Mechanisms of Tim-3 Signal Transduction in the Modulation of Downstream TCR Signaling

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Mechanisms of Tim-3 Signal Transduction in the Modulation of Downstream TCR

Signaling

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T-cell immunoglobulin and mucin 3 protein (Tim-3) is a type-I transmembrane protein known to negatively regulate Th1 and Th17 CD4 T cells, and CD8 T cell mediated immune responses. Tim-3 expression level correlates with the severity of T cell exhaustion under conditions of chronic viral infection and tumor burden and Tim-3 antibodies can reverse this dysfunctional phenotype. Exploring the signal transduction mechanism of Tim-3, I demonstrated that ectopic expression of Tim-3 enhanced NFAT/AP-1, NFAT and NF-κB reporter activity induced by TCR stimulation, and enhanced AP-1 reporter activity independent of TCR stimulation. Two tyrosines in the cytosolic tail of Tim-3 are responsible for the potentiation of signal transduction by TCR in a redundant manner. I demonstrated that the Src family kinase (SFK) Fyn can phosphorylate Y256 and Y263 residues of Tim-3. An SH2 domain screen and co-immunoprecipitation identified p85 PI3K interaction with Tim-3 in a tyrosine phosphorylation-dependent manner. I also found that Tim-3 expression upregulates phosphorylation of PLC-γ1 and MAP kinases ERK1/2, p38, and JNK. Finally, I demonstrated that Tim-3 upregulates phosphorylation of ribosomal protein S6, a downstream target of PI3K and ERK, and IL-2 secretion induced by TCR stimulation. I conclude that potentiation of NFAT/AP1 and NF-κB activity by Tim-3 is mediated by enhancement of the PI3K, PLC-γ1 and ERK pathways. Thus, we have demonstrated a paradoxical activating function of Tim-3 in TCR signaling, while Tim-3 is known as a negative regulator of Th1 and Tc1 T cell mediated immune responses. This augmentation of TCR

signaling by Tim-3 may contribute to driving and/or maintaining T cell exhaustion. Alternatively, Tim-3 may have a dual activation and inhibition role depending on the ligands available at a particular stage of T cell activation.

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1.0   INTRODUCTION

1.1 TIM FAMILY

There are 8 TIM family genes identified in mouse, which are clustered in chromosome 11, and there are 3 TIM family genes in human, in 5q33. Murine Tim-1, Tim-2, Tim-3 and Tim-4 encode functional proteins, but Tims-5 to 8 are predicted genes. Sequences of murine Tim-1 and 2 are homologous to human TIM-1, and sequences of murine Tim-3 and 4 are homologous to their corresponding human TIM-3 and 4. All TIM family genes encode type I transmembrane glycoproteins which consist of an IgV domain, a mucin domain, transmembrane domain and a cytosolic tail. The cytosolic tails of murine Tim-1, 2 and Tim-3 have putative tyrosine phosphorylation sites, possibly mediating signal transduction, but the cytosolic tail of Tim-4 does not [1, 2]. Since the first gene of Tim family has been identified as a hepatitis A virus cellular receptor [3], regulatory roles of Tim family in immune conditions such as autoimmune disease, allergy, and infection and cancer have been reported.

1.1.1 Tim-1

Tim-1 was discovered and cloned as a hepatitis A virus cellular receptor (HAVCR) in African green monkey kidney cells and in human organs including liver and kidney [3, 4]. Another group identified Tim-1 as kidney injury molecule 1 (KIM-1), which was upregulated after ischemic

injury in damaged and regenerating proximal tubule epithelial cells [5]. Soluble KIM-1/Tim-1 is used as a urinary biomarker for acute tubule necrosis. Since it was shown that Tim-1 blockade (with mAb RMT1-10) was protective against renal ischemia-reperfusion injury, decreasing infiltration of immune cells and production of cytokines, Tim-1 was suggested as a therapeutic target, not only a biomarker, for renal injury [6].

An immunoregulatory role of Tim-1 was first suggested by a study showing the genetic relationship between Tim-1 and airway hyperreactivity. In an attempt to identify the locus responsible for the reduced airway hyperreactivity phenotype in HBA (DBA/2) mice, positional cloning identified a locus named *Tapr* (T cell and airway phenotype regulator) on chromosome 11. The locus was associated with Th2 cytokine production, which was genetically separable from IL-4 cytokine gene cluster and other nearby cytokine genes. Within the locus, Tim family genes were found. In addition, major polymorphisms in Tim-1 and Tim-3 were found [7].

Tim-4 and phosphatidylserine (PS) were identified as natural ligands of Tim-1. Tim-4, which is expressed in APC but not in T cells, interacts with both the IgV and mucin domains of Tim-1, and the Tim-1/Tim-4-Ig interaction induces hyperproliferation of T cells [8]. Both Tim-1 and Tim-4 interact with PS, which is mediated by the metal ion dependent ligand binding site (MILIBS), and Tim-1 and Tim-4 play roles in phagocytosis of apoptotic cells and exosomes [9-11]. Tim-1 also can form a homodimer with another Tim-1 molecule expressed on a neighboring cell surface (*in trans*).

In T cells, Tim-1 is predominantly expressed in Th2 cells as compared to Th1 cells [12], and functions as co-stimulator for T cell activation induced by TCR stimulation. Transient overexpression of Tim-1, or its crosslinking with agonistic antibody or with recombinant Tim-4, upregulate T cell proliferation and Th2 cytokine production [8, 13]. Tim-1 is constitutively

expressed in bone marrow-derived mast cell (BMMC) and anti-Tim-1 antibodies (3B3, RMT1-4, and RTM1-17) and recombinant Tim-4 enhance Th2 cytokine (IL-4, 6, and 13) production in IgE-sensitized BMMC [14]. B cells express both Tim-1 and Tim-1L, Tim-1 expression is upregulated by stimulation with IgM and anti-CD40, but not LPS, while Tim-1L expression in B cells is constitutive, according to the staining with anti-Tim-1 antibody and Tim-1-Ig binding. Anti-Tim-1 antibody (RMT1-17) treatment of B cells stimulated with IgM and anti-CD40 enhances proliferation and differentiation to plasma cells, and as a consequence, antibody production is increased [15].

Xiao et al. generated Tim-1$^{\Delta mucin}$ mice and demonstrated the role of Tim-1 in development of regulatory B cells. The number of FoxP3^{+} regulatory T cells and IL-10 production in CD4 T were normal in the mutant mice. In wild type mice, the frequency of IL-10 producing B cells increased as the mice aged (< 6 months vs. >10 months). Moreover in a comparison of young WT mice and Tim-1$^{\Delta mucin}$ mice, the frequency of IL-10 producing B cells was lower in Tim-1$^{\Delta mucin}$ mice. In addition, in Tim-1$^{\Delta mucin}$ mice, the number of IL-10-producing B cells minimally increased as they aged over 10 months [16]. These data suggest that Tim-1 functions as a stimulatory molecule in B cells, but is also required for development of regulatory B cells.

de Souza et al. revealed that Tim-1 functions as a co-stimulatory molecule and began to define its signaling mechanism in T cells. Ectopic expression of Tim-1 upregulated NFAT/AP-1 reporter activation induced by TCR/CD28, but not NF-κB reporter stimulation, in which Y256 was responsible for this Tim-1 function[13]. According to an SH2 domain array, the phosphotyrosine residue in Tim-1 cytosolic tail interacts with p85, Fyn, and tentatively other molecules with SH2 domains [17].

1.1.2 Tim-2

Tim-2 is a member of Tim family protein which is also homologous to human TIM-1. Similar to Tim-1, Tim-2 is expressed preferentially on Th2 T cells, but Tim-2 expression was not detectable on B cells, macrophages, and dendritic cells [18]. Tim-2 functions as an inhibitory molecule for Th2 immune responses. *In vivo* administration of Tim-2-Ig, to block the Tim-2/Tim-2L interaction, led to increased production of Th2 cytokines IL-4 and IL-10. In a Th1 type EAE mouse model, Tim-2 blockade delayed the onset of disease and ameliorated its severity, suggesting that Tim-2 negatively regulates Th2 cells and shifts the balance between Th1 and Th2 immune responses [18]. Consistently, Tim-2 deficient mice displayed exacerbation of lung inflammation in a Th2 type airway atopic response model [19].

On the contrary, Tim-2 ligand Sema4A, which is expressed by dendritic cells and B cells, upregulated CD4 T cell proliferation and activation. Sema4A-Fc treatment enhanced production of IFN-γ and IL-4 under Th1 and Th2 polarization conditions, respectively, *in vitro*. In an EAE model, blockade of Sema4A enhanced CD4 T cell proliferation and production of IFN-γ and IL-4, and suppressed the development of EAE [20]. It is possible that Tim-2 and Sema4A might have other partners and the effect of blockade of Sema4A is not equivalent to blockade of Tim-2.

The investigation on Tim-2 signal transduction revealed that ectopic expression of Tim-2 in Jurkat T cells suppresses calcium mobilization and ERK activation, leading to inhibition of activation of NFAT/AP-1 transcription factor [21]. Tim-2 in can homo-dimerize via its IgV domain (*in cis*) and the Tim-2 dimerization prevents the in trans-homophilic interaction of Tim-1 [11]. Therefore, Tim-2 may inhibit the co-stimulatory function of Th1 in an extrinsic manner as well. It has been found that Tim-2 in oligodendrocytes recognizes H-ferritin and facilitates iron

transfer, but whether the Tim-2/H-ferritin axis has a role in the immune system has not been determined yet.

1.1.3 Tim-4

Tim-4 is expressed on APCs, including CD11c$^+$ dendritic cells, stromal cells, CD19$^-$CD11b$^+$ macrophages, CD19$^+$CD11b$^+$B cells, but not in in CD19$^+$CD11b$^-$ conventional B cells [8, 22-24]. To date, two known major functions of Tim-4 are to activate Th2 cells as a Tim-1 ligand and to mediate phagocytosis of apoptotic cells and exosomes as a PS receptor. Tim-1/Tim-4-Ig ligation regulates T cell activation and Th2 mediated immune responses. Conjugation of Tim-1 by Tim-4-Ig induces proliferation of T cells and phosphorylation of LAT, Akt and ERK, and expansion of CD3$^+$ T cells [8, 25]. A significant role for Tim-4 signaling in activation of Th2 type immune responses and development of intestinal food allergy was shown [24]. These studies demonstrated the activating function of Tim-4 as a ligand of Tim-1. However, the Tim-4 signal via Tim-1 was inhibitory in naïve and pre-activated T cells, suggesting that Tim-1/Tim-4 signaling has a bimodal function depending the stage of T call activation [26].

Tim-4 recognizes PS exposed on apoptotic cells and exosomes, to mediate the engulfment by phagocytic cells [9, 10, 27]. Recent studies suggested that the Tim-4-mediated removal of apoptotic bodies has implications for autoimmunity and tolerance. Tim-4 deficiency in mice resulted in hyperactivation of T cells and B cells, and elevated serum immunoglobulins and autoantibodies to dsDNA [22]. It has been shown that clearance of antigen-specific T cells, mediated by Tim-4 expressing medullary macrophages, is involved in respiratory tolerance [28]. Genetic studies in a Chinese Han population found an association of TIM-4 polymorphisms with asthma and rheumatoid arthritis [29-31].

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Unlike other Tim family proteins Tim-4 does not have a putative tyrosine phosphorylation site in its cytosolic tail, and the transmembrane and the cytosolic domains of Tim-4 were dispensable for phagocytosis of apoptotic cells. Therefore Tim-4 may serve as a binding receptor and a ligand for Tim-1, rather than a signaling receptor [32].

1.2 TIM-3 GENE (*HAVCR2*)

The gene encoding Tim-3 (*Havcr2*) was found within the *Tapr* locus of HBA mice, along with the genes encoding Tim-1 and Tim-4. Mouse Tim-3 gene contains seven exons [7], with segmentation of functional domain of the protein roughly equivalent to segmentation of the exons. The signal peptide sequence is encoded by exon 1, and the IgV domain is encoded by exon 2. The mucin and transmembrane domains are encoded by exons 3 to 5, and the cytosolic domain is encoded by exons 6 and 7 (Figure 1). Polymorphisms in seven amino acid residues between BALB/c and HBA mice were found, which are clustered in the IgV domain [7]. Polymorphisms in the BC loop of the IgV domain led to the loss of a potential O-linked glycosylation site in the HBA allele of Tim-3, which might affect binding of Tim-3 to its ligand(s).

Tim-3 gene also encodes a shorter isoform of Tim-3, which is generated by alternative splicing [33]. This shorter form of Tim-3 lacks exon 3, 4 and 5 and, as a consequence, lacks the mucin and transmembrane domains [33]. Expression of a soluble form of Tim-3 (sTim-3) was detectable in splenocytes, but not in non-lymphoid organs, and stimulation with HSP70-peptide complex induced the upregulation of sTim-3 expression in splenocytes. Several previous studies demonstrated that Tim-3-Ig fusion protein blocked the inhibitory function of Tim-3 [33, 34]. By

contrast, sTim-3 inhibited T cell mediated immune responses [35]. However, which cells express sTim-3, and the mechanisms by which sTim-3 and sTim-3-Ig differently regulate Tim-3, are not known. The involvement of Tim-3 polymorphisms in human immune-related diseases includes asthma, atopic dermatitis, and rheumatoid arthritis [36-40]. Several known polymorphisms are depicted in Figure 1 and more details will be discussed in the later part of this chapter (1.6.2 and 1.6.3).

1.3 TIM-3 STRUCTURE

Similar to other Tim family members, Tim-3 is a type I transmembrane protein. The extracellular domain of Tim-3 consists of IgV and mucin domains. The cytosolic tail of Tim-3 contains six tyrosine residues, four of which are predicted phosphorylation sites. The extracellular domain of Tim-3 has multiple putative N- and O-glycosylation motifs. Among them, there are two N-glycosylation sites and one O-glycosylation site in the IgV domain (no O-glycosylation motif in HBA), which potentially serve as galectin-9 binding sites. The Tim-3 IgV domain consists of two sheets formed by A,C,C',C",F and G β-strands and by B, E and D β-strands, with the two sheets facing each other like a sandwich. The main structure of the IgV domain is stabilized by a disulfide bond tethering B and F strands (Cys38-Cys111) and two hydrogen bonds (Trp52-Val94, and Tyr109-Asp105), which are common features in Ig superfamily proteins. Two disulfide bonds (Cys52-Cys63 and Cys58-Cys100) hold the CC' loop to C and C' β-sheet, shaping the cleft lined by CC' and FG loop. The CC' and FG loops form a narrow pocket in the IgV domain.

Since potential glycosylation sites (T44, N74, N100) (Figure 2, blue and red, Figure 3, blue) are located distantly from the pocket, Cao et al. proposed the pocket as a non-galectin-9

ligand binding site [41]. The pocket surrounded by CC'-FG loops, conserved in Tim-1 and Tim-4, interacts with PS in coordination with metal ion, and has been termed the metal ion-dependent ligand binding site (MILIBS) [11, 42]. DeKruyff et al. determined the structure of PS bound to the MILIBS (Figure 3, magenta and black) from the Tim-3 IgV domain, in coordination with a calcium ion. There are seven polymorphic amino acid differences between BALB/c and HBA, four of them clustered in the A' stand (HBA1) and four clustered in the BC loop (HBA2) (Figure 2 and Figure 3 green).

The BALB/c IgV domain interacts more avidly with PS in liposomes than does the HBA allele. Introduction of polymorphisms in the BC loop (HBA2) to BALB/c IgV reduced the binding affinity as low as that of HBA IgV, and point mutation of R22A in Tim-3 IgV and R25A in Tim-4 IgV domain decreased the binding affinity to PS. Although R22 does not reside in the MILIBS, R22 in the BC loop mediates electrostatic interactions with the charged phosphate head of membrane phospholipids [43].
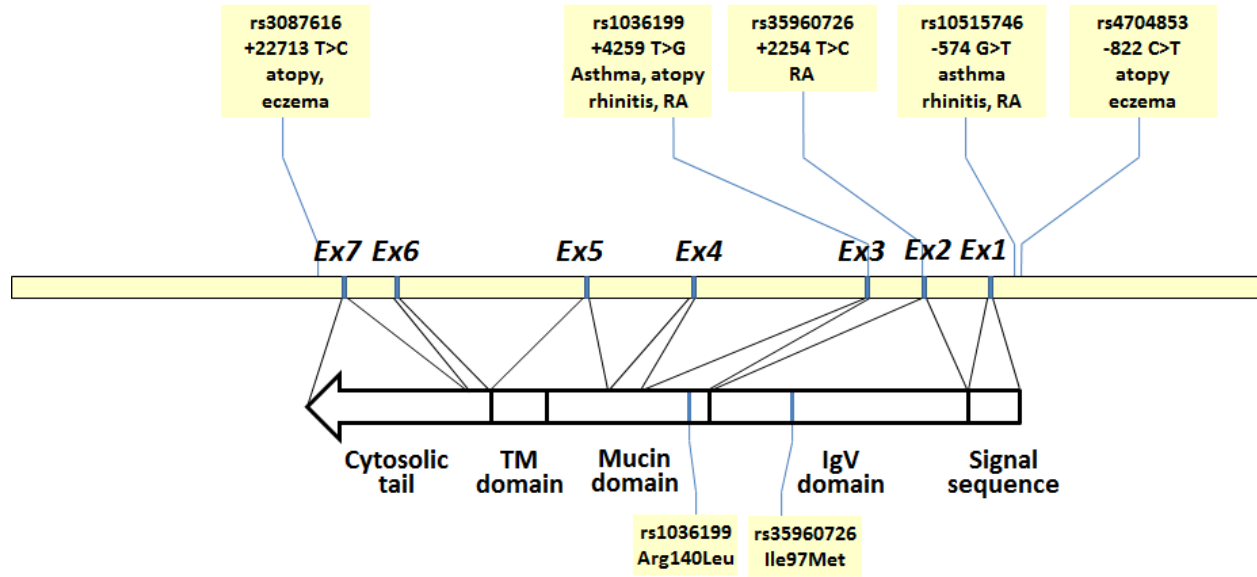
Figure 1. Schematic figure of Tim-3 gene and polymorphisms

```
                                        A'          B                        C
>BALB/c   MFSGLTLNCVLLLLQLLLARSLEDGYKVEVGKNAYLPCSYTLPTSGTLVPMCWGKGFCPW  60
>HBA      MFSGLTLNCVLLLLQLLLARSLENAVFEVGKNAYLPCSYTLSTPGALVPMCWGKGFCPW  60
          ********************:.* .***************.*.:*************

           C'       C"      D                 E            F
>BALB/c   SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN  120
>HBA      SQCTNELLRTDERNVTYQKSSRYQLKGDLNKGDVSLIIKNVTLDDHGTYCCRIQFPGLMN  120
          ***********************************************************

           G   IgV domain
>BALB/c   DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA  180
>HBA      DKKLELKLDIKAAKVTPAQTAHGDSTTASPRTLTTERNGSETQTLVTLHNNNGTKISTWA  180
          ***********************************************************

          Mucin domain        Transmembrane  domain
>BALB/c   DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG  240
>HBA      DEIKDSGETIRTAIHIGVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGG  240
          ***********************************************************

                             Intracellular domain
>BALB/c   LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS  281
>HBA      LANAGAVRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS  281
          *****************************************
```

Signal peptide

Polymorphism
O-Glycosylation
N-Glycosylaiton

Figure 2. Amino acid sequence of BALB/c and HBA Tim-3h

Amino acid sequence of BALB/c and HBA Tim-3 is aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). O-and N-glycosylation sites are predicted with NetOGlyc and NetNGlyc program (http://www.cbs.dtu.dk/services/NetOGlyc/, http://www.cbs.dtu.dk/services/NetNGlyc/). Polymorphisms and glycosylation sites are highlighted as indicated, and β-strands are shown with lines over the corresponding sequences.
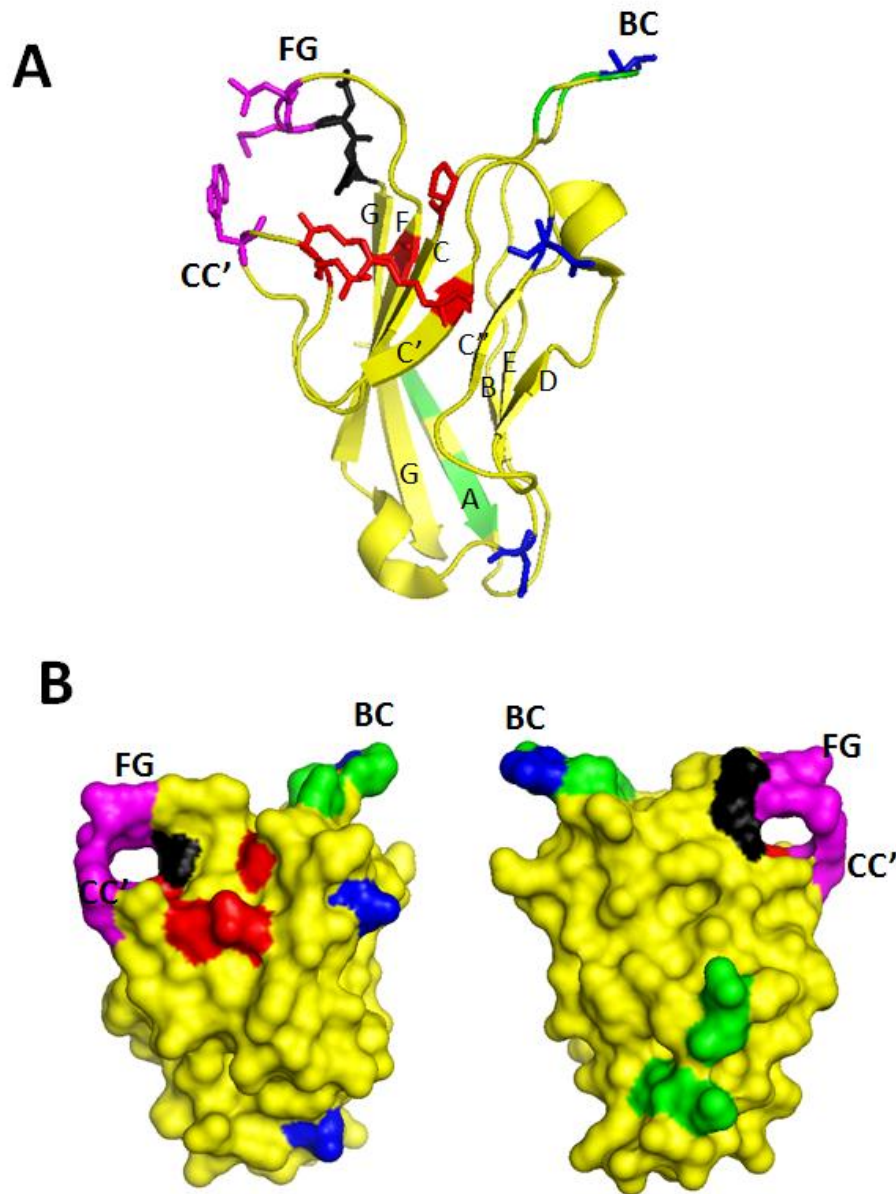
Figure 3. Tim-3 IgV domain structure

Non-galectin-9 ligand binding residues (red), MILIBS residues engaged metal ion coordination (black), hydrophobic residues in the tip of FG and CC' loop (magenta), glycosylation site (blue), polymorphisms (green) Tim-3 expression and function. Diagrams were generated using PDB ID: 2OYP using the PyMol software.

1.4 TIM-3 EXPRESSION AND FUNCTIONS

1.4.1 T cells

Tim-3 is expressed on activated Th1 cells, but not Th2 cells [44]. Blocking Tim-3/Tim-3L interaction, using blocking antibody or Tim-3 Ig fusion proteins, induced hyperproliferation and increases in Th1 cytokine secretion in T cells during development of autoimmunity and allograft rejection. Therefore, the concept is widely accepted that Tim-3 is an inhibitory regulator in Th1 immunity [33, 34, 44]. Although lower than in Th1 cells, Tim-3 is expressed in activated Th17 cells. When human primary CD4 T cells were stimulated with anti-CD3/CD28 antibodies, addition of Tim-3 "antagonistic" antibody enhanced the secretion of IFN-γ, IL-12, and IL-17 and IL-6, but not of Th2 cytokines. In psoriasis, a Th1- and Th17-mediated autoimmune disease of the skin, induction of Tim-3 expression in Th1, Th17 and Tc1 cells upon activation was less efficient in patients than in healthy donors [45]. Soluble galectin-9 treatment reduced the number of IFN-γ and IL-17 producing T cells, and mitigated the skin inflammation and thickness [46].

When strong antigen stimulation persists for a long period, as in chronic infection or tumors, Tim-3 expression is upregulated on CD8 T cells over time along with other inhibitory receptors, such as PD-1. This appears to cause virus/tumor-specific CD8 T cells to undergo a gradual loss of function, called exhaustion. Tim-3 and PD-1 double positive cells displayed more severe dysfunctional phenotype than Tim-3 or PD-1 single positive cells. Consistently, blocking Tim-3 and PD-1 restore CD8 T cell functions more efficiently than single blockade of Tim-3 or PD-1 [47-49], suggesting that Tim-3 and PD-1 are involved in induction of CD8 T cell exhaustion, cooperatively.

Tim-3 expressing CD4$^+$FoxP3$^+$ regulatory T cells were found in tumor tissues, and allograft tissues, and HCV infected patients. Among the tumor infiltrating T cells (TILs) in non-small cell lung cancer (NSCLC), 70% of TIM-3$^+$CD4$^+$ T cells are FoxP3$^+$ and 60% of FoxP3$^+$ TILs are Tim-3+ [50]. Among allograft infiltrating T cells, CD4$^+$FoxP3$^+$Tim-3$^+$ T cells express more CTLA-4, IL-10 and TGF-β and are more potent in suppression of effector T cells than CD4$^+$FoxP3$^+$Tim-3$^-$ T cells. However, possibly due to the expression of Tim-3, Tim-3$^+$ regulatory T cells were more prone to apoptosis induced by galectin-9 [51]. In HCV infection, Tim-3/galectin-9 seems to be involved in development and proliferation of regulatory T cells. Ji et al. showed that galectin-9 induces development of regulatory T cells from activated CD4 T cells, and Moorman et al. showed a positive correlation between Tim-3 expression and regulatory T cell proliferation. FoxP3$^+$ regulatory T cells are less susceptible to activation-induced cell death in a Tim-3 dependent manner than FoxP3$^-$ effector T cells. These results suggest that Tim-3 signaling is suppressive to the effector T cell, but favors function and/or survival of regulatory T cells.

1.4.2 Mast cells

Tim-3 is constitutively expressed on mouse peritoneal mast cells, and bone marrow derived cultured mast cells (BMMC), and expression was increased after IgE and antigen stimulation. [14, 52]. Treatment with polyclonal Tim-3 antibody upregulated the IL-4, IL-6, and IL-13 production and inhibited IL-3 withdrawal-induced apoptosis in BMMC sensitized with IgE and antigen. However, the same polyclonal Tim-3 antibody did not influence mast cell degranulation or activation of ERK and JNK MAP kinases [14].

1.4.3 NK cells

Tim-3 is expressed heterogeneously on immature $CD56^{bright}CD16^-$ NK cell subsets, and is expressed in all human $CD56^{dim}CD16^+$NK cell subsets in blood from healthy donors, Therefore Tim-3 was proposed as a marker for mature NK cells [53]. Elevated expression of Tim-3 was found in hepatitis B virus (HBV) infected patients, and atherosclerosis patients. Blockade of Tim-3 enhanced IFN-γ production and cytotoxicity of NK cells from HBV patients [54] and there was an inverse correlation between Tim-3 expression and number of peripheral NK cells [55]. Analysis of NK cells from healthy donors demonstrated that Tim-3 expressing cells were normal in cytokine production and cytotoxic function, but these responses were inhibited by cross-linking of Tim-3 [53]. According to these studies, Tim-3 is an inhibitory receptor on NK cells, nonetheless, there is a contradictory report as well. Thus, overexpression of Tim-3 on NK cell lines showed increase in IFN-γ production, while Tim-3 blockade decreased IFN-γ production [56].

