TIM-3 and Galectin-9 Regulation of Effector T Cell Activation and Function

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The T cell Immunoglobulin Domain and Mucin Domain 3 (TIM-3) is a type I glycoprotein expressed primarily on the surface of activated T cells and myeloid cells. The extracellular domain of TIM-3 consists of an IgV domain and a mucin domain with several sites for N- and O-linked glycosylation. The IgV domain is important for binding of TIM-3 to two of its known ligands, a β-galactoside binding lectin known as galectin-9 (Gal-9) and phosphatidylserine, a marker of early apoptosis. The cytoplasmic tail of TIM-3 has six conserved tyrosines, although their role in modulating downstream signaling pathways has yet to be determined. TIM-3 is widely regarded as a negative regulator of effector T cell function and viability. TIM-3 is also upregulated on exhausted T cells and is postulated to have a role in the development and/or maintenance of T cell exhaustion.

However, the exact regulation of T cells by TIM-3 has not been fully established for several reasons. TIM-3 and at least one of its ligand is expressed on both T cells and antigen presenting cells (APC). Therefore, it is not clear whether TIM-3 antibodies or Tim-3 Ig fusion proteins block the ligation of TIM-3 on T cells or on APCs to enhance effector T cell function. Additionally, gal-9 can also induce apoptosis in cells lacking the expression of TIM-3 and has been shown to positively regulate other cell types such as dendritic cells and mast cells. As TIM-3 is becoming an increasingly attractive therapeutic target because of its ability to reverse exhaustion in T cells, it is important to determine the regulatory nature of TIM-3 on T cells. To

do this, we expressed Tim-3 ectopically in Tim-3- Jurkat T cells and observed that Tim-3 enhances instead of inhibits signaling downstream of the T-cell receptor and co-stimulator, CD28. Then, using a series of truncation and point mutants of Tim-3, we determined that Y256 and Y263 are the most crucial of the six conserved tyrosines in mediating Tim-3 signaling. Another unexpected finding was that in addition to apoptosis, gal-9 also induces the secretion of pro-inflammatory cytokines from T helper subsets independently of Tim-3.

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I would like to dedicate this thesis to a few individuals whose presence in my life has allowed me to be where I am today.

To my parents, Too Kiat and Jee Fun, who both came from humble beginnings and understood that a good education was the best gift they could ever give my sisters and I. It is with your love, support and unwavering belief in me that on the days that are the most trying, I dare to dream. I also want to thank you both for teaching me the importance of happiness, for that is a lesson few learn in their lifetime.

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And finally, Larry. Thank you for your guidance and support throughout this entire process. Research is mired with a great deal of uncertainty and frustration. Nevertheless, you have allowed me to pursue my ideas and gut instincts freely while keeping a steady watch. For that, I am truly grateful.

1.0 THE TIM GENE FAMILY

Atopic diseases such as asthma and atopic dermatitis arise in genetically predisposed individuals under varying environmental conditions [1, 2]. Therefore, identifying a single chromosomal region that confers susceptibility to atopy has been challenging due to the contribution of both genetic and environmental factors. Nevertheless, in 2001, the T cell and Airway Phenotype Regulator (*Tapr*) region was identified in HBA mice, a congenic mouse strain produced by crossing asthma-susceptible BALB/c mice with asthma-resistant DBA/2 mice [3]. *Tapr* protects against airway hyperreactivity in HBA mice by limiting Th2 responses. However, it is genetically separable from the IL-4 cytokine gene cluster. It was within the *Tapr* that the T-cell, immunoglobulin domain and mucin domain (TIM) gene family was cloned. In mice, the TIM gene family is found on chromosome 11B1.1 which encodes for Tim-1, Tim-2, Tim-3 and Tim-4, while the human chromosome 5q33.2 contains genes for for TIM-1, TIM-3 and TIM -4. With the exception of Tim-4, all Tim proteins are expressed on T cells.

TIM proteins share a common architecture (Figure 1); an extracellular domain consisting of an IgV domain and a mucin domain with multiple sites for both N-linked and O-linked glycosylation, followed by a transmembrane domain and a cytoplasmic tail[2]. With the exception of TIM-4, the cytoplasmic tails of all TIM proteins have at least one tyrosine residue. Crystal structure definition reveals that the TIM IgV domains consists of two anti-parallel β-

sheets formed by B,E and D strands in one sheet (BED) and G,F,C,C' and C'' strands in the other (GFC). The first and last of the six cysteine residues that are conserved in all TIM proteins, form a disulphide bond linking the BED and GFC β-sheets. Disulphide bonds formed between the four remaining cysteine residues, fold the long CC' loop onto the GFC β-sheet to create a binding cleft unique to TIM-1, TIM-3 and TIM-4 [4, 5]. In canonical IgV domains, the CC' loop does not fold onto the GFC β-sheet, allowing it to form other intermolecular interactions [6]. This unique binding cleft is stabilized by a hydrogen bond formed between an arginine residue in the F β-strand and lysine residue in the G β-strand [6].

Upon closer examination, asparagine and arginine residues were found to coordinate a calcium ion within this cleft, and has therefore been named the metal ion-dependent ligand binding site (MILIBS)[7] (see Fig. 2A). Thus far only one ligand has been shown to bind to the MILIBS – phosphatidylserine (PS). PS is usually found in the inner leaflet of the plasma membrane but is redistributed to the outer leaflet during apoptosis or transiently during T cell activation [8, 9]. The acidic phosphate group of PS coordinates with the calcium ion deep within the MILIBS while the carboxylate group of PS forms a hydrogen bond with a serine residue conserved in the CC' loop of most TIM proteins. Recognition of PS in the outer leaflet of the plasma membrane requires hydrophobic residues located in either the CC' and FG loop [10]. Residues in the BC loop also contribute towards the binding of TIM proteins to PS. Binding studies have shown that Tim-1 and Tim-4 bind to PS with a much higher affinity than Tim-3[10]. This is most likely due to variations within the BC, CC' and FG loops. Thus far, PS remains the only common ligand to TIM-1, TIM-3 and TIM-4.

Apart from their association with atopic diseases, TIM-1, TIM-2 and TIM-3 have also been implicated in autoimmune diseases, allograft tolerance, chronic viral infections and cancer,

indicating that TIM proteins have a broader role in immune regulation [11-16]. As this introduction is focused on the regulation of T cells by TIM family members, TIM-4 is will not be discussed in detail in this introduction. Also, when referring to human TIM family members, TIM will be in uppercase while mouse Tim family members will be in lowercase.

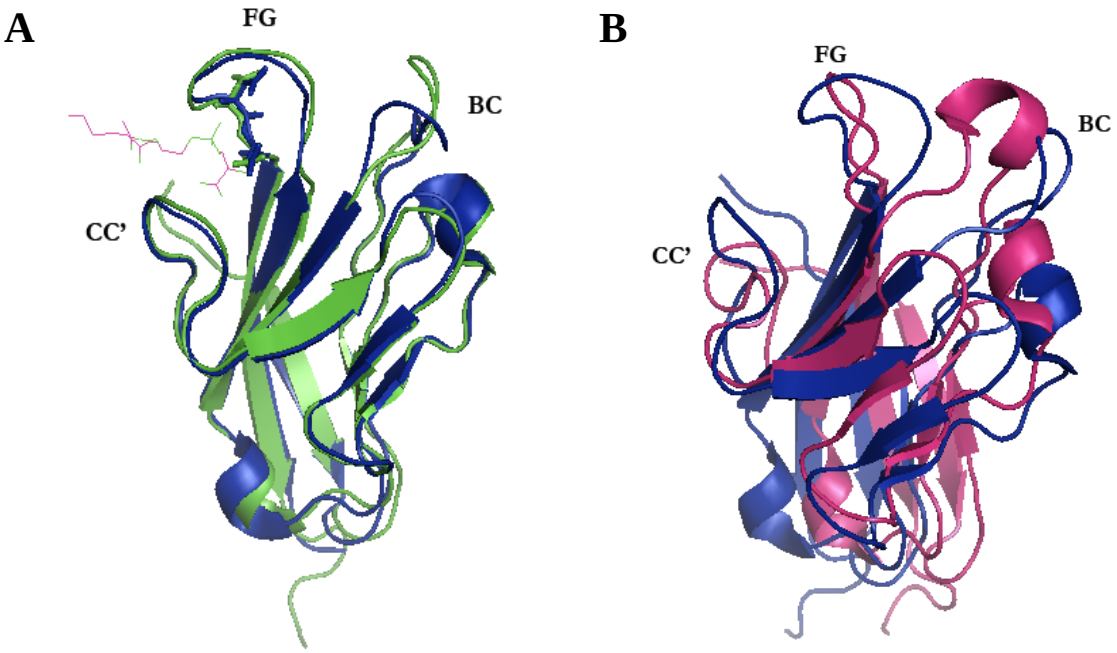

Figure 1: Comparison of Tim family IgV domains

(A) Tim-1 IgV domain (green) superimposed onto Tim-4 IgV domain (blue). Phosphatidylserine is seen in the MILIBS, which is conserved in Tim-1, Tim-3 and Tim-4 **(B)** Tim-1 IgV domain (blue) superimposed onto the Tim-2 IgV domain (purple). Unlike Tim-1, the CC' loop is not folded onto the GFC β-sheet.

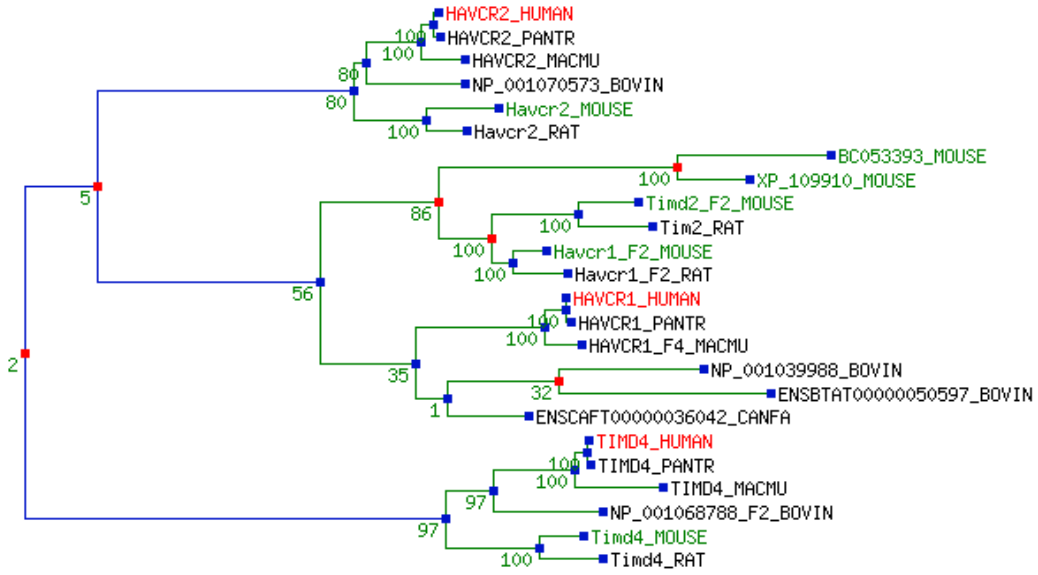

Figure 2: Phylogenic tree of mammalian TIM homologs.

Diagram above shows the evolutionary relationship of TIM family members from various mammalian species to mouse Tim-1.Mouse Tim proteins bear the greatest homology to rat Tim proteins whereas human TIM proteins are evolutionarily closest to chimpanzee (PANTR). TIM-2 is the most closely related TIM family member to TIM-1 (HAVCR-1), followed byTIM-3 (HAVCR2) and TIM-4.

1.1 TIM-1

1.1.1 Structure and function

TIM-1 is preferentially expressed on Th2 cells and activated T cells. Epidemiological studies that date back to as early as 1997, have reported an association between HAV infection and reduced

incidence of allergy and asthma [17, 18]. In humans, the ortholog of mouse Tim-1 is the hepatitis A virus receptor (HAVCR1)[3]. An insertion/deletion polymorphism in TIM-1 has been found to confer protection against atopy in individuals who have been infected previously with the hepatitis A virus (HAV) [19]. With this revelation, we now appreciate how HAV may have a direct role in protection against atopic diseases and does not just serve as an indicator of poor hygiene. Another ortholog of mouse Tim-1 is rat Kim1, a kidney injury molecule that has been proposed as a biomarker for renal cell carcinoma [20]. There are two splice variants of human KIM-1, both of which are lacking some portion of the cytoplasmic tail – TIM-1a, which lacks the tyrosine phosphorylation motif and Tim-1b, which has the signaling motif but is susceptible to cleavage by metalloproteinases [21, 22]. Tim-1a is found primarily in the liver while Tim-1b is the variant most expressed in the kidney [22]. Apart from HAV, TIM-1 also binds to TIM-4, another TIM family member expressed primarily on dendritic cells, and phosphatidylserine (PS), a marker of early apoptosis[10, 23]. Recently, it was shown that the interaction between TIM-1 and TIM-4 is mediated by exosomes and therefore, is indirect [24].

The extracellular domain of TIM-1 is predicted to have sixty glycosylation sites, a majority of which are O-linked [3]. In both mice and humans, TIM-1 polymorphisms are localized within the mucin domain (Figure 4 and Table 1: Human TIM-1 polymorphisms). Two residues, His64 and Glu67 of the TIM-1 DE loop are thought to be important for this trans-TIM-1 binding, since mutating histidine at position 64 to glutamic acid leads to significant reductions in homophilic Tim-1:Tim-1 binding and partially decreased levels of Tim-1:Tim-4 binding[4]. This could be of potential biologic relevance because this homophilic binding is conserved in humans. The authors also demonstrate that Tim-1 clusters mostly in the cytoplasm but relocates to the cell surface upon ionomycin or phorbol ester treatment[4]. Interestingly, the localization of

Tim-1 to these clusters appears to be dependent on the four critical residues of the MILIBS, another conserved feature of Tim proteins[7].

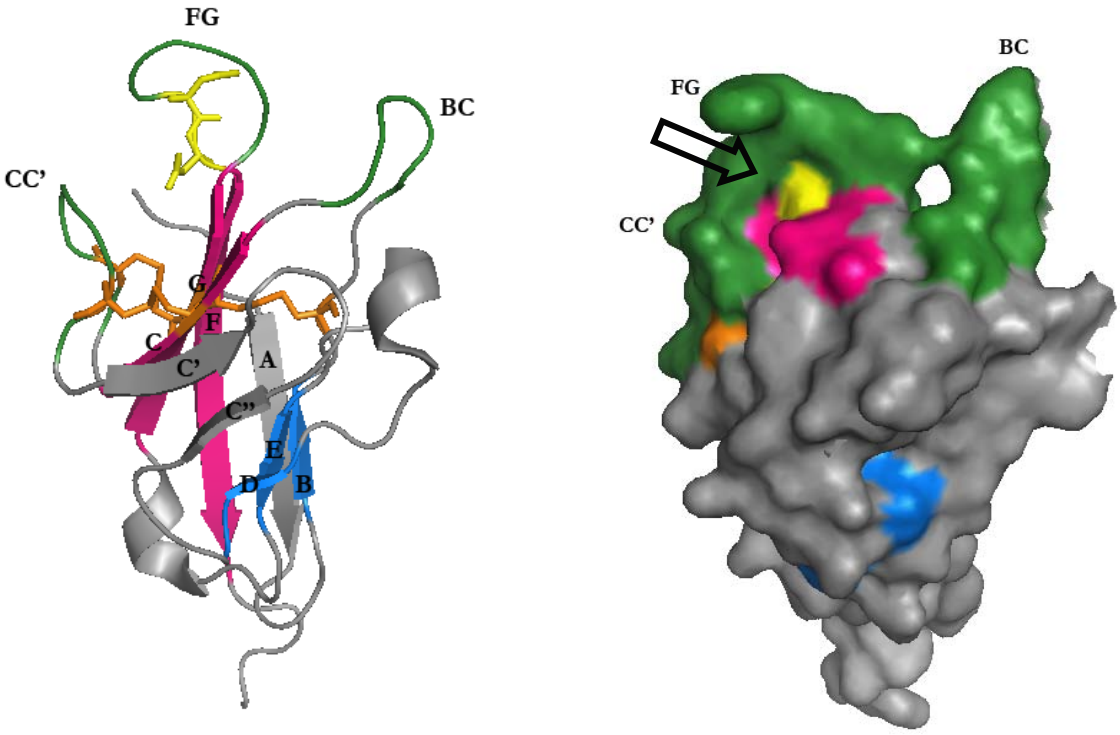

Figure 3: The Tim-1 IgV domain

Ribbon diagram of the Tim-1 IgV domain is shown on the left while the surface diagram is shown on the right. The BED β-sheet is highlighted in blue and GFC β-sheet in pink. Disulphide bonds are highlighted in orange, while the CC', FG and BC loops are highlighted in green. The two residues that coordinate the Ca2+ ion in the MILIBS are highlighted in yellow. A polymorphism in mkTim-1 (Lys88Gln), indicated approximately by the arrow, is required for binding of HAV to mkTim-1. Diagrams were generated using PDB ID: 2OR8 using the PyMol software.

Figure 4: Alignment of TIM-1 sequences.

There is a 42% sequence identity between mouse Tim-1(C57Bl6) and human TIM-1.β-strands are underlined, transmembrane domain highlighted in red, and conserved tyrosine residues highlighted in green . Residues that coordinate the Ca2+ ion in the MILIBS are highlighted in blue while the homophilic residues that allow Tim-3 to bind to PS on the membrane surface are highlighted in purple. Polymorphisms between C57Bl/6 and BALB/c highlighted in orange.

