three times.

6.3.2. TIM-1 co-stimulation requires proximal TCR signaling molecules: Lck, SLP-76 and ZAP-70.

As previously demonstrated, the conserved tyrosine 276 present in TIM-1 cytoplasmic tail is required for TIM-1dependent NFAT/AP-1 activation and TIM-1 can be inducibly tyrosine phopshorylated [40]. Tyrosine 276 is contained in a motif favorable for recognition by src family tyrosine kinases [88]. In T cells, Lck serves as the prominent src kinase member essential for initiating TCR signaling cascades. Furthermore, we show in chapter five of this thesis, that TIM-1 requires Lck for tyrosine phosphorylation. Thus, we performed a reporter assay in Lck deficient Jurkat cells (J.Cam.1) with transiently transfected TIM-1or vector along with the NFAT/AP-1 reporter, to assess its functional

role in mediating TIM-1dependent signals. As shown in **Figure 41**, there is no detectable NFAT/AP-1 activation in J.Cam.1 mutant Jurkat cells with either TIM-1 or vector transfection, whether stimulated though the TCR and CD28 or left unstimulated. Of note, this is in contrast to the effects observed in TCR –deficient cells where partial activation was obtained with TIM-1 expression alone or with TIM-1 transfected cells stimulated through CD28. JCam.1 cells that are stably reconstituted with Lck, however, were able to recapitulate the effects observed with TIM-1 in parental Jurkat cells. This indicates that Lck is of paramount importance in mediating TIM-1dependent signaling, and this is reminiscent of its pivotal role in initiating TCR signaling pathways.

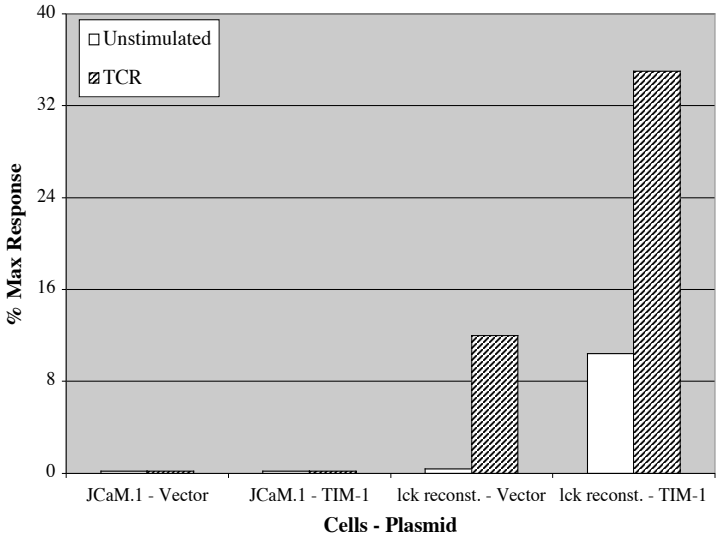


Figure 41: Requirement of Lck for TIM-1-mediated NFAT/AP-1 activation.

J.Cam.1 (Lck deficient) and Lck-Reconstituted J.Cam.1 Jurkat variants were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. Results are representative of the average of triplicates from a single experiment performed three times.

The requirement for surface expression of the TCR as well as the tyrosine kinase Lck to mediate optimum TIM-1 co-stimulatory signals suggested that TIM-1 dependent signals were in part, intersecting with TCR proximal signaling pathway components. In the TCR cascade, phosphorylation of the TCR ITAM's (immunoreceptor tyrosine based activation motifs) by Lck or Fyn, recruits ZAP-70 to the membrane by binding to the phsophorylated ITAMS [171]. Lck in turn phosphorylates and activates ZAP-70 tyrosine kinase [172,173]. Therefore we predicted that TIM-1-dependent signals would also require ZAP-70. Consistent with the NFAT/AP-1 activation profile observed in TCR deficient cells that was induced by TIM-1 (**Figure 40**), there was also a similar trend detected in ZAP-70 deficient Jurkat cells (p116) (**Figure 42A**). Low activation levels were observed with TIM-1expression alone and this was increased when treated with

TCR and CD28 stimulation. The fold increase in TIM-1 transfected cells over vector-transfected cells was also comparable to the observations in TCR-defiicient Jurkat cells (**Figure 40).** When transfected with ZAP-70, p116 cells now exhibited greatly increased activation levels of NFAT/AP-1 induced by TIM-1. Thus optimal enhancement of NFAT/AP-1 dependent transcription by TIM-1 is also ZAP-70 dependent.

The cytosolic adaptor protein SLP-76 is another key proximal TCR signaling component which when bound to the membrane adaptor LAT (SLP-76-LAT complex), forms a molecular scaffold that can interact with several important signaling molecules thereby linking proximal mediators to downstream signaling pathways. We have examined its requirement for TIM-1 function as well, using SLP-76 deficient mutant Jurkat cells (J14). Again, as observed with ZAP-70, TIM-1 shows only partial activation of the reporter in J14 cells and this is raised to a much higher level in SLP-76 reconstituted cells (**Figure 42B**). TIM-1 co-stimulates activation at 3-5 fold higher than vector transfected cells in either cell line.

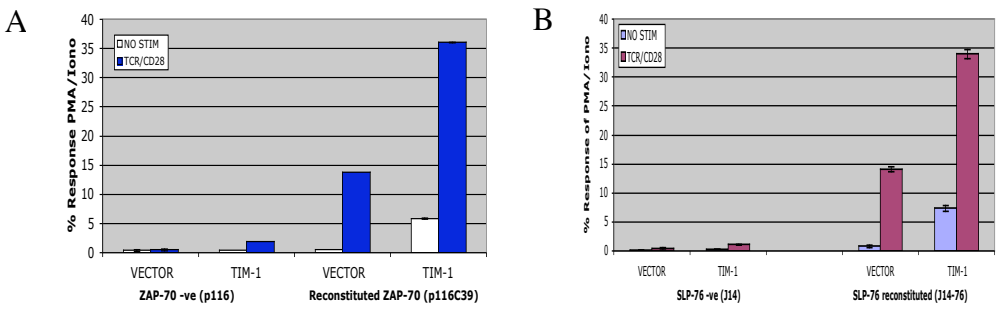


Figure 42: Requirement of ZAP-70 and SLP-76 for TIM-1-mediated NFAT/AP-1 activation.
(A) p116 (ZAP-70 deficient) and ZAP-70 Reconstituted p116 or (B) (A) J14 (SLP-76 deficient) and SLP-76 Reconstituted J14 Jurkat variants were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. Results are representative of the average of triplicates from a single experiment performed three (A) and two (B) times.