1.4.4 Dendritic cells, macrophages, and monocytes.

Tim-3 is expressed by many subsets of dendritic cells, macrophages, and monocytes. Anderson et al. first demonstrated that Tim-3 enhances TNF-α production in innate immune cells, and differentially activates signaling in dendritic cells and Th1 cells, supporting the concept that Tim-3 plays negative or positive roles in adaptive and innate immunity respectively [57]. However, recent studies showed that Tim-3 also has an inhibitory role in innate immune cells. Thus, Tim-3 is constitutively expressed by resting human $CD14^+$monocyte/macrophage, and Tim-3 expression was downregulated upon TLR stimulation [58, 59]. There was an inverse

correlation between Tim-3 expression and IL-12 production, and antibody blockade of Tim-3 signaling upregulated TLR-mediated IL-12 production [58, 59]. Tim-3 expressed on macrophages, monocytes, and splenic dendritic cells recognizes phsophatidylserine exposed on the cell surface and mediates the engulfment of apoptotic cells. This Tim-3 mediated phagocytosis is involved in cross-presentation of antigens from the apoptotic cells. Apparently, via a similar mechanism, Tim-3 expressing macrophages and dendritic cells contribute to fetomaternal tolerance. This Tim-3 blockade results in accumulation of apoptotic bodies, inflammatory granulocytes and macrophages at the uteroplacental interface [60].

1.5 TIM-3 LIGANDS

1.5.1 Galectin-9

Galectins are soluble β-galactoside-binding lectins, preferably binding to N-acetyllactosamine and polylactosamine chain. Galectins are evolutionally conserved in animals and widely distributed. Galectins can be classified into three types, based on their structure. Prototypic galectins (Galectin-1,-2-,-5,-7,-13,-14,-15) contain a single conserved carbohydrate recognition domain (CRD) and form dimers. Tandem repeat galectins (galectin-4,-6,-8,-9-,-12) consist of two CRD domains separated by a linker domain. Galectin-3 is the only chimeric galectin, which consists of one CRD domain in the C-terminus linked with proline and glycine rich tandem repeats in the N-terminus [61].

Galectins are known to play roles in the regulation of many parts of the immune response. Galectins are synthesized in the cytosol and secreted by an unknown mechanism despite having

no classical signal peptide sequence for targeting to the ER. The secreted galectins bind to multivalent glycan ligands on the cell surface or extracellular matrix to form lattices. The galectin-glycoproetin lattices can control TCR signaling, cytokine signaling, metabolism, and apoptosis by reorganizing membrane microdomains and localization of glycoproteins in the membrane and suppressing endocytosis [62, 63]. Found in the cytosol and nucleus, galectins can bind to intracellular ligands to participate in intracellular processes, including signal transduction, trafficking, apoptosis and mRNA splicing [64-66].

Zhu et al first identified galectin-9 as a ligand of Tim-3, binding to carbohydrate chains in Tim-3 and mediating an immune regulatory role in a Th1 cell mediated autoimmune condition [67]. *In vitro* galectin-9 enhanced calcium flux and cell death in Th1 cells, which was partially dependent on Tim-3. *In vivo* administration of galectin-9 led to elimination of myelin oligodendrocyte glycoprotein (MOG) peptide (MOG 35-55) specific IFN-γ producing Th1 cells in an EAE model, and ameliorated the severity of the disease [67]. However, galectin-9 can regulate T cell function in autoimmune conditions through both Tim-3-dependent and − independent effects. In EAE and collagen-induced arthritis (CIA) models, galectin-9 treatment reduced the number of Tim-3$^+$CD4 Th1 cells and Th17 cells, but also suppressed Th17 differentiation by inducing differentiation of regulatory T cells, in a Tim-3 independent manner [68, 69]. In NOD model of diabetes, galectin-9 binds to CD40 expressed on CD4^{lo}CD40$^+$T cells to prevent proliferation and to induce cell death, Tim-3 independently[70].

Galectin-9 also has implication in infectious diseases. Thus, galectin-9 has a suppressive role in CD8 and Th17 T cell responses against infection by HSV [71], influenza A virus [72], HBV [73], HCV [74] and *Klebsiella pneumoniae* [75]. The role of galectin-9 in tumor biology is not linear, but more complex. Galectin-9 has an anti-tumor function by inducing apoptosis of

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tumor cells [76-80] and preventing metastasis [81-84]. However, in the regulation of tumor immunity, galectin-9 has both activating and suppressive roles. In general, it seems that galectin-9 negatively influences anti-tumor T cell responses. Galectin-9 induces apoptosis of CD4 and CD8 T cells [85-87] and inhibits anti-tumor function of T cells [87-89]. On the other hand, there are reports that galectin-9 administration induces expansion of plasmacytoid DC-like macrophages, by which NK cells or CD8 T cells are activated and prolong the survival of tumor bearing mice [56, 90-92]. Galectin-9 can mediate both positive and negative role in T cell immunity. Independently of Tim-3, galectin-9 could induce apoptosis and inhibit proliferation at higher doses and induced pro-inflammatory cytokine production and proliferation at lower doses [70, 93].

1.5.2 Phosphatidylserine (PS)

PS is a glycolipid that comprises 3-10% of total membrane lipids and consists of a glycerol backbone esterified with two acyl chains at sn-1 and sn-2 positions and a polar head group attached to the sn-3 position [94]. PS is found exclusively on the inner leaflet of the cell membrane, which is actively maintained by an ATP-dependent amino phospholipid lipase, and exposed on outer leaflet of the apoptotic cells providing an 'eat me' signal for phagocytic cells [95]. It was shown that Tim-1 and Tim-4 are receptors for PS, which facilitates engulfment of apoptotic cells to help prevent autoimmunity and for the recognition of exosomes involved in intercellular signaling [9, 10, 96].

Crystallography studies of the PS/Tim-4 IgV domain complex revealed that PS binds to metal ion-dependent ligand binding site (MILIB) built by the CC' and FG loops of Tim-4 IgV domain, which are conserved in Tim-1 and Tim-3 as well. A crystal structure of the PS/Tim-3

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IgV domain complex revealed that PS bound to the MILIBS in coordination with calcium. Mutation of either amino acid residues interacting with PS or the metal ion resulted in a large decrease in the affinity of the Tim-3 IgV domain for PS [43]. Although the affinity of PS to Tim-3 was much lower than affinity to Tim-1 and Tim-4, Tim-3 is crucial for the clearance of apoptotic cells and cross-presentation of dying cell-associated antigens [97].

1.5.3 HMGB1

HMGB1 (high mobility group protein B1) is a nuclear protein with two DNA binding motifs. Intracelluarly, HMGB1 regulates DNA repair, V(D)J recombination, transcription and organization of DNA structure [98-100]. When released to the extracellular space, HMGB1 functions as a pro-inflammatory molecule. HMBGB1 binds to RAGE by itself to induce chemotaxis and proliferation, and also forms complexes with IL-1, nucleosome, and LPS to induce stronger inflammatory responses initiated by IL-1R and surface TLRs [101]. Extracellular HMGB1-nucleic acid complex activates endosomal TLR3, 7 and 9, suggesting that HMGB1 plays a role in delivery of extracellular nucleic acids into the cytosol [102, 103].

Recently, Tim-3 was found as a receptor of HMGB1. Chiba et al. demonstrated that Tim-3 bound HMGB1 and the interaction is abrogated by single amino acid mutation near the FG loop of Tim-3 IgV domain that forms the metal-ion dependent ligand binding site (MILIBS). Tim-3 binds to the A-Box of HMGB1 to compete with B-DNA for binding to HMGB1, by which Tim-3 may inhibit the delivery of nucleic acids to endosomes, and suppress innate immune responses mediated by nucleic acids in DC. In this study, it was not directly tested whether PS and HMGB1 compete for binding to Tim-3 IgV domain, although AnnexinV treatment did not alter the Tim-3 dependent inhibition of nucleic acid-mediated IFN-β1

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production [104]. Thus, HMGB1 may bind to a motif on Tim-3 different from where PS binds. Although point mutation of an amino acid residue on the tip of FG loop of the Tim-3 IgV domain impaired the interaction with HMGB1, it may be because of more severe disruption in the IgV structure.

1.5.4 Unknown ligand

When the crystal structure of the Tim-3 IgV domain and the existence of a non-galectin-9 ligand were reported by Cao et al, a cleft lined with CC' and FG loops drew attention as a novel ligand binding site. Later studies demonstrated that PS and HMGB1 interact with the IgV domain of Tim-3, and the interaction was abrogated when residues near the cleft were mutated. Yet it is not clear whether the unknown ligand described in Cao's study was PS or HMGB1. In Cao's study Tim-3 IgV tetramer bound to almost all CD4 and CD8 T cell, and macrophages, while in DeKruyff's study, Tim-3 transfected cells specifically bound to apoptotic thymocytes but, not to live thymocytes. Although, in these studies, expression of putative Tim-3 ligands was analyzed using different reagents (Tim-3 IgV tetramer vs. Tim-3 expressing cells), surface expression/exposure profiles of the ligands between the two studies seemed different from each other. HMGB1 exists as an intracellular or secreted protein, but not as a membrane-bound protein, and LPS treatment induces HMGB1 release in macrophage [105]. However, LPS and IFN-γ treatment did not affect Tim-3 IgV tetramer binding to the surface of macrophage according to Cao's study [41]. Given all those finding, there is the possibility that another unknown ligand of Tim-3 exists still.

1.6 TIM-3 FUNCTION

1.6.1 Multiple sclerosis/EAE

The function of Tim-3 in regulation of immune responses was first described in EAE, a mouse model for multiple sclerosis (MS). Tim-3 was expressed in active Th1 T cells but not in Th2 cells, after *in vitro* polarization. During development of EAE in SJL mice by immunization with myelin proteolipid protein (PLP) peptide, Tim-3 expression was upregulated on CD4 and CD8 T cells and at low levels on CD11b$^+$ cells. The upregulation of Tim-3 expression in lymph nodes preceded the disease onset and Tim-3 expression in the brain peaked at disease onset. Then Tim-3 expression in both lymph node and brain was downregulated to near basal levels at the maximal disease score. Treatment with blocking anti-Tim-3 mAb exacerbated disease severity and mortality inducing proliferation and activation of CD11b$^+$/F4/80$^+$ macrophages *in vivo*. Treatment of EAE induced mice with galectin-9, a Tim-3 ligand, induced Th1 cell death *in vivo*, including IFN-γ producing MOG peptide-specific T cells, and ameliorated EAE symptoms[67].

Not only in the mouse model, but also in human MS, the role of Tim-3 was examined. T cell clones isolated from cerebrospinal fluid (CSF) of MS patients expressed less Tim-3 and produced more IFN-γ. Functionally, the clones from MS patients were more resistant to the tolerance induction by CD28 blockade during TCR stimulation. In addition to the inverse correlation between Tim-3 expression and cytokine production in T cells, more direct evidence of the suppressive role of Tim-3 in T cell function was revealed. When Tim-3 expression in human T cells was reduced by siRNA, proliferation and IFN-γ production were enhanced in CD4 T cells [106]. The CD4 T cells from PMBCs of healthy control and MS patient responded differently to Tim-3 blockade. Tim-3 antibody treatment augmented IFN-γ production in CD4 T

cells from healthy controls upon TCR stimulation, while IFN-γ production in CD4 T cells from untreated MS patients was not affected. In contrast to the untreated MS patients, T cells from treated MS patients were responsive to TIM-3 blockade, in which restoration of TIM-3 expression was found [107]. Therefore, the loss of immunoregulation by TIM-3 was suggested as a contributing factor to the development of multiple sclerosis.

1.6.2 Rheumatioid arthritis (RA)

Since rheumatoid arthritis (RA) is thought to be a Th1-and Th17-mediated autoimmune disease, the protective role of Tim-3 in rheumatoid arthritis is expected. Galectin-9 deficient C57BL/6J mice are susceptible to bovine type II collagen (BCII) induced collagen-induced arthritis (CIA), while wild type C57BL/6J mice is resistant to CIA. Administration of galectin-9 reduced the clinical score and the symptoms of CIA in a dose dependent manner, lowering the level of pro-inflammatory cytokines (IL-1β, IL-6, MCP-1, and MIP-2) and Th1 and Th17 cytokines (IL-12, IFN-γ, and IL-17) in joints and eliminating Tim-3$^+$CXCR3$^+$CD4$^+$ Th1 and Th17 T cells, but not CCR4$^+$T1ST2$^+$CD4 Th2 T cells. Moreover, galectin-9 induced the differentiation of regulatory T cells but repressed differentiation of Th17 cells [68].

In human samples, there was an inverse correlation between Tim-3 expression and joint disease activity score in RA patients [108]. Tim-3 expression and susceptibility to galecitn-9 induced apoptosis was lower in the CD4 T cells isolated from RA patients, compared to CD4 T cells from healthy controls, suggesting that loss of negative regulation in Th1 cells in RA patients contributes to the disease development [109]. Two genetic studies of Korean RA patients showed that the polymorphisms -574G>T, 4259T>G, rs35960726 were associated with RA [40, 110]. Another study in a Chinese population suggested that the 4259G>T polymorphism

is associated with RA in both Hui and Han populations while -1541C>T or -574T>G were associated Hui and Han population respectively [111].

1.6.3 Allergic diseases

Tim-3 is preferentially expressed in Th1 cells, versus Th2 cells, and is known to negatively regulate Th1T cell responses. However, Tim-3 may also be able to affect Th2-driven allergic diseases by indirectly modulating the balance between Th1 and Th2 type responses. Administration of anti-Tim-3 antibody decreased the production of Th2 cytokines and infiltration of eosinophils and Th2 T cells, preventing allergen-induced airway inflammation in a mouse model of asthma [112]. Tim-3 is constitutively expressed by mast cells and Tim-3 polyclonal antibody treatment enhanced production of Th2 cytokines such as IL-4, IL-6 and IL-13 from mast cells, without inducing degranulation [14].

Several genetic studies on TIM-3 polymorphisms have suggested that TIM-3 is involved in the susceptibility to Th2 driven allergic diseases. In Korean populations, the -574T allele was found only in asthma and rhinitis patients (1 and 1.5% respectively), and the 4259T allele was found more frequently in rhinitis patients [36]. In a study on children from white or Hispanic parents, 22713A>G and 882C>T were shown to be associated with atopic dermatitis, but 4259T>G was not [37]. Another study conducted on Chinese children-parent trios did not find evidence of an effect of individual TIM-3 polymorphisms on asthma susceptibility. However, haplotype analysis for the combination of three TIM-3 polymorphisms revealed that the G-G-G haplotype for rs10053538G4T, rs13170556A4G and rs9313441G4A was undertransmitted to asthmatic children [38]. Still, the role of TIM-3 in the pathogenesis of Th2 type allergic diseases is unclear.

1.6.4 Tolerance induction

Tim-3 regulates tolerance in autoimmune models and allograft transplantation. Binding assays of full-length Tim-3-Ig and soluble Tim-3-Ig to immune cells revealed that Tim-3 ligands were expressed in terminally differentiated resting Th1 and Th2 CD4 T cells [34]. When CD4 T cells were activated *in vitro*, Tim-3L expression was downregulated in $CD4^+CD25^-$ T cells, but retained in $CD4^+CD25^+$ regulatory T cells. Blockade of Tim-3/Tim-3L interactions using Tim-3-Ig fusion protein or anti-Tim-3 monoclonal antibody accelerated spontaneous development of diabetes in NOD mice. Induction of an allograft tolerance by blockade of co-stimulation (anti-CD40L) and tolerance induction to a specific antigen by high dose antigen treatment were both impaired by blockade of Tim-3 or Tim-3 deficiency [33, 34]. It was found that galectin-9 is expressed on regulatory T cells, and blockade of Tim-3/galectin-9 suppressed the function of regulatory T cells [113, 114]. Not only Tim-3L but also Tim-3 itself is expressed on regulatory T cells, and the number of $CD4^+FoxP3^+Tim-3^+$ T cell increases during allograft rejection. $CD4^+FoxP3^+Tim-3^+$ T cells express more suppressive molecules like CTLA-4, IL-10 and TGF-β and are more potent in suppression of allo-specific effector T cells than $CD4^+FoxP3^+Tim-3^-$ T cells. However, the suppressive capacity of $Tim-3^+$ regulatory T cells was less than that of $Tim-3^-$ regulatory cells due to higher sensitivity to galectin-9-mediated cell death, and poorer survival of $Tim-3^+$ regulatory T cells [51].

1.6.5 Chronic viral infection

1.6.5.1 HIV

A correlation between Tim-3 expression and dysfunctional phenotype of T cells in chronic viral infection was demonstrated first in HIV [115]. Tim-3 expression was upregulated in CD4 and CD8 T cells from PMBCs of early/acutely HIV-1 infected individuals and chronic progressors, compared to uninfected individuals and HIV-1 infected controllers. Specifically, Tim-3 expression was upregulated in HIV-1 specific CD8 T cells compared to HIV-1 non-specific CD8 T cells. The effect of anti-viral therapy on Tim-3 expression on CD8 T cell was examined. Among the seven subjects whose viral load was reduced after highly active antiretroviral therapy (HAART), the frequency of Tim-3$^+$ cells was either declined or highly maintained in three and four subjects, respectively. However, in both groups, Tim-3 expression strongly correlated with CD38 expression (T cell activation marker) during the course of the therapy, implying that Tim-3 expression relates to on-going T cell activation, rather than viral load.

In the comparison of Tim-3 expressing and non-expressing T cells, Tim-3 expressing CD4 and CD8 T cells from HIV infected patients were dysfunctional in terms of proliferation and production of IFN-γ. Also it was found that STAT5, ERK and p38 activation were impaired *in vitro*, due to elevated basal phosphorylation of them. Similar to EAE/MS, Tim-3/Tim-3L blockade restored IFN-γ production and proliferation in HIV-1 specific T cells [115]. Tim-3 also appeared to regulate cytotoxic function of CD8 cells in HIV infection. Tim-3$^+$ CD8 T cells contained more perforin in association with granules than Tim-3$^-$ CD8 T cells, but there was a defect in degranulation, as shown by low surface expression of CD107a (lysosomal-associated membrane protein 1, LAMP1). Blockade of Tim-3 enhanced the expression of CD107a in CD8 T cells, and as a consequence, the cytotoxic activity against HIV-1 infected CD4 T cells was

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restored [116]. In contrast to the negative effect of Tim-3 in HIV-immunity via suppression of HIV-specific T cells, inhibition of CD4 T cells by Tim-3 can be protective from HIV infection. Galectin-9 binding to Tim-3 downregulated surface expression of HIV co-receptors like CCR4, CXCR, α4β7 which are also activation markers of T cell, and upregulated p21, an inhibitor of cell cycle progression, inhibiting HIV entry to activated CD4 T cell and viral replication in already infected CD4 T cells [117].

1.6.5.2 Herpes simplex virus (HSV) infection

Recurrent herpes simplex virus infection is very common in humans and HSV establishes latency in peripheral neurons that lasts for the life of the host. Under certain conditions such as disease, stress, and UV, HSV reactivates and causes lesions in the peripheral tissue or asymptomatic shedding of virus [118]. In the HSV infected trigeminal ganglion (TG), CD8 T cells infiltrate to prevent HSV-1 from reactivation. The local HSV-specific CD8 T cells produce anti-viral IFN-γ, TNF-α, and granzyme B to suppress viral replication immediately upon reactivation [119, 120]. Since a major cause of HSV-related blindness is the lesions from recurring inflammation, Tim-3 might be the useful therapeutic target for prevention of blindness. After HSV-1 infection, Tim-3 is upregulated in CD4 T and CD8 T cells. Tim-3 blockade using monoclonal antibody (RMT3-23) and galectin-9 treatment, respectively, increases and reduces the severity of the stromal keratitis. Galectin-9 administration suppresses the keratitis by induction of apoptosis of both CD4 and CD8 T cells. Moreover, galectin-9 treatment promotes differentiation of FoxP3^{+} regulatory T cells, and the expansion of myeloid suppressor cells [121, 122].

1.6.5.3 Lymphocytic Chorimeningitis virus (LCMV) infection

The phenomenon of CD8 T cell exhaustion during chronic infection was first identified in LCMV infection model in mice [123]. Jin et al. demonstrated that Tim-3 participates in CD8 T cell exhaustion in synergy with PD-1. When C57BL/6 mice were acutely or chronically infected with LCMV Armstrong strain and clone-13, respectively, Tim-3 expression in GP33-specific (LCMV epitope) CD8 T cells was upregulated in both mice at the initial phase of infections. Over time, Tim-3 expression on the virus specific CD8 T cells decreases in acutely infected mice but Tim-3 expression was maintained over 60 days in chronically infected mice. Consistently, the frequency of Tim-3 and PD-1 double positive cells among GP33-specific CD8 T cells isolated from spleen, lung, and liver was upregulated and sustained during chronic infection. When the splenocytes from chronically infected mice were re-stimulated with LCMV peptide, a significant decrease in the frequency of IFN-γ, TNF-α, and IL-2 producing cells in Tim-3-PD-1^{+} CD8 T cells, and further decrease in Tim-3^{+}PD-1^{+} CD8 T cells was found, in comparison to Tim-3^{-}PD-1^{-} virus-specific CD8 T cells. Administration of anti-Tim-3 and anti-PD-1 into chronically LCMV infected mice for two weeks synergistically enhanced the proliferation and cytokine production in the virus-specific CD8 T cells. In addition, the dual blockade of Tim-1 and PD-1 synergistically reduced the viral load, suggesting that blockade of Tim-3 along with PD-1, is a promising measure to achieve better control for chronic viral infection [47].

1.6.6 Cancer

Tumor causes persistent antigen stimulation, similar to chronic infection, resulting in dysfunction of tumor-specific CD8 T cells, thus Tim-3 negatively regulates antitumor activity of CD8 T cells. In mice, upregulation of Tim-3 and PD-1 expression was found in TILs from mice implanted

with CT26 solid colon adenocarcinoma. The frequency of IFN-γ, IL-2, and TNF-α producing CD8 TILs was lower among Tim-3$^+$PD-1$^+$ TILs than Tim-3$^-$PD-1$^-$ and Tim-3$^-$PD-1$^+$ TILs. Blockade of Tim-3 and PD-1 effectively restored the IFN-γ production upon *ex vivo* stimulation with anti-CD/CD28, and tumor growth *in vivo* [49]. Study of human samples, consistently, showed that Tim-3 expression was upregulated in NY-ESO-1, a tumor antigen,-specific CD8 T cells isolated from PBMCs of patient with advanced melanoma. Tim3$^+$PD-1$^+$NY-ESO-1$^-$specific CD8 T cells were more dysfunctional than Tim-3$^-$PD-1$^+$ and Tim-3$^-$PD-1$^-$ NY-ESO-1$^-$specific CD8 T cells in terms of IFN-γ, IFN-α, and IL-2 production upon *ex vivo* stimulation and Tim-3$^+$PD-1$^+$NY-ESO-1 specific CD8 T cells were in high activation/differentiation status according to the activation marker expression such as CD38, HLA-DR, and CD57. Tim-3 blockade with anti-Tim-3 alone or with co-blockade of PD-1 restored the frequency of cytokine producing tumor-specific CD8 T cells upon stimulation with tumor antigen peptide (NY-ESO-1$_{157-165}$) for six days. In NSCLC, majority TILs expressing Tim-3 were regulatory T cells [50], and positive correlation between Tim-3 expression and poor prognosis was found[124].

Recently, Tim-3 was shown to be expressed in tumor infiltrating dendritic cells and to bind to HMGB1, which inhibited the innate immune responses mediated by nucleic acid-sensing TLRs [104]. Kikushige et al. discovered that Tim-3 was expressed on most acute myeloid leukemic (AML) stem cells but not in normal hematopoietic stem cells and proposed Tim-3 as a promising surface molecule to specifically target acute myeloid leukemia. However, it was not determined whether Tim-3 has a role in the development of AML.

1.7 TIM-3 SIGNALING

The cytosolic tail of Tim-3 has six tyrosine residues, Y256 of mouse Tim-3 (Y265 in human Tim-3) and the amino acid sequence surrounding Y256 is conserved. Van der Weyer et al. suggested tyrosine phosphorylation of the conserved tyrosine may be important in Tim-3 signal transduction. Y265 of the ectopically expressed human TIM-3 was phosphorylated by treatment with pervanadate, a potent inducer of phosphorylation, in HEK 293 cells. Phosphorylation of Y265 was upregulated by co-expression of Itk or galectin-9, suggesting that ligation of Tim-3 with its ligand may induce tyrosine phosphorylation of Y265 and Itk may mediate the tyrosine phosphorylation [125]. Lee et al, showed human Tim-3 expression suppressed the activity of NFAT and transcription of AP-1 in Jurkat and human CD4 T cells when they were stimulated with PMA/ionophore, as a consequence, suppressed IL-2 production[126]. The inhibitory function of Tim-3 required the cytosolic tail of Tim-3, especially the conserved region with Y265 and Y262. Rangachari *et al*, reported that BAT3 as a molecule regulating Tim-3function. BAT3 associated with unphosphorylated Tim-3 and bound with active Lck. Ligation of Tim-3 induced the dissociation of BAT with Tim-3 and Lck. BAT3 deficiency or knock-down suppressed the development of EAE *in vivo*. IFN-γ and IL-2 production was decreased in BAT deficient cells, while increased in BAT3 overexpressing cells. Therefore, BAT3 interacts with Tim-3 suppressing function of Tim-3, and released from Tim-3[127]. These data suggest that tyrosine phosphorylation is crucial for regulation of inhibitory Tim-3 function. However, the mechanism by which the tyrosine residues of Tim-3 cytosolic mediate signal and downstream target of Tim-3 are not known.

1.8 T CELL RECEPTOR SIGNALING

1.8.1 T cell receptor complex

T cell receptor (TCR) complex consist of a TCR heterodimer, two ζ chains, and two CD3 heterodimers. TCR consists of highly variable α and β which recognize antigen epitope mounted on a MHC molecule. TCR α and β chains lack cytosolic tail, transmembrane domain of TCR α and β chains interact with ζ chains which have cytosolic tail containing three tyrosine phosphorylation motifs each. There are three CD3 chains, γ, δ, and ϵ, which form CD3γϵ and CD3δϵ heterodimers. ζ chains and CD3 chains contain conserved tyrosine phosphorylation motifs called an immunoreceptor tyrosine-based activation motif (ITAM, $YxxL/Ix_{(6-8)}YxxL/I$)[128], tyrosine phosphorylation of the motifs is induced by TCR-antigen engagement and mediate the recruitment of signaling molecules to TCR complex.

1.8.2 Src family kinases (SFK)

Lck and Fyn are members of SFKs, which phosphorylate ITAMs in TCR complexes. Both Lck and Fyn shares the common structures of SFK, which consist of SH2 domain, SH3 domain and a kinase domain [129]. Activity of SFKs is regulated by phosphorylation of dephosphorylation of tyrosine residues. Intracellular interaction between the C-terminal phosphotyrosine and SH2 domain limit the access of substrate [130]. In T cells, dephosphorylation of the inhibitory tyrosine residue by CD45 phosphatase and phosphorylation by Csk activates and inhibits kinase activity of the SFKs, respectively [131, 132]. When Lck or Fyn are released from inhibitory conformation, autophosphorylation of the tyrosine residue in the loop between N- and C-

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catalytic lobes leads to conformational changes in kinase domain resulting in full activation of the kinases.

Although Fyn has a partial role, Lck plays major role in initiation of TCR signaling by phosphorylating ITAMs in ζ chains and CD3. Lck constitutively interacts with CD4 or CD8 [133, 134], and Fyn interacts with CD3 [135]. It has been suggested that Lck is brought to proximity of TCR by co-aggregation of CD4/CD8 with TCR. Also localization of membrane microdomains regulates phosphorylation of TCR by Lck and Fyn [136-138]. However, it is still unclear how phosphorylation of TCR complexes by Lck and Fyn is regulated upon TCR-antigen ligation.