Table 1: Human TIM-1 polymorphisms

Location	*Polymorphism*	*Disease association*
Mucin	157insMTTTVP	Generates the "long" form of TIM-1 Protects against atopy only in individuals with prior HAV infection [19] Increased risk for severe hepatitis [25]
	5509-5511delCAA	Asthma[26] Rheumatoid arthritis [27, 28]
	5383-5397	Atopic dermatitis Rheumatoid arthritis [27, 28]
Promoter	-1637A>G	Rheumatoid arthritis [29]

1.1.2 Immune regulation

1.1.2.1 Effector T cells

Cross-linking Tim-1 in conjunction with TCR and CD28 enhances the proliferation of naïve CD4+ T cells and increases the production of IL-4 from Th2 cells [30]. When administered along with antigen, agonistic Tim-1 antibody abrogates the induction of respiratory tolerance by promoting the secretion of IL-4 and IFN-γ and proliferation of antigen-specific T cells [30, 31]. Therefore, Tim-1 is a co-stimulatory receptor with the ability to prevent the induction of tolerance by enhancing T helper effector function.

The ability for Tim-1 to modulate T cell activation has been studied from a mechanistic perspective. In reporter assays, the ectopic expression of Tim-1 leads to NFAT/AP-1 transcriptional activation which is dependent on Y276 in the cytoplasmic tail [32]. CD3 capping experiments showed that Tim-1 is recruited to the TCR signaling via its association with CD3. This study also showed that crosslinking of Tim-1 by an agonistic antibody induces the phosphorylation of Tim-1, ζ chain associate protein kinase 70 (ZAP-70) and IL-2-inducible T cell kinase (ITK). In addition, activation of Tim-1 recruits a complex consisting of ITK and phosphatidyl inositol-3 kinase (PI3K) to the TCR signaling complex [33]. More recent work has also suggested that the p85α subunit of PI3K is recruited to Y276 after it is phosphorylated by Lck [34]. Tim-4-Ig fusion proteins induces the phosphorylation of thymoma viral proto-oncogene 1(Akt), mitogen activated protein kinase ½ (ERK1/2) and pro-apoptotic factor, B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl2) in CD3+ T cells [35].

Although it appears that Tim-1 usually regulates T cell activation in a positive manner, this is not always the case. The agonistic (3B3) and antagonistic (RMT1-10) monoclonal antibodies to Tim-1 show that Tim-1 can have both positive and negative regulatory effects on T cells [36]. 3B3 increases secretion of IFN-γ and IL-17, exacerbating EAE in mice, while RMT1-10 promotes heart allograft tolerance by inhibiting the function of aggressive IL-17-secreting CD8+ T cells [37]. Further investigation revealed that while 3B3 and RMT1-10 both bind to the IgV domain of Tim-1 and induces CD3 capping, 3B3 binds to Tim-1 with much higher affinity and can cause cytoskeletal reorganization [36].

1.1.2.2 Mast cells and macrophages

Tim-1 is expressed constitutively on the surface of peritoneal mast cells and bone marrow-derived cultured mast cell but is downregulated following IgE and antigen stimulation. However, the addition of Tim-4 promotes the production of Th2 cytokines, such as IL-4, IL-6, and IL-13 without affecting degranulation, presumable through Tim-1[38]. In addition, macrophages also appear to be influenced by Tim-1. Treatment of the macrophage cell line, RAW264.7 with recombinant mouse Tim-1 (rmTim-1) resulted in increased production of TNF-α, IL-6, and IL-10 and upregulation of B7-1, B7-H1, B7-H2 and PD-L2. It is important to note that rmTim-1 was comparable to LPS in its ability to promote the upregulation of B7 family members in RAW264.7 cells[39].

1.1.2.3 Tolerance and Regulatory T cells (Tregs)

The ability for Tim-1 to either promote or suppress the development of Tregs is also dependent on the nature of the crosslinking antibody. *In vivo*, the agonistic Tim-1 antibody, 3B3, drives the polarization of alloreactive T cells towards Th1 and Th17 phenotypes but deprograms Tregs. 3B3 abrogates tolerance induced in mice by co-stimulatory blockade, resulting in allograft rejection[40]. In contrast, the antagonistic anti-Tim-1 Ab, RMT1-10, prolonged the survival of fully-MHC mismatched cardiac by inhibiting Th1 responses while promoting Th2 responses. Unlike the agonistic antibody, RMT1-10 inhibited the function of allospecific effector T cells without modulating the function or development of Tregs [16].

1.1.2.4 Invariant NKT cells (iNKT)

iNKT cells comprise a small subset of T cells that express both natural killer cell (NK) lineage markers and a semi-invariant TCR[41]. Recognition of glycolipid antigens is restricted to the non-classical class I MHC molecule, CD1d [42]. When activated, iNKT cells are able to secrete high levels of IL-4 and IFN-γ. Recently, iNKT cells were found to constitutively express Tim-1 and were able to bind without engulfing eryptotic red blood cells (ERBC)[43]. Tim-1 was shown to co-stimulate the activation of iNKT cells by CD1d and α-galactosylceramide. Likewise, ERBCs could also induce iNKT cells to proliferate. The *in vivo* relevance of iNKT cell recognition of apoptotic cells was further explored by using the agonistic anti-Fas mAb (Clone Jo2) to induce apoptosis in the liver and lung.

In the liver, treatment with anti-Fas mAb increased the number of iNKT cells – an effect that was abolished with the co-administration of anti-Tim-1 antibody (Clone 3D10) [43]. Anti-Tim-1 antibody alone did not increase the number of iNKT cells in the liver. To assess the involvement of iNKT cells in the development of hepatitis, serum ALT levels was measured in wild-type and Cd1d-/- mice following treatment with anti-Fas mAb and/or anti-Tim-1 antibody. The administration of anti-Tim-1 antibody decreased serum ALT levels induced by anti-Fas mAb treatment. CD1d-/- mice, which lack iNKT cells, had significantly reduced ALT levels following anti-Fas mAb treatment. Together, this indicates that iNKT cells play a role in promoting liver injury and that the ability for iNKT cells to recognize apoptosis hepatocytes is central to this role. A follow up study has showed that iNKT cells can also contribute towards hepatitis by binding to HAV[25]. Binding of HAV to TIM-1 improves the cytolytic function of iNKT cells against HAV-infected hepatocytes by inducing the production of IL-4, IFN-γ and granzyme B.

Intranasal administration of anti-Fas mAb induced airway inflammation as characterized by an increase in inflammatory infiltrate in the peribronchial space and the number of iNKT cells and neutrophils in the bronchoalveolar lavage fluid (BAL)[43]. This increase in the number of cells in the BAL fluid was not observed in mice treated with both anti-Tim-1 antibody and anti-Fas mAb, as well as in anti-Fas mAb treated Cd1d-/- mice. To determine if iNKT cell activation by apoptotic lung epithelial cells could lead to airway hyperreactivity (AHR), airway resistance in the presence of increasing doses of metacholine was measured in mice treated with saline only, or with anti-Fas mAb and/or anti-Tim-1 antibody[43]. This study showed that similar to airway inflammation, anti-Fas mAb treatment induced AHR depended on Tim-1 and iNKT cells. Using cytokine knockout mice, it was determined that AHR induced by anti-Fas mAb induce requires the secretion of IL-4, IL-17, IL-17 and IFN-γ[43].

Therefore, iNKT cells are rapidly emerging as an important player in the induction of both liver injury and AHR. The next step would be to compare the signaling pathways downstream of TIM-1 in iNKT cells when it is stimulated by PS and HAV.

1.1.2.5 Tim-1 as PS receptors

The removal of apoptotic bodies is generally associated with the induction of tolerance as the phagocytosis of apoptotic bodies by dendritic cells can lead to T cell anergy and development of Tregs[44]. Additionally, impairment in the uptake of apoptotic bodies results in the abrogation of peripheral tolerance and induction of autoimmunity [45, 46]. Therefore, the identification of TIM-1 as a PS provides an additional pathway through which TIM-1 can promote peripheral tolerance. Both human and mouse TIM-1 have been shown to bind to PS. NIH3T3 fibroblasts transfected with TIM-1 gained the ability to engulf apoptotic cells [10]. This ability was

diminished in the presence of TIM-1 mAb, which shows the engulfment is mediated TIM-1 dependent. In injured kidneys, Kim-1 expressing, tubule epithelial cells were found to phagocytose apoptotic bodies and necrotic debris. Confocal imaging studies show that Tim-1 co-localizes around the site of engulfment to facilitate the internalization of injured tubule cells expressing PS and oxidized lipoproteins [47].

Tim-1 and Tim-4 crystal structures suggest that the MILIBS is constructed by CC' and FG loops in the IgV domain is responsible for the recognition of PS. The hydrophilic phosphate head of PS can enter the cavity and interact with the Ca^{2+} ion while the fatty acid tail can interact with the aromatic residues of the FG loop [7]. Single mutations of residues required for the coordination of the Ca^{2+} ion decreases Tim-1 and Tim-4 binding to liposomes containing PS, while a double mutation completely abolished PS binding[7]. The identification of calcium as the divalent cation in the MILIBS is consistent with a prior observation that binding of PS to Tim-1 is abolished in the presence EDTA, which chelates calcium ions [7, 47]. Although purified fusion proteins of Tim-1 and Tim-4 binds only to PS and not to other phospholipids, kidney epithelial cells have been found to recognize both PS and phosphatidylethanolamine (PE) [47].

1.2 TIM-2

1.2.1 Structure and Function

Unlike Tim-1 and Tim-3, Tim-2 is the only mouse Tim family member that does not have a human ortholog. For this reason, there are comparatively fewer studies on Tim-2. Tim-2 is highly glycosylated and only has one tyrosine residue which is part of a tyrosine phosphorylation motif (RTRCEDQVY). Similarly to Tim-1, Tim-2 is upregulated on activated T cells and Th2 cells [48]. Its known ligands are Sema4A[49], a transmembrane protein expressed on dendritic cells and B cells, and H-ferritin[50], a component of an iron storing molecule. Structural analyses suggest that Tim-2 exists as a dimer which may facilitate its binding to multivalent ligands[4].

1.2.2 Immune regulation

Overexpression of Tim-2 in Jurkat T cells was shown to inhibit TCR/CD28 induced NFAT/AP-1 activity, suggesting that Tim-2 is a negative co-stimulator [51]. This is consistent with the observation that administration of Tim-2 Ig proteins ameliorates EAE in mice by generating splenocytes with high basal rate of proliferation and increased production of IL-4, IL-5 and IL-10[48]. Administration of Sema4A Ig also ameliorated EAE in mice [52]. CD4+ T cells from the lymph nodes of Tim-2 deficient mice challenged with keyhole limpet hyalocynin (KLH) emulsified in CFA secreted much higher levels of Th2-associated cytokines, IL-4, IL-5, IL-6 and IL-10[53]. The levels of IL-2 and TNF-α were similar in culture of wild-type and Tim-2

deficient mice. Interestingly, anti-CD3 stimulation elicited similar levels of Th2-cytokine production from both wild type and Tim-2 deficient mice[52].

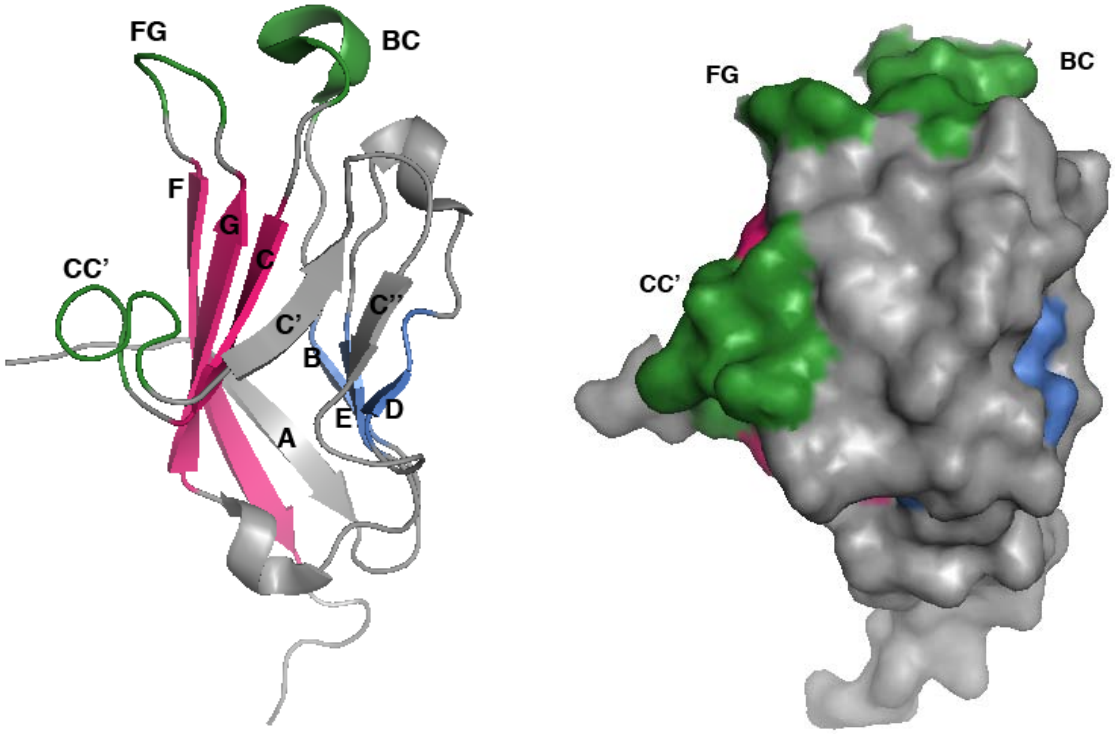

Figure 5: Tim-2 IgV domain

Ribbon diagram of Tim-2 is shown on the left and surface diagram is shown on the right. The GFC β-sheet is highlighted in pink while the BED sheet is highlighted in blue. The CC', FG and BC loops are highlighted in green. Although Tim-1 and Tim-2 have a high sequence identity(62%), Tim-2 does not have the unique binding cleft conserved in Tim-1, Tim-3 and Tim-4 primarily because its CC' loop is seen folded downwards and away from the GFC β-sheet. Replacement of the HLG sequence in the Tim-2 BC loop with YR sequence of the Tim-1 BC loop significantly reduced dimerization of Tim-2. Diagrams generated using PDB ID 2OR7 using the PyMol software.

1.3 TIM-3

1.3.1 Structure and function

In comparison to TIM-1 and TIM-2, TIM-3 is the most distantly related family member (Figure 2). Although it was originally identified through a screen for Th1-specific markers, TIM-3 has been detected on T cells under inflammatory conditions and is constitutively expressed on immature myeloid cells [54-56]. Thus far, TIM-3 has two known ligands - a β-galactoside binding lectin, which binds to in a carbohydrate-dependent fashion and phosphatidylserine (PS), an early marker of apoptosis, which binds in a non-carbohydrate dependent manner [57, 58]. However, other non-gal-9 ligand(s) for TIM-3 have also been detected on naive, effector, memory, regulatory T cells and dendritic cells [5, 59]. Interestingly, a putative ligand for TIM-3 is downregulated following activation in CD4+CD25-T cells but maintained on CD4+CD25+ T cells [60].

There are both membrane-bound and soluble forms of TIM-3. The membrane-bound form of TIM-3 includes an N-terminal IgV domain, a mucin domain followed by a transmembrane domain and a short cytoplasmic tail. And although the soluble form, which is a splice variant of Tim-3 that lacks both mucin and transmembrane domains, it still possesses the ligand binding specificity of the membrane-bound form [59]. Similarly to TIM-1, TIM-2 and TIM-4, the IgV domain of TIM-3 consists of a two-layered β-sheet sandwich (Figure 6, left) held

together and stabilized by disulphide bonds between the B and F strands (Cys-38-Cys-111), hydrogen bonds (Trp-53 of the C-strand and Val-94 of the E-strand, Tyr-109 of the F-strand and Asp-105 of preceding F-strand) and a salt bridge (Arg-82 and Asp-105 preceding D and F strands)[5]. These interactions are found in other canonical IgV domains. However, two inter-sheet disulphide bond formed by noncanonical cysteine residues invariant within the TIM family creates a unique cleft between the CC' loop and FG loop. Mutations of residues within the cleft either reduced or abolish binding of Tim-3 Ig to putative ligands on the surface of 3T3 cells and naïve CD4+ T cells (Figure 6, right). Interestingly, this cleft does not have any potential N- or O-linked glycosylation sites and therefore, is not required for binding to gal-9[5].

There are seven predicted polymorphic residues between the asthma resistant (HBA) and asthma susceptible (BALB/c) alleles of Tim-3[3]. These residues are located in the IgV domain but are positioned away from the unique binding cleft. Recently, it was shown that three of these residues which are located in the BC loop, contribute towards the differential binding of PS [58]. HEK293 cells expressing the BALB/c allele of Tim-3 were better at phagocytosing apoptotic thymocytes than those expressing the HBA allele [58]. As with Tim-1 and Tim-4, the binding of PS also requires the metal-ion ligand binding site (MILIBS), located within the unique binding cleft [7]. This binding site is formed between Asn, Asp and a calcium ion which allows coordination with the charged head of PS (Figure 7). The binding of PS to Tim-3 on T cells has no known effect.