Collectively, these studies denote a clear requirement for the TCR proximal signaling components Lck, SLP-76 and ZAP-70 to mediate optimal TIM-1 dependent co-stimulatory signals. Nevertheless, in Jurkat cells there is some residual activation obtained in mutant cells deficient for these molecules, implicating distinct signaling pathways that are triggered by TIM-1. The studies discussed here have all been performed in variants of the Jurkat cell line to maintain consistency in method for a more accurate comparison.

6.3.3. Modulation of NFAT Activation by TIM-1

NFAT dependent transcription is facilitated by increased nuclear residency of NFAT. In T cells the canonical pathway for NFAT activation is through increased intracellular calcium levels which binds calmodulin. Calmodulin in turn activates the calmodulin

dependent phosphatase Calcineurin, which dephosphorylates NFAT leading to its activation and nuclear translocation [102,204]. We have examined different components of this downstream signaling pathway to determine the extent of their modulation by TIM-1. As shown in **Figure 43A**, Jurkat cells transiently expressing TIM-1 show a greater level of calcium mobilization in the absence of any external stimulation. When coupled with TCR stimulation, TIM-1 expressing cells also exhibit a higher level of intracellular cytosolic free calcium as determined by flow cytomtery using a calcium sensitive indicator. This is sustained for a significant period of time before dropping down to vector levels, but before the latter has reached base line. Of interest, while TIM-1 induces a sustained rise in calcium levels, this is a modest increase.

The other critical regulator of calcium signaling is the phosphatase calcineurin, which directly activates NFAT. Since the activity of calcineurin is controlled in part by calcium levels, the preceding data indicated a role for this enzyme in TIM-1 signaling. A luciferase-reporter assay was performed as before, but in the presence of FK506 (tacrolimus)- a pharmacological inhibitor of calcineurin. Jurkat cells transfected with vector alone show no detectable levels of NFAT/AP-1 activation at resting levels, while activation is observed with TCR and CD28 stimulation. In conjunction with FK506, this activation is completely inhibited and reverts to basal levels (**Figure 43B**). As described previously, TIM-1 transfected cells show 5-7-fold increase in activation of the reporter without any external stimulation and ligation of TCR and CD28 enhances TIM-1 co-stimulation. When combined with FK506, reporter activation is decreased significantly across the board. However, in TIM-1 expressing cells, FK506 does not complete inhibit TCR/CD28- induced NFAT/AP-1 activation, but rather lowers it only to a level that is

observed at resting state with TIM-1 alone. Therefore, there is partial activation of NFAT despite the inhibitor. These studies thus suggest that while the calcium-calcineurin pathway is critical for TIM-1 mediated NFAT activation; alternate mechanisms are also employed to sustain NFAT residency in the nucleus.

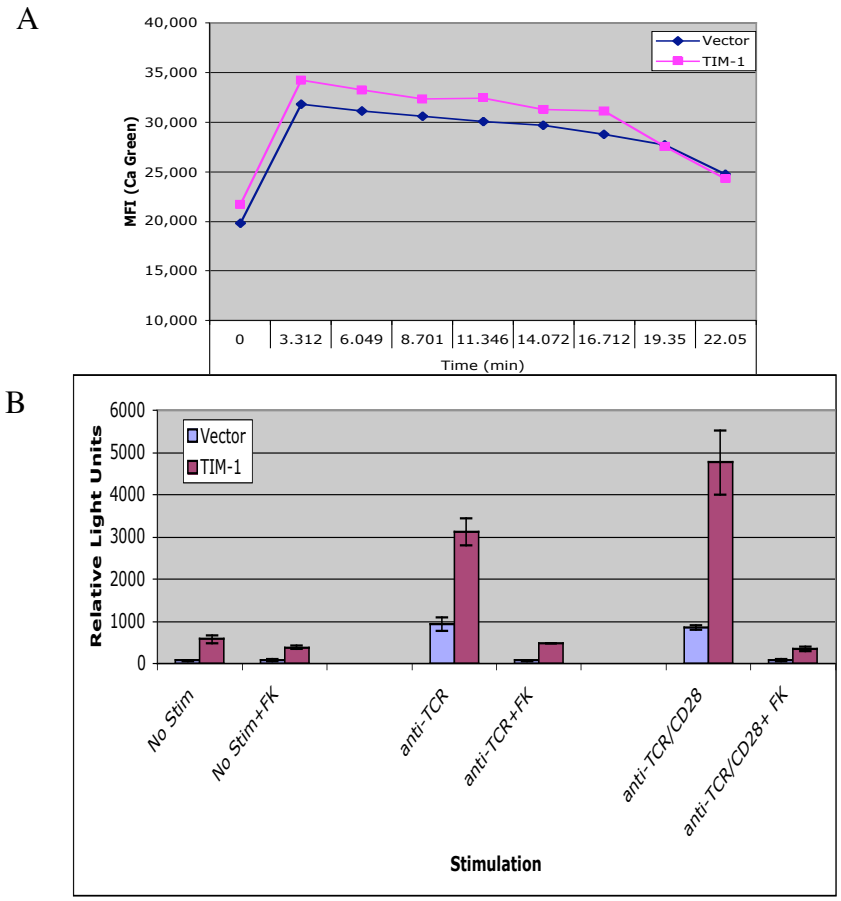


Figure 43: Modulation of NFAT activation by TIM-1 through Calcium mobilization and Calcineurin

A, Jurkat T cells were transfected with empty vector or Flag-TIM-1. Calcium mobilization was assessed the next day, as described in *Materials and Methods*. Results are presented as the mean fluorescence intensity (MFI) of all cells (vector) or Flag-positive cells (TIM-1), and are representative of two experiments. (B) Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, then stimulated the next day as indicated +/- FK506 (FK), and luciferase activity was determined. Results are representative of the average of triplicates from a single experiment performed three times.

The primary mechanism employed by the TCR to induce a rise in intracellular free calcium levels is through PLCγ1. Activated PLCγ1 hydrolyzes PIP2 (phosphatidylinositol-4,5- bisphosphate) to IP_3(inositol-1,4,5-trisphosphate) and DAG (diacylglycerol) [192]. IP_3 initiates opening of intracellular calcium channels, which triggers the opening of CRAC channels in the plasma membrane. In order to determine whether TIM-1 modulated activation states of PLCγ1 we assessed its phosphorylation by flow cytometry using a phospho-specific PLCγ1 antibody. As demonstrated in **Figure 44**, TIM-1-transfected Jurkat cells show higher phosphorylated PLCγ1 in comparison to vector transfected cells when stimulated through the TCR and detected at two minutes post-stimulation. This is consistent with previous findings in Jurkat cells where maximum phosphorylation of PLCγ1 is obtained very early, around 1-2 minutes following TCR stimulation. Thus, TIM-1 positively regulates activation of PLCγ1, which is also a central mediator of TCR signaling.