1.8.3 ZAP-70 and phosphorylation of adaptor proteins

When Lck phosphorylates ITAMs in the intracellular domains of ζ chains, ZAP-70 is recruited to the phosphotyrosine via its SH2 domains [139] and activated by Lck [140]. The activated ZAP-70 phosphorylates adaptor molecules LAT and SLP-76, which leads to assembly of LAT-GADS-SLP-76 scaffolding complex [141, 142]. LAT-GAD-SLP-76 complex nucleate the formation of signalosomes which control activation of PLC-γ1 pathway, MAP kinase pathway, and cytoskeleton reorganization.

1.8.4 PLC-γ1 and activation of transcription factors

PLC-γ1 [143, 144] and Itk, a Tec kinase which phosphorylates and activates PLC-γ1 [145] are recruited to a LAT-GADS-SLP-76 complex. PLC-γ1 hydrolyze phosphatidylinositol 4,5-bisphosphate(PIP_2) to generate second messengers inositol 1,4,5-trisphosphate (IP_3) PIP2 and diacylglycerol (DAG). IP_3 induces a Ca^{2+} release from ER. Association of Ca^{2+} bound

calmodulin activates phosphatase activity of calcineurin. The activated calcineurin dephoaphorylates NFAT transcription factors leading to nuclear translocation of NFAT [146]. PKC-θ is also activate by and Ca^{2+}. PKC-θ is recruited to DAG in plasma membrane, where PKC-θ phosphorylates Carma1. In turn, the phosphorylation facilitates formation of Carma1-Bcl10-Malt1 complex resulting in activation of NF-κB pathway. DAG also recruits RasGRP to activate Erk pathway. Then the activation of Erk pathway induces expression and activation of c-fos, which dimerizes with c-Jun leading to formation of AP-1 transcription factor.

1.9 T CELL EXHAUSTION

In the initial stage of infection, virus-specific naïve CD8 T cells, that encounter antigen differentiate into effector cells, which efficiently proliferate and produce effector molecules like IFN-γ, TNF-α, perforins and granzymes. After the infection is cleared acutely, the virus-specific CD8 T cell population shrinks and subsets of the T cells leave as memory cells, which can last a long-term period without a requirement for homeostatic cytokines IL-7 and IL-15, and can respond rapidly to the secondary antigenic challenge. On the contrary, if the infection is not able to be cleared but becomes chronic, the virus-specific CD8 T cells gradually lose their function: loss of ability to produce cytokine, less responsiveness to homeostatic cytokines, loss of proliferative potential, and apoptosis [147, 148]. During chronic infection, viral-specific T cells lose their polyfunctionality of cytokine production in a hierachial manner. For example, at the early stage of exhaustion, IL-2 and TNF-α production is diminished in the effector T cells while IFN-γ production was retained. Eventually, T cells lose production of IFN-γ at the late stage of exhaustion [149].

Antigen specific stimulation of T cells seems to drive exhaustion. There were correlation between the viral load and the severity of loss of function [149]. mRNA microarray analysis revealed that the mRNA profile of exhausted CD8 T cells is similar to the profile of effector CD8 T cells than that of naïve or memory T cells [150]. Higher level of NFATc1 mRNA was found exhausted CD8 T cells [150], and NFATc1 binds to promoter of PD-1 and regulates transcription of PD-1 [151].

During chronic infection several inhibitory receptors such as PD-1 and CTLA-4 are expressed on the surface and mediate the dysfunction T cell dysfunction [152-157]. PD-1 is a transmembrane receptor which is expressed on activated T cells but also expressed on B cells, NKT cells [158]. PD-1 has two ligand, PD-L1 is broadly expressed both in lymphoid and non-lymphoid cells, while PD-L2 is expressed in activated macrophage and DCs [159, 160]. PD-1 contains immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM) in its cytosolic tail. Ligation of PD-1 using anti-PD-1 inhibited activation of PI3K and Akt, which was dependent on a ITSM than a ITIM [161, 162]. SHP-1 and SHP-2 interaction with the cytosolic tail of PD-1 was found in primary murine B cells [163], Jurkat T cells [164], and in vitro yeast two hybrid system [165]. SHP-2 phosphorylation was upregulated by TCR/PD-1 ligation in primary human CD4 T cells [164]. However, it is still unclear whether PD-1 signaling is mediated by SHP-1 or SHP-2 in T cells, because SHP-1/2 interaction with PD-1 in T cells was not demonstrated in physiological conditions. In addition, since SHP-1 and SHP-2 are involved in several other inhibitory signaling of T cell activation, there is a difficulty to prove whether inhibitory effect of PD-1 signaling is SHP-1/2-dependent using SHP-1/2 deficient model or by knock-down [166].

CTLA-4 is an inhibitory molecule shown to inhibit CD28 co-stimulation, whose deficiency in mouse resulted in spontaneous lymphoproliferation and fatal tissue destruction within 3-4 weeks of age [167, 168]. The cytosolic tail of CTLA-4 lacks ITIM or ITSM motif but has GVYVKM motif for AP-2 binding [169]. YVKM motif of CTLA-4 mediates the binding of PI3K, SHP-2 and PP2A [170]. Although CTLA-4 inhibits Akt activation, ligation of CTLA-4 did not inhibited PI3K. Inhibition of Akt by CTLA-4 was suppressed by treatment with okadaic acid, a PP2A inhibitor, suggesting CTLA-4 inhibits Akt via PP2A [161]. Cell-extrinsically, CTLA-4 can competitively inhibit CD28 co-stimulation by binding to CD80 and CD86, ligands of CD28, with greater affinity. CLTA-4 binding to CD80 and CD86 can mediate the removal and degradation of CD80 and CD86 from APC by trans-endocytosis to T cells [171-173].

1.10 STATEMENT OF THE PROBLEM

Tim-3 has been implicated in the modulation of various immune conditions mediated by Th1 and Th17 CD4 T cells and CD8 T cells such as multiple sclerosis [44, 67, 107], type I diabetes [34], allograft rejection [33], chronic viral infection [47, 115, 116, 121], and tumor [48, 49, 104]. Moreover, Tim-3 is expressed by innate immune cells, and modulates production of Th2 cytokines by mast cells [14], and LPS- and nucleic acid-recognizing TLR responses in APC [57, 104, 174]. In general Tim-3 seems to function as an inhibitory receptor on T cells. However, depending on the type of cells and response it appears that Tim-3 has positive roles as well. Tim-3 is an emerging therapeutic target for treatment of autoimmune disease, tumors, and infectious disease. However, the mechanisms by which Tim-3 signaling suppresses activation and effector cytokine production in T cells and how Tim-3 results in different outcomes in different type of

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cells are not understood. Because of the promiscuity of the known Tim-3 ligands, various antibodies were developed and used to characterize the function of Tim-3 in place of the natural ligands of Tim-3. These antibodies have been described as "agonistic" or "antagonistic" by comparing their effects to the consequences of the treatment with Tim-3-Ig fusion protein, assuming that Tim-3-Ig blocks Tim-3/Tim-3L interaction. However, due to a lack of knowledge of Tim-3 function at cellular and molecular level, there is still confusion of the definition of "agonistic and antagonistic" antibody.

To investigate the mechanism how Tim-3 signals are initiated and regulated, we generated and studied wild type Tim-3 constructs with serial deletion or point mutation at putative tyrosine phosphorylation sites. We intended to examine the influence of Tim-3 expression on TCR signaling and to identify the residues which are required for the Tim-3 function using the Tim-3 constructs. We also intended to identify the proteins interacting with the cytosolic tail of Tim-3, and to analyze the mode of the interaction. Then we aimed to identify the signaling molecules downstream of the TCR modulated by Tim-3 expression. The results that we collect from these experiments will help us to understand how Tim-3 modulates T cell signaling and exert inhibition of T cell function. Also this study may help to identify therapeutic targets for T cell mediated immune conditions like autoimmune diseases, cancer and, chronic infection.

2.0 TIM-3 TYROSINE PHOSPHORYLATION AND STRUCTURE FUNCTION

2.1 INTRODUCTION

Numerous studies revealed that Tim-3 functions as a negative regulator of Th1 and Th17 CD4 T cells and CD8 T cell mediated immune responses. Tim-3 expression is upregulated on activated T cells, and blockade of the Tim-3/Tim-3L using recombinant Tim-3 fusion protein or antagonistic Tim-3 antibody increases the production of cytokines such as IFN-γ, IL-2, IL-17, and TNF-α and [45, 47, 48, 72, 75, 175], suggesting that Tim-3 is an inhibitory receptor. During prolonged T cell activation, such as chronic infection, inhibitory receptors like PD-1 and 2B4 are expressed on the surface of T cells leading to suppression of T cell function [147]. Some of these inhibitory receptors contain ITIM or ITSM, which recruit phosphatases upon tyrosine phosphorylation [162, 176, 177]. However, Tim-3 does not have conventional activating or inhibitory motifs, like ITAM, ITIM or ITSM in its cytosolic tail. It has not been determined yet whether Tim-3 generates inhibitory signaling like PD-1, or 2B4. In addition, it was not known how Tim-3 signal transduction is initiated and how Tim-3 signaling leads to inhibition of T cell function. Our group previously showed that tyrosine phosphorylation (pY276) of Tim-1 cytosolic tail is required for the co-stimulatory function of Tim-1 and recruitment of downstream molecules [13, 17]. When examining the sequence of Tim-3, we found that the amino acid residue sequences surrounding the tyrosine residue in Tim-1 are conserved in Tim-3. Therefore, we hypothesized that the tyrosine residues are important for Tim-3 function. van de Weyer et al.

demonstrated that galectin-9 treatment induces phosphorylation of TIM-3 at Y265 in 293T cells [125]. However, the function of Tim-3 in T cell activation, the role of tyrosine phosphorylation in the regulation of Tim-3 function, and the identity of the kinase responsible for the tyrosine phosphorylation were not determined.

In this section I will describe the function of Tim-3 in modulation of downstream TCR signaling in T cell lines, the domains and tyrosine residues required for Tim-3 function, and regulation of phosphorylation of the tyrosine residues in Tim-3 cytosolic tail.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines and cell culture

Jurkat T cells and Jurkat variants were cultured in RPMI-1640 (Mediatech) supplemented with 5% bovine growth serum (BGS, Hyclone) 100U/ml penicillin and 100μg/ml streptomycin. D10 Th2 T cell clone was cultured in RPMI-1640 (Mediatech) supplemented with 10% BGS, 100U/ml penicillin, 100μg/ml streptomycin, nonessential amino acid, 1mM sodium pyruvate, 55μM 2-mercaptoechanol (Life sciences), and 10mM HEPES (Fisher). Tet-on Tim-3 Jurkat T cells were maintained in 0.8μg/ml puromycin, and Tim-3 expression was induced with 1.5μg/ml doxycycline for 2 days.

2.2.2 Antibodies and reagents

C305 antibody to Jurkat TCR was obtained from Dr. Arthur Weiss (UCSF, CA), human anti-CD28 were from Life Sciences. Biotinylated anti-mouse CD3 (2C11) and CD28 (37.51) were from eBioscience, and anti-mouse CD4 (L3T4) was from eBioscience. Streptavidin was from Zymed Laboratories. Anti-Flag M2 antibody, anti-Flag M2 agarose, and β-actin antibody were from Sigma Aldrich, polyclonal goat anti-mouse Tim-3 antibody was from R&D systems. anti-phospho-tyrosine (4G10) was from Millipore. HRP conjugated goat anti-mouse antibody and donkey anti-goat antibody were from Thermo scientific and Jackson Immunoresearch respectively. PMA, ionomycin and doxycycline were from Calbiochem and D-luciferin was from Thermo scientific. Peptide N-glycosidase F (PNGaseF) was from New England Biolabs.

2.2.3 DNA constructs

Murine Tim-3 sequence corresponding to each full-length and deletion Tim-3 without signal sequence were amplified by PCR and cloned in to pCDEF3 vector with CD8 signal sequence and Flag-tag sequence in N-terminus side. Point mutant constructs were generated using Quickchange site-directed mutagenesis kit (Stratagene). CD2-Tim-3 chimera was generated by fusing the ectodomain and transmembrane domains of human CD2 with the cytoplasmic tail of murine Tim-3. Sequences of all constructs were verified by automated sequencing. Tetracycline-inducible Tim-3 construct was generated by PCR amplification of Flag-tagged Tim-3 and cloning into pBI-EGFP vector (Clontech)

2.2.4 Generation of Tet-on inducible Tim-3 Jurkat cell line

Parental Jurkat T cells were transfected with Tet-on transactivor (rtTA) vector and empty pCDNA 3.1 vector with Neo selection marker, and stable cells were isolated by G418 selection. The rtTA stable Jurkat T cells were transfected with pBI-EGFP-Tim-3 vector along with pIRES-puro (Clontech) and clones were selected with limiting dilution and 1μg/ml puromycin selection. Inducible expression of Tim-3 in the selected clones was screened by flow cytometry.

2.2.5 Luciferase reporter assay

2×10^6 Jurkat or D10 cells were transfected with 15ug of NFAT/AP-1, NF-κB, NFAT, or AP-1-luciferase reporter, and Tim-3 constructs by electroporation at 260V/950μF(Jurkat) or 250V/950μF(D10). The day after transfection, 5×10^5 cells were stimulated with anti-human TCR (C305)/CD28 antibodies (Jurkat) or biotinylated anti-mouse CD3/CD4/CD28 and streptavidin (D10) for six hours in round bottom 96 well plates. After freezing and thawing cycle, luciferase assay was performed in 5mM ATP, 10mM $MgCl_2$, and 1mM D-luciferin using luminometer (Berthold)

2.2.6 Co-immunoprecipitation and western blotting

293T cells transfected with Flag-Tim-3 constructs and Jurkat T cells inducibly expressing Flag-Tim-3 (Tet-on Tim-3 Jurkat) were stimulated with pervanadate or monoclonal antibody specific for Jurkat TCR (C305.2), then lysed with ice-cold 1% NP-40 lysis buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride,

sodium orthovanadate, and β-glycerophosphate. Lysates were then incubated on ice for 10 min before being cleared by centrifugation. The lysates were incubated with anti-Flag agarose for overnight. After beads were washed with lysis buffer the incubated with 2X Sample buffer (Bio-Rad, Hercules, CA) at 37'C for 10min. the supernatants were loaded onto 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, probed with anti-Tim-3, or anti p-Tyr 4G10 antibody, and detected with anti-mouse HRP or anti-goat HRP. Blots were imaged on a Kodak ImageStation 4000R system.

2.3 RESULTS

2.3.1 Tyrosine 256 and 263 are responsible for Tim-3 function to enhance NFAT/AP-1 and NF-κB reporter activity

The amino acid sequence of cytosolic tail of Tim-3 is highly conserved and both mouse and human Tim-3 contain six tyrosine residues in their cytosolic tails at residues 219, 256, 263, 271, 272, and 274 (positions in mouse Tim-3, Figure 4A). Previously, we demonstrated that Y276 of Tim-1 is critical for co-stimulatory function of Tim-1 and its phosphorylation was induced by SFK Lck [13]. Comparing the sequence of Tim-3 and Tim-1, we could find that the conserved tyrosine residue at the position of Y256 analogous to Y276 of Tim-1 is surrounded by acidic amino acid residues. When we searched for possible phosphorylation sites in the cytosolic tail of Tim-3 with a motif prediction algorithms, both Y256 and Y263 were predicted to be

phosphorylated (Figure 4B). Therefore, we hypothesized that the conserved region surrounding the two tyrosine residues may be required to couple Tim-3 to downstream signaling.

To determine the requirement of the conserved sequence for Tim-3 signaling, we generated Flag-tagged serial deletion mutants of Tim-3. Truncation 1 construct (T1) contains two tyrosine residues in the conserved region (Y256 and Y263), but lacks three terminal tyrosine residues (Y271, Y272, and Y274). Truncation 2 construct lacks all five tyrosine residues from the C-terminus leaving only a membrane proximal tyrosine (Figure 5). Jurkat T cells which do not endogenously express Tim-3 were transfected with Flag-tagged full-length Tim-3, or the serial deletion mutants of Tim-3, along with NFAT/AP-1 luciferase reporter, and stimulated the T cells with anti- human TCR and CD28 antibodies. Although Tim-3 was known as an inhibitory molecule in Th1 immune responses, surprisingly, we found that ectopic expression of WT Tim-3 in Jurkat T cells resulted in enhancement of NFAT/AP-1 reporter activity induced by TCR/CD28 stimulation rather than inhibition. T1 Tim-3 mutant not only retained the ability to augment NFAT/AP-1 activation by TCR/CD28 stimulation, but slightly, and reproducibly enhanced NFAT/AP-1 activation more than WT full-length Tim-3. Meanwhile, the T2 deletion completely abolished the ability of Tim-3 to augment NFAT/AP-1 reporter activity, suggesting that the conserved region near Y256 and Y263 is crucial for function of Tim-3 in the modulation of downstream TCR signaling, and that the C-terminus, containing three terminal tyrosine residues, may be involved in negative regulation of Tim-3 function (Figure 6A).

Since we observed a functional requirement for the Tim-3 conserved region around Y256 and Y263, which are predicted as tyrosine phosphorylation sites, we hypothesize that tyrosine phosphorylation of the two tyrosine residue mediates Tim-3 signal. To determine the role of the tyrosine residues in Tim-3, I point mutated the tyrosine residues at position of 256 and 263 to

phenylalanine, either individually or together (Figure 5). The full-length and T1 Tim-3 constructs carrying either a Y256F or Y263F point mutation enhanced NFAT/AP-1 activation by TCR/CD28 stimulation, at levels equivalent to WT full-length, or T1 Tim-3. However, the Y256/263F double point mutant of Tim-3 significantly, but not completely, lost this function. Although the T1 truncation enhanced Tim-3 activity, paradoxically, when the Y256/263F double mutation was combined with T1 truncation I observed a loss of Tim-3 function, which was more severe effect than seen with the full-length Y256/Y263F double mutation (Figure 6B). We also examined the modulation of NF-κB luciferase reporter activity by Tim-3 expression in Jurkat T cells, and found it to be very similar to the results obtained with an NFAT/AP-1 reporter (Figure 7C).

Consistently, we observed the same effects of expression of each Tim-3 construct in a both NFAT/AP-1 and NF-κB reporters in non-transformed D10 Th2 T cell clones (Figure 7B and D). In addition, although we observed similar modulation of basal NFAT/AP-1 and NF-κB reporter activity by WT and Tim-3 mutants in Jurkat T cells, in the absence of TCR/CD28 stimulation, the extent of modulation by Tim-3 constructs was profoundly different in the presence of TCR stimulation. (i.e. co-stimulation). These results suggest that the two tyrosine residues Y256 and Y263 are responsible for Tim-3 function to enhance NFAT/AP-1 and NF-κB activation, by amplifying TCR signaling, and that Tim-3 signaling converges with TCR signaling via common signaling pathways upstream of NFAT/AP-1 and NF-κB.

I tested whether the conserved region near Y256 and Y263 and C-terminus region of Tim-3 cytosolic tail are sufficient for Tim-3 function. I generated another Tim-3 mutant lacks the region between transmembrane domain and conserved region (Δ216-244, Figure 5) and tested its effect to NFAT/AP-1 reporter activity in D10 T cells. Δ216-244 deletion of Tim-3 led to

complete loss of the positive Tim-3 function equivalent to T2 deletion and Δcyto deletion, which

lacks most of the cytosolic tail of Tim-3 (Figure 8).

A

```
         218                                              T2          T1
                                                         ↓256   263  ↓  271,2,4
mTim-3 WYSCKKKKLSSLSLITLANLPPGGLANAGAVRI RSEENIYTIEE NVYEVENSNEYYCYVNS-QQPS
hTim-3 WYSHSKEKIQNLSLISLANLPPSGLANAVAEGI RSEENIYTIEE NVYEVEEPNEYYCYVSSRQQPSQPLGCRFAMP
mTim-1 YILMKR-KSASLSVVAFRVSKIEALQNAAVVHSRAEDNIYIVEDRP
hTim-1 KYFFKK-EVQQLS-VSFSSLQIKALQNAVEKEVQAEDNIYIENSLYATD
```

B

Site	Sequence	Score
Y256	EENIYTIEE	0.95
Y263	EENVYEVEN	0.957
Y272	SNEYYCYVN	0.977
Y274	EYYCYVNSQ	0.611

Site	Sequence	Score
S226	KKKLSSLSL	0.783
S251	VRIRSEENI	0.992

C

Site	Sequence	Score	Kinase
Y274	EVENSNEYYCYVNSQ	0.4164	EGFR kinase
Y263	YTIEENVYEVENSNE	0.4757	Src kinase
Y256	IRSEENIYTIEENVY	0.525	Src kinase

Figure 4. Sequence of Tim-1 and Tim-3 cytosolic tails and prediction of phosphorylation motifs

(A) Sequence of Tim-1 and Tim-3 cytosolic tails. (B) phosphorylation sites were predicted with NetPhos (http://www.cbs.dtu.dk/services/NetPhos/) (C) kinase-specific prediction of phosphorylation sites was done with NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/)
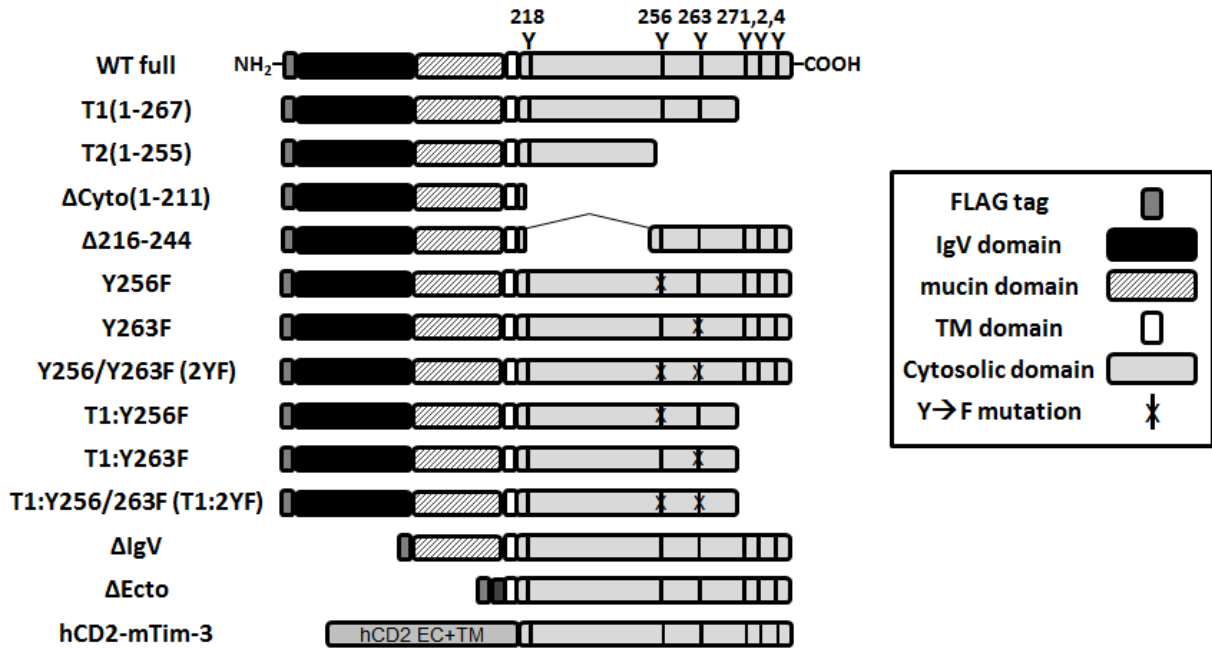
43

Figure 5. Schematic diagram of Flag-tagged Tim-3 and mutant Tim-3 constructs
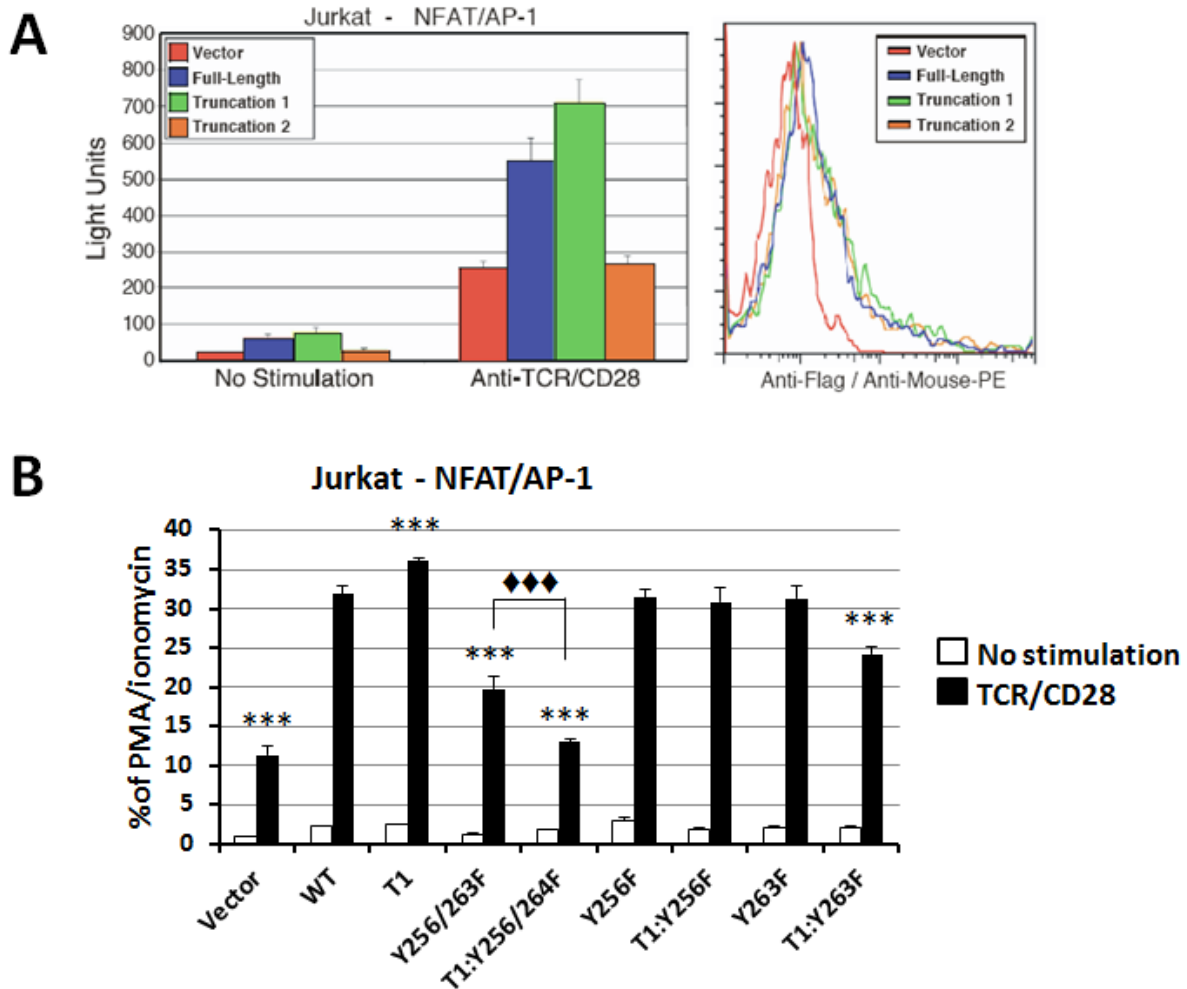
Figure 6. Modulation of NFAT/AP-1 reporter activity by WT and mutant Tim-3 expression in Jurkat T cells