The signaling pathways downstream of Tim-3 have yet to be dissected. There are six tyrosines in the cytoplasmic tail that do not constitute any obvious signaling motifs [61]. These tyrosines are well conserved in both mouse and human homologs of Tim-3 and have been shown to be inducibly phosphorylated when ectopically expressed in HEK293 cells [62].

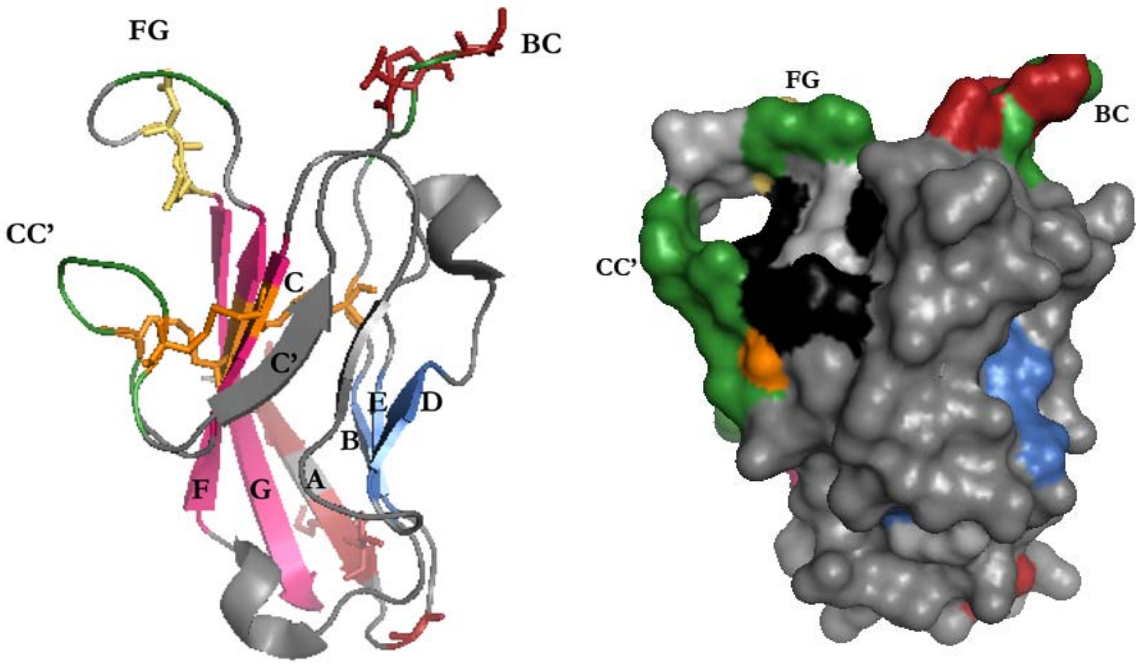

Figure 6: Tim-3 IgV domain

The ribbon diagram of Tim-3 is shown on the left and the surface diagram, on the right. The BED β-sheet is highlighted in blue and the GFC β-sheet is highlighted in pink. Disulphide bonds are highlighted in orange while residues required for the binding of PS on the outer membrane surface and coordination of the Ca2+ are highlighted in red and yellow respectively. In the surface diagram, residues highlighted in black are required for binding of Tim-3 Ig to non-galectin-9 ligands. Diagrams generated using PDB ID: 2OYP using the PyMol software.

Figure 7: Alignment of mouse and human TIM-3 sequences

There is a 63% sequence identity between mouse Tim-3 (C57Bl/6) and human TIM-3. β-strands are underlined and named, the transmembrane domain is highlighted in red, and conserved tyrosine residues are highlighted in green . Residues that coordinate the Ca^{2+} ion in the MILIBS are highlighted in blue while the homophilic residues that allow Tim-3 to bind to PS on the membrane surface are highlighted in purple. Published amino acid polymorphisms between C57Bl/6 and BALB/c mice are highlighted in orange.

Table 2: Human TIM-3 polymorphisms

Location	*Polymorphism*	*Disease association*
Mucin	4295G>T	Allergic rhinitis Rheumatoid arthritis[28]
Promoter	-574T>G	Asthma Allergic rhinitis Rhematoid arthritis

1.3.2 Reagents and mouse models used to examine Tim-3/Tim-3L interactions

1.3.2.1 Antibodies and Tim-3 fusion proteins

To examine the role of Tim-3 both *in vivo* and *ex vivo*, a variety of reagents and antibodies have been used **(**Table 4). In general, both antibodies and Tim-3 fusion proteins are thought to modulate Tim-3 activity on T cells by blocking interaction with its ligand(s). Although all these antibodies appear to improve the capacity of T cells to secrete cytokines and proliferate following antigen receptor stimulation, no study has yet to test the possibility that these antibodies are blocking and/or agonistic. Additionally, whether these antibodies specifically interfere with Tim-3 binding to gal-9 or one of its other non-gal-9 ligand is not known.

Table 3: Antibodies and fusion proteins used to study TIM-3 function

Antibody/ Fusion protein	Species	Outcome
Tim-3 Ig	Mouse	Prevented the induction of tolerance by co-stimulation blockade; accelerated diabetes in NOD mice[63] Generated CD4+ T cells that secreted IL-2 and IFN-γ spontaneously and had a high basal rate of proliferation[64]
5D12	C57Bl/6	Induce tyrosine phosphorylation in AE7 Th1 cell line and D2SC/1 dendritic cell line; induce NF-κB activation in D2SC/1 cells[65] Prevented the development and expansion of MDSC in Tim-3 Tg mice[66]
Tim-3 polyclonal antibody (R&D)	C57Bl/6	Induced secretion of Th2 cytokines by IgE –sensitized mast cells; protect from IL-3 withdrawal apoptosis[38]
RMT3-23		Accelerated allograft, enhanced effector function of allospecific T cells, prevented the expansion of allospecific Tregs, promoted Th1 and Th17 polarization[67]
8H7	BALB/c	Significantly reduced airway hyperreactivity transfer of OVA-specific Th2 cells and OVA challenge[68]
8B.2C12	BALB/c	Exacerbated EAE[61]
2E2	Human	Restored function to exhausted T cells from HIV chronic progressors and patients with advanced melanoma *ex vivo* [69, 70] Reinvigorated PD-1+Tim-3+ CD8+ T cells from mice infected chronically with LCMV[13]

1.3.2.2 Galectin-9 (Gal-9)

The interaction between Tim-3 and gal-9 was first recognized when Tim-3 Ig but not Tim-2 Ig precipitated bands migrating around 85 kilodaltons (kDa) and 40-60 kDa from the lysates of surface biotinylated CD8+ mouse lymphoma cells (TK-1)[57]. N-glycosidase (PNGase) treatment of these precipitates resulted in a sharper band migrating around 39kDa, which was identified as gal-9 by mass spectrometry. Gal-9 was then shown to induce apoptosis in *in vitro* polarized Tim-3+Th1 cells and ameliorated EAE in mice by eliminating IFN-γ-secreting, MOG (35-55)-specific T cells[57]. These observations helped shape the current hypothesis that Tim-3 terminates Th1 immunity.

Gal-9 belongs to galectin, a family of mammalian lectins that recognize the basic structure of N-acetyllactosamine (Galβ1-4GlcNac)[71, 72]. Galectins are classified according to their structure. There are prototype galectins (galectin-1,-2,-5,-7,-10,-11 and -13), chimera-type galectins (galectin-3) and tandem-repeat galectins (galectins-4,-6,-9, and -12)[73]. Prototype galectins exist as monomers or non-covalent homodimers, chimera-type galectins consist of a non-lectin domain attached to a single carbohydrate recognition domain (CRD), while tandem-repeat lectins consist of two CRD linked together by a polypeptide chain. Although galectins bind to N-acetyllactosamine with relatively low affinity (K_d = 90-100μM), they are able to engage receptors decorated with polylactosamine residues with a much greater affinity ($K_d \approx$ 1μM)[72]. The multivalency of galectins also allows them to cross-link several cell-surface receptors simultaneously[74].

Apart from being a Tim-3 ligand, gal-9 is also known as a neutrophil chemoattractant and a pro-apoptotic agent of activated CD4+T cells and cancer cells [75, 76]. In mice, gal-9 administration ameliorates EAE as well as collagen-induced arthritis by inducing the apoptosis of alloreactive T cells and synoviocytes respectively [57, 77]. However, gal-9 has also been shown to have positive regulatory effects on immune cells. Treatment of *in vitro* derived myeloid dendritic cells with gal-9 induces their maturation [78, 79]. Additionally, administration of gal-9 to tumor bearing mice suppressed tumor growth by improving CD8+ T cell cytolytic function and DC maturation[80]. It is possible that T cells and myeloid cells respond differently to gal-9 because they have different glycophenotypes [81].

Gal-9 requires both CRDs to bind to Tim-3. However, an R to A mutation a position 64 in the N-terminal CRD reduces the ability for gal-9 to bind to Tim-3 Ig to a greater degree than a similar point mutation as position 238 in the C-terminal CRD[57]. Although this suggests that the N-terminal CRD of gal-9 may be more important for binding to Tim-3, a recent study showed that the C-terminal CRD is more important for the induction of apoptosis in EL-4 thymoma cells [82].

1.3.2.3 Mouse models

Tim-3-/- mouse [64]

The Tim-3-/- mouse was generated by targeted deletion of the entire first exon and part of the second exon of the gene encoding Tim-3. Chimeric mice were then generated from Tim-3 deficient embryonic stem cell clones and crossed with BALB/c females for six to eight generation to produce Tim-3-/- mice. Tim-3-/- mice are viable with a no gross abnormalities. Thymic development was comparable to wild-type mice, producing normal numbers and ratio of

peripheral CD4+ and CD8+ T cells. Tim-3 deficiency *in vivo* did not appear to affect the development of Th1 responses as both Tim-3-/- and wild-type mice had comparable contact hypersenstivity. When cultured with allogeneic C57Bl/6 stimulators, T cells from Tim-3-/- mice secreted IFN-γ and IL-4 at levels comparable to T cells from wild-type mice. It is possible that because the Tim-3-/- mouse was generated on a BALB/c background and BALB/c mice are inherently biased towards Th2 responses, the loss of Tim-3 *in vivo* will not have a measurable effect.

Tim-3 Tg mouse[66]

To generate Tim-3 Tg mice, the BALB/c Tim-3 cDNA was placed under the control of the human CD2 promoter on the C57Bl/6 genetic background. These mice were viable, fertile, and did not exhibit any gross alterations in the size of lymphoid organs. Tim-3 is expressed on double negative thymocytes in the thymus but only 30-40% of CD4+SP and CD8SP+ thymocytes. In comparison to control littermates, there was a slight decrease in the frequency of double negative thymocytes in Tim-3 Tg+ mice. In the periphery, Tim-3 is also expressed on only 30-40% of CD4+ T cells and CD8+ T cells and is absent from Tregs. However, the number and frequency of CD4+, CD8+ and regulatory T cells were no different than wild-type littermates. Peripheral T cells that express or lack the expression of Tim-3 will hereby be referred to as Tim-3 Tg+ and Tim-3 Tg-. The frequency of $CD44^{high}$ cells was higher in Tim-3 Tg+ population of both CD4+ and CD8+ T cell while the frequency of $CD62^{low}$ cells was higher only in the Tim-3 Tg+ CD8+ T cell population. Interestingly, the percentage of myeloid suppressor cells ($CD11b+Gr-1+F4/80^{low}$) cells was higher in the spleens of Tim-3 Tg mice in comparison to WT mice.

Splenocytes from both Tim-3 Tg and Gal-9 Tg mice were suppressed in their ability proliferate and secrete IFN-γ in response to anti-CD3 stimulation. In gal-9 Tg mice, gal-9 expression is driven by the actin-promoter [66]. The suppressive cell type in these splenocyte cultures was identified to be myeloid-derived suppressor cells (MDSC), which are expanded in both Tim-3 Tg and Gal-9 Tg mice. MDSC are a heterogeneous population of myeloid cells that are expanded under high inflammatory conditions[83]. They are known to exert their immunosuppressive abilities onto T cells through IFN-g, ROS and arginine metabolism. Arginase II was highly upregulated on granulocytic MDSC and chemical inhibition of arginase (NOR-NOHA) abolished the ability for granulocytic MDSC from Gal-9 Tg mice to suppress T cells. The exacerbation of EAE in Tim-3 Tg mice treated with anti-Tim-3 Ab and Tim-3KO mice correlated with reduced frequencies of myeloid suppressor cells. Incubation of myeloid suppressor cells from gal-9 Tg mice (Gal9 Tg) with WT CD4+ T cells Their expansion and function were negatively affected in Gal-9 Tg X Tim-3-/- mice. The expansion of myeloid suppressor cells is dependent on Tim-3 expressing CD4+ T cells. Rag-/- mice that had received Tim-3 Tg+ CD4+ T cells had a greater frequency of myeloid suppressor cells than those that had received Tim-3 Tg- CD4- T cells. Tim-3+CD4+ T cells from Tim-3 Tg mice also helped stimulate anti-microbial activity of *Mycobacterium tuberculosis* (Mtb)-infected macrophages. This anti-microbial activity was shown to be dependent on caspase-1 and IL-1β but not dependent on IFN-γ or iNOS.

1.3.3 TIM-3 in Immune regulation

1.3.3.1 EAE/MS

The role of Tim-3 *in vivo* was first tested in experimental autoimmune encephalomyelitis (EAE), the mouse model of MS [61]. The choice of antigen used to induce EAE in these studies was proteolipid (PLP 139-151) emulsified in incomplete Freund's adjuvant administered in combination with pertussis toxin. Tim-3 was upregulated on CD4+ and CD8+ T cells infiltrating the CNS at the onset of disease but was downregulated with disease progression, indicating a possible role for Tim-3 in the initiation of EAE. Treatment with Tim-3 antibody did not alter the time of onset of EAE but accelerated disease progression [54]. To determine if activated macrophages were responsible for this hyperacute phenotype, splenocytes were harvested after onset of disease and stained for a variety of cell-specific markers. As expected, there was a marked increase in the percentage of CD11b+ cells in splenocytes of anti-Tim-3 treated mice. By selectively eliminating CD11b+, B220+ and CD3+ splenocytes from mixed cultures, the expansion of CD11b+ cells from anti-Tim-3 treated mice was found to require direct interaction between T cells and non-T cells.

Although the pathogenesis of EAE and MS are not identical, an initial study using T cell clones derived from the cerebrospinal fluid (CSF) of human patients with MS revealed an inverse correlation between Tim-3 expression and IFN-γ secretion[15]. T cell clones from the CSF of MS patients have lower levels of Tim-3 transcript but secrete higher levels of IFN-γ in comparison to those derived from healthy patients. As proof of principle, silencing Tim-3 expression in CD4+ T cells from healthy subjects was sufficient to increase their secretion of IFN-γ in response to anti-CD3 stimulation[15]. However, CD4+ T cells isolated from peripheral

blood mononuclear cells (PBMC) of MS patients had lower levels of Tim-3 expression than those from healthy subjects but did not secrete higher levels of IFN-γ. Blocking Tim-3/Tim-3L on CD4+ T cells from PBMC CD4+ T cells from MS patients did not improve their secretion of IFN-γ in response to anti-CD3 stimulation[84]. These studies suggest that in MS patients, Tim-3 regulates IFN-γ production only by CD4+ T cells in the CSF but not those circulating in the blood.

1.3.3.2 Tolerance

There are several mechanisms through which tolerance can be achieved *in vivo* – deletion of antigen-specific T cells, anergy, suppression by regulatory T cells and ignorance. Therefore, to investigate whether Tim-3 can modulate transplantation tolerance, several different tolerizing regimes have been employed. Sánchez-Fueyo et. al.used a combined treatment of donor specific transfusion (DST) and anti-CD154 (CD40L), which relies on CD4+CD25+ T cells to provide donor-specific allograft tolerance. In an accompanying paper, Sabatos et. al. treated mice with tolerogenic doses of PLP (139-151), a regimen that renders T cells unresponsive to stimulation. Administration of Tim-3-Ig fusion proteins to DST/anti-CD154 tolerized mice led to the rejection of donor islet allografts. The authors showed that Tim-3-Ig could only prevent tolerance towards allografts *in vivo* when present during and not after, CD4+CD25+ T cells have acquired their suppressive abilities[60]. It is not clear whether Tim-3-Ig treatment skews the ratio of effector:regulatory T cells or prevents the expression of an immunosuppressive cell-surface or secreted factor by CD4+CD25+ T cells.

In a model of antigen-induced tolerance, Tim-3-Ig treatment generates splenocytes that proliferate and secrete high levels of IL-2 and IFN-γ independently of PLP (139-151) antigen

restimulation. Interestingly, the spontaneous proliferation and cytokine secretion by T cells can only been seen in mixed splenocyte cultures. When purified, CD3+ T cells from mice treated with Tim-3-Ig only displayed high basal proliferation but were unable to secrete detectable levels of IL-2 or IFN-γ. Addition of either B220+ or CD11b+ cells from mice treated with either Tim-3-Ig or hIgG enhanced the proliferation of CD3+ T cells above that of splenocyte cultures and promoted their secretion of IL-2 and IFN-γ[59]. These biological responses could not be titrated with increasing concentrations of PLP (139-151), suggesting that Tim-3-Ig prevents tolerance in this model by lowering the threshold of activation of both antigen-specific and non-specific T cells, resulting in enhanced effector responses.