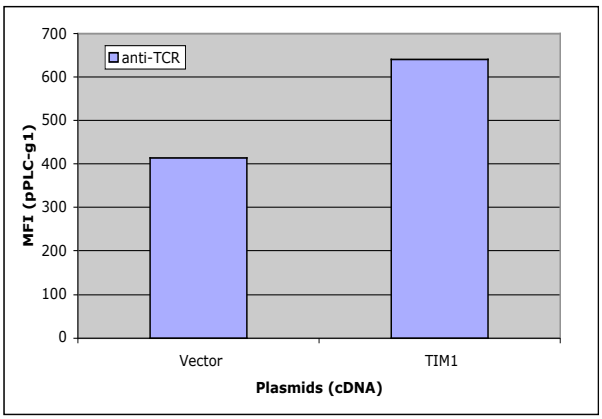


Figure 44: Effect of TIM-1 on PLC-γ1 phosphorylation

Jurkat T cells were transfected with empty vector or Flag-TIM-1, plus pMax-GFP, then stimulated the next day with anti-TCR antibody for 2 minutes. Intracellular staining was performed to analyze PLCγ1 phosphorylation and is represented as the APC channel MFI within the GFP+ gate.

6.3.4. Modulation of AP-1 activation by TIM-1

We have previously shown that TIM-1 can transduce signals to co-stimulate activation of a reporter comprising of the NFAT/AP-1 composite transcription factor elements [40,103]. AP-1, which consists of FOS and JUN transcription factors [205], is the main transcriptional binding partner of NFAT during T cell activation, and this interaction is of crucial importance for T cell activation [103,206]. In the absence of AP-1, NFAT regulates different gene expression patterns [207,208]. We have also shown previously that TIM-1 can independently activate an AP-1 reporter [40]. Induction of the c-FOS element of AP-1 is regulated by ELK-1 transcription factor [209], which in turn is regulated by MAP kinases [210-213]. In order to understand TIM-1's role in potentiating MAP kinase signaling cascades for AP-1 activation, we first assessed its effect on ELK-1. As shown in **Figure 45**, TIM-1 induces an increased trans-activation of ELK-1 at resting levels and can also co-stimulate the reporter under TCR and CD28 stimulation. In contrast, a version of TIM-1 lacking the cytoplasmic tail (TIM-1delta cyto) is significantly impaired in its ability to mediate these signaling outcomes and exhibits a phenotype similar to vector transfected cells. These differences are directly attributed to signaling differences between full-length TIM-1 versus TIM-1-delta-cyto since PMA, which is a pharmacological activator of the MAP kinases and serves as positive control, induces relatively similar levels of activation with each of the different constructs tested. Therefore, TIM-1 can potently induce MAP kinase activation through signaling mechanisms mediated via the cytoplasmic tail.

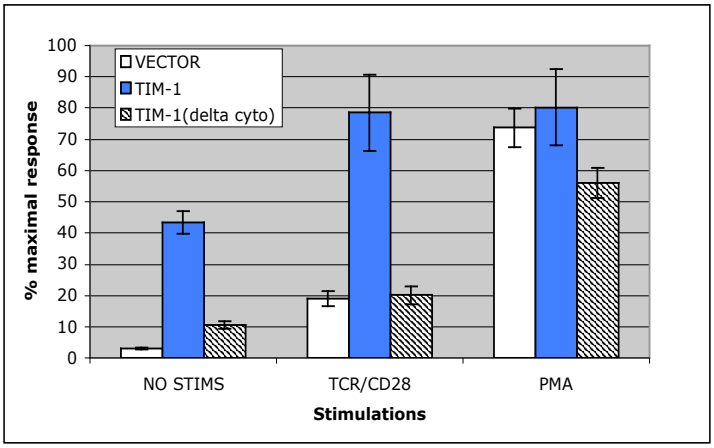


Figure 45: TIM-1-mediated activation of Elk-1.

Jurkat T cells were transfected with an Elk-1 transactivating luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. Results are representative of the average of triplicates from a single experiment performed more than five times.

While ELK-1 is primarily activated by ERK phosphorylation, depending on the stimulus, all three MAP kinases- ERK, JNK and p38 can regulate its activation [210-213]. Furthermore, induction of the c-JUN element of AP-1 is mediated by JNK phosphorylation [205]. Using a phospho-specific antibody, TIM-1-mediated activation of ERK was determined by flow cytometry. Consistent with our finding discussed above that TIM-1 alone induces high levels of ELK-1 activation (**Figure 44**), Jurkat cells expressing TIM-1 also demonstrated elevated levels of ERK phosphorylation in the absence of any external stimuli. A kinetic analysis in the presence of TCR stimulation showed an overall augmentation of phosphorylated ERK induced by TIM-1, with greatest effect within ten minutes following TCR stimulation (**Figure 46A**). A similar analysis of the MAP kinase JNK revealed a much higher level of phosphorylated JNK with TIM-1 alone, in comparison to activated ERK under similar conditions (**Figure 46A and 46B**).

Interestingly, in conjunction with TCR and CD28 stimulation, unlike activated ERK profiles, TIM-1-induced co-stimulation of phosphorylated JNK was optimally observed at 10 minutes following stimulation and this was sustained till at least 30 minutes post-stimulation(**Figure 46B**). TIM-1mediated signaling also resulted in JNK phosphorylation in primary murine CD4+ T cells when ligated with an agonistic anti-TIM-1 antibody (**Figure 46C**) and in a manner that required p85 recruitment (**Figure 46D**).Collectively, TIM-1 can co-stimulate phosphorylation of both ERK and JNK MAP kinases for AP-1 activation, with slightly differing kinetics of activation.