(A) Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter, plus the indicated Tim-3 constructs. The next day, cells were left unstimulated or stimulated with anti-TCR/CD28 mAb's. Luciferase activity is presented as relative light unit (A) and the percentage of the maximal response, obtained with PMA/ionomycin stimulation (B). (A, right panel) Surface expression of the indicated Tim-3 constructs was analyzed by anti-Flag staining and flow cytometry.   Average results (± SD) from triplicates are shown (P-value vs. WT: NS, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.01) (♦♦♦, P<0.001)
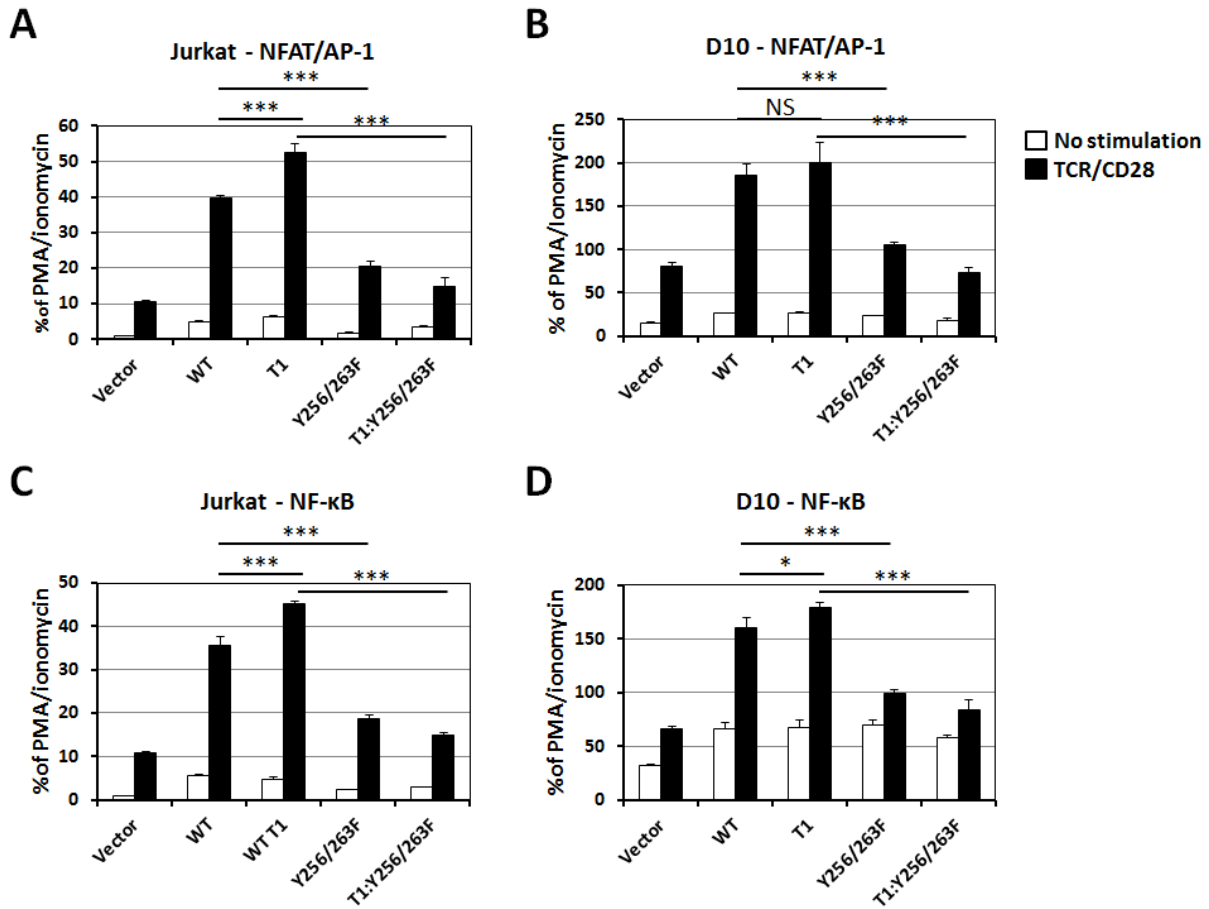
Figure 7. Requirement of Y256 and Y263 of Tim-3 to potentiate TCR signaling

(A, C) Jurkat T cells were transfected with an NFAT/AP-1 (A) or NF-κB (C) luciferase reporter, plus the indicated Tim-3 constructs. (B, D) D10 T cells were also transfected with an NFAT/AP-1 (B) or NF-κB (D) reporter and the indicated Tim-3 constructs. The next day, cells were left unstimulated (white bars) or stimulated with anti-TCR/CD28 mAb's (black bars). Luciferase activity is presented as the percentage of the maximal response, obtained with PMA plus ionomycin. Average results ($\pm$ SD) from triplicates are shown (*, P<0.05; **, P<0.01; ***, P<0.01)
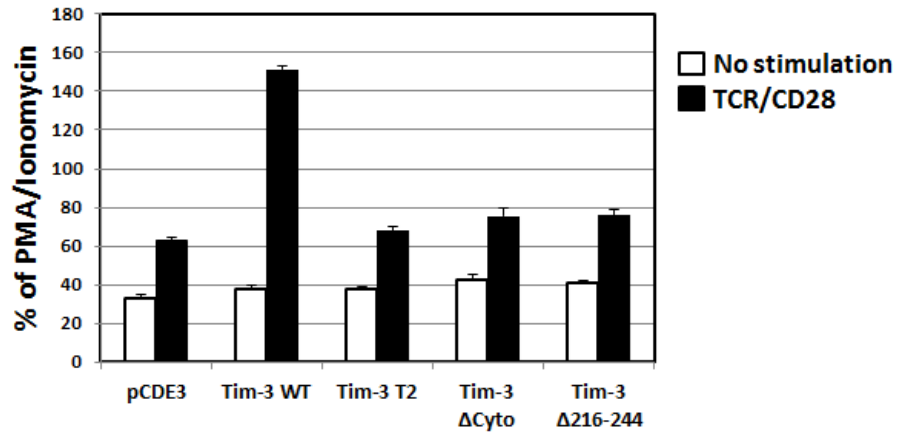
Figure 8. Requirement of region between transmembrane domain and the conserved domain

D10 T cells were also transfected with an NFAT/AP-1 reporter and the indicated Tim-3 constructs. The next day, cells were left unstimulated (white bars) or stimulated with anti-TCR/CD28 mAb's (black bars). Luciferase activity is presented as the percentage of the maximal response, obtained with PMA plus ionomycin. Average results (± SD) from triplicates are shown.

2.3.2 Tim-3 induces AP-1 reporter activity independently of TCR signaling.

Both NFAT and AP-1 are downstream of TCR signaling, NFAT is regulated by the Calcium signaling pathway and AP-1 is regulated by MAP kinase pathway. To further dissect the signaling pathways downstream Tim-3, I examined the role of Tim-3 in activation, of NFAT and AP-1 separately. I performed luciferase reporter assays for NFAT and AP-1 as described above. The results from NFAT reporter assays were similar to NFAT/AP-1 and NF-κB reporter assays. That is, ectopic expression of WT and T1 Tim-3 were capable to augment NFAT reporter activity in Jurkat T cells, while T2 was not (Figure 9A and B).

Interestingly, WT Tim-3 expression in Jurkat T cells led to upregulation of AP-1 reporter activity independently of TCR/CD28 stimulation (Figure 9C and D). T1 Tim-3 was at least as efficient as WT Tim-3 in enhancing NFAT activity, but T1 Tim-3 was less potent in enhancement of AP-1 reporter activation compared to WT Tim-3. These data suggest that the region near Y256/263 of cytosolic tail of Tim-3 mediate the enhancement of calcium-NFAT pathway in a TCR stimulation-dependent manner and that the C-terminal region of the cytosolic tail of Tim-3 containing Y271/272/264 partially mediate the enhancement of MAP kinase-AP-1 pathway in a TCR dependent manner.
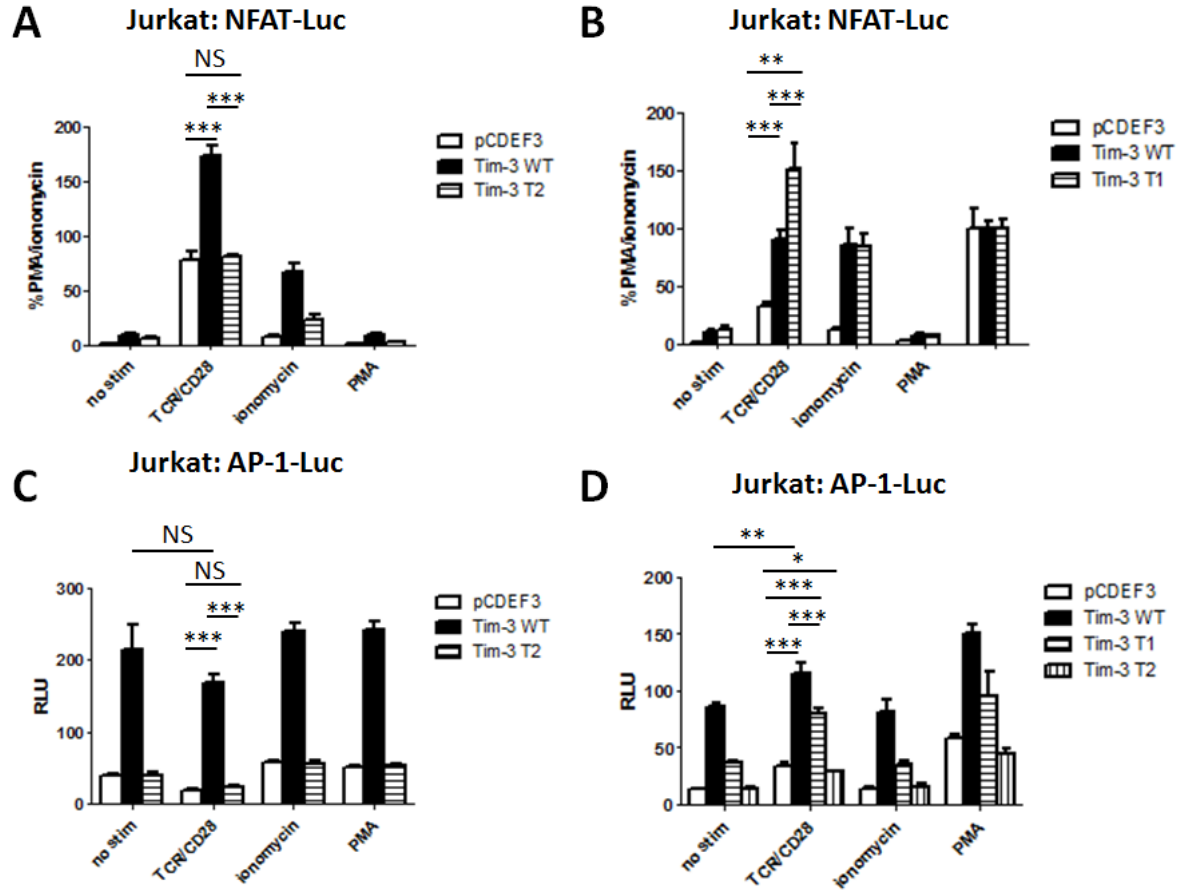
Figure **9. Differential activation of NFAT and AP-1 reporter activity by Tim-3**

(A, B) Jurkat T cells were transfected with an NFAT and plus the indicated Tim-3 constructs. (C, D) Jurkat T cells were transfected with an AP-1 and plus the indicated Tim-3 constructs. The cells were stimulated anti-TCR/CD28 antibodies, PMA or ionomycin. Luciferase activity is presented as the percentage of the maximal response, obtained with PMA/ionomycin (A, B) or relative light unit (C, D). Average results (± SD) from triplicates are shown (*, P<0.05; **, P<0.01; ***, P<0.01)

2.3.3 Cytosolic tail of Tim-3 is sufficient to induce Tim-3 signaling

We demonstrated above that the cytosolic tail of Tim-3 mediated signal transduction to modulate TCR signaling. I next assessed if expression of the cytosolic tail is sufficient for Tim-3 signaling. I generated Flag-tagged Tim-3 constructs lacking IgV domain (ΔIgV), and most of the ectodomain (Δecto). Despite the deletion, both ΔIgV and Δecto Tim-3 constructs retained the ability to enhance TCR signaling, to activate NFAT/AP1 (<u>Figure</u> 10A). When I crosslinked the surface-expressed Tim-3 using anti-Flag-antibody, this did not alter the function of WT, but a slight enhancement in function was observed with the Tim-3 ΔIgV construct. However, this crosslinking effect was not seen with the Tim-3 Δecto construct. Expression of a chimeric protein consisting of the echo-and transmembrane domains of an unrelated protein CD2 and the Tim-3 cytosolic tail did upregulate NFAT/AP-1 reporter activity in a dose-dependent manner, suggesting that ectopic expression of the Tim-3 cytosolic tail in Jurkat T cells is sufficient to initiate Tim-3 signaling (Figure 10B).
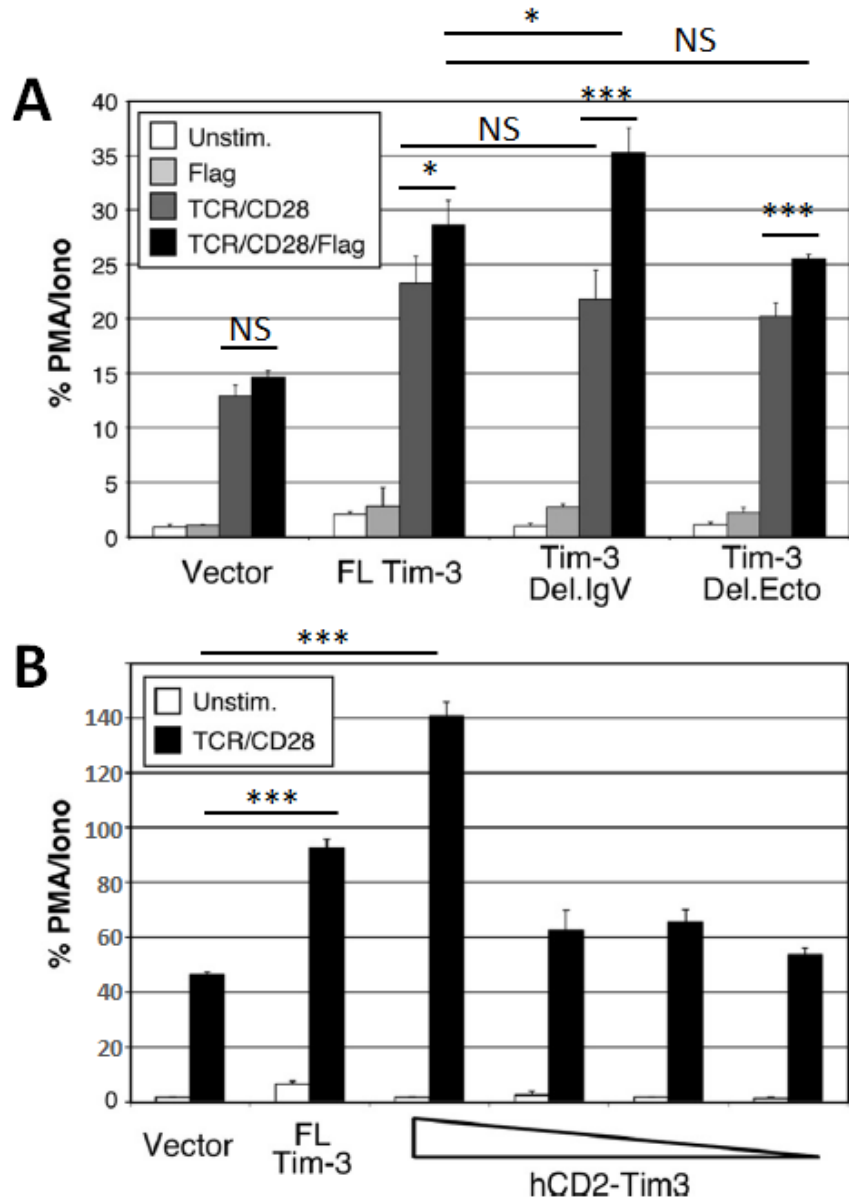
Figure 10. Role of Tim-3 cytosolic tail in Tim-3 signaling function.

(A) Effect of IgV and ectodomain deletions on Tim-3 co-stimulation of NFAT/AP-1. (B) Co-stimulation of NFAT/AP-1 activation by a CD2-Tim3 chimera. Jurkat T cells were transfected with an NFAT/AP-1 reporter and the indicated constructs and then stimulated as indicated before determination of luciferase activity.). Average results ($\pm$ SD) from triplicates are shown (*, P<0.05; **, P<0.01; ***, P<0.01)

2.3.4 Tyrosine 256 and 263 are available for tyrosine phosphorylation

To address how tyrosine phosphorylation might mediate Tim-3 signaling, I next examined the tyrosine phosphorylation of the Tim-3 cytosolic tail. I stimulated 293T cells transiently expressing Flag-tagged WT, T1 and T2 Tim-3 proteins and immunoprecipitated (IP'd) with anti-Flag agarose. After SDS-PAGE and western blotting, the IP'd Tim-3 proteins were probed with anti-phosphotyrosine antibody and anti-Tim-3 antibody. Tim-3 proteins appear as large smear over 50kDa (glycosylated) and a condensed band under 40kDa (unglycosylated). Phosphorylation of WT Tim-3 was induced by pervanadate treatment and the phosphorylation was partially diminished in T1 Tim-3 and completely abolished in T2 Tim-3 (Figure 11A).

To focus on the phosphorylation of Y256 and Y263, I assessed the phosphorylation of point mutants of Tim-3 in the T1 deletion background in 293T cell. Again, I observed pervanadate-induced tyrosine phosphorylation of T1 Tim-3. The Y256F mutation, but not Y263F mutation, resulted in drastic loss of tyrosine phosphorylation of T1 Tim-3, indicating that Y256 may be the major phosphorylation site for Tim-3. However, considering the residual phosphorylation in T1:Y256 mutant and complete loss of the phosphorylation in T1:Y256/263F (2YF), I conclude that Y263 is still available for phosphorylation (Figure 11B).

Finally, I investigated tyrosine phosphorylation in Jurkat T cells, which does not express endogenous Tim-3. For these experiments, I generated Jurkat T cells in which expression of Tim-3 is inducibly driven by a Tet-on transactivator (Tet-on Tim-3 Jurkat cell). The Tet-on Tim-3 Jurkat T cells were treated with doxycycline to induce the Tim-3 expression and stimulated with anti-TCR and CD28 crosslinking antibody. IP'd Tim-3 was incubated with PNGaseF to remove glycosylation, then probed with antibody to phosphotyrosine and Tim-3. I observed that tyrosine residue(s) in Tim-3 were basally phosphorylated in the unstimulated Tet-on Tim-3

Jurkat T cells, and the tyrosine phosphorylation was modestly upregulated by crosslinking of

TCR and CD28. Here I demonstrated that tyrosine residues, especially Y256 and Y263 were

available for phosphorylation, and tyrosine residues of Tim-3 were basally, and also inducibly,

phosphorylated in Jurkat T cells (Figure 11C).
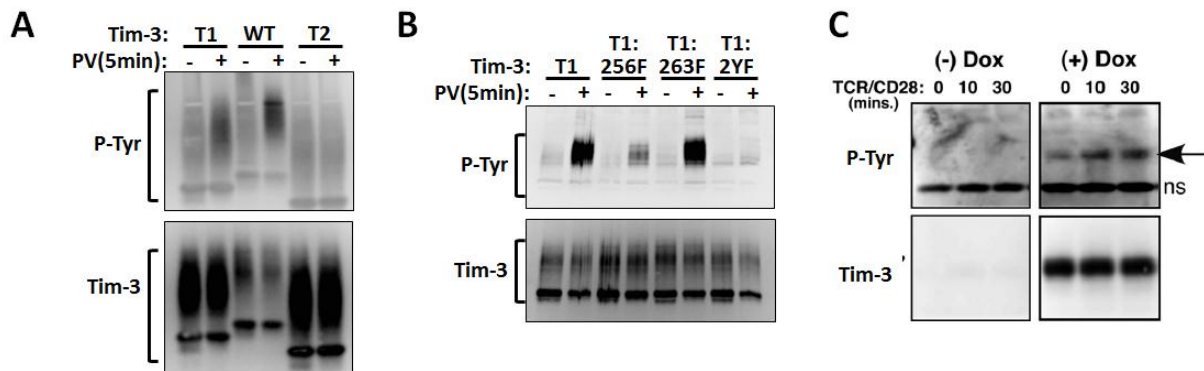
Figure 11. Tyrosine phosphorylation of Tim-3 cytosolic tail

(A and B) 293T cells were transfected with the indicated Flag-tagged Tim-3 constructs and then split and left unstimulated or treated with pervanadate. Lysates were prepared and subjected to IP with an anti-Flag antibody, followed by SDS-PAGE and Western blotting. Blots were first probed for Tim-3 (lower panel) and then stripped and reprobed with an anti-phosphotyrosine antibody (upper panel). (C) Parental Jurkat T cells or Tet-on Tim-3 Jurkat T cells were stimulated as indicated and prepared for IP and Western blotting as described above. After IP, samples were treated with PNGaseF F prior to SDS-PAGE

2.4 DISCUSSION

Studies of Tim-3 signaling have been very limited and the downstream targets of Tim-3 remained unknown. Cross-linking of Tim-3 with a monoclonal agonistic antibody (5D12) induced phosphorylation of ERK in a Th1 T cell clone AE7 and in a D2SC1 dendritic cell line [57]. However, since Tim-3 is known as a negative regulator of Th1 and CD8 T cell responses, it was expected that Tim-3 signaling would play a suppressive role in T cell signaling. Surprisingly, we found that ectopic expression of Tim-3 upregulated activation of major transcription factors involves in T cell function, like NFAT, AP-1, and NF-κB not only in Jurkat T cells but also in D10 a non-transformed T cell clone. Tim-3 expression could enhance basal NFAT, NFAT/AP-1 and NF-κB reporter activities at low levels in the absence of TCR/CD28 stimulation, but the extent of the enhancement of the reporter activities was dramatically increased in the presence of TCR/CD28 stimulation. T1 constructs were more potent in enhancement of NFAT/AP-1, NFAT and NF-κB reporter activity, suggesting that the C-terminus region, containing three tyrosine residues, has an element which is involved in inhibition of Tim-3 function. However, as somewhat paradoxically, the T1:Y256/Y263 mutation led to severe ablation of Tim-3 function. Unlike other reporters we tested, activation of a pure AP-1 reporter by Tim-3 did not require the TCR/CD28 stimulation. In addition T1 deletion resulted in decreased Tim-3 function to upregulate AP-1 reporter activity. Thus, we hypothesize that the conserved region around Y256 and Y263 recruits one or more signaling molecules intersecting downstream of the TCR pathway, and the C-terminal region of Tim-3 mediates signal transduction to a MAP kinase pathway bypassing TCR signaling (Figure 12).

For the positive function of Tim-3, two tyrosine residues two tyrosine residues, Y256 and Y263, surrounded by a conserved sequence, were required and the two tyrosine residues were available for phosphorylation. These results suggest that phosphorylation of Y256 and Y263 couples Tim-3 to TCR signaling pathways and Tim-3 signaling intersects to the common upstream of NFAT and NF-κB pathway (Figure 12).

Note that Tim-3 expression alone was sufficient to initiate Tim-3 signaling. One possibility is that a Tim-3 ligand is expressed on Jurkat T cells. Galectin-9 expression is detectable in resting Jurkat T cells and upregulated by PMA stimulation [178] and exposure to PS on the surface of dead cells after transient transfection is expected. However, I observed that expression of Tim-3 constructs lacking the IgV domain or ectodomain could also initiate the activating signaling. Due to excess number of molecules on the surface, formation of Tim-3 complexes may occur in the transient overexpression conditions bypassing the requirement of ligand. Another possibility is that Tim-3 has dual function. Tim-3 may induce activating signals in the presence of ligand but inhibitory signals in the absence of ligand. We observed when Tim-3 was retro-virally expressed in primary murine CD4 T cells, augmentation of IFN-γ production by Tim-3 was inhibited by agonistic Tim-3 antibody (5D12) [179]. I attempted to crosslink of Flag-tagged Tim-3 in Jurkat T cells, but the crosslinking of Tim-3 constructs with anti-Flag mAb failed to enhance or repress the positive function of full-length and Δecto Tim-3 constructs, rather, crosslinking slightly enhanced the function of ΔIgV domain Tim-3 constructs, while IgV domain interacts with the known ligands. These results might imply that the IgV domain might inhibit multimerization of Tim-3 or association of Tim-3 with other surface molecules or that Tim-3 ligand binding to IgV domain is involved in the inhibitory signal.

Next I determined that the two tyrosine residues (Y256 and Y263) responsible for positive Tim-3 function were available for tyrosine phosphorylation and that tyrosine phosphorylation of Tim-3 is inducible by TCR/CD28 stimulation in Jurkat T cells. These observations support the model that tyrosine phosphorylation of its cytosolic tail couples Tim-3 to enhancement of T cell activation. Interestingly, Tim-3 was basally phosphorylated in resting Jurkat T cells and the extent of the TCR-inducible phosphorylation was relatively moderate, while the impact of Tim-3 expression on the activation of TCR signaling was more profound. Thus, there might be other mechanisms in addition to tyrosine phosphorylation that regulate the Tim-3 function, like its localization or the regulation of molecules recruited to Tim-3.
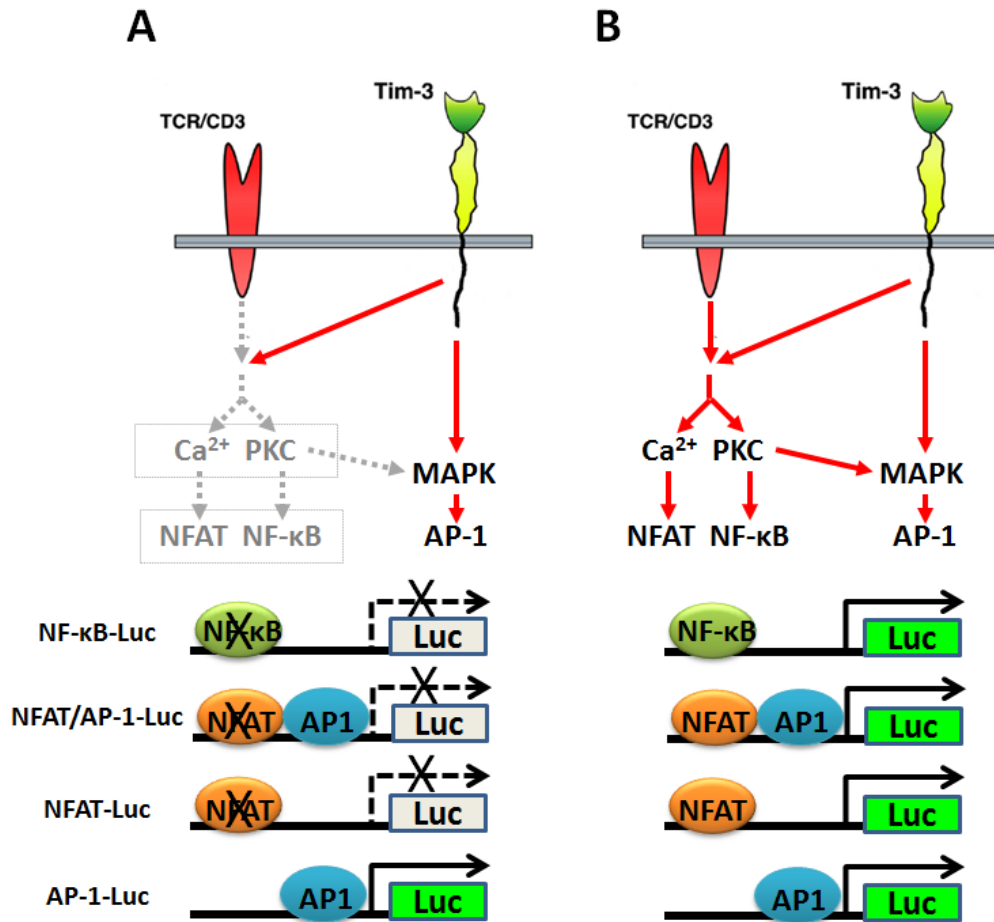
Figure 12. Model of activation of transcription factors downstream TCR signaling

Augmentation of NF-κB and NFAT activity by Tim-3 signaling requires activation of proximal TCR signaling, while augmentation of AP-1 activity by Tim-3 is TCR-independent. (A) In the absence of TCR signaling, Tim-3 signaling upregulates AP-1 reporter activity is upregulated by Tim-3, but cannot upregulated NF-κB, NFAT/AP-1 and NFAT reporter activity. (B) In the presence of TCR signaling,, Tim-3 signaling enhances NF-κB, NFAT, and NFAT/AP-1 activity in a TCR signaling dependent manner and AP-1 reporter activity in both TCR-dependent and –independent manner.