In studies using gal-9 to modulate Tim-3 activity, mice receiving gal-9 were able to delay the rejection of fully allogeneic skin grafts for up to 6 days[85]. This delay was attributed to the ability for gal-9 to inhibit proliferation of lymphocytes in response to anti-CD3/CD28 stimulation and reduce the number of CD8+Tim3+ T cells in the draining lymph nodes of gal-9 treated mice day 7 post-transplantation. The authors also noted a reduction in serum IFN-γ levels of gal-9 treated mice with a slight increase in both IL-2 and IL-4 levels. In an accompanying paper, the authors showed that gal-9 prolongs skin allograft survival inducing apoptosis in CD8+CD44^{high}CD62L^{low} T cells and reducing the cytolytic ability of those that survive[86].

Therefore, Tim-3 is required for the induction of peripheral tolerance as it negatively affects the function and viability of allospecific T cells and is possibly required for the development of peripheral Tregs.

1.3.3.3 TIM-3 in Immune Response to Cancer

The relationship between Tim-3 and cancer has only been explored recently. Spontaneous NY-ESO-1-specific CD8+ T cells from patients with advanced melanoma were found to upregulate Tim-3[70]. NY-ESO-1 is a cancer germline antigen (CGA) that drives spontaneous cellular and humoral responses and is only detectable in patients with advanced NY-ESO-1 expressing cancer. Unlike Gag-specific CD8+ T cells from chronic HIV progressors, spontaneous NY-ESO-1-specific CD8+ T cells that expressed Tim-3 were also positive for PD-1 expression. Therefore, Tim-3 does not demarcate a distinct population from PD-1-expressing T cells in this disease setting. However, similarly to chronic viral infections, the Tim-3+PD-1+ subset in patients with advanced melanoma was the most defective in terms of IL-2, TNF-α and IFN-γ production. Tim-3+PD-1+ NY-ESO-1-specific CD8+ T cells were also highly activated, as assessed by CD38, HLA-DR, CCR7 and CD45RA expression. Although the Tim-3-PD-1+ subset constitutes the majority of NY-ESO-1-specific CD8+ T cells, blockade of Tim-3/Tim-3L interactions had a greater restorative effect than blockade of PD-1/PD-1L alone. Consistent with previous reports, combined blockade of both receptors and their ligands had an even better restorative effect. Combined blockade could even rescue the ability of NY-ESO-1-specific CD8+ T cells to secrete IL-2, which is a cytokine that is the most sensitive to exhaustion.

Most recently, Tim-3 was found to be highly upregulated on leukemia stem cells (LSC)[87]. LSCs are postulated to be the most likely cause for relapse of patients with acute myeloid leukemia (AML) and therefore, are the primary targets in attempts to cure human AML. Reconstitution of NOD-SCID mice with Tim-3+LSCs but not Tim-3-LSCs promoted the development of human AML. In xenotransplanted mice, a human anti-TIM-3 monoclonal antibody was successful at halting the development of AML but not normal hematopoiesis was.

Therefore, Tim-3 is an ideal target for the removal of LSCs, which will reduce the incidence of relapse amongst patients with AML.

1.3.3.4 Chronic Viral infections

Human Immunodeficiency Virus (HIV)

The role of TIM-3 in chronic viral infection was first explored in the context of HIV. This study sought to examine the relationship between TIM-3 expression and T cell dysfunction in subjects infected with HIV [69]. Subjects were divided into three categories based on duration of infection, CD4+ T cell count and viral load. Acute/early subjects were individuals who had been infected with HIV within 4 months prior to the study. Chronic progressors had been infected for more than 1 year and had a CD4+ T cell count that declined at >50 cells/mm3/year while viral controllers were defined as individuals who also had been infected with HIV for more than a year but had no decline in CD4+ T cell count and had a viral load of <5,000 copies/mL branched-chained DNA.

In comparison to uninfected subjects, the frequencies of TIM-3+ CD4+ and TIM-3+CD8+ T cells were significantly higher in both acutely and chronically infected subjects. However, the frequency of TIM-3+ T cells was not statistically different between uninfected subjects and viral controllers [69]. Amongst treatment-naïve, HIV-infected subjects, there was a significant correlation between the frequency of TIM-3+ T cells with either viral load or CD38, a T cell activation marker. Taken together, it would be reasonable to conclude that TIM-3 frequency positively correlates with viral load. However, it was found that following HAART therapy, TIM-3 frequency remained significantly correlative with CD38 expression but not with viral load.

Staining for both TIM-3 and another receptor associated with exhaustion, PD-1, revealed three distinct populations - TIM-3+PD-1+, TIM-3+PD-1- and TIM-3-PD-1+. However, when this same analysis was applied to viral-specific CD8+ T cells, a majority of Pol-specific CD8+ T were TIM-3+PD-1- while Nef-specific CD8+ T cells were primarily PD-1+CD8- T cells. It has been shown that during chronic viral infection, CD8+ T cells specific for the immunodominant epitope are the first to be deleted[88]. Therefore, TIM-3 and PD-1 may demarcate CD8+ T cells at different stages of exhaustion.

The most important finding of this study was that by blocking TIM-3/TIM-3L interactions with anti-TIM-3 antibody (clone 2e2) or TIM-3 fusion proteins, it is possible to restore the ability for viral specific T cells to proliferate and secrete cytokines in response to antigen receptor stimulation. This has important implications for the disease management in chronic progressors of HIV.

Lymphocytic choriomeningitis virus (LCMV)

Two strains of LCMV – Armstrong (Arm) and Clone 13 (Cl-13), which differ at only 2 bases in the entire genome, has allowed researchers to study the role of inhibitory receptors during acute and chronic infections[89]. LCMV Arm infects mice acutely while LCMV Cl-13 infects mice chronically[90]. In mice infected with LCMV Arm, Tim-3-expressing GP33+ CD8+ T cells could be detected in in the spleen, lung and liver by day 8. By day 30, the frequency of Tim-3+GP33+ T cells was greatly reduced in these organs [91]. However, in mice infected with LCMV Cl-13, Tim-3+GP33+ T cells were detected in spleen, lung and liver up to day 60. The Tim-3+PD-1+ population comprised the majority of total GP33+ CD8+T cells and were the most deeply exhausted, as assessed by the inability to secrete IL-2, TNF-α or IFN-γ upon antigen

restimulation. However, this subset had the highest frequency of IL-10 producing cells when compared to GP33+ Tim-3-PD-1+ and Tim-3-PD-1 CD8+ T cells. Recent work suggests that IL-10 is involved in driving an infection from acute to chronic [92, 93].Therefore, whether Tim-3 enhances the production of IL-10 at the later stages of an acute infection to allow viral persistence remains to be seen.

Hepatitis Simplex Virus (HSV)

CD4+ T cells in draining lymph nodes (DLN) and spleens of mice infected ocularly with HSV upregulated Tim-3 at 8 days post infection (DPI)[94]. Tim-3 expression levels peaked at 15 DPI but was significantly diminished by 40 DPI. Notably, the total number of Tim-3+CD4+ T cells peaked in the spleen 8 DPI but only in the DLN on 15 DPI. On 8 DPI, approximately 50% of CD4+ T cells that invaded HSV-infected ocular and trigeminal ganglion were also Tim-3+. Administration of Tim-3 Ab (RMT3-23) every other day beginning 3 DPI until 13 DPI, exacerbated HSV-induced lesions in the cornea, and increased the percentages of both CD4+ T cells in the cornea and cytokine producing HSV-specific CD4+ T cells in both spleen and DLN. Therefore the authors reasoned that if Tim-3 is an inhibitory receptor, treatment of HSV-infected mice with its ligand, gal-9 would reduce the severity of HSK-induced lesions and extent of neovascularization. Although this hypothesis was accurate, gal-9 did not reduce the severity of HSK lesions by suppressing the function and proliferation of HSK-specific CD4+ T cells. Instead, it reduced the number of both total and Tim-3+CD4+ T cells in the cornea but increased the number and percentage of FoxP3+CD4+ T cells in the spleens of HSV-infected mice. The authors show that CD4+ T cells from HSV-infected mice were more sensitive to gal-9 induced apoptosis but did not show whether this was because of Tim-3 expression. Gal-9 treatment also

expanded the percentage of myeloid suppressor cells in the cornea and spleens of HSV-infected mice.

In a follow up paper, mice were infected with HSV in the hind footpad instead of the cornea. This time, Tim-3 expression was shown to be upregulated specifically on SSIEFARL-specific CD8+ T cells in both lymph nodes and spleens as early as 3 DPI (here forth referred to as HSV-specific CD8+ T cells)[95]. SSIEFARL is the immunodominant epitope in HSV-infected C57Bl/6 mice [96]. A majority of HSV-specific CD8+ T cells were also $CD44^{high}$ and $CD62L^{low}$, which is reflective of an effector/activated phenotype. The authors strived to show that gal-9 specifically induced apoptosis of Tim-3+CD8+ T cells and consequently, HSV-specific CD8+ T cells. *Ex vivo*, Tim-3+ and HSV-specific CD8+ T cells were treated with increasing concentrations of gal-9 and then co-stained for Tim-3 and annexin-V. Although the percentage of Tim-3+annexin-V- cells decreased with increasing concentrations of gal-9, there was not a concomitant increase in the percentage of Tim-3+annexin-V+ cells. Additionally, the authors did not compare the susceptibility of Tim-3+ and Tim-3+ CD8+ T cells gal-9. The authors then compared the percentage and function of HSV-specific CD8+ T cells in HSV-infected WT mice to gal-9 KO mice. Despite an increased in the percentage of Tim-3+ HSV-specific CD8+ T cells, this increase was also observed with Tim-3- HSV-specific CD8+ T cells. Interestingly, administration of exogenous gal-9 negatively affected HSV-specific CD8+ T cell response and delayed viral clearance.

These are the only two studies that employed both gal-9 and anti-Tim-3 antibody to modulate immune responses *in vivo*. As highlighted in the statement of the problem (38), the Tim-3 antibody enhanced effector T cell function while gal-9, did not inhibit effector function, which would be consistent with it being a Tim-3 agonist. Rather, it lowered the number of total

CD4+ T cells through apoptosis. Several possibilities that could explain these observations exists. The first is that Tim-3 is capable of inhibiting effector T cell function and apoptosis and that these effects are dependent on the site at which it is ligated or the affinity of binding. Second, the negative signal delivered to T cells relies on the binding of the Tim-3 ligand on T cells by Tim-3 on antigen presenting cells. Lastly, gal-9 binds to another receptor apart from Tim-3 to induce apoptosis in effector T cells.

More than just a biomarker

About a decade ago, seminal studies by Giorgi et. al. led to the current use of CD38 as a biomarker for disease progression in patients with HIV[97]. CD38, a T cell activation marker, has far greater prognostic value than CD4+ T cell count and viral load because under conditions of chronic immune activation, CD4+ and CD8+ T cells often become “exhausted”[98]. Exhausted T cells have an activated phenotype but are unable to proliferate and secrete cytokines that promote antiviral responses when stimulated through the TCR [99]. Therefore, CD4+ T cell counts may not be reflective of the anti-viral potential of the host’s immune system. T cell receptors associated with the exhaustion phenotype now include CD27, CTLA-4, PD-1, LAG-3, KLRG-1 and TIM-3[100-102]. Upregulation of more than one of these receptors on a T cell is correlative with greater functional impairment [13, 100]. Antibodies that block the interaction between some of these receptors and their ligands have been shown to restore TCR-responsiveness to exhausted, antigen-specific T cells [13]. However, whether these receptors are actively contribute towards the development of exhaustion remains an active field of investigation.

1.4 SUMMARY

Since its identification, the TIM family has emerged as an important regulator of adaptive and innate immune responses (Table 4). In mouse, Tim-1 activation by an agonistic antibody ameliorates EAE, exacerbates airway hyper-reactivity and prevents the development of peripheral tolerance. These effects are dependent on the co-stimulatory function of Tim-1 which enhances cytokine production and proliferation of Th2 cells but deprograms Tregs. Tim-2, which is also preferentially expressed on Th2 cells, inhibits their effector function. Tim-3, which was identified through a screen for Th1-specific markers, adversely affects Th1 production of IFN-γ, proliferation and viability. Inhibition of Tim-3/Tim-3L interactions exacerbates EAE and prevents the induction of allograft tolerance by co-stimulatory blockade. Tim-1 and Tim-3 can also enhance IgE and antigen-induced production of IL-4, IL-6 and IL-13 by bone marrow-derived cultured mast cells (BMCMC)[38]. However, only Tim-3 engagement could protect BMCMC from undergoing apoptosis in the absence of IL-3.

TIM-1 and TIM-3 have also been proposed as biomarkers. KIM-1(TIM-1) is not expressed in normal kidney tissue but is upregulated and shed by injured proximal tubule epithelial cells undergoing dedifferentiation, such as in the case of acute renal failure (ARF)[103, 104]. TIM-1 levels rise in the urine of patients with ARF as soon as 12 hours following an ischemic episode and it is the only biomarker that increases as a result of ARF and not because of other forms of kidney injury. It is important to have a reliable marker for ARF as early intervention is postulated to reduce mortality[103]. TIM-1 has also been suggested as a biomarker for renal cell carcinoma which can only be diagnosed early with the use of costly imaging methods[20]. Recently, several groups have observed that TIM-3 is upregulated on

dysfunctional CD4+ and CD8+ T cells from chronic progressors of HIV and is significantly lower in spontaneous controllers [69, 105] During chronic viral infection, T cells become exhausted due to persistent stimulation and are unable to complete their differentiation from effector to long-lived memory T cells. This system of clonal deletion ensures that viral-specific T cells remain unresponsive to antigen receptor stimulation until severe exhaustion sets in and they undergo apoptosis. However, blocking TIM-3/TIM-3L interactions has been shown to be effective at reversing exhaustion in T cells, which makes TIM-3 not only a good biomarker for diseases progression but can also serve as a therapeutic target.

Although we now have a broader understanding of how TIM-1 and TIM-3 modulate immune responses in mice and to a much lesser extent humans, we are still not equipped to design therapies around these cell-surface receptors for the management or prevention of atopic diseases, autoimmune diseases, cancer and chronic viral infections. The wide distribution of TIM-1 and TIM-3 as well as their ligands across various immune and non-immune cell types requires that we perform a more rigorous analysis on how TIM-1 and TIM-3 signaling impacts the function of each specific cell type under various inflammatory conditions. Also, we need to better characterize the ligands binding sites of each receptor and the downstream signals that result from that interaction. Modulating the function of a co-receptor *in vivo* is a precarious task as it can tip the immunological balance towards either autoimmunity or peripheral tolerance, and can lead to disastrous outcomes [106].

Table 4: TIM-1 and TIM-3 regulation of immune responses

Disease/ Cell type	TIM-1	TIM-3
EAE **Multiple sclerosis**	Ameliorates/Exacerbates[36] Tim-1 mRNA upregulated in mononuclear cells in cerebrospinal fluid (CSF)[107]	Exacerbates[57, 61] Transcript correlates with higher IFN-γ production only in T cells from the CSF and not peripheral blood[15, 107]
Allograft	Promotes rejection[40] Prolongs survival[16]	Prolongs survival[63]
Airway hyperreactivity	Exacerbates[108] Ameliorates[11, 12]	Ameliorates[68]

1.5 STATEMENT OF THE PROBLEM

TIM-3 appears to negatively regulate effector T cells through two primary modes – apoptosis and inhibition of effector function. However, apoptosis is only observed when TIM-3+ T cells are treated with gal-9 [57, 94, 95] while the inhibition of effector T cell function can only be relieved in the presence of reagents that perturb TIM-3/TIM-3L interactions[63, 64, 69]. The lack of cross-regulation between gal-9 and TIM-3/TIM-3L blocking reagents has brought several important issues into focus. Firstly, gal-9 also binds to CD44, a receptor that is commonly used to distinguish between naïve and effector T cells in mice [109, 110]. Gal-9 is also able to induce apoptosis in cells lacking the expression of TIM-3 and has been shown to positively regulate dendritic cells and mast cells [38, 78, 111]. Secondly, TIM-3 and at least one of its ligand are expressed on both T cells and antigen presenting cells (APC) [5]. Therefore, it is not clear whether TIM-3 antibodies or soluble TIM-3 proteins block the ligation of TIM-3 on T cells or APCs to relief inhibition of effector T cells. Lastly, antibodies to human TIM-3 and its mouse homolog are poorly characterized. The binding epitopes of a majority of these antibodies are unknown and whether blocking antibodies can also induce TIM-3 signaling has not been determined.

As TIM-3 is becoming an increasingly attractive therapeutic target, it is important to establish the regulatory nature of TIM-3 on T cells and APCs. To do this, we used anti-TIM-3 antibodies and gal-9 to dissect the signaling pathways downstream of TIM-3 in T cells and APCs. Then we constructed a series of wild-type, truncation and tyrosine to phenylalanine point mutants of mouse TIM-3 to determine if any of these tyrosines, when phosphorylated, are required to modulate downstream signaling pathways of TIM-3 in both T cells and APCs. We

also intend to examine if signaling pathways downstream of TIM-3 can enhance or antagonize CD3/CD28 and Toll-like receptor-4 signaling in T cells and APCs respectively.

Results from these experiments we help us identify molecular mediators downstream of TIM-3 that allows it to negatively regulate effector T cells and positive regulate of APCs. We can then proceed to determine which of these signaling mediators contribute towards either the induction of apoptosis or inhibition of function in T cells. It will also help us determine if TIM-3 activation by both galectin-9 and anti-TIM-3 antibodies can induce apoptosis and inhibit the function of effector T cells. Finally, it will allow us to further explore the co-operativity between the signaling pathways downstream of TIM-3 and either TCR/CD28 (T cells) or TLR-4 (APCs).