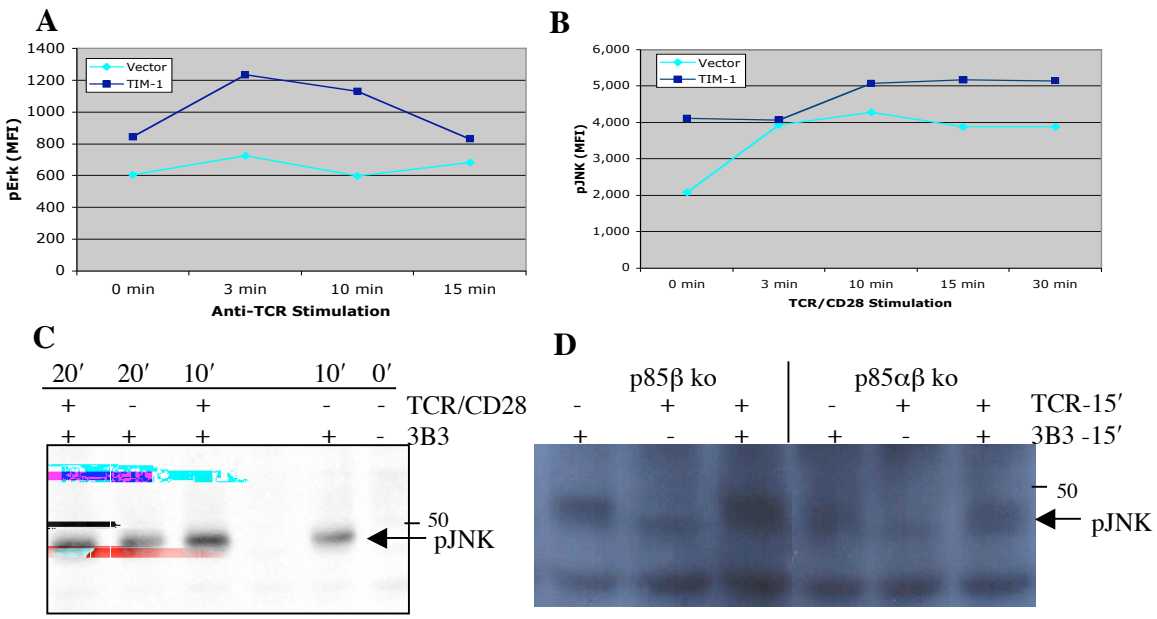


Figure 46: Modulation of MAP kinase phosphorylation by TIM-1

Jurkat T cells were transfected with empty vector or Flag-TIM-1, plus pMax-GFP, then stimulated the next day with anti-TCR/CD28 antibodies. Intracellular staining was performed to analyze Erk (A) and Jnk (B) phosphorylation and is represented as the PE channel MFI within the GFP+ gate. CD4+ T cells from C57BL/6 (C) mice or p85β KO or p85αβ KO (D) were stimulated as indicated and lysed. Blots were probed for p-JNK.

Small GTPases serve as the immediate activator for triggering MAP kinase signaling cascades ultimately leading to activation of ERK and JNK proteins. Of these, Ras and Rac have been implicated in signaling events mediating NFAT and AP-1 dependent transcription. In T cells, activated Ras arises mainly as a result of TCR signaling via PLCγ1 hydrolysis of PIP2, which in turn initiates DAG/PKC-theta/ RASGRP/ RAS pathway[214] . Another signaling mechanism for its activation is through activated LAT, which binds the adaptor Grb2 [215,216]. Grb2 activates SOS which subsequently activates RAS [217]. Thus, we have determined through a reporter assay whether inhibiting RAS action by means of a dominant negative version of RAS (RASN17) can abolish co-stimulation of NFAT/AP-1 mediated by TIM-1. As shown in **Figure 47**, co-transfecting RasN17 along with TIM-1 in Jurkat cells partially inhibits TIM-1-induced basal activation, and in conjunction with TCR stimulation or TCR and CD28 stimulation, reporter enhancement is also impaired. However, this impairment is achieved only to a level comparable to similarly stimulated vector- transfected cells in the absence of RASN17 and is not inhibited to the lower level observed when vector is combined with RASN17. The inhibition of reporter activation suggests that Ras is indeed an important mediator for TIM-1 co-stimulation and one conserved effector system in T cells is through Raf-Mek-Erk activation [218]. Nevertheless, these findings reveal that Ras GTPase is not sufficient to bring about optimal TIM-1 co-stimulation suggesting that other small GTPases such as RAC-1 may also be involved.

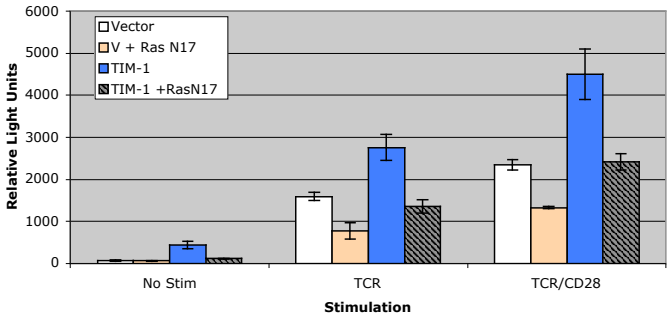


Figure 47: Effect of RasN17 on TIM-1 mediated activation of NFAT/AP-1.

Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. Results are the average of duplicates from a single experiment, representative of three experiments.

Several lines of evidence have shown that AP-1 induction is strongly sensitive to the action of the serine/ threonine kinase PKC-θ upon activation [219-221]. One signaling mechanism through which PKC-θ induces AP-1 is via activation of the guanine nucleotide exchange factor- RASGRP that in turn activates RAS [222] . PKC-θ has also been connected to modulation of NFAT activation by increasing intracellular free calcium, in a manner that involves PKC-θ-dependent PLCγ1 activation [116,117]. To evaluate TIM-1's requirement of PKC-θ for MAP kinase induction, two mutant versions of PKC-θ including a partially activated form (PKC-θ A/E) or a dominant negative form (PKC-θ dom. negative) were incorporated in the reporter assay along with an ELK-1 reporter. Use of PKC-θ dom. negative, decreased ELK-1 activation in vector transfected cells by half whether stimulated through the TCR or through TCR and CD28 (**Figure 48A**). However, PKC-θ dom. negative version had only a marginal effect on ELK-1 inhibition in TIM-1 transfected cells whether left alone or treated with different stimuli

(**Figure 48A**). Therefore, while PKC-θ is required for ELK-1 activation as indicated in vector-transfected cells, it does not however contribute tremendously to TIM-1-mediated co-stimulatory signaling.