3.0   IDENTIFICATION OF TIM-3 INTERACTING MOLECULES

3.1 INTRODUCTION

Phosphorylation of amino acid residues with side chains containing hydroxyl groups is one of the most common post-translational modifications regulating cell signaling. Phosphorylation can turn "on" or "off" the activity of proteins by changing the conformation of a proteins or creating a binding site for a protein-protein interaction. In antigen receptor signal transduction in immune cells, including T cells, tyrosine phosphorylation plays a key part. The signature motifs for tyrosine phosphorylation called immunoreceptor tyrosine-based activation motif (ITAM, $YxxL/Ix_{(6-8)}YxxL/I$) and immunoreceptor tyrosine-based inhibitory motif (ITIM, $S/I/V/LxYxxI/V/L$) are found in many activating or inhibitory receptors. For example, CD3-ζ of TCR contains three ITAM motifs [128]. Engagement of MHC molecules loaded with specific antigen induces the tyrosine phosphorylation of the ITAM motifs by SFK Lck or Fyn [134, 180]. Then the Syk family kinase ZAP-70 is recruited to the phosphotyrosine residues, leading to activation of TCR signaling, in which, the SH2 domains in ZAP-70 mediate the binding to phosphotyrosine [181]. The SH2 domain is a specialized domain that interacts with phosphorylated tyrosine, which is found in many kinases and adaptor proteins [182]. SH2 domain mediates inter-protein interactions to regulate the localization of proteins, and also mediate intra-protein interactions to regulate conformation of proteins. Although, Tim-3 does not

have a typical ITAM or ITIM motif, it has several putative tyrosine phosphorylation sites. Y276 of Tim-1, which is similar in context to Y256 of Tim-3, is available for tyrosine phosphorylation, and its phosphorylation mediates the recruitment of PI3 kinase [17]. Therefore, we hypothesized that phosphorylation of tyrosine residues in the cytosolic tail of Tim-3 recruits signaling molecules with SH2 domains.

In this study, we identify signaling molecules interacting with phosphorylated tyrosines of Tim-3 via SH2 domains, and a kinase responsible for tyrosine phosphorylation of Tim-3.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines and cell culture

293T cells were cultured in DMEM (Mediatech) supplemented with 10% bovine growth serum (BGS, Hyclone) and 100U/ml penicillin, 100μg/ml streptomycin. Parental Jurkat T cells and Jurkat variants were cultured in RPMI-1640 (Mediatech) supplemented with 5% bovine growth serum (BGS, Hyclone) and 100U/ml penicillin, 100μg/ml streptomycin.

3.2.2 Antibodies and reagents

C305 antibody to Jurkat TCR was obtained from Dr. Arthur Weiss (UCSF, CA), human anti-CD28 were from Life Sciences. Anti-Flag M2 antibody, anti-Flag M2 agarose, and β-actin antibody were from Sigma Aldrich. Polyclonal anti-mouse Tim-3 was from R&D systems. Anti-Fyn and p85 antibodies were from Millipore. HRP conjugated goat anti-mouse antibody was

from Thermo scientific, and donkey anti-goat, donkey anti-rabbit antibody were from Jackson Immunoreserach respectively. HRP conjugated Protein A was from GE. PMA and ionomycin were from Calbiochem and D-luciferin was from Thermo scientific.

3.2.3 SH2 domain array

Membranes with 38 recombinant human SH2 domains were purchased Panomics (Santa Clara, CA) and blotted following the manufacturer's instruction. Biotinylated synthetic Tim-3 peptide was generated at the University of Pittsburgh peptide Synthesis core facility (biotin-SEENIpYTIEENVpYEVENSN) and openbiosystems (biotin-EVENSNEpYpYCpYVNSQQPS). Tim-3 peptides were incubated with the membrane at 0.33μg/ml with or without 200mM phenyl phosphate.

3.2.4 Co-immunoprecipitation and western blotting

293T cells transfected with Flag-Tim-3 constructs, Jurkat T cells stably expressing Tim-3, and JHM1 cells, a Jurkat variant expressing muscarinic receptor 1, transiently transfected with Tim-3 constructs were stimulate with pervanadate or monoclonal antibody specific for Jurkat TCR (C305.2), then lysed with ice-cold 1% NP-40 lysis buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and β-glycerophosphate. Lysates were then incubated on ice for 10 min before being cleared by centrifugation and supernatant were incubated with anti-Flag M2 agarose for 4hr or overnight. After Agarose beads were washed with lysis buffer supplemented with protease inhibitor and phosphatase inhibitor, 2X Sample buffer (Bio-Rad, Hercules, CA) was added to the

beads and incubated at 37'C for 10min to elute the capture protein. The supernatant was subjected onto 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were probe with Fyn or p85 antibodies and detected with HRP conjugated protein A

3.2.5 NMR spectroscopy

Fyn-SH2 domain was expressed as N-Term-GST fusion protein in BL21 cells in ^{15}N minimal media, with 18hrs induction at 17'C with Isopropyl β-D-1-thiogalactopyranoside(IPTG). The protein is purified on GSH column, following which the GST-tag is cleaved by thrombin cleavage at RT. The Fyn-SH2 protein is further enriched by size exclusion chromatography to >95% purity (50mM Tris pH8.0, 150mM NaCl, 2mM DTT, 0.02% NaN$_3$). Purified Fyn was concentrated and dialyzed against the NMR buffer (50mM Na$_2$HPO$_4$ pH 6.5, 5mM DTT). Unphosphorylated and phosphorylated Tim-3 peptides (SEENIYTIEENVYEVENSN and SEENIpYTIEENVpYEVENSN) were generated by OpenBiosystems (Huntsville, AL). ^{15}N HSQC of the purified Fyn SH2 domain (200uM) alone or with either unphosphorylated Tim-3 peptide(1.2mM) or phosphorylated Tim-3 peptide (1.12mM) were analyzed as previously described [183].

3.2.6 Kinase assay

Flag-tagged full-length Tim-3 was expressed from 293T cells, IP'd with anti-Flag M2 agarose, and eluted with Flag peptide. Purified Tim-3 was mixed with recombinant active Fyn (gifted from Dr. Smithgall, University of Pittsburgh) in kinase buffer (5mM morpholinepropanesulfonic

acid (MOPS), pH 7.2, 2.5mM glycerol 2-phosphate, 4mM MgCl2, 5mM MnCl2, 1mM EGTA, 0.4mM EDTA, 0.25mm dithiothreitol(DTT), 20μM ^{32}P-ATP (200 μCi/ml). Reactions were carried out at 30'C and terminated by adding 2X SDS sample buffer. Samples were separated by 10% SDS-PAGE and blotted to PVDF. Integration of ^{32}P-ATP was detected by autoradiograph, then, Tim-3 was probed by immunoblotting with anti-Tim-3 antibody.

3.3 RESULTS

3.3.1 The tyrosine residues in Tim-3 cytosolic tail interacts with SH2 domains of signaling molecules

Having identified tyrosine residues involved in Tim-3 function and showing that they were available for phosphorylation, we hypothesized that signaling proteins containing SH2 domains are recruited to the phosphotyrosine sites of the Tim-3 cytosolic tail. To screen for signaling molecules interacting with phosphotyrosine in the cytosolic tail of Tim-3, we performed an SH2 domain array experiment with two synthetic biotinylated peptides: a tyrosine phosphorylated peptide containing the conserved region with Y256 and Y263 (pY256/263 Tim-3 peptide), and one containing C-terminus region containing three Y271, Y272 and Y274 (pY271/272/274 Tim-3 peptide) (Figure 13A and B).

The synthetic pY256/263 Tim-3 peptide strongly bound to SH2 domains of Fyn, the regulatory subunit of phosphoinositol-3 kinase (PI3K) p85α and p85β and RasGAP (N-term). The pY256/263 Tim-3 peptide also weakly interacted with SH2 domains of SFKs Hck, Yes, and with those of PLC-γ1, and the other SH2 domain of RasGAP (C-term). The pY271/272/274

Tim-3 peptide strongly interacted with SH2 domains of Fyn, p85α, p85β, and PLC-γ1, and weakly interacted with SH2 domains from Abl, Hck, Yes, RasGAP, GRAP, and Grb2 (Figure 13C and D, upper panels). When 10^6 molar excess of phenylphosphate, an analogue of phosphorylated side chain of tyrosine, was incubated together with the phosphorylated Tim-3 peptides to compete for phosphotyrosine specific interaction, interaction of the both synthetic Tim-3 peptides to most of the SH2 domains on the membrane was drastically blocked. In contrast to other SH2 domains, the Fyn SH2 domain interaction with the Tim-3 peptides remained strong in the presence of phenyl phosphate competition (Figure 13C and D, lower panels). This suggests that phosphorylation of tyrosine residues in the cytosolic tail of Tim-3 mediates recruitment of signaling proteins to Tim-3 via SH2 domain, but that Fyn may interact with Tim-3 independently of tyrosine phosphorylation.
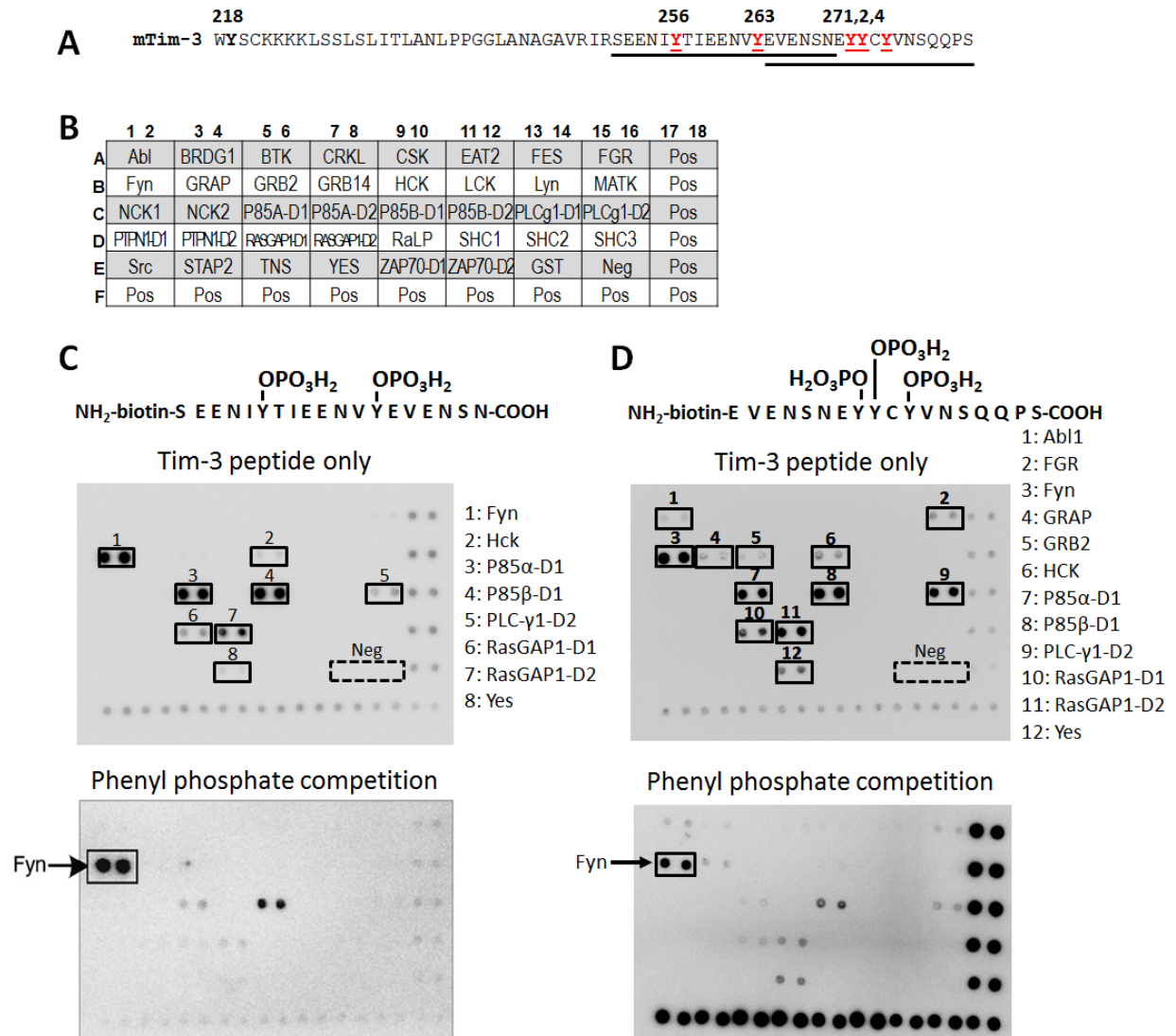
Figure 13. SH2 domain binding to phosphorylated Tim-3 cytoplasmic tail peptides

(A) Sequence of Tim-3 cytosolic tail and location of sequences corresponding to the synthetic Tim-3 peptides. (B) Layouts of the SH2 domain array. SH2 domains are present as duplicated spots. For proteins with more than one SH2 domain, "D1" indicates the more N-terminal SH2. (C, D) Sequence of the synthetic peptide used to determine the SH2 specificity of phospho-tyrosine residues and results of the SH2 domain array screen. Peptide binding to SH2 domains was detected by streptavidin-HRP.

3.3.2 Tim-3 is co-IP'd with p85 subunit of PI3kinase and Fyn

According to the SH2 domain array experiment, the SH2 domains of Fyn and p85 interacted with both pY256/263 Tim-3 peptide and pY271/272/274 Tim-3 peptide. Next, we confirmed the interaction of Tim-3 with p85 and Fyn by co-IP. To test tyrosine phosphorylation dependent interaction of endogenous p85 and Tim-3, JHM1, a Jurkat variant expressing human muscarinic receptor1, cells transfected with WT Flag-Tim-3, Flag-Tim-3 Y256/263F (2YF), or T2 truncation Tim-3 constructs were stimulated with pervanadate, to induce robust tyrosine phosphorylation. The WT and the mutant Tim-3 proteins were then IP'd with anti-Flag agarose. p85 bound to WT Tim-3, but p85 binding to 2YF or T2 Tim-3 protein was drastically diminished (Figure 14). These results indicate that p85 recruitment to the Tim-3 cytosolic tail is dependent on the phosphorylation of tyrosine residues in the cytosolic tail of Tim-3.

Fyn was the candidate whose SH2 domain on the array membrane most strongly interacted with Tim-3 phosphopeptides (Figure 13C and D). To confirm the Fyn interaction to Tim-3, WT Tim-3 or mutant Tim-3 was co-expressed with Fyn in 293T cells and co-IP'd Fyn was probed. Fyn associated with both WT and the 2YF mutant of Tim-3 to a similar extent, and induction of Tim-3 phosphorylation with pervanadate treatment did not detectably alter the Tim-3-Fyn interaction (Figure 15B). I observed that tyrosine phosphorylation of Tim-3 was modestly upreguated upon TCR stimulation in Jurkat T cells (Figure 11C), so I decided to examine whether TCR stimulation can affect Fyn-Tim-3 interaction in Tim-3 expressing Jurkat T cells. Fyn constitutively interacted with Tim-3 in the resting state and there were no apparent changes in the Fyn-Tim-3 interaction after TCR stimulation or pervanadate treatment (Figure 15C). To determine the region of the Tim-3 cytosolic tail that interacts with Fyn, I performed co-IP experiment of Fyn with various Tim-3 deletion mutants. Thus, Fyn association with not only WT

but also T1 truncation Tim-3 indicates that Fyn binds to the conserved region of the cytosolic tail of Tim-3 containing Y256 and Y263. Unexpectedly, we also observed Fyn bound to the T2 mutant of Tim-3 even though the T2 deletion construct does not have any known motif interacting with SFKs such as a tyrosine phosphorylation sites or a proline rich motif (Figure 15D). Although T2 deletion constructs still has a single remaining tyrosine residue (Y219) in proximity to the predicted transmembrane domain, the residues was not conserved in other Tim family genes, or not predicted as a phosphorylation site. Thus, we assumed that the tyrosine residue is not functional for recruitment of SH2 domain, however, we cannot completely exclude the possibility that the tyrosine residue mediates the interaction with Fyn. Although Δ216-244 Tim-3 construct efficiently bound to Fyn, whose function to upregulate NFAT/AP-1 luciferase reporter activity was impaired (Figure 8), this may suggest that the region located between transmembrane domain and the conserved region surrounding Y256 and Y263 is required to recruit downstream signaling molecules or maintain the functional structure of Tim-3.

*Tim-3-p85 co-immunoprecipitation experiment was done by Ee Wern Su (Lawrence Kane's Lab, University of Pittsburgh)
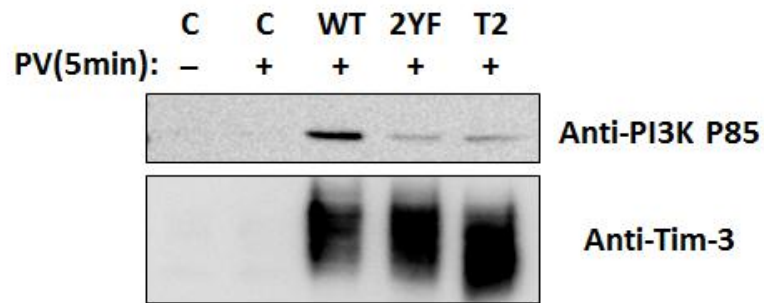
Figure 14. p85 was co-IP'd with Tim-3

JHM1 cells were transfected with wild type or mutant Flag-tagged Tim-3 constructs. The cell were simulated with pervandate for 5min, Tim-3 proteins in the lysates were pulled down with anti-Flag-M2 agarose. The blot was probed with anti-p85.
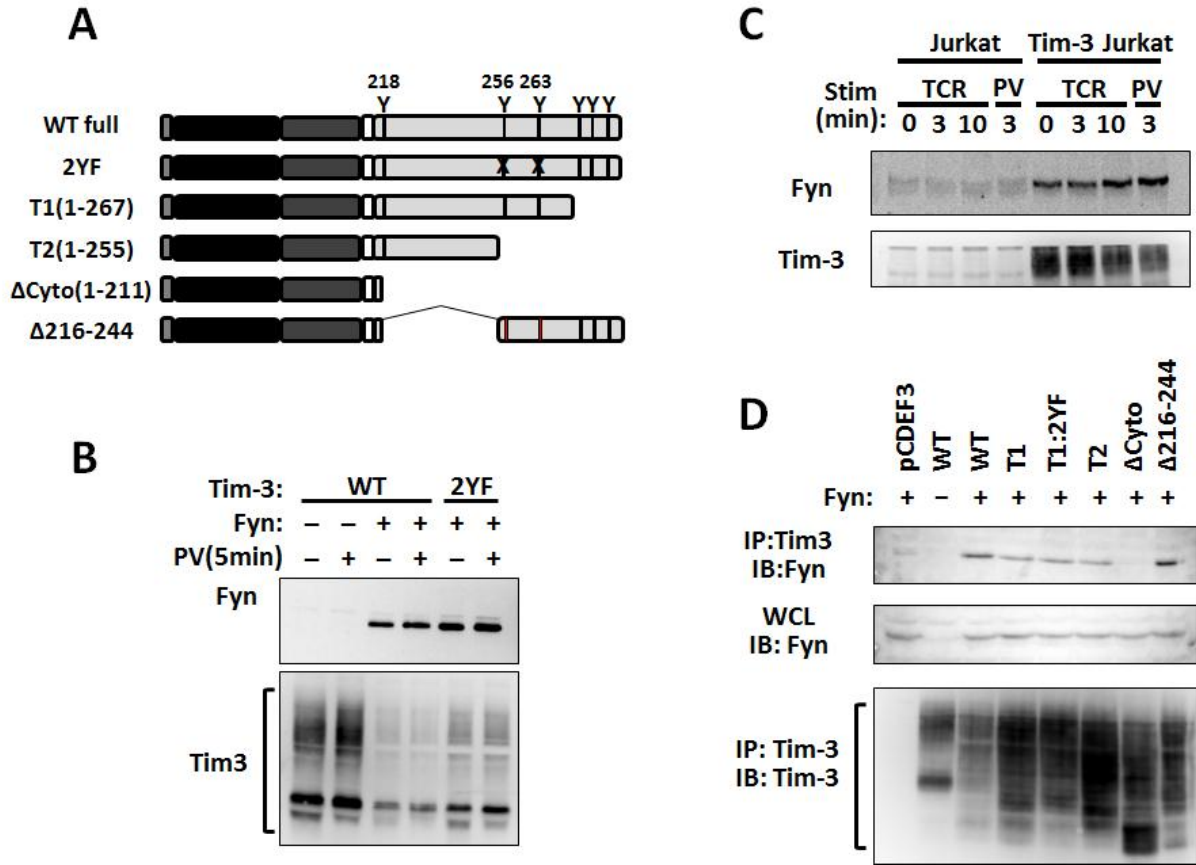
Figure 15. Fyn co-IP'd with Tim-3

(A) Schematic <u>figure</u> of Flag-Tim-3 constructs. (B) 293FT cells were transfected with either Tim-3 constructs or Fyn alone, or both, and stimulated with pervanadate. Flag-Tim-3 was pulled down with anti-Flag-M2 agarose. The blot was probed with anti-Fyn, and anti-Tim-3. (C) Parental Jurkat T cells or Tim-3 stably transfected Jurkat T cells were lysed and Tim-3 and endogenous Fyn were pulled down with anti-Flag-M2 agarose. (D) 293FT cells were transfected with Tim-3 constructs and Fyn. Flag-Tim-3 was pulled down with anti-Flag-M2 agarose. The blot was probed with anti-Fyn, and anti-Tim-3.

3.3.3 The Fyn SH2 domain interacts with both an un-phosphorylated and phosphorylated peptide of Tim-3 corresponding to region around Y256/Y263

To better understand the mode of Tim-3-Fyn SH2 domain interaction, we decided to identify which amino acid residues in Fyn SH2 participate in the interaction with the region around Y256 and Y263 in the cytosolic tail of Tim-3. We analyzed ^{15}N HSQC spectrum of recombinant Fyn SH2 domain with phosphorylated or unphosphorylated Y256/Y263 Tim-3 peptide. The NMR spectroscopy revealed that residues surrounding the conserved pY binding pocket and variable pY$^+$3 binding pocket of Fyn SH2 domain interacted with the phosphorylated Tim-3 peptides. The residues surrounding pY binding pocket of Fyn SH2 domain did not participate in the interaction with the unphosphorylated Tim-3 peptide, but still the residues surrounding the pY$^+$3 binding pocket mediated interaction with the unphosphorylated peptide (Figure 16). Considering that pY pocket binding to phosphotyrosine is the primary force of the interaction mediated by SH2 domain, this binding of unphosphorylated Tim-3 peptide to pY$^+$3 binding pocket appears to be unique and may have implications for regulation of Tim-3 function by Fyn.

*NMR analysis was done by Jagannathan Alagurajan (Amy Andreotti's lab, Iowa State University)
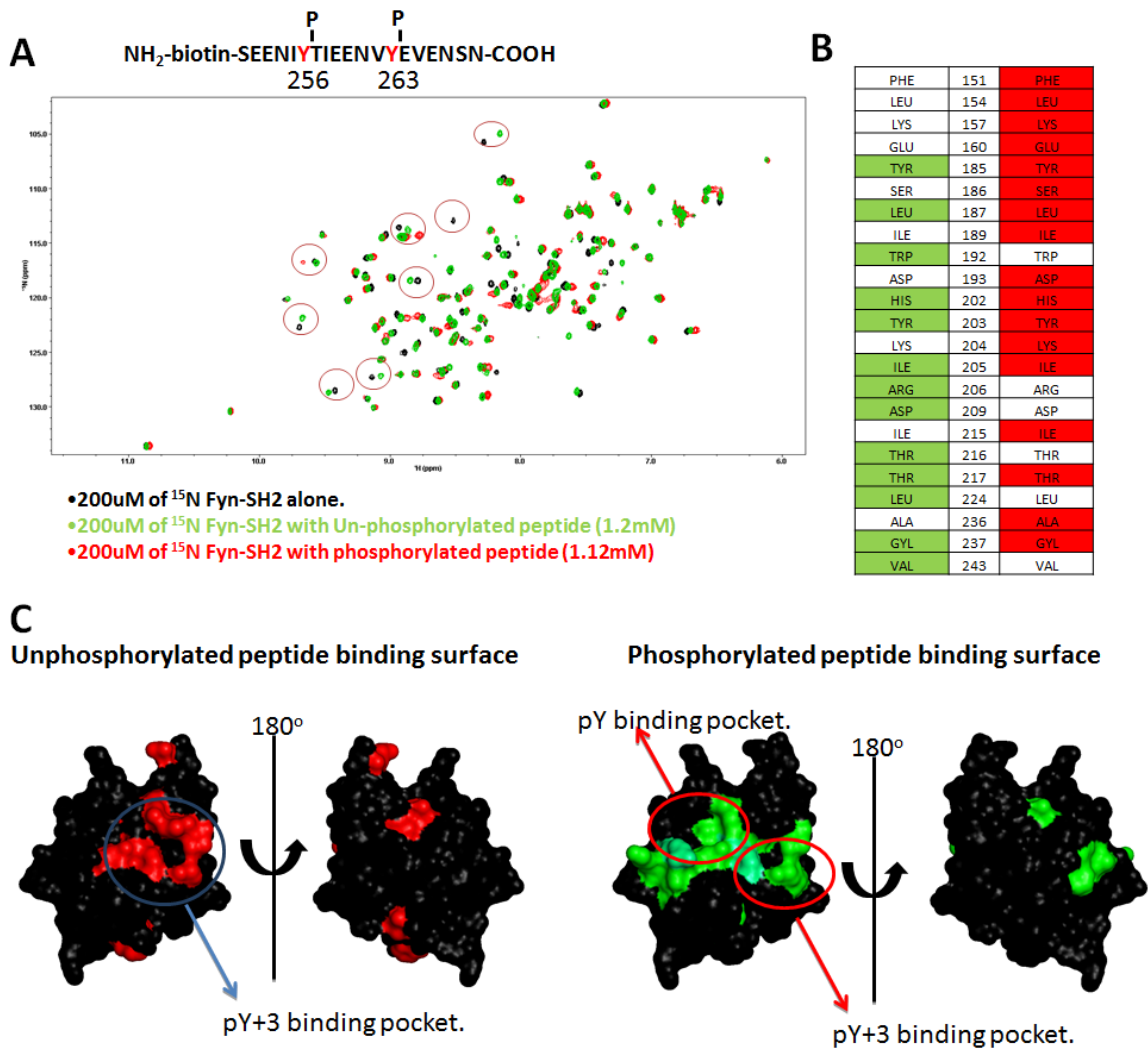
Figure 16. Identification of amino acids in Fyn SH2 interacting with phosphorylated and un-phosphorylated Tim-3 peptides

(A) The ^{15}N HSQC of the Fyn-SH2 alone(black) was compared to the in presence of un-phosphorylated(green) or phosphorylated peptides(red). The HSQC from each of the sample was overlaid to determine the peaks that show shift and thus determine the amino acids that interact with the peptides. (B) List of amino acids in Fyn SH2 domain that interact with un-phosphorylated(green) and phosphorylated(red) Tim-3 peptide. (C) Surface mapping of amino acids in Fyn SH2 domain interacting with un-phosphorylated(red) and phosphorylate(green) Tim-3 peptides.