2.0 DISSECTING TIM-3 SIGNALING PATHWAYS IN T CELLS AND MYELOID CELLS

2.1.1 Introduction

Prior to the demonstration that gal-9 was able to induce TIM-3+Th1 cells to undergo apoptosis, several *in vivo* studies had already set the precedent that TIM-3 was a negative regulator of effector T cells[61, 63]. However, gal-9 has also been shown to positively regulate other cell types. In the presence of gal-9, human monocyte-derived dendritic cells (moDC) upregulated CD40, CD54, CD80, CD83, CD86 and HLA-DR in a dose-dependent manner. Gal-9 matured moDC also secreted IL-12 and were able to polarize allogeneic CD4+ T cells towards a Th1 phenotype [78]. A Tim-3 polyclonal antibody was able to enhance the production of IL-4, IL-6 and IL-13 by IgE-sensitized mast cells and protect them from apoptosis resulting from IL-3 withdrawal [38]. *In vivo,* gal-9 administration to tumor bearing mice enhanced cytolytic function of CD8+ T cells and induced the maturation of dendritic cells, leading to prolonged survival[80].

Thus, we were interested in understanding how TIM-3 could possess such a Jekyll and Hyde effect on immune cells. There are six conserved tyrosines in both mouse and human TIM-3 that when phosphorylated, could serve as docking sites for proteins with SH2-domains. As myeloid cells and lymphocytes have vastly different mechanisms for activation, it is conceivable that TIM-3 signaling can either enhance or antagonize Toll-like receptor (TLR) and T- cell

receptor pathways [112-115]. As an example, the SH2-doman containing phosphatase 1(SHP-1), has been reported to inhibit T cell activation through lipid rafts but promotes the production of IL-12p70 by APCs in a phosphatidylinositol-3-kinase (PI3K) manner [116-119]. Therefore, we hypothesize that TIM-3 recruits different signaling mediators in T cells and APCs, which leads to the apoptosis/inhibition of effector function in T cells and activation/differentiation in myeloid cells.

2.1.2 Materials and Methods

Antibodies and reagents

A recombinant protease-resistant human gal-9 was obtained from Dr. Mitsuomi Hirashima, Kagawa University, Japan. TCA-extracted LPS (O26:B6) was obtained from Sigma-Aldrich, St. Louis, MO.Anti-Tim-3 monoclonal antibody (Clone 5D12) was a gift from Dr. Vijay Kuchroo, Harvard University. Rabbit polyclonal antiserum for Clnk was obtained from Dr. André Veillette from McGill University, Montreal, Canada. This antiserum does not recognize SLP-76 [120]. For stimulating T cell and dendritic cell lines, 24-well plates were first coated with anti-syrian hamster IgG (Jackson Immunoresearch, West Grove, PA) for binding to anti-CD3ε (Clone 500.A2) and anti-CD28 (Clone 37.51) and goat anti-mouse IgG (Fc) (Pierce/ Thermo Scientific, Rockford, IL) for binding to anti-Tim-3 antibody. When working with dendritic cells and monocytes, endotoxin –free reagents and equipment were used.

Cell lines

The mouse splenic DC cell line (D2SC/1) was obtained from Dr. Vijay Kuchroo (Harvard University) and was maintained in Iscove's modified Dulbecco's medium supplemented (IMDM) with 10% FCS. AE7 Th1 cells were re-stimulated with mitomycin-C (50µg/mL, Sigma Aldrich, St. Louis, MO) treated B10.A splenocytes and PCC (30µg/mL, Sigma Aldrich, St. Louis, MO) every 21 days. AE7 cells were maintained continually in the presence of recombinant human IL-2 (50IU/mL).

Isolation of CD14+ human monocytes

Buffy coats were spun over a Ficoll gradient to isolate peripheral blood mononuclear cells (PBMC). CD14+ monocytes were selected from PBMCs using the CD14+ monocyte isolation kit (Miltenyi Biotec). To induce their maturation, CD14+ monocytes were cultured with media alone, galectin-9 and LPS for 20 hours in 24-well plates. Cells were then harvested and stained with CD14, CD16, CD40, CD80 and CD86 to be analyzed by FACS. Supernatants were collected and kept at -80ºC until ready for analysis with Luminex.

Intracellular staining for phosphorylated-ERK

AE7 Th1 cells were spun onto 24 well plates coated with anti-Tim-3 antibody for 30 seconds (-0.5s). At the stipulated times, paraformaldehyde (PFA) was added to these cultures to fix cells at a final concentration of 1.5% PFA. These cells were then permeabilized with cold methanol and then stained with anti-phospho ERK antibodies. Cells were then washed with FACS buffer twice before FACS analysis.

2.1.3 Results

To address whether Tim-3 engagement induces distinct signaling in innate and adaptive immune cells, we stimulated a Tim-3–expressing CD4+ T cell clone and a DC cell line with anti–Tim-3 and examined them for tyrosine phosphorylation. Differences were observed in the proximal signaling pathways triggered by Tim-3 in T cells and DCs (Figure 8). Specifically, tyrosine phosphorylation was induced in two molecules after Tim-3 engagement in T cells but not in DCs, and phosphorylation of a third molecule was induced in DCs but not in T cells. In contrast, engagement of Tim-3 led to similar degrees of extracellular signal–regulated kinase activation and degradation of the NF-κB inhibitor IκBα in the two cell types (Figure 8B and C). Although the magnitude of ERK phosphorylation induced by Tim-3 appeared to be lower in the DCs, phosphorylation induced with a positive control stimulus phorbol 12-myristate 13-acetate (PMA) was also lower in these cells.

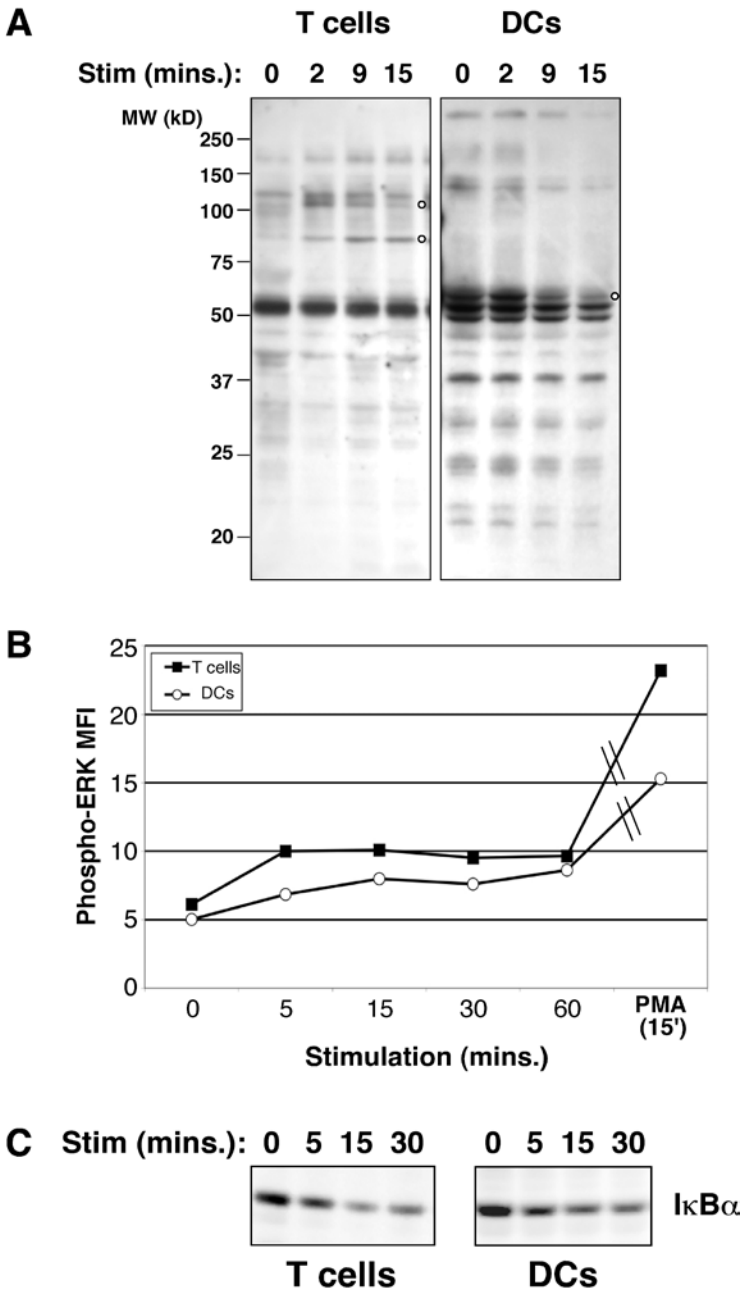

Figure 8: Differential Tim-3 signaling in T cells versus DCs

T cells (TH1 clone AE.7) or DCs (D2SC1) were stimulated for the indicated times with anti–Tim-3. (A) Cells were lysed with NP-40 lysis buffer and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and Western blotting with phosphotyrosine mAb 4G10 (Upstate/Chemicon). The top two circles in the T cell lanes indicate the position of tyrosine-phosphorylated substrates uniquely induced in T cells; the bottom circle in the DC lanes

indicates a substrate uniquely induced in DCs. (B) Cells were fixed with paraformaldehyde and permeabilized with cold methanol for intracellular staining with a mAb to phospho-ERK (BD Biosciences). As a control, a sample of each cell type was also stimulated with PMA. (C) Cells were lysed with NP-40 lysis buffer and analyzed by SDS-PAGE and Western blotting for total IκBα (Cell Signaling Inc.). Data in (A), (B), and (C) are representative of three independent experiments. This work appears in Anderson et. al. Promotion of Tissue Inflammation by the Immune Receptor Tim-3 Expressed on Innate Immune Cells. (2007) *Science* 318 (5853): 1141-1143. (**http://www.sciencemag.org/content/318/5853/1141.full**)

Identification of tyrosine phosphorylated bands in Tim-3 stimulated AE7 Th1 lysates

To identify the proteins that were phosphorylated in AE7 cells by Tim-3, we incubated lysates of AE7 that were left unstimulated or stimulated for 5 minutes with either plate-bound anti-Tim-3 mAb or anti-CD3/CD28, with 4G10 beads. Tyrosine phosphorylated proteins immunoprecipitated from these lysates were first separated on a 10% polyacrylamide gel and then silver stained (Figure 9A). A band migrating around 100 kDa that was precipitated only from the lysates of AE7 Th1 cells under anti-Tim3 mAb stimulation and not in other stimulation conditions was excised and sent for mass spectrometry. However, mass spectrometry analysis did not yield any promising leads.

Since one of the proteins that was inducibly phosphorylated in both anti-Tim-3 and anti-CD3/28 stimulated AE7 cells migrated around 75 kDa, we decided to blot 4G10 immunoprecipitates for SH2 domain containing leukocyte protein of 76kD (SLP-76). SLP-76 is an adaptor protein that is required for the nucleation of signaling mediators downstream of the TCR during T cell activation [121]. Although we did not consistently immunoprecipitate a greater amount of SLP-76 from AE7 cells stimulated with anti-Tim-3 antibody than unstimulated

controls, we did observe a faster migrating band that was inducible tyrosine phosphorylated by both anti-Tim-3 and anti-CD3/CD28 stimulations (Figure 9). As there are no known splice variants if SLP-76, we scoured the literature for a protein that shared the N-terminal domain of SLP-76, which was the immunogen used to generate the SLP-76 antibody. We found that apart from SLP-76, the SLP-76 family of adaptor proteins consists of Blnk (SLP75 or Bash) and Clnk (MIST)[121]. All three share a common architecture, including the N-terminal domain that carries the tyrosine phosphorylation site. We decided to probe for Clnk as it is expressed in T cells maintained in the presence of IL-2 while Blnk is only expressed in B cells [120, 122]. However, a polyclonal rabbit antiserum for Clnk failed to recognize any of the 4G10 immunoprecipitates of anti-Tim-3 antibody stimulated lysates (data not shown). As we also failed to detected Clnk in unstimulated AE7 whole lysates, it is possible that AE7 Th1 cells do not express Clnk or that the antiserum was no longer effective in recognizing Clnk.

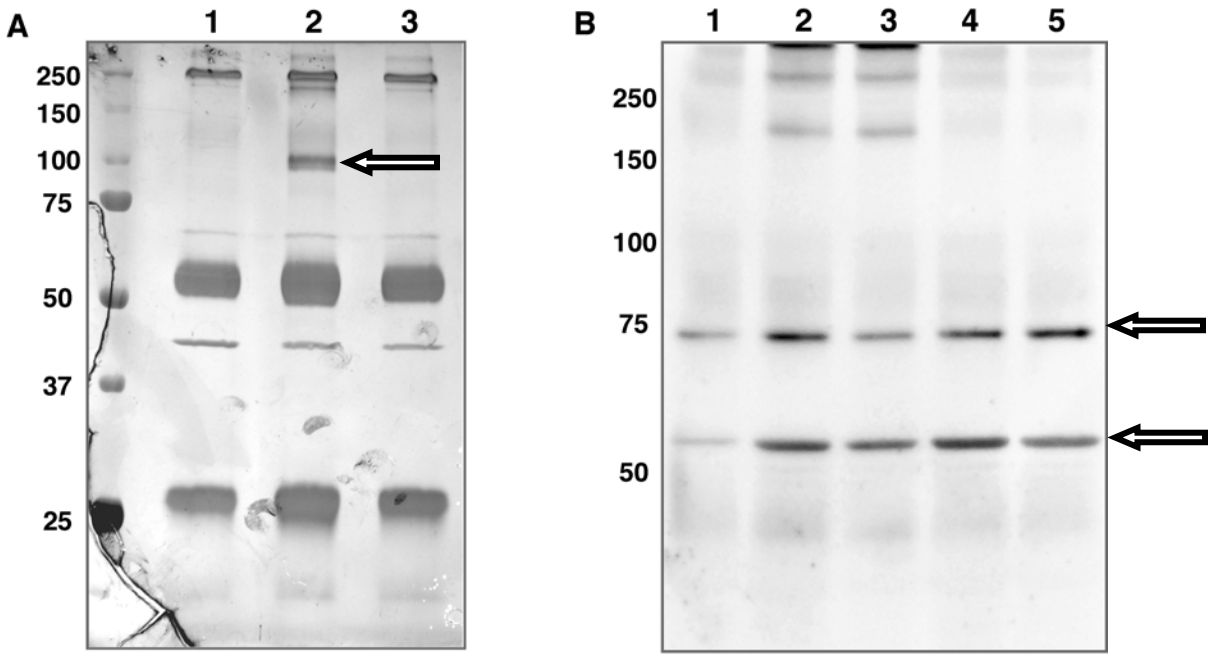

Figure 9: Identification of tyrosine phosphorylated bands in AE7 Th1 cells

A) AE7 Th1 cells were left unstimulated (1), stimulated with either anti-Tim-3 mAb (2) or anti-CD3/28 (3) for 5 minutes. Cells were then lysed and cleared by centrifugation and protein-G beads. Tyrosine phosphorylated proteins were immunoprecipitated (IPs) from lysates with 4G10 beads. IPs were run on a 10% polyacrylamide gel and silver stained. Band indicated by an arrow was excised and sent for mass spectrometry. **B)** AE7 Th1 cells were left unstimulated (1), stimulated for 4 and 8 minutes with either anti-Tim-3 mAb (2,3) or anti-CD3/28 (4,5). Lysates were then cleared and IPed with 4G10 beads. IPs were ran on a 10% polyacrylamide gel and blotted for SLP-76 indicated by the upper arrow. The lower arrow points towards the band that represents what was thought to be Clnk. Result representative of three independent experiments.

Gal-9 activates human CD14+ monocytes phenotypically but not functionally

To dissect the signaling pathway in myeloid cells using gal-9, we decided to use human CD14+ monocytes for practical reasons. The effects of gal-9 on human monocyte-derived dendritic cells

(moDC) had already been explored and human monocytes express TIM-3 constitutively without any need for further differentiation [78, 87].

The first thing we wanted to do was determine if monocytes could modulated by gal-9 as well. We treated CD14+ monocytes with gal-9 for 20 hours and then examined their expression of CD14 and CD16 (subset markers) as well as CD40, CD80 and CD86 (maturation markers). In untreated monocyte cultures, we observed a heterogeneous population of CD14+CD16- and CD14+CD16+ monocytes. However, in the presence of high doses of gal-9 or LPS, expression of CD16 was lost (Figure 10, upper panel). High doses of gal-9 also induced the upregulation of CD40 and CD80, and to a much lesser extent, CD86 (Figure 10, lower panel). At a concentration ten times lower, LPS but not gal-9 could still induce the downregulation of CD14 and upregulation of CD40 and CD80. As our collaborators found that Tim-3 and LPS can have a synergistic effect on the secretion of TNF-α by splenic dendritic cells, we treated monocytes with low doses of gal-9 and LPS. However, instead of synergy, we found that gal-9 prevented the ability for LPS to modulate monocytes.

We also decided to examine the cytokine profile of monocytes treated with gal-9 and/or LPS (Figure 11). Consistent with surface markers, we detected high levels of IL-6, IL-8 and IL-10 in the cell supernatant of monocytes treated with both high and low doses of LPS. However, even though high doses of gal-9 could modulate the expression of surface markers, we could not detect secretion of any cytokines in the panel. Unexpectedly, the cytokine profile of monocytes treated with low doses of LPS and gal-9 was similar to monocytes treated with LPS only.