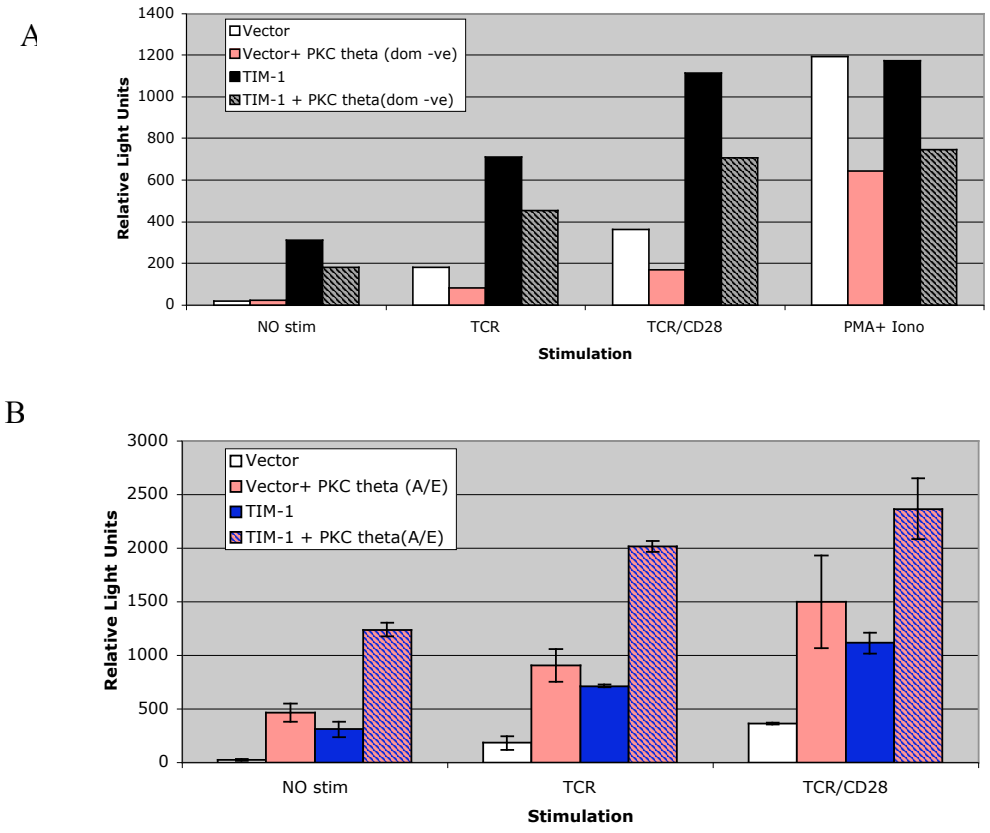


Figure 48: Modulation of TIM-1 mediated activation of ELK-1 with PKCθ mutant constructs

Jurkat T cells were transfected with an ELK-1 luciferase reporter and the indicated constructs, then stimulated the next day, as indicated, followed by determination of luciferase activity. Results are representative of the average of duplicates from a single experiment done three times.

Co-transfection of the partially activated form -PKC- θ (A/E)- led to a significant induction of ELK-1 in vector-transfected cells itself, in the absence of any external stimuli (**Figure 47B**). In combination with TCR or TCR and CD28 stimuli, this effect is

enhanced even more. Interestingly, co-transfection of PKC-θ (A/E) with TIM-1 instead, exerted a profound increase on ELK-1 induction that surpassed the potentiation effected by vector transfection with PKC-θ (A/E), under all conditions examined (**Figure 48B)**. Therefore, TIM-1signaling can synergize with signals originating from PKC-θ activity. Yet, the findings with PKC-θ dom. negative, suggest that PKC-θ is not absolutely required to mediate TIM-1 dependent co-stimulation (**Figure 48A**). Therefore, Figure 6B implies that TIM-1 might amplify signals that are triggered downstream of PKC-θ activity.

6.4. DISCUSSION

The findings presented in this study provide evidence that TIM-1 mediates optimal activation of NFAT/AP-1-dependent transcription in a manner that requires a functional TCR as well as proximal components of TCR signaling such as the tyrosine kinases Lck, ZAP-70 and the adaptor protein SLP-76. These signaling mediators are essential for optimal induction of NFAT/AP-1 whether induced by TIM-1 alone or when TIM-1 transfected cells are coupled with stimulation through TCR or TCR and CD28. Nevertheless, with the exception of src family tyrosine kinases such as Lck, mutant cell lines deficient for each of the other components do not completely abolish TIM-1 stimulation and co-stimulation of NFAT/AP-1 in at least variants of parental Jurkat used in these studies. A partial effect is still retained. Therefore TIM-1 can initiate alternate signaling pathways that are probably further amplified by TCR dependent signaling. One

such mechanism is likely to be through a coupling of TIM-1 to distinct phospho-tyrosine dependent signaling pathways.

We show in chapter five that TIM-1-mediated T cell activation requires recruitment of p85 and PI3K activity in a manner that is dependent on a conserved TIM-1 tyrosine in its tail. This is consistent with our earlier findings in chapter three which underscore the importance of the conserved tyrosine 276 in TIM-1 tail for its function. Given that Lck is required for TIM-1 tyrosine phosphorylation (chapter five) and that there is complete abrogation of NFAT/AP-1 induction in Lck- deficient cells observed in this study, this further supports the notion that TIM-1 is connected to phosphotyrosine dependent signaling pathways which synergize with signaling components induced by the TCR. One explanation for the residual activation in mutant Jurkat cells that is consistent with a requirement for p85, is the existence of a mutant form of the lipid phosphatase PTEN in Jurkat cells rendering it non-functional [223]. PTEN serves as a natural inhibitor of PI3 kinase by de-phosphorylating its catalytic products. Consequently, Jurkat cells have high resting levels of PI3K catalytic products, namely the PIP2 and PIP3 lipid second messengers that can initiate effector pathways. Aberrant functioning of this pathway is therefore more evident in cells lacking a functional TCR hinted by residual NFAT/AP-1 activation mediated by TIM-1. Nevertheless, this also reveals an important role for a p85-PI3 kinase mechanism in TIM-1 signaling.

Consistent with TIM-1's requirement for proximal TCR signaling molecules, this study also provides evidence that TIM-1 positively modulates the canonical pathway employed by the TCR for NFAT induction. Thus we show an elevated PLCγ-1 activation followed by increased calcium influx leading to activation of calcineurin, which in turn

allows nuclear translocation of NFAT. Nevertheless, these modulations are modest. While TIM-1 can sustain moderately elevated levels of free calcium over vector transfected cells, they are terminated even before vector transfected cells have reached basal levels of calcium flux. Furthermore, partial NFAT activation is retained in the presence of a calcineurin inhibitor -FK506. Therefore, TIM-1 might adopt other mechanisms as well, to sustain the residency of NFAT in the nucleus. We show in chapter five that TIM-1 induces Akt activation and Akt is required to mediate NFAT/AP-1 activation by TIM-1. One mechanism for NFAT induction involving activated Akt is through phosphorylation of Akt and subsequent inhibition of the kinase GSK-3. GSK-3 which normally functions to expedite NFAT nuclear export by phosphorylating NFAT, is thereby inhibited [224,225].