3.3.4 Fyn directly phosphorylates Tim-3

I showed that tyrosine residues, especially Y256 and Y263, in the Tim-3 cytosolic tail are available for phosphorylation (Figure 11), and Y256 and Y263 were predicted as SFKs phosphorylation sites by the Scansite motif scan program (Figure 4). As shown in Figure 15, I demonstrated a physical interaction between Fyn and cytosolic tail of Tim-3. Therefore I decided to examine whether SFKs can phosphorylate tyrosine residues in the Tim-3 cytosolic tail. To test induction of tyrosine phosphorylation of Tim-3 by SFKs, I transfected 293T cells with full-length WT Tim-3 alone or co-transfected with either Lck or Fyn, then treated with pervanadate. When Tim-3 alone was expressed in 293T cells, tyrosine phosphorylation induced by pervanadate treatment was detectable likely due to endogenous kinases in 293T cells. When Tim-3 was co-expressed with Lck, both basal and pervandate-induced phosphorylation level were significantly increased. Similarly, co-expression of Fyn upregulated tyrosine phosphorylation of Tim-3. However, there was a more profound increase in basal phosphorylation by Fyn compared to basal phosphorylation in the presence of Lck. These results suggest that SFKs Lck and Fyn can induce tyrosine phosphorylation of Tim-3, but that Fyn may be more efficient in phosphorylation of Tim-3, compared with Lck (Figure 17A).

Next, I tested whether Fyn kinase can directly phosphorylate tyrosine residues of Tim-3 by performing *in vitro* kinase assay. IP'd WT Tim-3 and a Tim-3 mutant with all 5 tyrosine residue mutated to phenylalanine (5YF) were incubated with purified active Fyn. WT Tim-3 was efficiently phosphorylated by Fyn, while 5YF Tim-3 was not phosphorylated (Figure 17B). *In vitro* kinase assay using the T1 Tim-3 construct with or without point mutation of Y256 and Y263 (T1 and T1:2YF) showed that T1 Tim-3, but not T1:2YF, was phosphorylated by Fyn

(Figure 17C). These results provide direct evidence that Fyn able to directly phosphorylate Tim-3 at Y256 or Y263, or both.

*The purified active Fyn kinase used for *in vitro* kinase assay was provided by Jamie A. Moroco (Thomas Smithgall's lab, University of Pittsburgh)
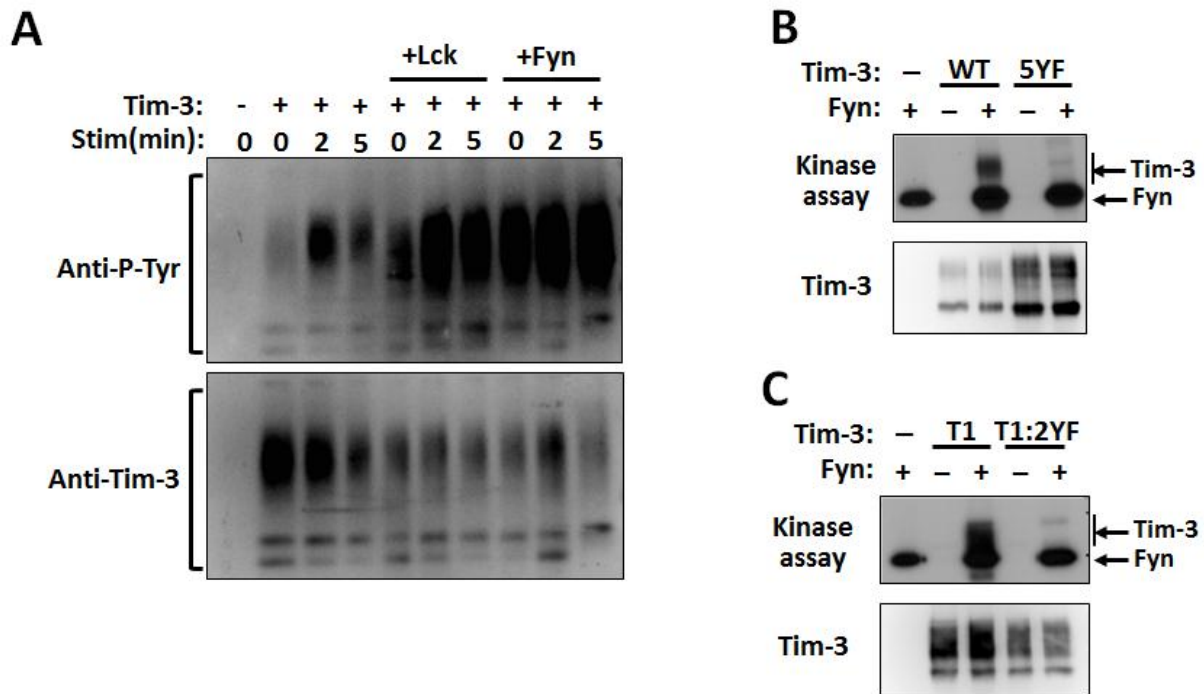
Figure 17. Fyn directly phosphorylated Tim-3

(A) 293T cells were transfected with Flag-tagged wild type Tim-3 or mutant constructs. Flag-mTim-3 protein was IP'd with anti-Flag M2-agarose and blotted with mTim-3 antibody. The blot was stripped and reprobed with anti-phosphotyrosine 4G10 antibody(B,C) wild type or Tim-3 mutant ectopically expressed in 293FT cells were pulled down with anti-Flag-M2 agarose bead and eluted with Flag-peptide. The eluted Tim-3 proteins were incubated with purified active Fyn and ^{32}P-γ-ATP. Phosphorylation was detected by autoradiography (top panel) and wild type and mutant Tim-3 were probed with anti-Tim-3(bottom panel) (5YF: Y256/263/271/272/274F mutant)

3.4 DISCUSSION

SH2 domains are protein modules specialized in recognizing phosphorylated tyrosine residues. Conventional SH2 domains have a conserved pY interacting pocket and a variable pY$^+$3 interacting pocket conferring substrate specificity [182]. Here we identified signaling molecules

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interacting with the Tim-3 cytosolic tail in a tyrosine phosphorylation-dependent manner. An SH2 domain array revealed that signaling molecules involved in TCR signaling, such as p85 PI3K, PLC-γ, and RasGAP may be recruited to the cytosolic tail of Tim-3 via their SH2 domains upon tyrosine phosphorylation of Tim-3. In particular, we demonstrated tyrosine phosphorylation-dependent co-immunoprecipitation of p85 with Tim-3 in Jurkat T cells.

We also found binding of SH2 domain of Fyn to the cytosolic tail of Tim-3. Unlike other candidate molecules we identified from the SH2 domain array, the Fyn interaction occurs in a tyrosine phosphorylation-independent manner. Fyn interaction with the phospho-Tim-3 peptides was not efficiently inhibited by excess phenylphosphate and mutation of Y256 and Y263 did not disrupt the Fyn interaction with Tim-3. In addition, NMR spectroscopy revealed that the Fyn SH2 domain can interact with the unphosphorylated Tim-3 peptide via the SH2 $pY^{+}3$ binding pocket. Therefore, I suggest a model whereby Fyn constitutively interacts with, and phosphorylates, tyrosine residue(s) in the cytosolic tail of Tim-3, to induce the recruitment of p85 to Tim-3. A similar mode of signal transduction was reported previously. Thus, Fyn bound to Cbl via both SH2 and SH3 domains of Fyn but independently of tyrosine phosphorylation, leading to Fyn-mediated tyrosine phosphorylation of Cbl, to which p85 was recruited [184].

I demonstrated that both Y256 and Y263 of the Tim-3 cytosolic tail are available for tyrosine phosphorylation, and that Fyn phosphorylates Y256 and/or Y263 of the Tim-3 cytosolic tail. There could be several possible mechanisms for this Tim-3 phosphorylation by Fyn. As Fyn binds to several region of the Tim-3, Fyn may interact with one motif in the Tim-3 cytosolic tail then phosphorylate tyrosine residues in the same Tim-3 molecule (*cis*-phosphorylation). However, efficient phosphorylation of the T1 Tim-3 mutant construct (Figure 11) implies that the C-terminal region containing Y271/272/274 may be dispensable for recruiting Fyn to induce

phosphorylation of Y563/Y263. As shown in Figure 15D, the Fyn SH domain binds to the region between the transmembrane domain and the conserved region near Y256 and Y263, deletion of the region (Δ216-244) abolished Tim-3 function (Figure 8). However, which domains of Fyn and the specific motifs in the Tim-3 cytosolic tail are involved in this interaction were not determined yet, or whether the functional impairment of the Δ216-244 construct was caused by impairment of the Fyn interaction or general disruption of Tim-3 structure.

Since Fyn interaction with the region near Y256/263 of the Tim-3 cytosolic tail seemed stable and the Fyn SH2 domain bound to both phosphorylated and unphosphorylated peptide of the corresponding sequence, Fyn may bind to unphosphorylated Y256/Y263 residues of Tim-3 and phosphorylate tyrosine residues in the adjacent Tim-3 molecule (*trans*-phosphorylation). Precisely, which unphosphorylated or phosphorylated tyrosine residues among Y256 and Y263 participate in the interaction with Fyn SH2 domain was not identified yet. To address this, co-immunoprecipitation and functional assays with more fine deletion and mutational analyses of Tim-3 cytosolic tail and a fine structural study of the Fyn SH2 domain-Tim-3 complex are required.

Another remaining question regarding the Fyn-Tim-3 interaction is whether this interaction regulates Fyn kinase activity. It is possible that Fyn binding to unphosphorylated or phosphotyrosine of Tim-3 leads to conformational change of Fyn to enhance its kinase activity. Alternatively, Tim-3 may regulate the localization of Fyn and thus access to substrates. Although I did not provide evidence of direct phosphorylation, Lck co-expression also induced phosphorylation of Tim-3. In addition, van de Weyer et al. reported that co-expression of Itk phosphorylates Y265 of human Tim-3 (corresponding to Y256 in murine Tim-3) in 293T cells. Therefore, it is possible that other kinases positively or negatively regulate Tim-3 function by

phosphorylation. The role of these kinases in Tim-3 function, specific target sites of the kinase, and the stoichiometry of phosphorylation have not been determined.

RasGAP is one of the interesting candidates identified in an SH2 domain array experiment. Although RasGAP1 is an inhibitory regulator of the Ras-MAP kinase pathway, it at has a positive role in T cell activation independent of Ras-MAP kinase pathway. In cardiac myocytes, RasGAP1 bound to the PH1 domain of Akt and knockdown of RasGAP1 resulted in inhibition of Akt phosphorylation [185]. Lapinski et al demonstrated that RasGAP1 was negative regulator in ERK pathway in thymocytes during positive selection, but a positive regulator in homeostasis of naïve T cells in periphery but dispensable for ERK activation [186]. Tim-3 is known as an inhibitory receptor, however, our data show that Tim-3 upregulates T cell activation, at least during the short-term stimulation. Thus, RasGAP may be the interesting candidate which can mediate inhibitory and activation function depending on the different cellular context.

In this study I focused mainly on proteins binding to the conserved Tim-3 region with Y256 and Y263, which seems to have an important role in regulation of TCR signaling. However, binding of signaling molecules to the C-terminus of Tim-3, containing Y271/272/274, was not extensively studied. As shown in Figure 13, I found that the pY271/272/274 Tim-3 peptide, but not the pY256/263 peptide, interacted the with SH2 domains of two adaptor proteins, GRAP and Grb2, which couple tyrosine phosphorylation to the Ras-MAP kinase pathway. In the previous chapter, I proposed the role of C-terminus region containing three terminal tyrosine residues in the activation of MAP kinase pathway and AP-1 transcription factor (Figure 9C and D). Thus, GRAP and Grb2 may be responsible for the signaling from this domain of Tim-3

4.0 TIM-3 DOWNSTREAM SIGNALING AND MODULATION OF TCR SIGNALING

4.1 INTRODUCTION

During short-term immune stimulation, such as acute viral infection, antigen-specific naïve CD8 T cells develop effector T cells producing effector cytokines and cytolytic molecules. When the source of the antigen is cleared acutely, the subsets of antigen-specific T cells remain as memory T cells. However, under the immune conditions become chronic, such as chronic viral, bacterial and parasitic infection, and tumor, CD8 T cells gradually lose their effector function and the potential to proliferate and to form memory subsets. It appeared that surface expression of inhibitory receptors such as PD-1, LAG-3 and CTLA-4 are involved in the functional loss in T cells.

Recently, role of Tim-3 in T cell exhaustion has been suggested. In the various chronic infections such as HIV, HCV and LCMV, [47, 115, 116, 187] and in the tumor tissue[49, 188], upregulation of Tim-3 expression in antigen-specific T cells and positive correlation between Tim-3 expression and dysfunctional phenotype of T cells were found. T cells expressing both PD-1 and Tim-3 showed more severe loss of effector function compared to the T cells expressing PD-1 or Tim-3 alone. In addition, blockade of PD-1 and Tim-3 restore the exhausted T cell function synergistically [47, 49, 188].

Because of the synergy of Tim-3 and PD-1, it has been assumed that Tim-3 also transmits an inhibitory signal to T cells. However, the elements of Tim-3 signaling were not known. In the previous chapters I showed that Tim-3 upregulated activation of transcription factors downstream of TCR in T cell lines, and that Tim-3 interacts with Fyn and PI3 kinase. In this chapter, I further explore the signaling pathways downstream of Tim-3. Thus I will examine the requirement for TCR-the proximal signaling molecules in the function of Tim-3, and determine whether Tim-3 enhances or inhibits the activation of signaling molecules downstream of TCR and PI3 kinase.

MATERIALS AND METHODS

4.1.1 Cell lines and cell culture

Jurkat T cells and Jurkat variants were cultured in RPMI-1640 (Mediatech) supplemented with 5% bovine growth serum (BGS, Hyclone) and 100U/ml penicillin, 100μg/ml streptomycin. Murine primary T cells were cultured in RPMI-1640 (Mediatech) supplemented with 10% BGS, 100U IL-2, 100U/ml penicillin, 100μg/ml streptomycin, non-essential amino acid, 1mM sodium pyruvate, 55μM 2-mercaptoechanol (Life sciences), and 10mM HEPES (Fisher). Tet-on Tim-3 Jurkat T cells were maintained in 0.8μg/ml puromycin, and Tim-3 expression was induced with 1.5μg/ml doxycycline for 2 days.

4.1.2 Antibodies and reagents

C305 antibody to Jurkat TCR was obtained from Dr. Arthur Weiss (UCSF, CA), anti-human CD28 (10F3), Anti-mouse CD3 (37.51), and anti-mouse CD28 (2C11) were from Life Sciences. Anti-Flag M2 antibody and β-actin antibody were from Sigma Aldrich, polyclonal anti-mouse Tim-3 antibody was from R&D systems. Anti-PLC-γ1 was from Millipore and phospho-PLC-γ (pY783) antibody was from BD bioscience. ZAP-70, phospho-ZAP-70 (Y319), Alexa647 conjugated phospho-S6 (S235/S246) antibodies were from Cell Signaling. Alex647 conjugated ERK (T202/Y204), p38 (T180/182), and JNK (T183/185) were from BD bioscience. HRP conjugated goat anti-mouse antibody and donkey anti-goat antibody were from Thermo scientific and Jackson Immunoresearch respectively. PMA and ionomycin were from Calbiochem and D-luciferin was from Thermo Scientific.

4.1.3 Luciferase reporter assay

$2x10^6$ Jurkat and Jurkat variants were transfected with 15ug of NFAT/AP-1, NF-κB, NFAT, or AP-1-luciferase reporter, and Tim-3 constructs by electroporation at 260V/950μF (Jurkat). The day after transfection, $5x10^5$ cells were stimulated with anti-human TCR (C305)/CD28 antibodies for six hours in round bottom 96 well plates. After a freezing and thawing cycle, luciferase assay was performed in 5mM ATP, 10mM $MgCl_2$, and 1mM D-luciferin using luminometer (Berthold)

4.1.4 Western blotting

Tet-on Tim-3 Jurkat T cells were incubated with or without 1.5μg/ml doxycycline for 2 days. Tet-on Tim-3 Jurkat T cells were pervanadate or monoclonal antibody specific for Jurkat TCR (C305.2), then lysed with ice-cold 1% NP-40 lysis buffer (20mM Tris-HCl, pH 7.5, 150mM NaCl) supplemented with AEBSF, aprotinin, leupeptin, pepstatin, sodium fluoride, sodium orthovanadate, and β-glycerophosphate. Lysates were then incubated on ice for 10 min before being cleared by centrifugation. 6X Sample buffer (Bio-Rad, Hercules, CA) was added to the cleared lysates before loading onto 10% SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, probed with anti-PLC-γ1, anti-phospho-PLC-γ, anti-ZAP-70, or anti-phospho-ZAP-70 antibodies. Then, the binding of the primary antibodies detected with HRP conjugated anti-mouse or anti-rabbit antibody. Blots were imaged on a Kodak ImageStation 4000R system.

4.1.5 Flow cytometry and ELISA

Tet-inducible Tim-3 Jurkat T cells were induced for 2 days with doxycycline and then stimulated with TCR antibody or PMA/ionomycin. For intracellular phospho-flow analysis, cells were fixed in PFA immediately after stimulation for the appropriate times and permeabilized with methanol, then processed for staining with antibody specific for phospho-ERK, phospho-p38, phospho-JNK or phospho-S6. All flow cytometry was carried out on a BD LSR II instrument. Data were exported and analyzed in the FlowJo software program (Treestar, Ashland, OR). For IL-2 ELISA, cells were stimulated for 24 h with anti-TCR/CD28 antibodies or PMA/ionomycin. Cell-free

supernatants were analyzed by ELISA for human IL-2, using OptEIA matched antibodies (BD Bioscience).

4.1.6 Retroviral infection

Purified murine CD4   T cells were stimulated under Th1 conditions and infected with recombinant MSCV retroviruses expressing the indicated constructs, essentially as previously reported [189]. Infected T cells were then restimulated, again under Th1 conditions, followed by intracellular cytokine staining 2 days later for IFN-γ.

4.2   RESULTS

4.2.1 Proximal TCR signaling molecules are required for enhancement of NFAT/AP-1 and NF-κB reporter activity by Tim-3

Tim-3 expression augmented basal NFAT/AP-1 and NF-κB activation, but the extent was more profound in the presence of TCR stimulation (Chapter 2.0). Therefore I decided to investigate the requirement of TCR-proximal signaling molecules involving in the TCR signaling in the Tim-3 signaling. I took advantage of ZAP-70 deficient (P116) and SLP-76 deficient (J.14) Jurkat T cell lines, which display severe defects in TCR signaling [190, 191]. Expression of Tim-3 in ZAP-70 deficient Jurkat cells resulted in moderately increased basal NFAT/AP-1 reporter activity, and TCR/CD28 stimulation could not induce further enhancement of the reporter activity, as was seen in wild type Jurkat T cells. When the ZAP-70 mutant Jurkat T cells were

reconstituted with wild type ZAP-70, the extent of basal upregulation by Tim-3 expression was enhanced compared to ZAP-70 mutant Jurkat T cells. Distinct enhancement of the reporter activity by Tim-3 expression in the presence of TCR/CD28 stimulation was not observed in the ZAP-70 reconstituted mutant cells due to hyperresponsiveness of the reconstituted cells to TCR/CD28 stimulation (Figure 18A). In SLP-76 deficient cells Tim-3 expression modestly upregulated basal NFAT/AP-1 reporter activity, similar to ZAP-70 deficient Jurkat T cells, the reporter activity was not further enhanced by TCR/CD28 stimulation. Tim-3 expression in mutant Jurkat T cells reconstituted with SLP-76 led to a moderate increase in basal NFAT/AP-1 reporter activity and further upregulation of the activity upon TCR/CD28 stimulation, as shown in parental Jurkat T cells (Figure 18B). These results suggest that TCR signaling is required for full functionality of Tim-3 in upregulation of T cell activation, and that Tim-3 may converges with TCR signaling at the downstream of ZAP-70 and SLP-76.

In the previous chapter, I demonstrated that SFK, especially Fyn, induces tyrosine phosphorylation of Tim-3. Thus I assessed the role of SFKs in Tim-3 function to upregulate T cell activation using of an Lck-deficient Jurkat T cell line (JCaM.1) [192]. Although TCR signaling was severely abolished in the Lck deficient Jurkat T cells, Tim-3 expression could still weakly upregulate NFAT/AP-1 reporter activity in synergy with TCR/CD28 stimulation. Fyn might compensate for the loss of Lck in TCR signaling and mediate Tim-3 signaling in the Lck-deficient cells. Lck co-transfection restored the function of Tim-3 to upregulate TCR signaling to the level of wild type Jurkat T cells, while Fyn co-transfection led to only a slight increase in Tim-3 function (Figure 18C). This could be due to the fact that Fyn was already abundant in the Lck deficient Jurkat.
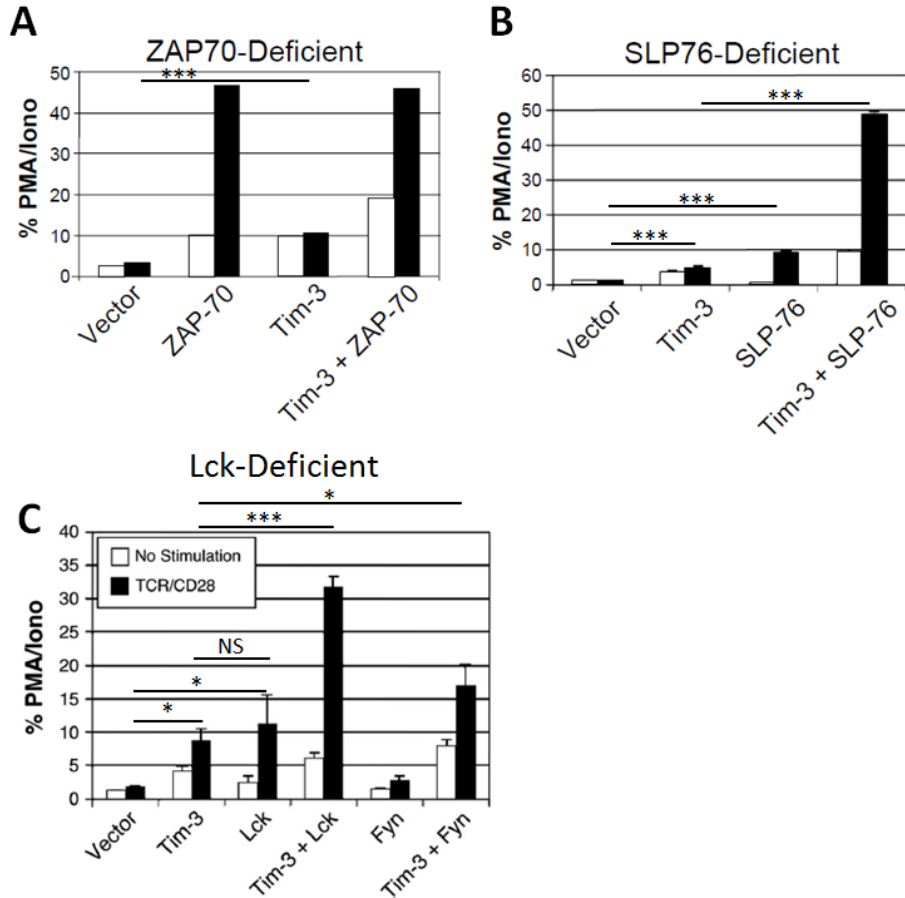
Figure 18. Requirement of ZAP-70, SLP-76 and Lck in Tim-3 signaling

Jurkat T-cell lines lacking expression of either ZAP-70 (A) or SLP-76 (B) and stably reconstituted mutant cells were transfected with NFAT/AP-1-luciferase and the indicated constructs. The next day, cells were left unstimulated (white bars) or stimulated with anti-TCR/CD28 monoclonal antibodies (mAbs) (black bars).

(C) Lck deficient Jurkat T-cell lines were transfected with NFAT/AP-1-luciferase reporter and indicated constructs, and analyzed as above. Luciferase activity is presented as the percentage of maximal stimulation obtained with PMA-ionomycin. Luciferase activity is presented as the percentage of the maximal response obtained with PMA plus ionomycin. Results shown are the average and SD of data for triplicate samples from a single experiment, representative of at least three that were performed in each case.). Average results (± SD) from triplicates are shown (*, P<0.05; **, P<0.01; ***, P<0.01)

4.2.2 Ectopic expression of Tim-3 enhances activation of PLC-γ

To determine whether expression of Tim-3 modulates TCR signaling, I examined differential induction of tyrosine phosphorylation upon TCR crosslinking in the presence and absence of Tim-3 expression in Jurkat T cells. I took advantage of Jurkat T cells stably transfected with reverse tetracycline-controlled transactivator (rtTA) and Tet-on inducible construct encoding Flag-tagged full-length Tim-3 (Tet-on Tim-3 Jurkat). Tim-3 expression in Tet-on Tim-3 Jurkat T cells was induced with doxycycline prior to TCR stimulation. The stimulated cells were lysed and subjected to western blotting, then probed with phosphotyrosine specific antibody. I observed that when Tim-3 was expressed, TCR-induced tyrosine phosphorylation of 70kDa and 150kDa proteins were enhanced (Figure 19). I speculated that these 70kDa and 150kDa proteins were ZAP-70 and PLC-γ1, respectively, which are proximal signaling molecules downstream of the TCR.