Then, to determine if the effects of gal-9 on monocytes were dependent on TIM-3, we treated monocytes with a human anti-TIM-3 antibody along with gal-9. This antibody blocks the interaction of TIM-3/TIM-3L interactions [69]. However, we were unable to establish whether

Tim-3 antibody had any effect on monocytes as treatment with endotoxin-free preparations of its isotype control, mouse IgG1 appeared to activate CD14+ monocytes (Figure 12).

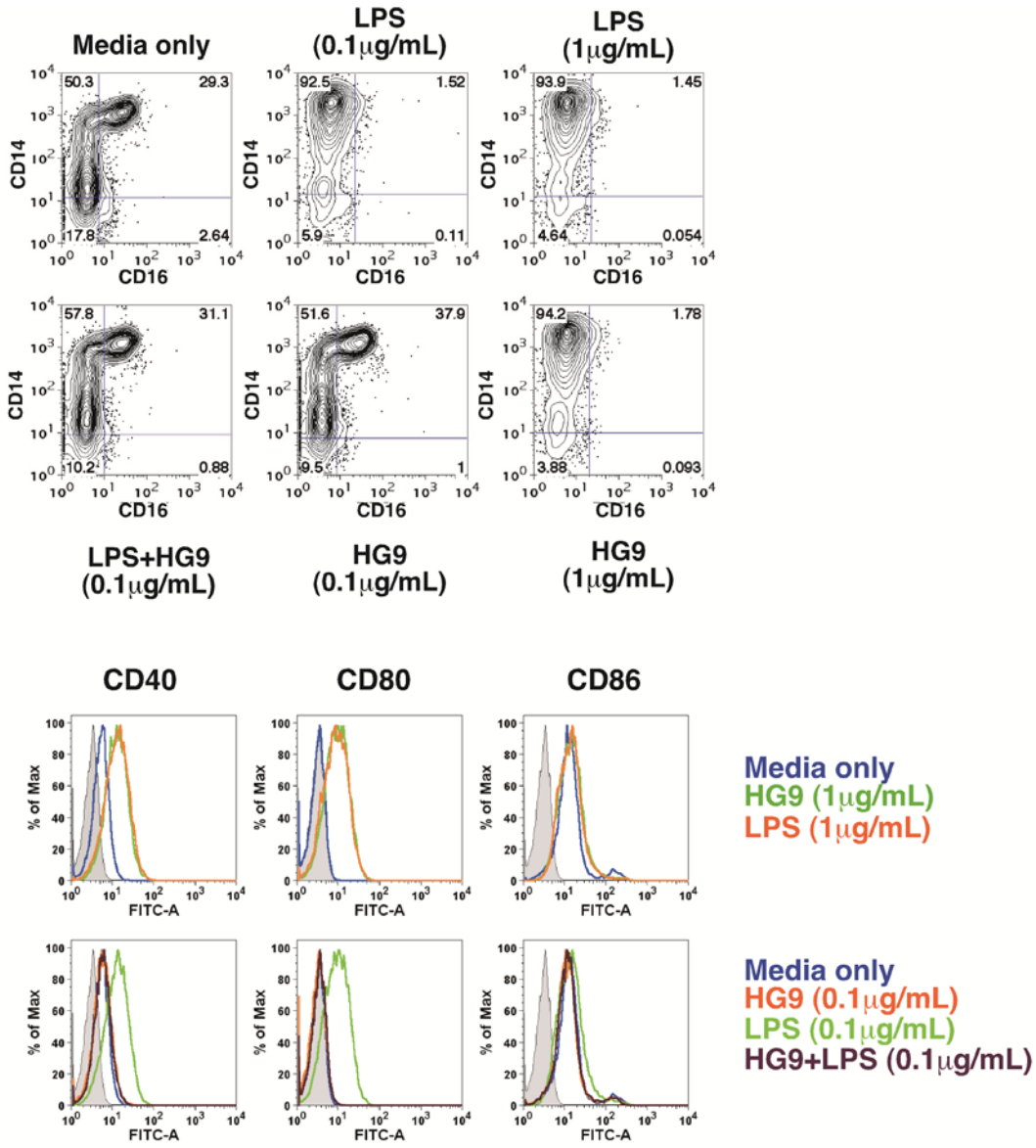

Figure 10: Gal-9 induces upregulation of maturation markers in CD14+ human monocytes

Human CD14+ monocytes isolated from human PBMC were left unstimulated, or stimulated with LPS and/or endotoxin-free gal-9 (HG9) for 20 hours. Cells were then harvested and stained for subset markers A) CD14 and CD16 and maturation markers B) CD40, CD80 and CD86. Grey filled histogram represents isotype control staining. Results are representative of at least three experiments.

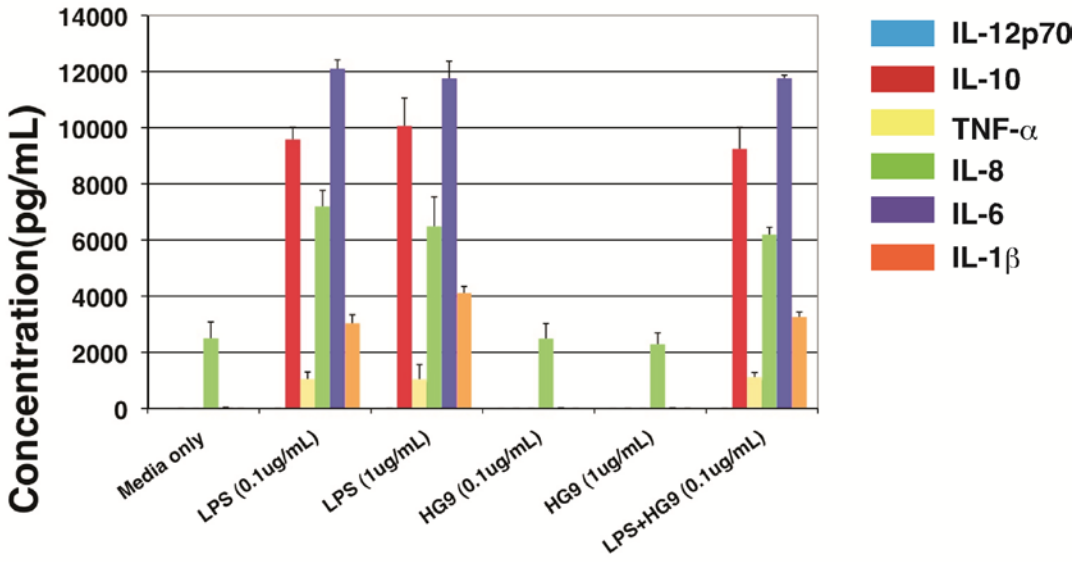

Figure 11: Cytokine profile of gal-9 activated human CD14+ monocytes

Supernatant from one experiment in Figure 10 was subjected to Luminex to assess the levels of pro- and anti-inflammatory cytokines listed in the upper right hand corner.

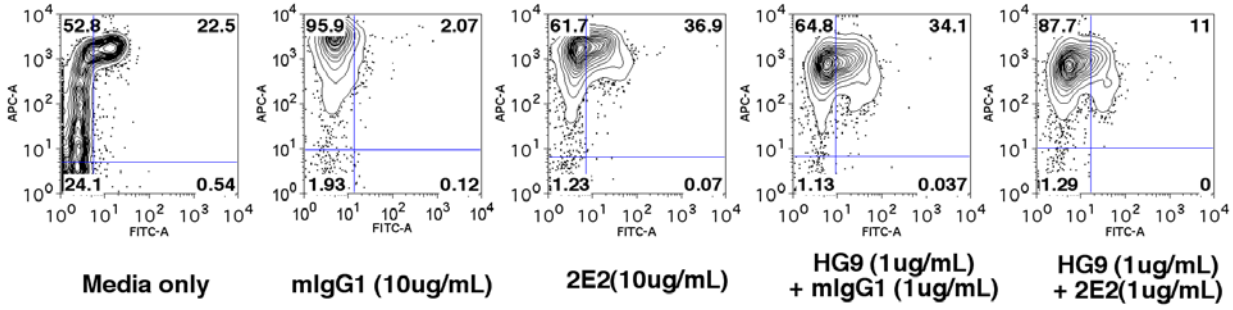

Figure 12: Stimulation of human CD14+ monocytes with anti-TIM-3

CD14+ monocytes isolated from PBMCs were treated on plate-bound anti-TIM-3 antibody or isotype control, mouse IgG1 either in the absence or presence of gal-9 for 20 hours. These cells were then stained for CD14 and CD16.

2.1.4 Discussion

During an infection, blood monocytes have the ability to exit from the blood stream into inflamed tissues where they differentiate into DCs and macrophages to replenish the population that was lost[123]. However, under steady state, monocytes remain undifferentiated and serve as important effector cells[124]. In humans, blood monocytes can be classified according to their expression of CD14 and CD16 [125, 126]. CD14 is required for loading of LPS onto the TLR-4/MD-2 complex while CD16, is also known as FcγRIII low-affinity receptor and assists in phagocytosis [127, 128] Of all blood monocytes, CD14+CD16- monocytes compose the majority and are considered the "classical" monocytes. Classical monocytes are highly phagocytic and produce IL-10 instead of pro-inflammatory cytokines when stimulated with TLR ligands [126, 129]. CD16+ monocytes can be further divided into CD14+CD16+ and CD14+^{dim}CD16+ subsets [130, 131]. CD14+CD16+ monocytes were first described as dendritic-cell like due to their high expression of class II MHC and noted capacity to secrete TNF-α in response to TLR ligands[132, 133]. Although CD14+^{dim}CD16+ are poorly phagocytic and lack the ability to secrete pro-inflammatory cytokines when treated with LPS, they are able to secrete TNF-α and IL-1β when treated with ligands to intracellular TLRs [131, 134].

We found that treatment of monocytes with gal-9 induced the upregulation of maturation markers and modulate the expression of CD14 and CD16 without inducing the secretion of either pro- or anti-inflammatory cytokines. In monocytes, the synergy between TIM-3 and TLR-4 pathways was not observed. Instead, gal-9 prevented LPS from modulating the expression of CD14, CD16, CD40, CD80 and CD86. However, gal-9 did not affect the ability for LPS to modulate cytokine production. We are puzzled by the finding that gal-9 can induce phenotypic

but not functional changes. CD14+^{dim}CD16+ monocytes are poor producers of cytokines when stimulated by LPS because they express low levels of CD14 [135] . However, if TIM-3 is downregulated by gal-9 treatment, we should see no effect at all both phenotypically and functionally.

Here, we also show that an anti-mouse Tim-3 monoclonal antibody (Clone 5D12) has the ability to induce different tyrosine phosphorylation patterns in T cell and dendritic cell lines. Although attempts to uncover the identity of these tyrosine phosphorylated proteins were not successful, we are the first to show that from a signaling perspective, Tim-3 has different downstream targets in T cells and dendritic cells. This may be a major reason for the diametric effects seen in gal-9 treatment of Th1 cells and dendritic cells. The next step is to determine whether this antibody can induce the same effect as gal-9 on T cells and myeloid cells using cytokine production, cell viability, proliferation and upregulation of activation/maturation markers. As outlined in the statement of problem, gal-9 and TIM-3 blocking reagents appear to modulate two different aspects of Th1-mediated immune responses. If TIM-3 can indeed induce both apoptosis and inhibit effector T cell function, it is important for us to understand how it does so both mechanistically and structurally.

3.0 GALECTIN-9 REGULATES T-HELPER FUNCTION INDEPENDENTLY OF TIM-3

3.1 INTRODUCTION

Galectin-9 (Gal-9) is a lectin composed of two non-identical carbohydrate recognition domains (CRD) separated by a linker peptide of variable lengths, depending on the isoform [136].Gal-9 is secreted through a non-classical route and can be found either in the cytoplasm or the extracellular matrix. Resting T cells express gal-9 but can further upregulate its expression when activated [137]. Although gal-9 is able to bind to CD44, another surface glycoprotein expressed by lymphocytes [109], its interaction with Tim-3 has been the most common focus in recent immunological studies.

Tim-3 is a type I glycoprotein whose expression on T cells is controlled in part by the Th1-transcription factor, T-bet [138], consistent with the observation that both human and mouse Tim-3+ T cells have a Th1/Tc1 phenotype. Interestingly, Tim-3 has also been detected on Th17 and regulatory T cells (Tregs), T lymphocyte subsets whose polarization does not depend on T-bet [56, 77]. In addition to gal-9, Tim-3 has at least one other ligand, which remains to be identified. This putative ligand is expressed on activated T cells, macrophages and dendritic cells, and is predicted to bind to Tim-3 at a site distinct from gal-9 [5].

Due to the lack of agonistic antibodies to Tim-3, gal-9 has been used in many studies focusing on the function of Tim-3. Administration of gal-9 to mice chronically infected with *Herpes simplex virus* reduces the number of CD4+ T cells but increases the percentage of Tregs and myeloid suppressor cells[94] . In tumor-bearing mice, gal-9 increases the percentage of Tim-3+ CD8+T cells and dendritic cells. [80]. Thus, gal-9 can have either positive or negative effects on different cell types or the same cell type in different inflammatory settings [139].

Another method commonly employed to study the function of Tim-3 is the blockade of Tim-3/Tim-3 ligand interactions. Antibodies that block Tim-3/Tim-3L interactions restore the ability of exhausted T cells isolated from human subjects with HIV or multiple sclerosis (MS) to respond to anti-CD3 stimulation [69, 84]. Tim-3 Ig fusion proteins prevented the induction of tolerance in mice and hastened the onset of diabetes in NOD mice [63, 64]. Collectively, these studies support the current model that Tim-3 can negatively regulate immune responses. However, they do not specifically address whether the gal-9/Tim-3 interaction is required for the effects described above. Thus, the target epitope for these blocking antibodies is still not known, and whether soluble Tim-3 proteins preferably disrupt the interaction of Tim-3 with gal-9 or its other ligand(s) has not been established.

In this study, we show that gal-9 can not only instruct T helper cells to undergo apoptosis but it can also induce them to secrete pro-inflammatory cytokines. These effects are dependent on the concentration of gal-9 but are independent of Tim-3. Thus, gal-9 can have either positive or negative effects on T helper cells, both of which can occur independently of Tim-3.

3.2 MATERIALS AND METHODS

3.2.1 Antibodies and reagents

Anti-mouse CD3ε (clone 500.A2), anti-FLAG (M2, Invitrogen, Carlsbad, CA), anti-mouse CD3ε (clone 2C11), anti-mouse CD28 (clone 37.51), anti-mouse IL-12p40 (clone 11B11) and anti-mouse IL-4 (clone C17.8, Biolegend, San Diego, CA), anti-mouse Tim-3 (Clone 8B.2C12, eBioscience, San Diego, CA), recombinant protease-resistant human gal-9 (Dr. Mitsuomi Hirashima, Kagawa University, Japan), biotin-human gal-9 (Dr. Linda Baum, UCLA, LA), anti-mouse IFN-γ (Clone XMG1.2) and recombinant mouse IL-12p70, GolgiPlug (BD Pharmingen, San Diego, CA) and anti-mouse Tim3 polyclonal Ab and recombinant mouse IL-4 (R&D Systems, MN). T-bet (4B10) and GATA-3 (HG3-31) antibodies obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

3.2.2 Cell lines

AD10 Th1 clone were re-stimulated with mitomycin-C (50µg/mL, Sigma Aldrich, St. Louis, MO) treated B10.A splenocytes and PCC (30µg/mL, Sigma Aldrich, St. Louis, MO) every 21 days. AD10 cells were maintained continually in the presence of recombinant human IL-2 (50IU/mL). A fast-growing variant of the D10 Th2 clone was obtained from M. Krummel (University of California, San Francisco, CA) and maintained as described [140].

3.2.3 FACS staining

AD10 Th1, D10 Th2, primary Th1 and Th2 cells were washed once with FACS buffer (1%FBS, 0.01% sodium azide in PBS) before incubation with either isotype controls, anti-Tim-3 or biotin-human gal-9 for 30 minutes on ice. Cells were then washed once with FACS buffer and then incubated for another 10 minutes with fluorescently conjugated secondary antibodies. Cells were then washed 3 times, before being resuspended in FACS buffer and analyzed on the BD LSR II Flow Cytometer. Entire procedure was performed at 4ºC.

3.2.4 Measurement of cytokine secretion

Intracellular cytokine staining - AD10 Th1, D10 Th2 cells, primary Th1 and Th2 cells were treated with various concentrations of gal-9 [141] in cell culture media containing 50 μM 2-mercaptoethanol unless noted otherwise. GolgiPlug was added 1 hour post-treatment. At the end of the stimulation period, cells were divided to stain for intracellular cytokines [142] or cell viability.

ELISA - AD10 Th1 cells and primary Th1 cells were stimulated in 96-well plates ($0.2X10^6$ cells/well) for 20 hours. Supernatants were then collected and frozen at -80ºC. Before analysis, supernatants were spun down at 1800 rpm to pellet cell debris. When necessary, supernatants were diluted with culture media before being analyzed with a mouse IFN-γ ELISA kit (BD Bioscience, San Jose, CA).

3.2.5 Measurement of apoptosis

To measure internucleosomal DNA fragmentation, manufacturer's protocol for Cell Death Detection ELISAPLUS was followed (Roche Diagnostics, Indianapolis, IN). Manufacturer's protocol was followed for 7-AAD/annexin V staining (BD Pharmingen, San Diego, CA).