Consistent with classical TCR signaling mechanisms, the findings presented here denote a requirement of TIM-1 for the small GTPase RAS to activate downstream signaling pathways to AP-1, through activation of Erk and Jnk. However, our observations with dominant negative RASN17 demonstrate that RAS is not sufficient for optimal TIM-1 signaling and sustained AP-1 activation may also require the other GTPase Rac-1. Several studies have implicated Rac-1for optimal activation of the JNK pathway [226-229]. Indeed, we show significantly elevated levels of JNK phosphorylation over levels observed with Erk, induced by TIM-1 alone. In primary cells deficient for p85, we demonstrated that TIM-1 mediated JNK phosphorylation requires p85.

The immediate activator for Rac-1 is the GEF Vav-1[230]. Significantly, both these signaling mediators are intimately connected with PI3K, a point that is of strong

relevance to our study given TIM-1's interaction with PI3K (Chapter five). Accumulating evidences have positioned Vav-1 both as an upstream regulator of PI3K as well as functioning downstream of PI3K through its PH domain interaction with PI3K catalytic products [231-235] .Thus Vav and PI3K activity seem to be co-coordinately regulated. Either way, for the most part Vav-1's control of gene transcription is dependent on Rac-1[197,226]. Indeed, Rac-1 can itself bind to the p85 regulatory sub-unit of PI3K, and enhance PI3K catalytic activity, as well as NFAT/AP-1 activation [131,236]. Rac-1 has also been linked to NFAT activation in mast cells by regulating NFAT dephosphorylation and nuclear import [237]. Additionally, several studies have provided evidence of a role for Vav-1 in the regulation of calcium mobilization [194,196,234,238-242].

Finally, we demonstrate that the serine/threonine kinase PKC-θ is not essential for TIM-1-mediated signaling, but TIM-1 can nonetheless synergize with signals originating from PKC-θ to enhance ELK-1 induction. Our findings suggest that these signals, which are amplified by TIM-1, lie downstream of PKC-θ. Of the different PKC isoforms, PKC-θ selectively contributes to strong ERK and JNK activation [220,222,243]. Furthermore, PKC-θ can co-operate with calcineurin and together their signals converge on (or upstream of) the GTPase Rac leading to potent JNK activation [243]. Recent studies have also suggested SPAK to lie downstream of PKC-θ in the regulation of AP-1 activation [244].

In conclusion, the study presented here is consistent with a model where TIM-1 associates with the TCR to amplify classical TCR dependent signaling cascades. As these studies were being performed, emerging evidence from another group showed that TIM-1 does indeed interact with the TCR-CD3 complex [119]. Therefore, it appears that TIM-1

might function as a co-receptor for the TCR, and this is dependent in part on the time in immune development when TIM-1 is expressed on T cell surface. While it augments TCR signaling cascades, it can also fine-tune signaling outcomes by initiating and sustaining distinct signaling mechanisms of its own that are phospho-tyrosine dependent. It will be interesting to observe how these molecular mechanisms vary in an *in vivo* setting and with different polymorphic variants of TIM-1 that are correlated with different immune effector responses.

7. CHAPTER 7: SUMMARY OF THE THESIS

The TIM proteins belong to a novel family of molecules contained within a single gene locus positioned on mouse chromosome 11B1.1 and human chromosome 5q33.2 [5]. This locus is devoid of intervening genes and is separate from nearby cytokine gene clusters. Early evidence from genetic, epidemiologic and immune functional studies implicated these members individually as being important regulators of Th1 or Th2 type immunity. Specifically, they were shown to regulate asthma and autoimmune diseases [5,6,8,22,245]. However, there was virtually nothing known about the biochemical basis for these varied functional outcomes.

At the time these studies commenced, the existing information on putative signaling motifs contained within TIM protein sequences, coupled with initial evidence of TIM-2 tyrosine phosphorylation [31] suggested that these molecules could serve as signal transducing cell surface receptors. This led us to propose that TIM-1 and TIM-2 could transduce intracellular biochemical signals in response to ligation of the receptor, by coupling to phosphotyrosine dependent signaling mechanisms in order to regulate T cell activation. This thesis study was thus designed to elucidate and characterize the molecular mechanisms that underlie the immune function of TIM-1 and TIM-2.

In order to address the signaling capabilities of TIM-1, a series of reporter experiments were performed in Jurkat and D10 T cell lines with different cytoplasmic tail

mutants of TIM-1, along with wild type TIM-1cDNA and a range of transcriptional reporters (Chapter three). These reporters contained either isolated DNA elements for different transcription factors contained in the IL-2 promoter or various cytokine promoters. Our findings indicated that expression of TIM-1 provides a co-stimulatory signal that augments the activation of the IL-4 promoter in the D10 Th2 T cell clone and the IFN-γ promoter in at least the Jurkat cell line, an effect that may occur through increased activation of composite NFAT/AP-1 transcription factors. Importantly, TIM-1-mediated signals were transduced in a manner that required the cytoplasmic tail of TIM-1 as well as conserved tyrosine 276 contained in the tail. Consistent with a role for TIM-1 in Th2 immunity, our studies also showed that ectopic expression of TIM-1, in primary murine T cells by retroviral transduction, increased the frequency of IL-4 but not IFN-γ producing T cells, when stimulated under non-polarizing conditions. For reasons that are still unclear, TIM-1 signaling effects observed in T cell occurred in the absence of an external ligand, and active cross-linking of TIM-1 had no additional positive or negative effect. Using a TIM-1-Ig chimera we provided evidence for a ligand on Jurkat cells, but blocking this putative interaction with TIM-1-Ig had a negligible effect on reporter activity. Recent evidence has indicated that TIM-1 can undergo homophilic interaction on neighboring cells at the intercellular junction. Thus, perhaps over-expressing TIM-1serves to naturally cross-link it by enhancing homophilic interaction, and thereby triggering TIM-1 signaling.