To test the hypothesis, again I took advantage of Tet-on Tim-3 Jurkat T cells and Jurkat T cells expressing the rtTA only but not Tet-on inducible Tim-3 construct (rtTA Jurkat) and performed western blotting to examine the tyrosine phosphorylation of PLC-γ1 and ZAP-70 using phospho-specific antibodies. Without stimulation, there was no difference in the level of PLC-γ1 phosphorylation (Y783) between doxycycline treated and non-treated Tet-on Tim-3 Jurkat T cells. However, I observed enhanced and sustained phosphorylation of PLC-γ1 induced by TCR in the Tet-on Tim-3 Jurkat T cells treated with doxycycline compared to the cells not treated (Figure 20, upper panels). As expected, I did not observe any difference in the level of PLC-γ1 phosphorylation between doxycycline-treated and non-treated rtTA Jurkat T cells (Figure 20, lower panels). In the same way, I examined tyrosine phosphorylation of ZAP-70

(Y319). I did not observe a significant Tim-3 dependent enhancement of ZAP-70 phosphorylation in Tet-on Tim-3 Jurkat T cells vs. rtTA Jurkat T cells (Figure 21).
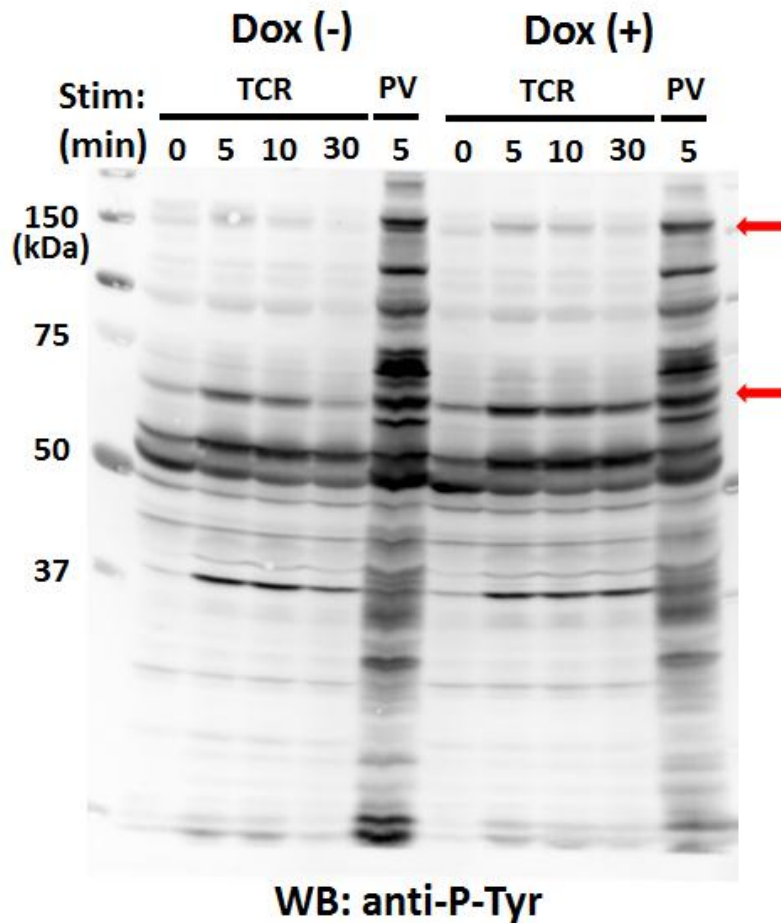
Figure 19. Differential induction of tyrosine phosphorylation in presence of Tim-3 expression

Tet-on Tim-3 Jurkat T cells were treated with doxycycline or left untreated, each cells were stimulated with anti-TCR/CD28 antibodies or pervanadate for indicated times. Whole cell lysates were subjected to SDS-PAGE and western blotting, and the membrane was probed with anti-phosphotyrosine antibody.
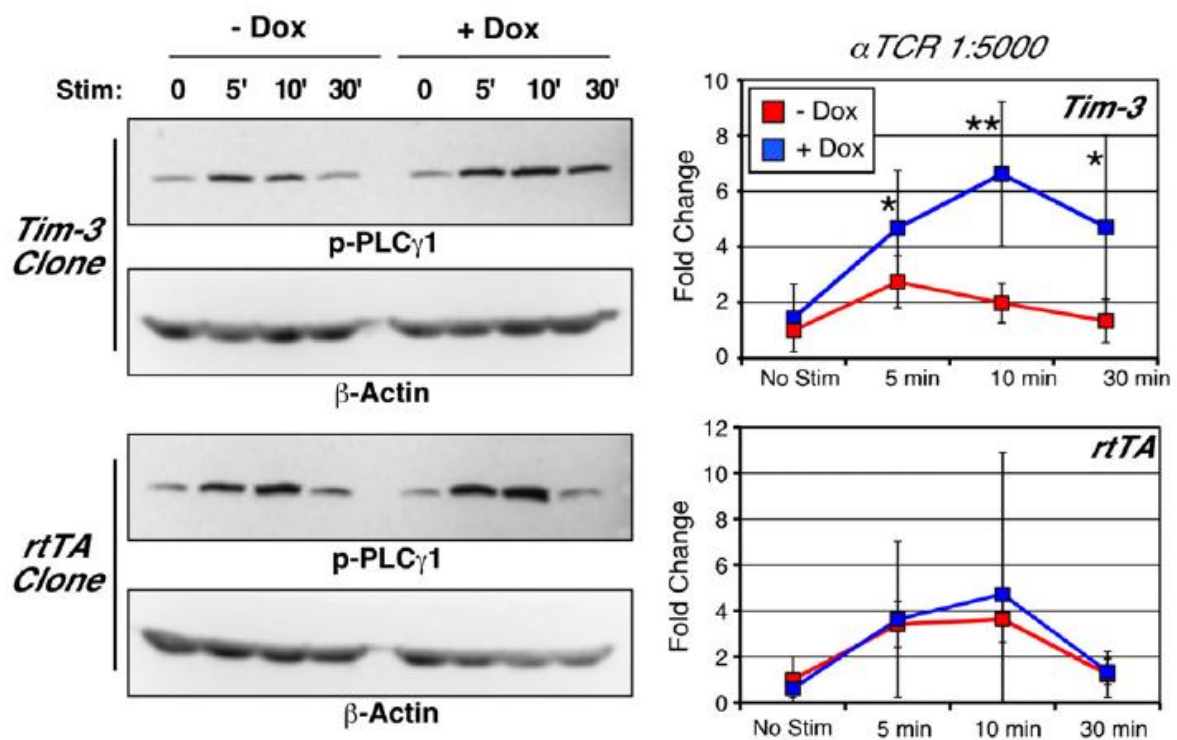
Figure 20. Upregulation of PLC-γ1 activation by Tim-3

Tet-on-Tim3 or parental Jurkat T cells expressing only the reverse tetracycline transactivator (rtTA) were cultured for 2 days without or with doxycycline (Dox). Cells were stimulated as indicated for analysis of phospho-PLC-γ1 (Y783) by Western blotting, followed by probing for beta-actin as a loading control (left). Average results and SD from five separate experiments are shown (*, P <0.05; **, P<0.01)

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Figure 21. No effect on ZAP-70 phosphorylation by Tim-3 expression

Tet-on-Tim3 or parental Jurkat T cells expressing only the reverse tetracycline transactivator (rtTA) were cultured for 2 days without or with doxycycline (Dox). Cells were stimulated as indicated for analysis of phospho-ZAP-70 (Y319) by Western blotting, followed by probing for beta-actin as a loading control (left). Average results and SD from five separate experiments are shown

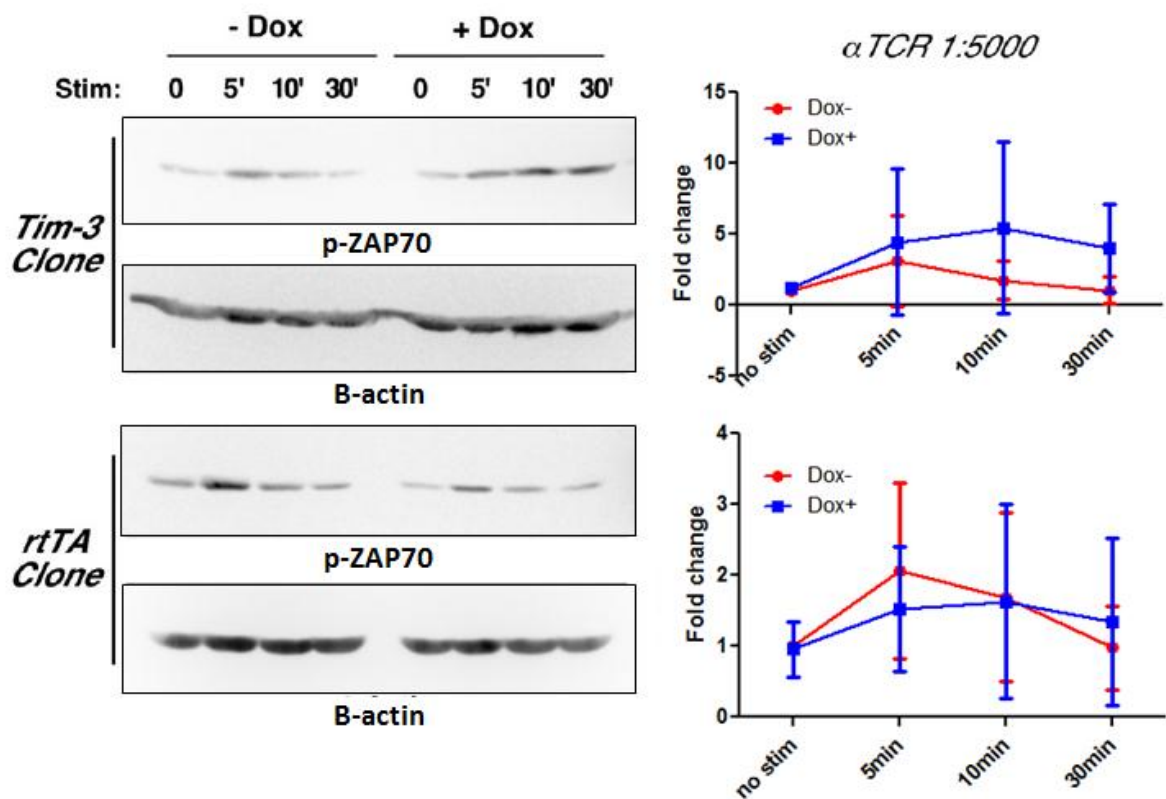**4.2.3 Ectopic expression of Tim-3 enhances activation of MAP kinases**

MAP kinase pathway is one of the major downstream pathways of TCR signaling and upstream of AP-1 transcription factor. In addition, we found NFAT/AP-1 and AP-1 reporter activity in Jurkat were enhanced by ectopic expression of Tim-3. To determine whether Tim-3 modulated MAP kinase pathway, I assessed phosphorylation of MAP kinases in Tet-on Tim-3 Jurkat T cells. I stimulated doxycycline treated and non-treated Tet-on Jurkat T cells with anti-TCR, then stained the cells with phospho-specific ERK, p38 and JNK antibody and analyzed by flow cytometry. Note that in Tet-on Tim-3 Jurkat T cells, co-expression of GFP and Tim-3 is driven by a bi-directional promoter upon doxycycline treatment. As the Tet-on Tim-3 Jurkat T cells contained both doxycycline-responsive and unresponsive populations, I separately analyzed Tim-3 expressing cells and non-expressing cell by gating the cells based on the level of co-expressed GFP.

As shown Figure 22, TCR-induced phosphorylation of ERK, p38 and JNK were enhanced in the Tim-3 expressing cells (Dox treated/GFP^{hi} cells) compared to the cells not expressing Tim-3 (Dox non-treated cells and Dox treated/GFP^{lo} cells), while basal phosphorylation of ERK, p38 and JNK (i.e. without TCR stimulation) were not significantly upregulated by Tim-3 expression. The upregulation of ERK phosphorylation was only seen with TCR stimulation, but not without TCR stimulation, while upregulation of AP-1 reporter activity by Tim-3 expression was independent of TCR stimulation.
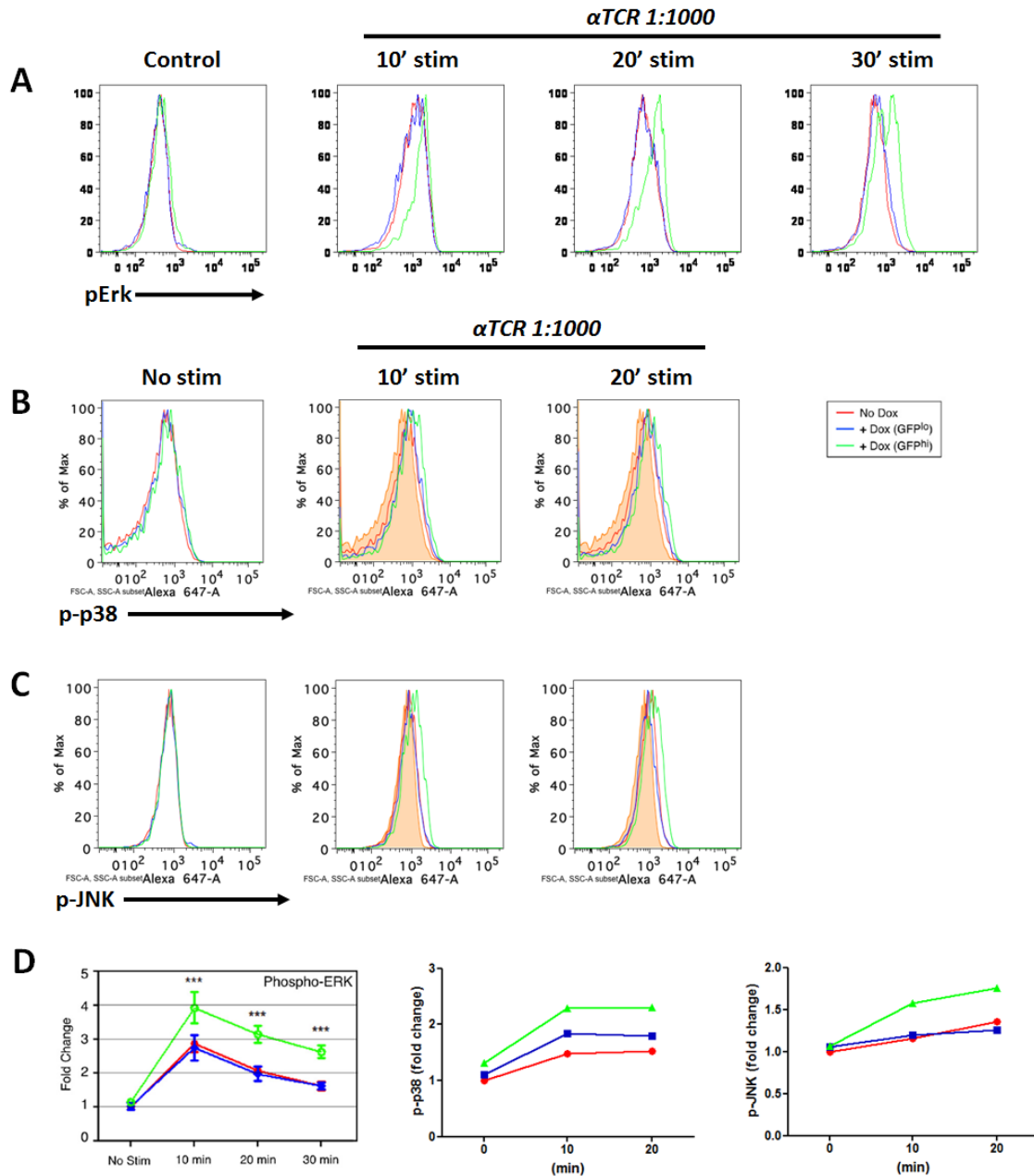
Figure 22. Tim-3 expression enhances phosphorylation of MAP kinases.

Tet-on-Tim3 Jurkat T cells were cultured for two days without or with doxycycline. Cells were then stimulated as indicated and processed for intracellular staining of phospho-ERK(A), phospho-p38(B), and phospho-JNK(C). (D) Graphical representation of MFI of each staining (*** P<0.005)

4.2.4 Ectopic expression of Tim-3 enhances phosphorylation of ribosomal protein S6

We demonstrated that p85 interacted with Tim-3 in a tyrosine phosphorylation dependent as well as upregulation of ERK activation manner by Tim-3 expression. Then we proceeded to investigate the modulation of further downstream target of the pathways by Tim-3 using Tet-on Tim-3 Jurkat T cells. Ribosomal protein S6 is regulated by p70S6 kinase and p90S6 kinase, which are downstream of PI3 kinase pathway and MAP kinase pathway, respectively. The frequency of pS6^{hi} cells was greater in the Tim-3 expressing cells (Dox treated/GFP^{hi} cells) than in the non-expressing cell (Dox non-treated and Dox treated/GFP^{lo} cells) when the cells were stimulated with an optimal dose of anti-TCR (Figure 23, upper panels). However, when the cells were stimulated with a suboptimal dose of anti-TCR, induction of S6 phosphorylation was minimal in the Tim-3-absent cells and the augmentation of S6 phosphorylation in the Tim-3 expressing cells was more dramatic (Figure 23, lower panels).

Then, I examined the contribution of Akt and MAP kinase pathway to the upregulation of phosphorylation of S6 by Tim-3 expression, using Akt inhibitor (Akti) and MEK inhibitor (U0126). Akt inhibitor partially inhibited the upregulation of S6 phosphorylation in Tim-3 expressing Tet-on Tim-3 Jurkat T cells (Dox treated/GFP^{hi} cells). MEK inhibited more potently than Akti did, but not completely to the basal level in the unstimulated cells without Tim-3 expression (Dox non-treated) (Figure 24). These data suggest that both Akt and MAP kinase pathway, at least partially, mediate Tim-3 signaling to upregulate S6 phosphorylation in Tet-on Tim-3 Jurkat T cells.
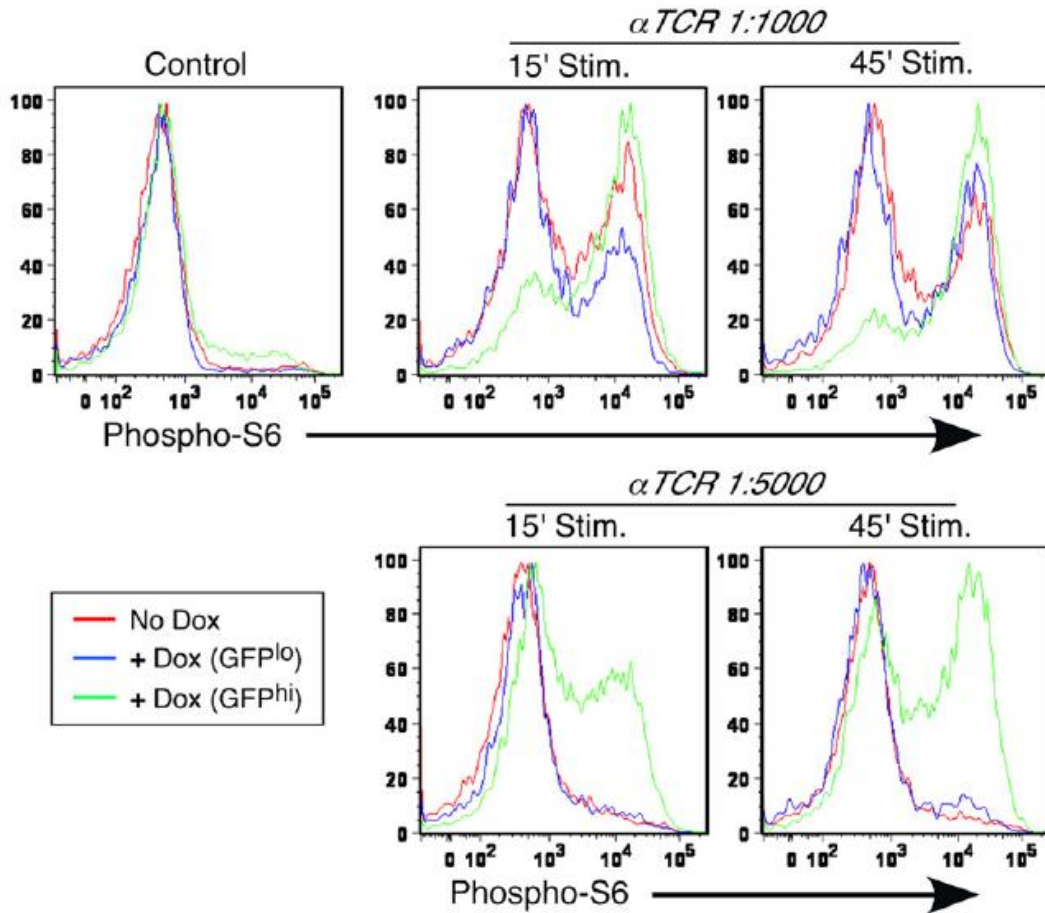
Figure 23. Ectopic expression of Tim-3 enhances pS6 phosphorylation induced by TCR signaling.

Tet-on-Tim3 Jurkat T cells were cultured for two days without or with doxycycline. Cells were then stimulated as

indicated and processed for intracellular staining of phospho-S6
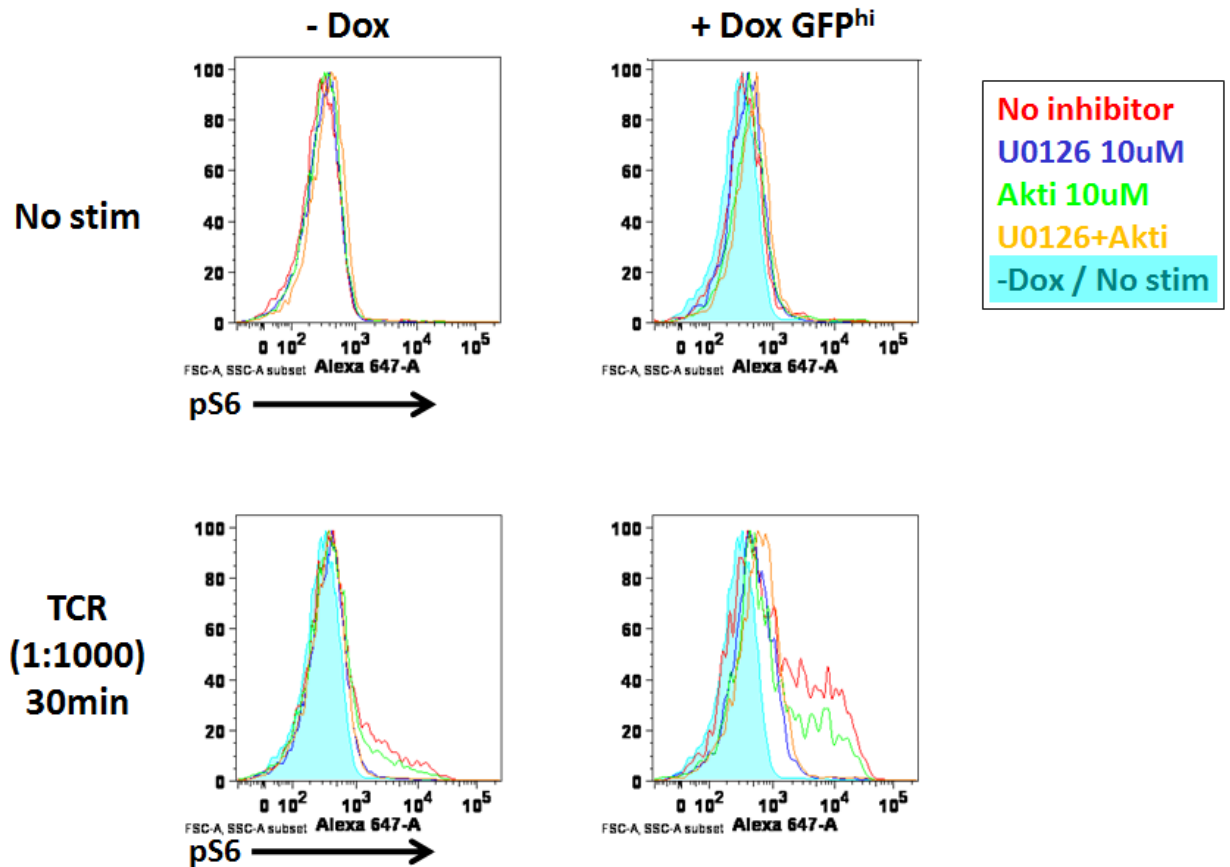
Figure 24. MAP kinase and Akt pathways mediate the enhancement of S6 phosphorylation by Tim-3 expression

Tet-on-Tim3 Jurkat T cells were cultured for two days without or with doxycycline. Cells were pretreated with the indicated inhibitors for 30min, and stimulated with anti-TCR antibody. Then processed for intracellular staining of phospho-S6

4.2.5 Tim-3 expression upregulated IL-2 and IFN-γ production

Next, we tested whether Tim-3 could upregulate IL-2 production in T cells. We incubated Tet-on Tim-3 Jurkat T cells and rtTA Jurkat T cells in the various dose of doxycycline to induce various amount of Tim-3 expression in Tet-on Tim-3 Jurkat. We then stimulated the cells with a

suboptimal dose of anti-TCR antibody and measured IL-2 production by ELISA. This doxycycline treatment induced expression of Tim-3 in Tet-on Tim-3 Jurkat T cells in a dose-dependent manner (Figure 25A), and IL-2 production was also upregulated in Tet-on Tim-3 Jurkat T cells, which correlated with Tim-3 expression level in the cells. In contrast with Tet-on Tim-3 Jurkat, doxycycline treatment did not induce IL-2 production in control rtTA Jurkat T cells, confirming that upregulation of IL-2 is Tim-3 dependent (Figure 25B).

Next we examined the effect of Tim-3 expression on IFN-γ production in primary CD4 T cells. Murine CD4 T cells were isolated from lymph nodes, activated under Th1 differentiation conditions, and infected with a retrovirus encoding full-length, T1, and T2 deletion of Tim-3. IFN-γ production upon restimulation with anti-CD3/CD28 was determined by intracellular cytokine staining. As show in Figure 26A and B, IFN-γ production was greatly increased in the CD4 T cells expressing full-length Tim-3 and even more in the CD4 cells expressing the T1 deletion, while this increase of IFN-γ production was not observed in T2 Tim-3 expressing CD4 T cells (Figure 26B).

I examine whether expression of Tim-3 suppresses the growth of the Tet-on Tim-3 Jurkat T cells. I treated Tet-on Tim-3 Jurkat T cells and rtTA parental Jurkat T cells with doxycycline or left untreated, and counted the number of the cells. The cell number was lower in Dox treated Tet-on Tim-3 Jurkat T cells compared untreated Tet-on Tim-3 Jurkat cells. However, we observed same trend from rtTA parental Jukat T cells. Thus, decreased in growth is at least partially due to off-target effect by doxycycline. High dose of doxycycline treatment suppressed Jurkat growth (>5μg/ml) and induces apoptosis (>10μg/ml) [193]. At the concentration we used to induce Tim-3 expression (1.5μg/ml), treatment of doxycycline did not affect growth of Jurkat

T cells for 4 days, but seemed to suppress the growth in the long-term. However, I did not further analyzed whether the cause is suppression of proliferation or induction of apoptosis.

*IL-2 ELISA was performed by Ee W. Su (Lawrence Kane's Lab, University of Pittsburgh) and IFN-γ intracellular staining experiment was done by Chen Zhu (Vijay Kuchroo's Lab, BWH, Harvard Medical School), respectively.
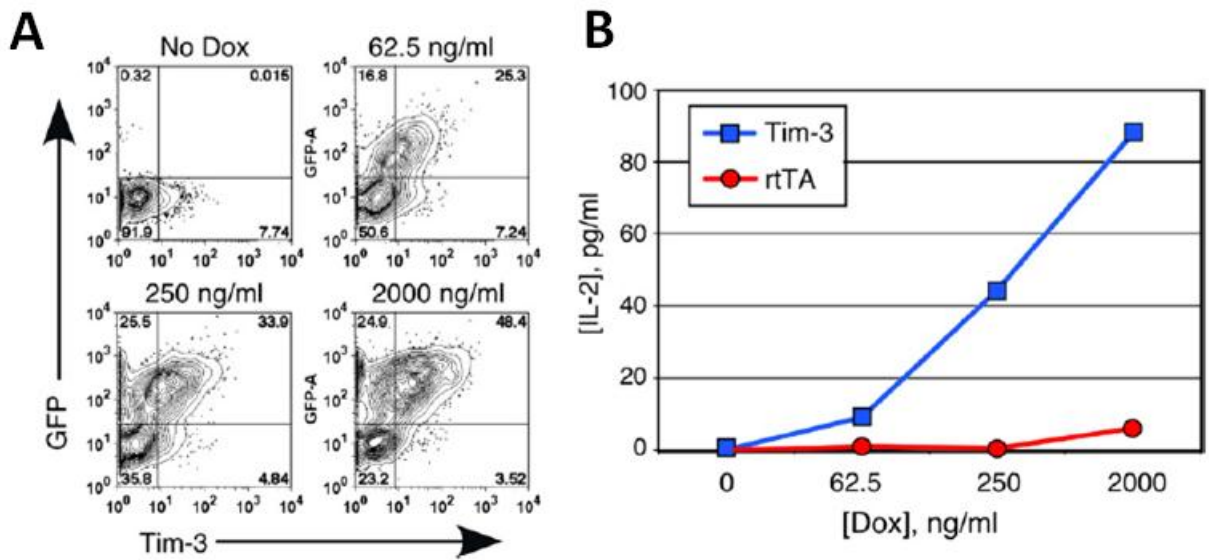
Figure 25. Upregulation of IL-2 production by Tim-3 expression

(A) Tet-on-Tim3 Jurkat T cells were cultured with the indicated concentrations of doxycycline and analyzed by flow cytometry for Tim-3 expression (x axis) and a co-expressed GFP reporter (y axis).