3.2.6 Th1 and Th2 polarization

Spleens harvested from C57Bl/6 mice or Tim-3 KO mice and wild-type littermates [63], were mechanically disrupted to liberate resident cells. CD4+ T cells were then isolated from splenocytes using magnetic bead isolation (CD4+ T cell isolation Kit II, Miltenyi Biotec, Auburn, CA). CD4+ T cells were then stimulated in 24 well plates coated with anti-CD3 (1 µg /mL) and anti-CD28 (5µg/mL) antibodies, under either Th1 polarizing conditions - anti-mouse IL-4 (10 µg /mL) and recombinant IL-12 p70 (5ng/mL) or Th2 polarizing conditions - anti-mouse IL-12p40 (10 µg g/mL) and recombinant IL-4 (10ng/mL) for 3 days. On day 4, CD4+ T cells were removed 24 well plates and expanded until day 10 in culture media containing recombinant human IL-2 (50 IU/mL). For secondary and tertiary stimulations, the same process was adopted except that anti-CD3 and anti-CD28 were plated at 2 µg /mL. Functional assays were performed either 7 days after first round of polarization or 11 days after third round polarization.

3.2.7 Western blotting

CD4+ T cells ($10X10^6$ cells) were subjected to nuclear fractionation after one round of polarization under Th1 or Th2 conditions using the NE-PER kit (Thermo Scientific, Rockford, IL). Nuclear lysates were divided, run on a 10% SDS-PAGE gel, and western blotted for either T-bet or GATA-3, after transfer to PVDF membrane. Equal loading was confirmed by blotting for lamin B.

3.3 RESULTS

3.3.1 Gal-9 modulates T helper cell function and viability in a dose-dependent manner

In the first description of the gal-9: Tim-3 interaction, Th1 cells were shown to undergo apoptosis when treated with gal-9 [57]. This report contributed significantly to the current model that Tim-3 regulates the immune system by terminating Th1 responses. Because galectins can have pleiotropic effects on immune cells [139], we wanted to determine if gal-9 could mediate other effects on T helper cells. We treated a Th1 clone (AD10) and a Th2 clone (D10) with various concentrations of gal-9, then assayed for cell viability and secretion of cytokines. AD10 Th1 cells are Tim-3+ while D10 Th2 cells are Tim-3- (Figure 13A, upper panels). Both cell lines however, stained with gal-9 (Figure 13A, lower panels). In agreement with Zhu et. al., AD10 cells treated with gal-9 underwent apoptosis (Figure 13B, lower panels). We also found that gal-9 could induce the production of IFN-γ by AD10 cells (Figure 13B, upper panels). Higher

concentrations of gal-9 were required to induce apoptosis, while the production of IFN-γ was still observed in AD10 cells treated with non-lethal concentrations of gal-9. The same dose range of gal-9 was also sufficient to induce Tim-3 negative D10 Th2 cells to secrete TNF-α and undergo apoptosis (Figure 13C). Therefore, in addition to being a pro-apoptotic factor, gal-9 can also induce T helper cells to secrete pro-inflammatory cytokines.

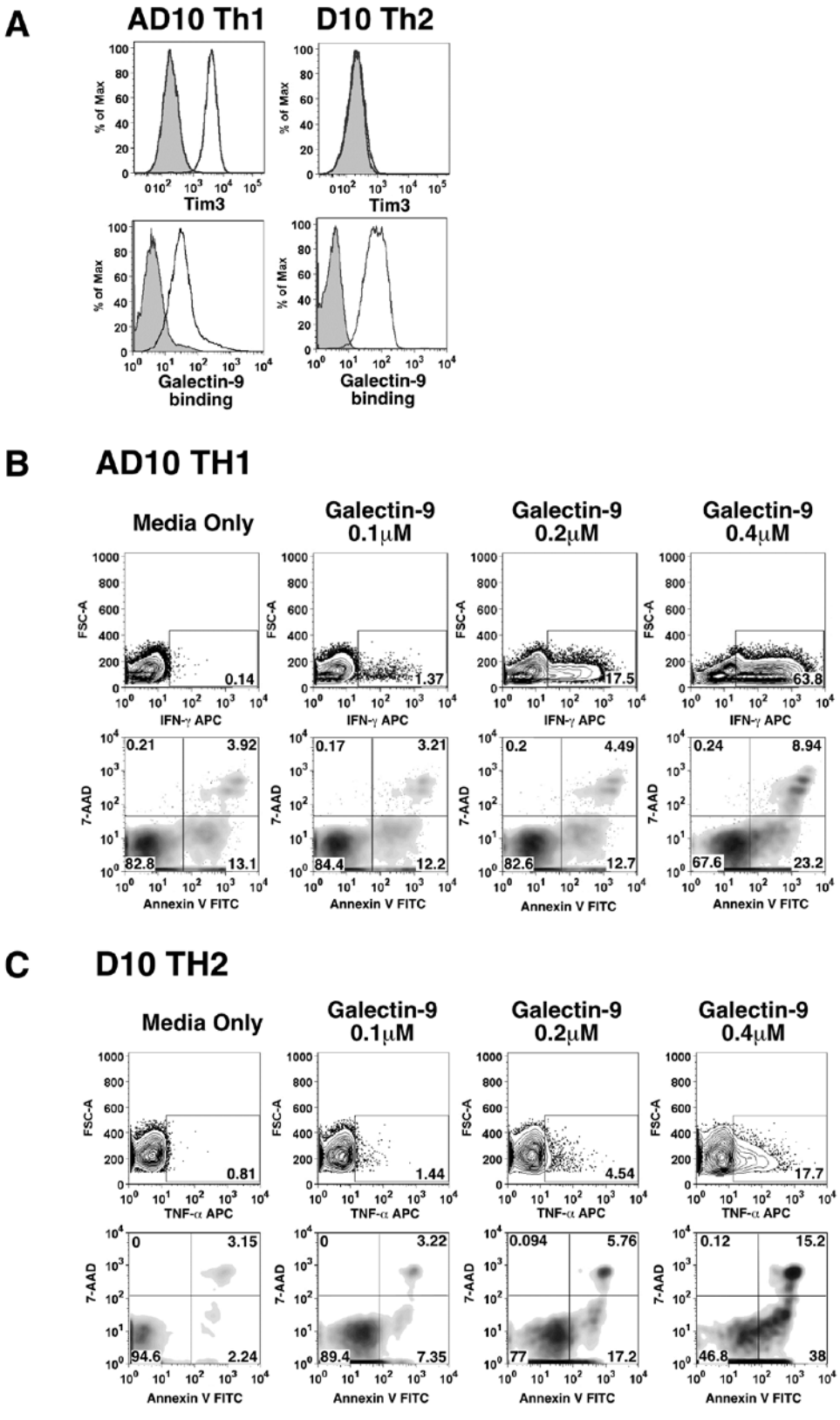
A
AD10 Th1
D10 Th2
% of Max
Tim3
Galectin-9 binding
B
AD10 TH1
Media Only
Galectin-9 0.1μM
Galectin-9 0.2μM
Galectin-9 0.4μM
FSC-A
IFN-γ APC
7-AAD
Annexin V FITC
0.14
1.37
17.5
63.8
0.21
3.92
82.8
13.1
0.17
3.21
84.4
12.2
0.2
4.49
82.6
12.7
0.24
8.94
67.6
23.2
C
D10 TH2
TNF-α APC
0.81
1.44
4.54
17.7
0
3.15
94.6
2.24
0
3.22
89.4
7.35
0.094
5.76
77
17.2
0.12
15.2
46.8
38

Figure 13: Gal-9 induces cytokine secretion and apoptosis in Th1 and Th2 cells.

(A) Staining of AD10 Th1 and D10 Th2 cells with isotype control (filled histogram) and Tim-3 pAb (empty histogram), upper panel, and streptavidin (filled histogram) and biotin-gal-9 (empty histogram). (B) AD10 Th1 cells were treated with gal-9 for 6 hours. Cells were then either fixed or permeabilized for staining with anti-mouse IFN-γ APC (top panel), or washed and stained with 7-AAD/annexin V (bottom panel). Data are representative of three independent experiments. (C) D10 Th2 were treated with gal-9 for 5 hours and stained as described for panel B, with anti-mouse TNF-α (upper panel) and 7-AAD/annexin V (bottom panel). Data are representative of two independent experiments. GolgiPlug was added to both AD10 and D10 cultures one hour post-stimulation to facilitate retention and detection of cytokines.

3.3.2 Gal-9 modulation of T helper cell function and viability is carbohydrate-dependent

Carbohydrate-dependent binding of gal-9 to its binding partners can be competitively inhibited by lactose [143]. To confirm that the effects of gal-9 described above were carbohydrate-dependent, we compared the ability of gal-9 treated AD10 Th1 cells to undergo apoptosis and secrete IFN-γ in the presence of PBS or lactose. We confirmed that gal-9 treatment induces IFN-γ which was detectable intracellularly by six hours (Figure 14A, left panel) and in cell culture supernatant by 20 hours (Figure 14B, left panel). The addition of lactose prevented the secretion of IFN-γ by AD10 Th1 cells treated with gal-9 (Figure 14A-B, left panels). By six hours, gal-9-treated AD10 cells displayed more internucleosomal DNA fragmentation than control cultures (Fig. 2A, right panel), which confirms that gal-9 induces apoptosis and not just PS exposure. However, by 20 hours, AD10 cells cultured in media only or with gal-9 were similarly enriched

with nucleosomes (Figure 14B, right panel). This suggests that the pro-apoptotic effects of gal-9 become less dominant when AD10 cells begin to succumb to other apoptosis-inducing conditions such as IL-2 withdrawal, between 6-20 hours. The apoptosis observed after six hours of gal-9 treatment was not dependent on IFN-γ since these experiments were performed in the presence of brefeldin A, which prevents cytokine secretion. Furthermore, the addition of blocking antibodies to IFN-γ did not diminish the capacity of gal-9 to induce apoptosis (data not shown).

Galectin-1, another member of the galectin family, has also been shown to induce apoptosis and cytokine secretion in Th2 cells [144]. However, galectin-1 has been reported to induce only PS exposure and not apoptosis, in the absence of reducing agents [145]. Therefore, we compared the ability of gal-9 to induce apoptosis in primary T cells cultured in Th1 conditions, in the absence or presence of 2-mercaptoethanol, which is usually present in our cell culture medium. In Figure 14C-D, we show that gal-9 is able to induce apoptosis in one-round polarized primary Th1 cells to the same extent, in the absence and presence of 2-mercaptoethanol. Therefore, unlike galectin-1, gal-9 can induce apoptosis of helper T cells independently of reducing agents.

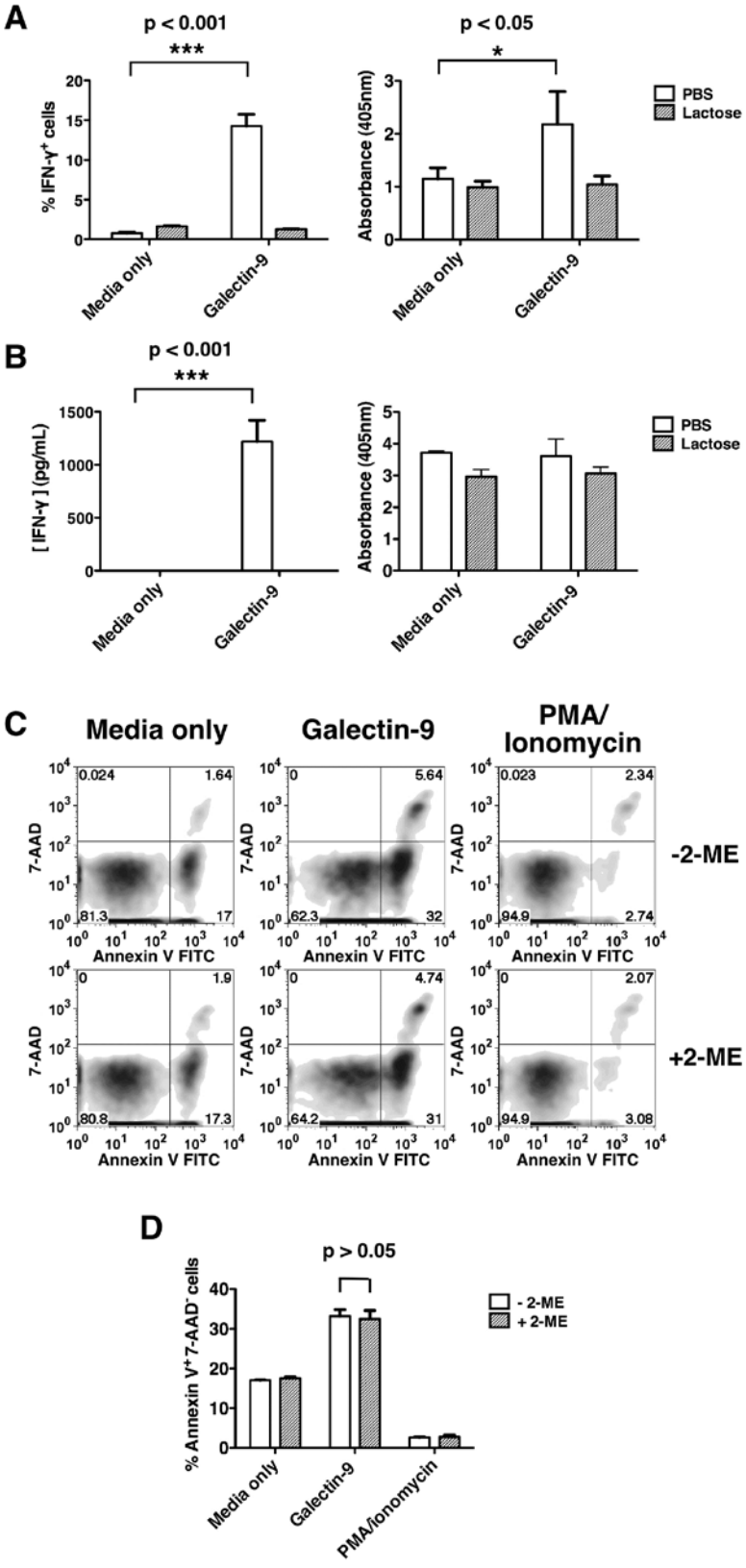
A
p < 0.001
***
% IFN-γ+ cells
Media only
Galectin-9
p < 0.05
*
Absorbance (405nm)
PBS
Lactose
B
p < 0.001
***
[IFN-γ] (pg/mL)
Absorbance (405nm)
C
Media only
Galectin-9
PMA/
Ionomycin
7-AAD
Annexin V FITC
-2-ME
+2-ME
D
p > 0.05
% Annexin V+ 7-AAD- cells
- 2-ME
+ 2-ME
PMA/Ionomycin

Figure 14: Carbohydrate dependence of gal-9 effects on T cells.

IFN-γ secretion was assayed at 6 hours with intracellular cytokine staining (A, left panel) and 20 hours with ELISA (B, left panel). Nucleosome enrichment was determined at 6 hours (A, right panel) and 20 hours (B, right panel). Data are representative of two independent experiments. (C-D) Apoptosis was examined in primary Th1 cells treated with gal-9 in the absence (upper panel) and presence (lower panel) of 2-mercaptoethanol (2-ME). Data are representative of three independent experiments. Error bars indicate S.D from replicate cultures. P values were calculated using two-way ANOVA.

Gal-9 induced apoptosis is not dependent on caspases or IFN-γ

Previously, gal-9 was found to induce apoptosis in human T cells through the calcium-caspase-calpain-1 pathway [146]. Therefore, we wanted to determine if caspases were also required to mediate gal-9 induced apoptosis. We pre-treated AD10 Th1 cells with a pan-caspase inhibitor, Z-VAD-FMK prior to treatment with gal-9 and found that unlike dexamethasone-treated cells, gal-9 treated cells were still able to undergo apoptosis (Figure 15A-B). Another factor that could induce apoptosis in gal-9 cultures is IFN-γ[147]. As brefeldin A blocks the secretory pathway of Th1 cells and is present in assays presented in Figures 13 and 14, it is highly unlikely that IFN-γ is responsible for gal-9 induced apoptosis. Therefore, the mechanism of gal-9 induced apoptosis in Th1 cells remains to be elucidated.