Murine TIM-2 shares the greatest homology with TIM-1, although they differ in the number of tyrosines present in the cytoplasmic tail. Surprisingly, a similar series of experiments performed with TIM-2 to evaluate the nature of signals mediated by it,

revealed that it can inhibit T cell activation, in contrast to TIM-1 (Chapter four). Expression of TIM-2 in human Jurkat and murine D10 cell lines significantly inhibited NFAT/AP-1 induction either by ligation of TCR or with PMA /Ionomycin, and in a manner that required the cytoplamsic tail. This block does not appear to be due to a general effect on cell viability, and the block can be bypassed by expression of activated alleles of Ras or calcineurin, or MEK kinase in the case of AP-1. Our findings suggested that TIM-2 exerts inhibition either at or just downstream of PLC-γ1. Interestingly, TIM-2 was also able to inhibit TIM-1 co-stimulation and this supports the notion that TIM-1 triggers more proximal signals. The inhibitory function of TIM-2 is consistent with another study that reported exacerbated and dysregulated Th2 responses in TIM-2 gene deficient mice [52]. Reminiscent of our findings with TIM-1, the effects observed with TIM-2 in our study occurred apparently in the absence of a ligand and active cross-linking of TIM-2 had no detectable effect. However, emerging X-ray crystal structure analysis of TIM-2 has revealed that neighboring TIM-2 molecules can homo-dimerize on the same cell surface, which could in turn contribute to ligand-independent function of TIM-2 upon transient over-expression, similar to that observed with CD45 [109].

Our finding in chapter three that the conserved tyrosine 276 of TIM-1 tail is important for TIM-1 signaling led us to examine in more detail the regulation of TIM-1 tyrosine phosphorylation and it's potential coupling to phosphoytrosine dependent signaling mechanisms for T cell activation (Chapter five). Our studies revealed a requirement for the src family kinase Lck for TIM-1 tyrosine phosphorylation, and subsequent NFAT/AP-1-dependent transcription. One mechanism by which phosphotyrosine-dependent signaling complexes are formed is through phosphotyrosine-

SH2 domain interactions. Thus, we showed that TIM-1 associates with endogenous p85 α/β, which is the regulatory sub-unit of PI3 kinase. Recruitment of p85 is mediated in a phospho-tyrosine dependent manner. Furthermore, the catalytic activity of PI3 kinase is also essential for TIM-1 co-stimulation, mediated in part through the serine/ threonine kinase Akt. Finally, using a genetic approach of p85 α/β-deficient T cells we show that p85-PI3K is necessary for TIM-1-induced up-regulation of surface activation markers CD69 and CD25 associated with T cell activation. While TIM-1 co-stimulatory effects were observed over a range of anti-TCR concentrations, the greatest effect was observed with low levels of TCR stimulation, suggesting that TIM-1 provides a complementary signal that is in part quantitative in nature and can thereby reduce the TCR triggering threshold. This is also consistent with the nature of other co-stimulatory molecules such as CD28 [168,169]. Conversely, therefore, an uncommonly strong signal from the TCR alone would compensate in part or totally for certain cellular responses and to a large extent would override the need for co-stimulatory signals. This is indeed the case for even the hallmark co-stimulatory molecule CD28 [168,246,247].

Finally, in chapter six we address how TIM-1 co-stimulatory signals intersect with the TCR signaling machinery for induction of NFAT/AP-1 dependent transcription. Where do these pathways merge and how do they synergize? To begin answering these questions, a series of reporter assays were performed either with mutant Jurkat cells deficient for proximal TCR signaling components or by using dominant negative constructs of downstream signaling mediators of the TCR signaling cascade. These studies revealed a profound requirement by TIM-1 for the TCR and for most of the key signaling components of the classical TCR pathway to mediate optimal co-stimulation of

NFAT/AP-1 induction. These included proximal components such as Lck tyrosine kinase, ZAP-70 tyrosine kinase, the adaptor protein SLP-76 and downstream components to NFAT induction via PLC-γ1/calcium /calcineurin, and to induction of AP-1 through Ras and Erk/JNK MAP kinase activation. Nevertheless, a disruption of the TCR-mediated chain of signaling events, through a loss of function in any of these components, did not completely abrogate TIM-1 signaling, with the exception of Lck. This suggests that TIM-1 also employs alternative mechanisms to amplify and sustain TCR signals. One mechanism that is Lck-dependent is the recruitment and activation of p85-PI3K (Chapter five) upon TIM-1 tyrosine phosphorylation. As a result of this, TIM-1 induces Akt activation and functions through at least Akt to mediate co-stimulatory activation. Among other effector functions, Akt can indirectly sustain NFAT nuclear residency by inhibiting GSK-3, which normally functions to expedite NFAT nuclear exit [224,225].Collectively, it appears that TIM-1 activates signaling mediators already mobilized by TCR signaling. One difference lies in the method of recruitment and activation of some of them in part through p85-PI3K.

Our findings indicate that TIM-1 can activate both NFAT and AP-1 transcription factors. Co-operative interaction of NFAT with AP-1 thus integrates two of the main signaling pathways required for T cell activation, resulting in the transcription of a specific set of genes characteristic of an activated T cell [103,206,248]. Indeed, activation of NFAT in the absence of the RAS-MAP kinase signaling pathway leading to AP-1 induction, results in a different set transcribed genes that accompany T cell anergy [207,208] .

This study thus establishes these molecules as cell-surface signal transducing receptors and provides an initial understanding of the molecular mechanisms mediated by TIM-1 and TIM-2. Our findings are consistent with a model where TIM-1 associates with the TCR to amplify classical TCR dependent signaling cascades. As these studies were being performed, emerging evidence from another group showed that TIM-1 does indeed interact with the TCR [119]. Therefore, it appears that TIM-1 may function as a co-receptor for the TCR, and this is dependent in part on the time in immune development when TIM-1 is expressed on T cell surface as well as the extent of it's inhibition by TIM-2. While TIM-1 augments TCR signaling cascades, it can also fine-tune signaling outcomes by initiating and sustaining distinct signaling mechanisms of its own that are phospho-tyrosine dependent. Thus, we have delineated a TIM-1-induced signaling pathway, starting with Lck-dependent phosphorylation of TIM-1, to recruitment and activation of PI3K and Akt, which is required for induction of NFAT/AP-1-dependent transcription and up-regulation of surface activation markers associated with T cell activation. Future studies call for a more detailed understanding of how p85-PI3K mediates TIM-1 function and how TIM-1 induces basal activation of the T-cell in the absence of external stimuli (discussed in chapter five).