(B) Tet-on-Tim3 or parental Jurkat T cells, or the parental Tet-on cells, were then stimulated for 24 hours with soluble anti-TCR/CD28 antibodies. Cell-free supernatants were harvested and secreted IL-2 was measured by ELISA. IL-2 production is presented as the percentage of the maximal response, obtained with PMA plus ionomycin.
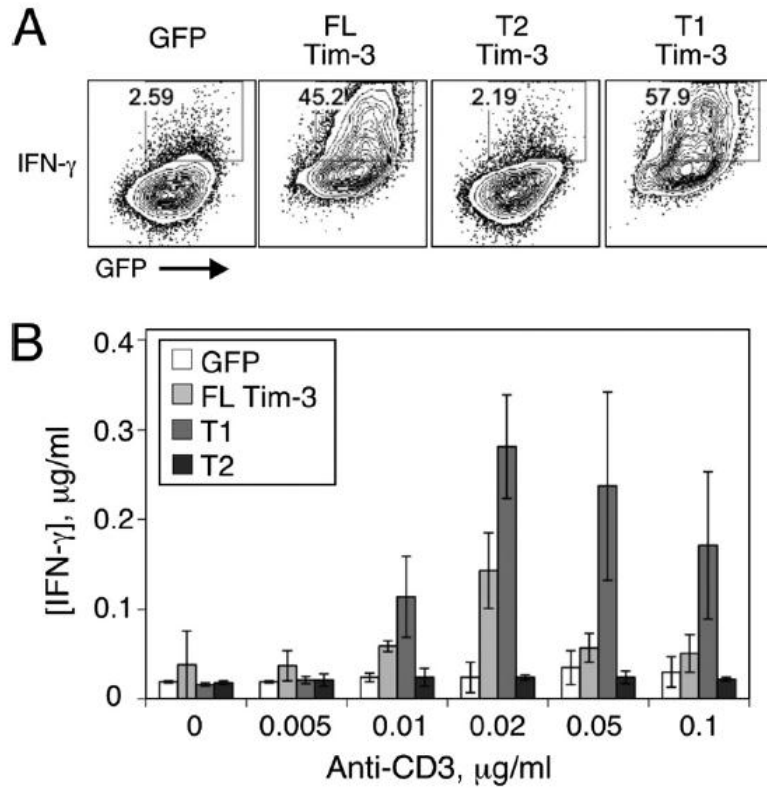
Figure 26. Upregulation of IFN-γ by Tim-3 in primary CD4 T cells

Naive murine CD4^{+} T cells were stimulated with anti-CD3/CD28 antibodies under Th1 conditions and then infected

With retrovirus encoding the indicated constructs and restimulated with the indicated concentrations of soluble anti-

CD3 antibody, again under Th1 conditions. Two days later, cells were processed for intracellular cytokine staining

and flow cytometry. (A) Representative flow cytometry of intracellular IFN-γ and retroviral coexpressed GFP. (B

and C) T cells were restimulated with the indicated concentrations of anti-CD3, and supernatant was collected for

determination of IFN-γ concentrations by ELISA.
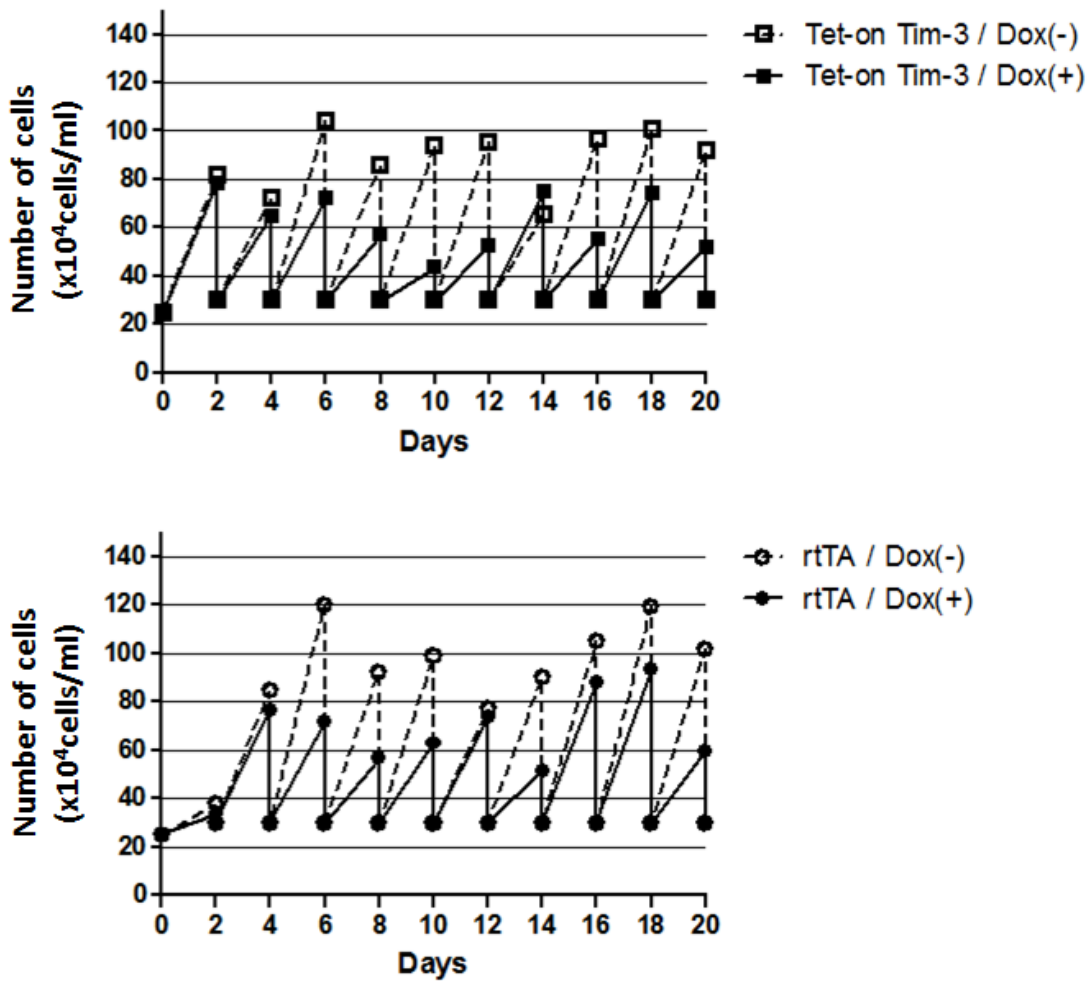
Figure 27. Inhibition of growth of Tet-on Tim-3 Jurkat cell by induction of Tim-3 expression was not found.

Tet-on Tim-3 Jurkat cells and parental rtTA Jurkat T cells were maintained in 0.8μg/ml puromycin and with or without 1ug/ml doxycycline. Every 48hrs, the number of the cells were counted and diluted at 0.3×10^6 cells/ml.

4.3 DISCUSSION

Here we first demonstrated that Tim-3 signaling enhances T cell activation by TCR signaling. In Chapte 2.0 , we demonstrated that Tim-3 can enhance the activity of major transcription factors such as NFAT/AP-1,NF-κB, AP-1 in Jurkat T cell lines and non-transformed D10 T cells. As we found that Y256 and Y263 are crucial for the positive function of Tim-3, we searched for the signaling molecules interacting with the cytosolic tail of Tim-3 near Y256 and Y263. While p85 interacted with Tim-3 in a Y256 and Y263 phsphorylation-dependent manner, Fyn interacted with Tim-3 in a Y256 and Y263 phosphorylation-independent manner. Moreover, Fyn could directly phosphorylate tyrosine residues of Tim-3. I also found Tim-3 upregulated AP-1 reporter activity independently of TCR stimulation and SH2 domain of GRAP and Grb2 on the array interacted with tyrosine phosphorylated peptide corresponding to C-terminus of Tim-3.Therefore, I hypothesize a model whereby Fyn phosphorylates Tim-3, in turn p85 is recruited to phosphotyrosines in the Tim-3 cytosolic tail to activate the PI3L-Akt pathway and that GRAP or Grb2 is recruited to the C-terminus of Tim-3 to activate MAP kinase pathways (Figure 28).

Since Tim-3 expression enhanced both NFAT/AP-1 reporter and NF-κB reporter activity induced by TCR stimulation, I speculate that Tim-3 signaling intersects TCR signaling at a place upstream of both NFAT and NF-κB pathways. I found the requirement of the common upstream signaling molecules Lck, ZAP-70, and SLP-76 for the upregulation of TCR stimulation-induced NFAT/AP-1 and NF-κB activation by Tim-3 ectopic expression. Indeed I found that the activation of PLC-γ, a common upstream of NFAT, AP-1, and NF-κB, was enhanced in Tim-3 expressing Tet-on Tim-3 Jurkat. I hypothesized that 70kDa protein whose phosphorylation by TCR stimulation was augmented in the Jurkat T cells expressing Tim-3 was ZAP-70, I examined the Y319 phosphorylation of ZAP70 only, which plays critical role in ZAP-70 catalytic activity

[140]. Although I did not observe the significant changes in Y319 phosphorylation of ZAP70 by Tim-3 expression, I cannot rule out the possibility that Tim-3 regulates the phosphorylation of other tyrosine residues in ZAP-70. ZAP-70 has other tyrosine phosphorylation sites. For example, phosphorylation of Y315 and Y493 regulate catalytic activity of ZAP70 [194] and recruitment of ZAP-70 to TCR [194], respectively.

Although we revealed that Tim-3 expression could upregulate TCR signaling, the mechanism how Tim-3 potentiates TCR signaling, specifically PLC-γ phosphorylation is unclear. I suspect that Fyn is at least partially responsible for Tim-3 enhancement of TCR signaling. Fyn is a TCR-proximal signaling molecule along with Lck, which phosphorylates ZAP-70, and *in vitro* Fyn can also directly phosphorylate PLC-γ1 [195]. Interestingly, it has also been shown that Fyn is able to regulate MAP kinase pathways both directly and indirectly. Thus, Fyn activates autokinase activity of Raf-1 in NIH 3T3 cells [196], and increases the magnitude of ERK signal through a pathway different from Lck [197]. In the previous chapter I discussed the possibility that Tim-3-Fyn interaction changes conformation of Fyn to primed status. However, I found that Fyn constitutively interacted with Tim-3, suggesting there is another regulatory mechanism. Note that, Up to ~50% of Lck were in primed and ~40% were active status in human CD4 T cells [198]. Lck constitutively interact with CD4 and CD8, and the co-localization with the co-receptors are thought as one of the mechanism that initiates TCR signaling [133, 134]. Similarly, Fyn mediated positive effect may be regulated by localization or higher-order organization of Tim-3 during TCR stimulation. Therefore, imaging the localization of Tim-3 during TCR stimulation or Tim-3 ligation would be a useful experiment to unravel the mechanism of Tim-3 signaling transduction. Alternatively, Fyn-Tim-3 may constitutively induce moderate activation lowering the threshold of TCR signaling.

AP-1 is a transcription factor consisting of c-Jun and c-Fos, whose activation and expression are regulated by MAP kinase pathway. I showed that Tim-3 expression enhances activation of Erk, p38 and JNK by flow cytometry, and AP-1 activation by reporter assay. The upregulation of pErk, p38 and JNK was detected only with TCR stimulation, while upregulation of AP-1 reporter activity was TCR stimulation independent, as shown in Figure 9C and D. This discrepancy may be due to involvement other factors to activate AP-1 other than MAP kinases or higher sensitivity of the luciferase reporter assay than flow cytometry.

Tim-3 expression upregulated phosphorylation of S6 which is regulated by both MAP kinase pathway and PI3K-Akt pathway, I hypothesized that PI3K pathway is as a major downstream of Tim-3. Akt inhibitor did not potently inhibited S6 phosphorylation upregulated by Tim-3 expression, while MEK inhibitor did. PDK1 could directly phosphorylate p70S6K at threonine in the activation loop and activated p70S6K [199, 200]. In CD8 T cells, deletion of PDK1 resulted in loss of S6 phosphorylation induced by IL-2 but treatment with Akti weakly inhibited S6K and S6 phosphorylation [201]. Moreover, Akt was dispensable for metabolism in CD8 T cells [201]. Thus, Tim-3 may be able to upregulated S6 phosphorylation via Akt-independent pathway. To address this, I need to test the effect of PI3K or PDK inhibition in S6 activation and investigate activation of p70S6K and p90S6K.

Functionally, Tim-3 not only upregulated IL-2 production in Jurkat T cells, but also upregulated IFN-γ production in primary murine T cells. Moreover, the consequence of T1 and T2 deletions of the cytosolic tail of Tim-3 on IFN-γ production were consistent with the results of NFAT/AP-1 and NF-κB reporter assays, indicating that Tim-3 exerts positive signaling that is not limited only to transformed T cell lines.
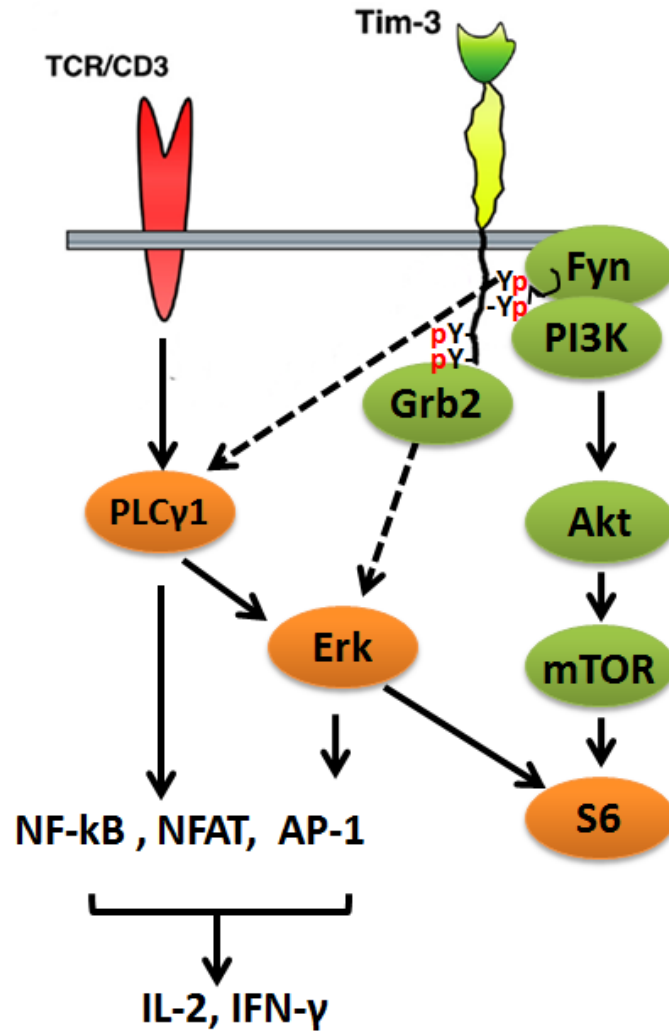
Figure 28. Model for acute enhancement of TCR signaling and activation by Tim-3

5.0   SUMMARY AND DISCUSSION

We demonstrate that ectopic expression of Tim-3 upregulated NFAT/AP-1 reporter, NF-κB and NFAT reporter activity induced by TCR signaling and AP-1 activity independently of TCR stimulation. Tyrosine residues at 256 and 263 were required for the positive function of Tim-3 and were available for phosphorylation. Tyrosine phosphorylation of Tim-3 was modestly induced by TCR stimulation. Then we searched for the signaling molecules interacting with the cytosolic tail of Tim-3. Through SH2 domain array we identified that proximal TCR signaling molecules like SFKs, p85 subunit of PI3 kinase, and PLC-γ1, and adaptor proteins involved in MAP kinase like Grb2, and GRAP. Then, we confirmed Tim-3 interacts with Fyn and p85 PI3K in a tyrosinephosphorylation dependent and independent manner, respectively. I revealed Tim-3 expression enhances activation of PLC-γ1, MAP kinase, and S6, leading to IL-2 and IFN-γ production.

Our data show that Tim-3 enhances, rather than represses, T cell activation, at least during short term stimulation (<6hr). Then, how does activation of T cell by Tim-3 mediate inhibition of T cell immune responses? One possibility is that Tim-3 may have both activating and inhibitory function, depending on the repertoire of the available ligands at the different stage of T cell differentiation. As discussed in Chapter 2.0 , Tim-3 may activate signaling by default, while association with certain ligands switches it to an inhibitory molecule. To-date, there are several ligands of Tim-3 identified, which alone may differentially influence T cells via different

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affinity or different conformational change. BAT3 interacted with unphosphorylated Tim-3 inhibiting negative regulatory function of Tim-3 [127]. Association of intracellular molecules in resting status may govern the default signaling in the absence of ligation.

It is also possible that a combination of Tim-3 with signaling from other receptors determines the consequence of Tim-3 signaling in T cells. During chronic exhaustion of T cells, several exhaustion receptors are co-expressed with Tim-3, especially PD-1, so Tim-3 may cooperatively induce T cell dysfunction during T cell exhaustion. Indeed, T cell function was more severely impaired in Tim-3$^+$PD-1$^+$ T cells than in Tim-3$^+$PD-1$^-$ or Tim-3$^-$PD-1$^+$ cells, and co-blockade of Tim-3 and PD-1 was more efficient in restoration of T cell function than Tim-3 or PD-1 blockade alone [47, 49, 115]. Given that the known Tim-3 ligands can interact with receptors other than Tim-3, the Tim-3 ligands may initiate Tim-3 independent signal or recruit other receptors to Tim-3. If so, the different combination of Tim-3 and non-Tim-3 signaling, or formation of different receptor complexes with Tim-3 may be able to differentially influence T cell function.

Another possibility is that augmentation of T cell activation by Tim-3 eventually leads to inhibition of T cell function. First, Tim-3 may accelerate negative feedback. Above, I discussed Fyn as a putative molecule responsible for enhancement of TCR signaling. Intriguingly, Fyn also has role in negative regulation of TCR signaling. There were reported lymphoproliferative and autoimmune phenotypes in Fyn-deficient mice [202, 203] and Fyn is upregulated in anergic T cells [204]. PAG is a membrane adaptor protein which recruits Csk, an inhibitor of SFKs. Fyn interacts with PAG and phosphorylates PAG more efficiently than Lck to regulate the recruitment of Csk to PAG. In addition PAG associated Fyn enhances Ca^{2+}signlaling, without MAPK activation leading to anergy induction in T cells [205-207].

Secondly, an activating signal by Tim-3 may contribute to induction or maintenance of T cell exhaustion. T cell exhaustion is the phenomenon whereby T cells lose their effector function over time, as T cells are chronically stimulated with persistent and high antigen load [208]. Gene expression array data show that of the genes differentially upregulated in exhausted CD8 T cells, a greater number of these genes overlap with effector T cells than with memory T cells. I looked up the expression profile of the candidate molecules of Tim-3 signaling in the gene array data [150]. Interestingly, activating molecules like Fyn, MAP3K1, and NFATc1 were upregulated and inhibitory molecules like RasGAP and Dgkα were downregulated, in the exhausted T cells. The observation that in chronic HIV patients, basal phosphorylation level of STAT5 and MAP kinases like ERK and p38 were elevated in Tim-3^{hi} CD8 T cells, compared to Tim-3^{lo} CD8 T cells [115], also supports the correlation between hyperactivation of T cells and dysfunctional phenotype in Tim-3 expressing T cells.

During immune activation as in infection, antigen-specific T cells initially expand and differentiate, then undergo contraction during which the activated effector T cells continue to differentiate to short-lived terminal effector or exhausted T cells and a subset of the effector cells become memory T cells [209]. At the level of single T cells, exhaustion is the loss of effector function and proliferative ability. In the scope of a T cell population, T cell exhaustion can be interpreted as depletion/loss of long-lived functional T cell population. Activating signal via antigen receptor is necessary for the differentiation of effector T cells and memory T cells during infection, there is evidence that strong activation, especially activation of metabolism pathway, skews the balance of T cell differentiation towards effector T cells, resulting in reduced memory T cell differentiation. Inhibition of mTOR with rapamycin during LCMV infection enhances the number and the quality of virus specific memory CD8 T cells [210], prolonged IL-2R signaling

promoted terminal differentiation of effector CD8 T cells at the expense of memory CD8 T cells [211, 212]. In addition, there were drastic increases in the frequency of effector memory (CD44^{hi}CD62L^{lo}) CD4 T cells from *Map3k2$^{-/-}$Map3k3$^{Lck-Cre/-}$*CD4$^+$ mice [213]. Interestingly these pathways overlap with the downstream signaling of Tim-3, that we identified.

Thus, S6 is downstream of mTOR and MAP kinases pathway, ERK is downstream of MEKK2/MEKK3 and p85 is downstream of IL-2. Analyzing the profile of activation and memory markers in Tim-3-PD-1$^+$ and Tim-3$^+$PD-1$^+$ TILs, the frequency of CD44^{int} cells (transition from naïve to effector) and CD44^{hi}CD62L^{lo} (effector memory) was similar and slightly higher, respectively, in Tim-3$^+$PD-1$^+$ TILs than Tim-3$^-$PD-1$^+$ TILs. However, the frequency of CD44^{hi}CD62L^{hi}cells (central memory) was much lower in Tim-3$^+$PD-1$^+$ cells [49]. Thus, I speculate that Tim-3 promotes T cell differentiation towards short-lived effector cells by contributing to stronger stimulation, resulting in loss of long-lived functional T cells.

While we propose an acute activating function of Tim-3 in Th1 and CD8 T cells, PD-1 is known to inhibit the PI3K/Akt pathway, then how might Tim-3 and PD-1 cooperates in T cell exhaustion? Recently, Macintyre et al demonstrated that IL-2-induced expression of CTL activation and metabolism were mediated by separate pathways. p110δ and Akt was necessary for the expression of CTL effector molecules like IFN-γ, perforins and granzymes, but dispensable for proliferation and metabolism in CD8 T cells. Instead, PDK1 is required for proliferation and metabolism by IL-2 [201]. Possibly, PD-1 inhibits the PI3K-Akt pathway to represses cytotoxic function of CD8 T cells, while Tim-3 activates metabolic pathways by activating ERK and S6, to promote T cell differentiation toward short-lived/exhausted T cells, at the expense of memory precursors (Figure 29). To address this issue, it will be very interesting to investigate whether Tim-3 modulates metabolism of effector T cells, and affects effector and

memory formation in chronic infection or tumor conditions using Tim-3 transgenic or knockout in vivo model.
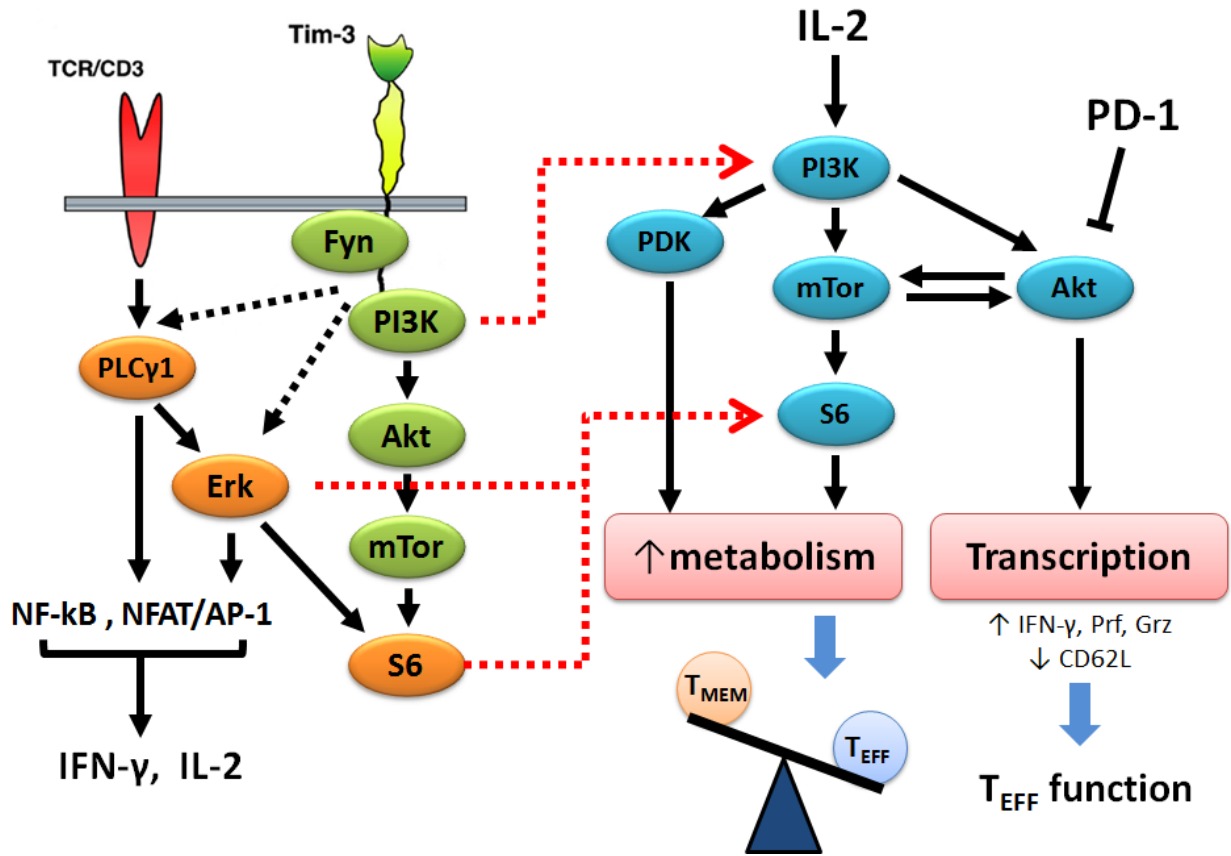
Figure 29. Model for a role of Tim-3 in effector/memory T cell differentiation.

Part of this research and all or part of Figure 6, Figure 7, Figure 10, Figure 11, Figure 13, Figure 14, Figure 15, Figure 17, Figure 18, Figure 20, Figure 22, Figure 23, Figure 25 and Figure 26 were originally published in Molecular and Cellular Biology. Judong Lee, Ee Wern Su, Chen Zhu, Sarah Hainline, Jiayao Phuah, Jamie A. Moroco, Thomas E. Smithgall, Vijay K. Kuchroo and Lawrence P. Kane. Phosphotyrosine-Dependent Coupling of Tim-3 to TCR Signaling Pathways. *Molecular and Cellular Biology.* 2011, 31(19):3963-3974. © the American Society for Biochemistry and Molecular Biology.

APPENDIX A

ABBREVIATIONS

AML   acute myeloid leukemia

AP-1   activator protein 1

BMMC   bone marrow-derived mast cell

CIA   collagen-induced arthritis

CRD   carbohydrate recognition domain

CTLA-4   cytotoxic T lymphocyte antigen 4

DAG   diacyl glycerol

EAE   Experimental autoimmune encephalomyelitis

HMGB1   high mobility group protein B1

IP   immunoprecipitation

IP_3 inositol 1,4,5-trisphosphate

ITAM   immunoreceptor tyrosine-based activation motif

ITIM   immunoreceptor tyrosine-based inhibitory motif

ITSM   immunoreceptor tyrosine-based switch motif

LAT   lymphocyte activting protein

LCMV	lymphocytic choriomeningitis virus
MILIBS	metal ion dependent ligand binding site
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa B
NFAT	nuclear factor of activated T cells
NOD	non-obese diabetic
NSCLC	non-small cell lung cancer
PD-1	programmed death 1
PDK	3'-phosphoinositide-dependent kinase
PI3K	phosphoinositol-3 kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	phosphoalipase C
PMA	phorbol 12-myristate 13-acetate
PMBC	peripheral blood mononuclear cell
PS	phosphatidylserine
RA	rheumatoid arthritis
SFK	Src family kinae
SLP-76	SH2 domain containing leukocyte protein of 76kDa
TIL	tumor infiltrating lymphocyte
TIM	T cell immunoglobulin and mucin domain protein
ZAP70	zeta-chain-associated protein kinase of 70 kDa

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