It will be interesting to observe how these molecular mechanisms vary in an *in vivo* setting and with different polymorphic variants of TIM-1 that are correlated with different immune effector responses associated with Th2-type immunity. Furthermore, X-ray crystal structure analysis of TIM-1 and TIM-2 predict different ligand binding modes [10,33]. Like other members of the TIM family, TIM-1and TIM-2 can bind to more than one ligand and with different functional outcomes [7,19,21,25]. It is also possible that

more than one ligand can bind concurrently on different faces of the molecules. In addition, TIM-1 and TIM-2 can undergo homophilic and homo-dimeric interactions, respectively [10,33]. Therefore, the ultimate outcome of their engagement may depend upon the nature of the ligand(s) with which they interacts, as well as the timing and stoichiometry of such interactions. Differences in functional outcomes after ligation may result from divergent effects of TIM ligation on T cell signaling pathways under such circumstances. Eventually, an understanding of the mechanistic function of a family of molecules implicated in Th1 and Th2 type immunity, in turn will present targets for therapeutic manipulation of immune responses in health and immune-mediated disease.

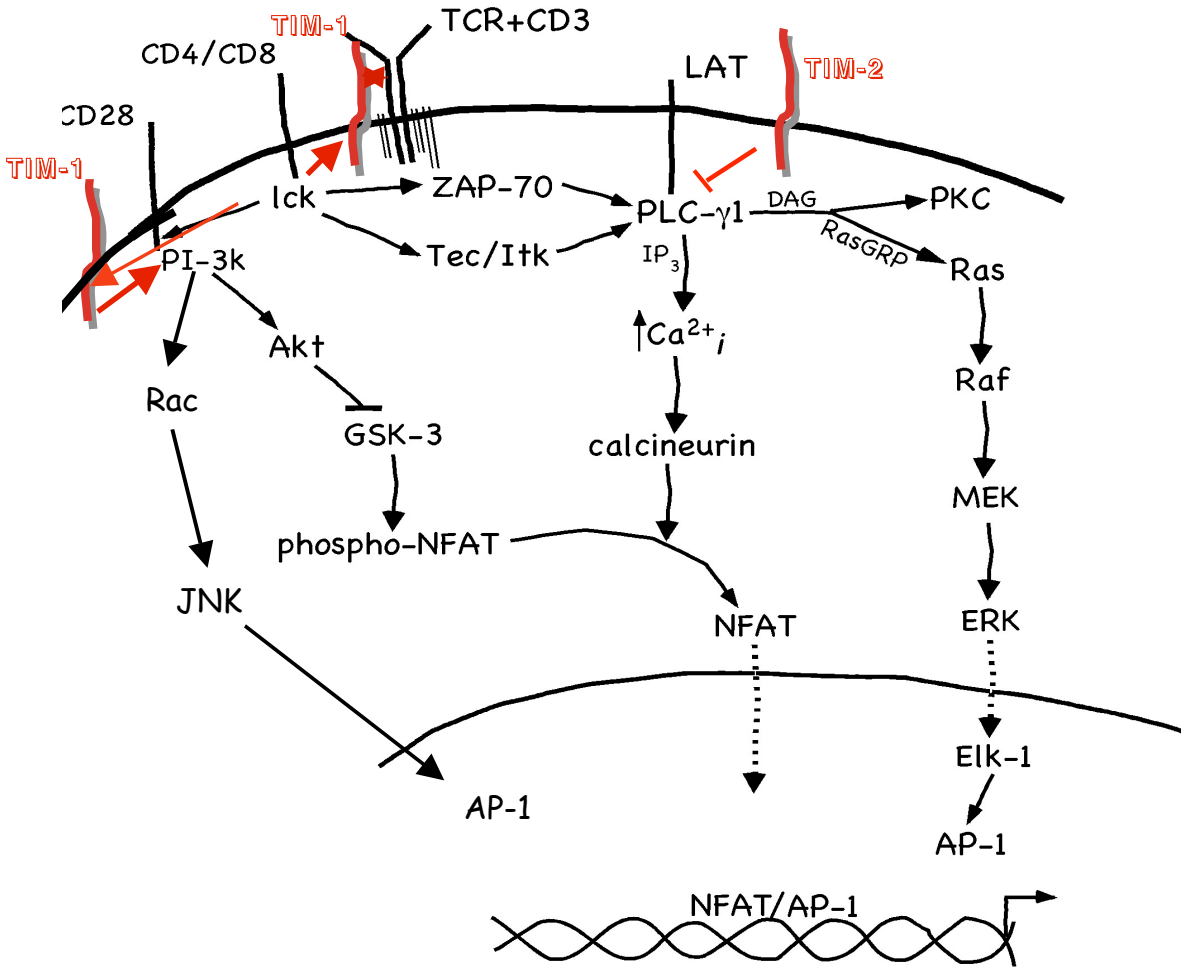


Figure 49: Model for TIM-1 mediated co-stimulatory signaling.

TIM-1 associates with the TCR (although not by a co-valent interaction) and amplifies TCR signaling cascades for NFAT/AP-1 dependent transcription. This occurs in a manner that requires contribution of most of the key signaling components elicited by the TCR as indicated in the figure. TIM-1 can also trigger distinct pathways that involve Lck-dependent tyrosine phosphorylation followed by recruitment of p85-PI3K and activation of PI3K activity for increased expression of surface markers associated with T cell activation. In this context TIM-1 requires Akt to enhance NFAT/AP-1 dependent transcription. TIM-2 on the other hand, exerts a block at or just below PLC-γ1 for NFAT/AP-1 inhibition.

APPENDIX A

PUBLICATIONS

1. **de Souza AJ,** Oriss TB, O'malley KJ, Ray A, Kane LP**.** T cell Ig and mucin 1 (TIM-1) is expressed on in vivo-activated T cells and provides a costimulatory signal for T cell activation. *Proc Natl Acad Sci U S A*. 2005 Nov 22; 102(47): 17113-8.

2. Knickelbein JE*, **de Souza AJ***, Tosti R, Narayan P, Kane LP. *Cutting edge*: Inhibition of T cell activation by TIM-2. *Journal of Immunology*. 2006 Oct 15; 177(8): 4966-70.
   *** co-first author**

3. **de Souza AJ,** Kane LP. Immune Regulation by the TIM gene family. *Immunological Research.* 2006; 36 (1-3): 147 –56

Manuscript submitted for publication

4. **de Souza AJ**, Oak J, Jordanhazy R, Fruman D, Kane LP. TIM-1-Mediated T Cell Activation Requires Recruitment and Activation of PI3 Kinase.

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