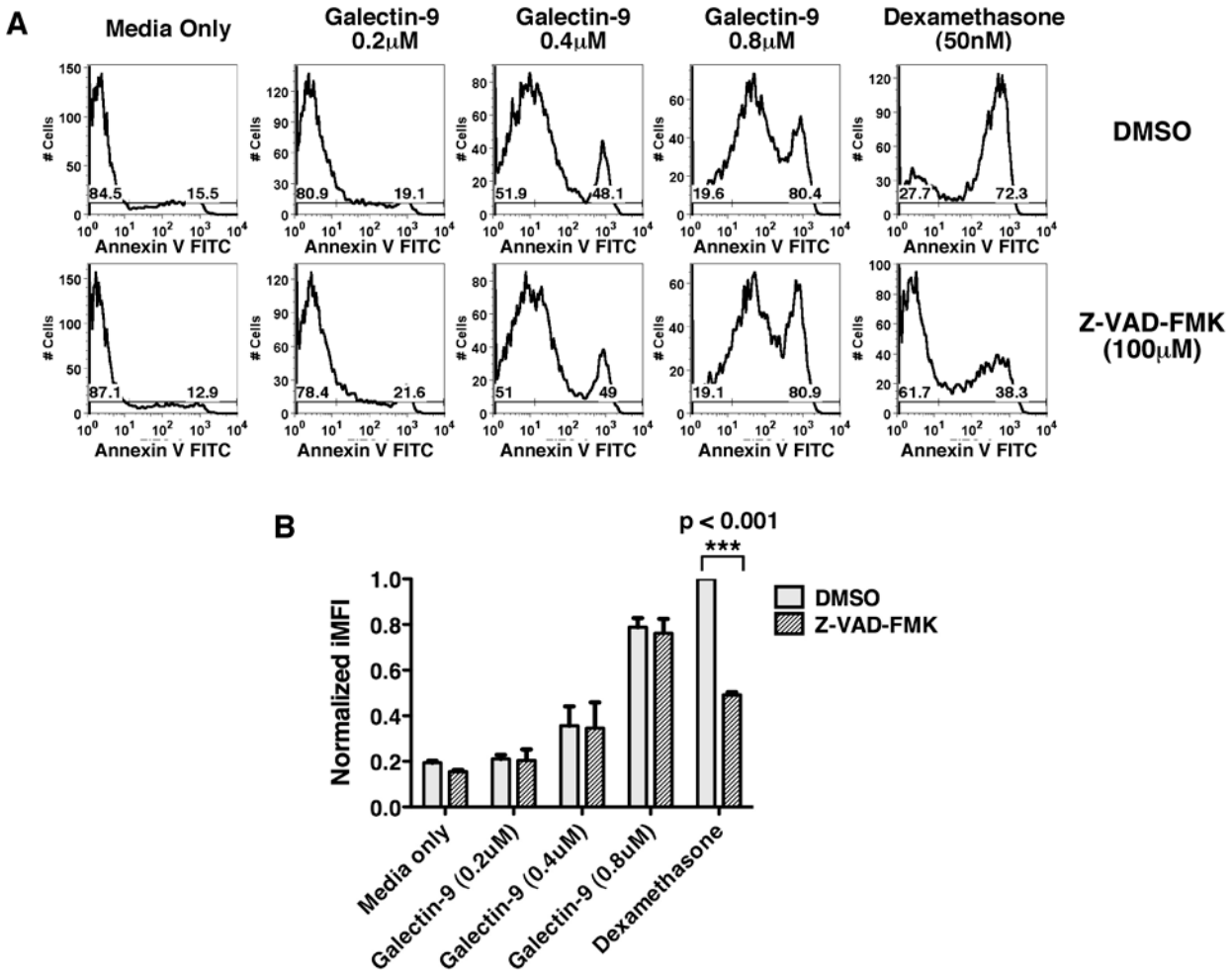

Figure 15: Gal-9 mediated apoptosis is not dependent on caspases

A) AD10 Th1 cells were pre-treated for 1 hour with either DMSO or Z-VAD-FMK prior to treatment with increasing doses of gal-9 or dexamethasone for 20 hours. Cells were then stained with 7-AAD and Annexin-V. B) iMFI was calculated by multiplying the % of Annexin V+ cells with the MFI of Annexin V+ cells. This value was then normalized to the iMFI of DMSO/dexamethasone treated AD10 Th1 cells. *p* values were calculated using two-way ANOVA. Error bars indicate standard deviation from two independent experiments.

3.3.3 Gal-9 modulates T helper cell function and viability in the absence of Tim-3

The results above suggest that Tim-3 may not be the major mediator for gal-9 on T helper cells. To address this more directly, we assessed the effects of gal-9 on CD4+ T cells stimulated only once under Th1 and Th2 polarizing conditions. As expected, T-bet and GATA-3 were upregulated in Th1 and Th2 cells, respectively (Figure 16A). In agreement with previous reports, we found that these cells do not express Tim-3 on their surface (Figure 16B). However, they stained positively with gal-9, which suggests that other binding partner(s) of gal-9 are expressed on the surface of T helper cells after only one round of polarization under either Th1 or Th2 conditions (Figure 16C). Despite the absence of Tim-3 on the cell surface, gal-9 treatment induced IFN-γ secretion (Figure 16D-E) and apoptosis (Figure 16F-G) in Th1 cells. Likewise, Th2 cells secreted TNF-α (Figure 16H-I) and underwent apoptosis (Figure 16J-K) in the presence of gal-9. These effects were abrogated in the presence of lactose (Figure 16D, F, H and J), consistent with our findings in the AD10 Th1 and D10 Th2 clones.

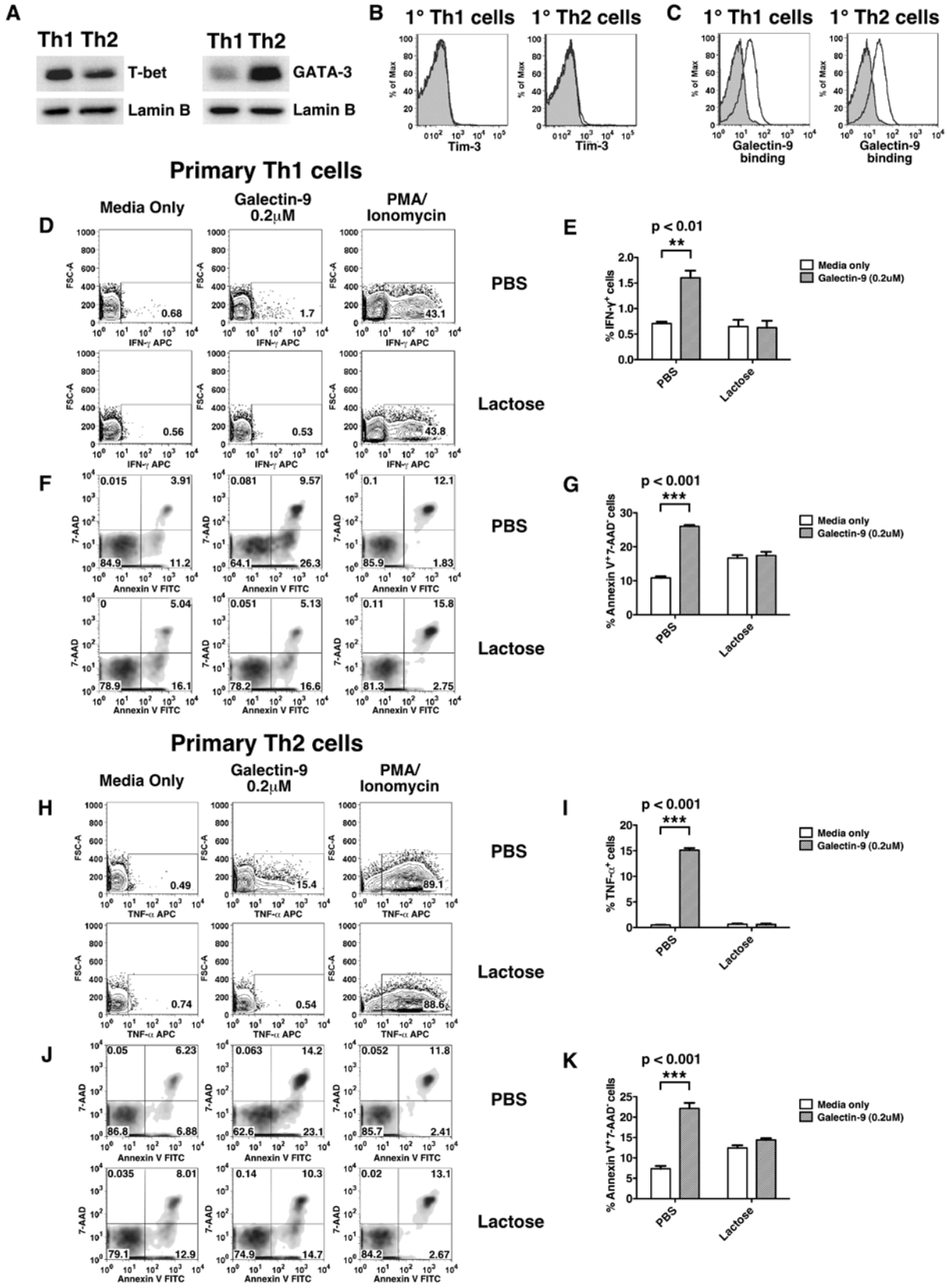
A
Th1 Th2
T-bet
Lamin B
Th1 Th2
GATA-3
Lamin B
B
1° Th1 cells
1° Th2 cells
% of Max
Tim-3
C
1° Th1 cells
1° Th2 cells
% of Max
Galectin-9 binding
Primary Th1 cells
Media Only
Galectin-9 0.2μM
PMA/ Ionomycin
D
PBS
Lactose
FSC-A
IFN-γ APC
0.68
1.7
43.1
0.56
0.53
43.8
E
p < 0.01
**
% IFN-γ+ cells
Media only
Galectin-9 (0.2uM)
PBS
Lactose
F
7-AAD
Annexin V FITC
0.015
3.91
84.9
11.2
0.081
9.57
64.1
26.3
0.1
12.1
85.9
1.83
0
5.04
78.9
16.1
0.051
5.13
78.2
16.6
0.11
15.8
81.3
2.75
G
p < 0.001
***
% Annexin V+7-AAD- cells
Media only
Galectin-9 (0.2uM)
PBS
Lactose
Primary Th2 cells
Media Only
Galectin-9 0.2μM
PMA/ Ionomycin
H
PBS
Lactose
FSC-A
TNF-α APC
0.49
15.4
89.1
0.74
0.54
88.6
I
p < 0.001
***
% TNF-α+ cells
Media only
Galectin-9 (0.2uM)
PBS
Lactose
J
7-AAD
Annexin V FITC
0.05
6.23
86.8
6.88
0.063
14.2
62.6
23.1
0.052
11.8
85.7
2.41
0.035
8.01
79.1
12.9
0.14
10.3
74.9
14.7
0.02
13.1
84.2
2.67
K
p < 0.001
***
% Annexin V+7-AAD- cells
Media only
Galectin-9 (0.2uM)
PBS
Lactose

Figure 16. Effects of gal-9 on primary Th1 and Th2 cells.

(A) T-bet and GATA-3 expression in nuclear lysates of primary Th1 or Th2 cells (upper panel). Lamin B expression is shown as a loading control (lower panels) (B) Primary Th1 and Th2 cells were stained with isotype control (filled histogram) and Tim-3 pAb (empty histogram), or (C) streptavidin (filled histogram) and biotin-gal-9 (empty histogram). Primary Th1 cells treated with gal-9 for 6 hours in the presence of PBS or lactose were either stained for intracellular IFN-γ (D) or 7-AAD/annexin V (F). Results are summarized in (E) and (G). Primary Th2 cells treated with gal-9 for 6 hours in the presence of PBS or lactose were either stained for intracellular TNF-α (H) or 7-AAD/annexin V (J). Results are summarized in (I) and (K). Error bars indicate S.D from replicate cultures. Data are representative of two independent experiments. p values were calculated using two-way ANOVA.

3.3.4 Tim-3 is not the major mediator of gal-9 effects on fully-differentiated Th1 cells

To further confirm that gal-9 does not require Tim-3 to modulate T helper function and viability, we stimulated CD4+ T cells from wild type and Tim-3 KO mice under Th1 polarizing conditions for three rounds to induce surface expression of Tim-3. We confirmed that only Th1 cells from wild type mice and not Tim-3 KO mice express Tim-3 (
Figure **17**A). After twenty hours of treatment with gal-9, we were able to detect significant amounts of IFN-γ in the supernatant of both wild type and Tim-3 KO Th1 cells (
Figure **17**B). We also found that wild type and Tim-3 KO Th1 cells were equally susceptible to gal-9 induced apoptosis as determined by 7-AAD/Annexin V staining (
Figure **17**C-D). Therefore, gal-9 does not require Tim-3 to induce cytokine secretion and apoptosis in fully differentiated Th1 cells.

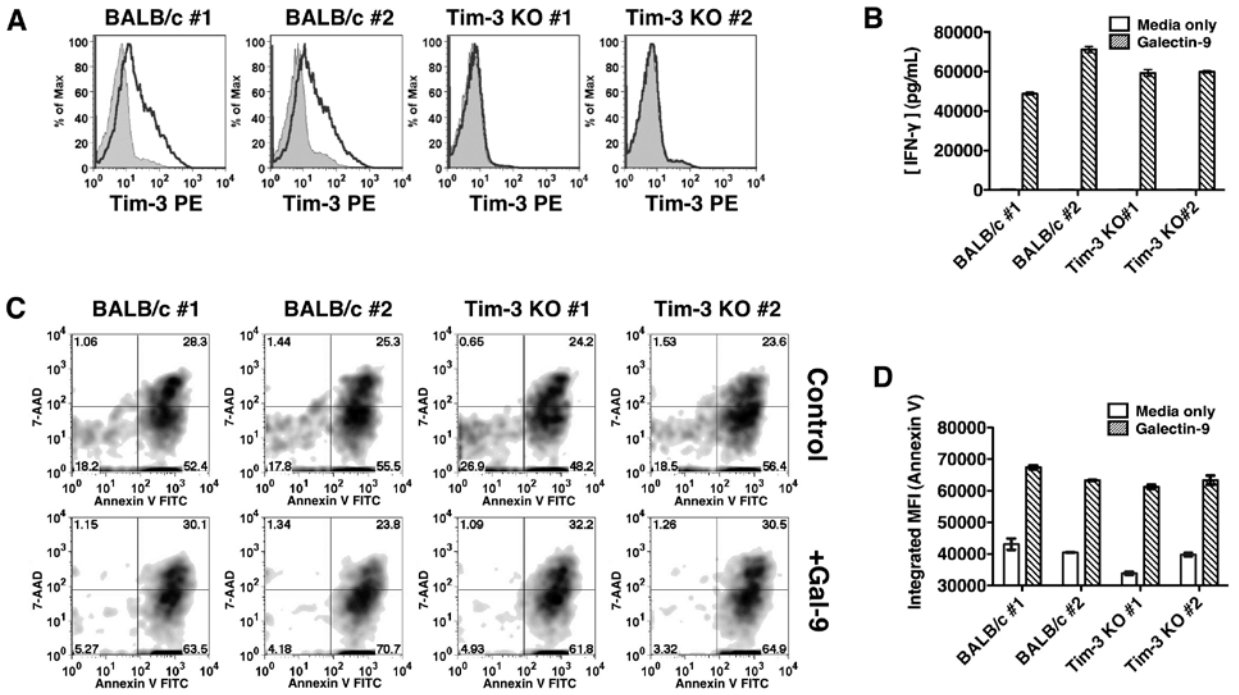

Figure 17: Effects of gal-9 on WT and Tim-3 KO fully differentiated Th1 cells.

(A) CD4+ T cells from spleens of wild type and Tim-3 KO mice were subjected to three rounds of Th1 polarization and stained with either rat IgG1 (filled histogram) or anti-Tim-3 (empty histogram). (B) Th1 cells from both BALB/c and Tim-3 KO mice were stimulated in duplicate sets for 20 hours. Supernatants were analyzed by ELISA for IFN-γ. (C) Th1 cells from (B) were stained with 7-AAD/annexin V. (D) Results from (C) are quantified as integrated MFI (% of annexin V+ cells X MFI annexin V+ cells) to reflect cells undergoing both early and late apoptosis. Increases in IFN-γ and apoptosis due to gal-9 treatment were found to be significant in both wild-type and Tim-3 KO Th1 cultures ($p<0.001$, two-way ANOVA).

3.3.5 Discussion

In this study, we show for the first time that in addition to inducing apoptosis, gal-9 can promote the production of pro-inflammatory cytokines by both Th1 and Th2 cells. These effects occur in a dose-dependent fashion and do not require Tim-3. The ability of gal-9 to bind and modulate both T helper subsets is noteworthy, as Th1 and Th2 cells have distinct glycophenotypes [144]. These findings clearly highlight the complex nature of the biological effects of gal-9, and call into question the centrality of the gal-9:Tim-3 interaction for such effects.

The pro-apoptotic activity of gal-9 was first identified using thymocytes, cells that are not known to express Tim-3 [148]. Following the identification of gal-9 as a ligand for Tim-3, numerous biological effects have been attributed to the gal-9:Tim-3 interaction. For example, gal-9 is thought to increase viral burden or ameliorate autoimmune disease in mice by inducing the apoptosis of Tim-3 expressing, antigen-specific, T cells [77, 95]. However, the loss of Tim-3+ cells in vivo due to gal-9 induced apoptosis has yet to be demonstrated directly. The enhanced susceptibility of Tim-3 expressing cells to gal-9 induced apoptosis has been addressed in vitro. Th1 cells polarized from Tim-3 KO mice displayed less nucleosomal enrichment than Th1 cells from wild-type mice following gal-9 treatment, up to twelve hours, the last time point analyzed [57]. Here, we show that twenty hours after gal-9 treatment, there were no significant differences in the ability of Th1 cells from wild type and Tim-3 KO mice to undergo apoptosis. However, there are several differences between our approaches. Zhu et. al. used CD4+ T cells polarized under Th1 conditions with peptide-loaded splenocytes while we employed plate-bound anti-CD3 and anti-CD28. Additionally, the previous study treated Th1 cells with mouse gal-9 (0.5μM) while we used human gal-9 (0.2μM). Lastly, Zhu et al. detected apoptosis using the nucleosome

enrichment assay, which we have found to be not as sensitive as 7-AAD/annexin V staining for detection of small changes in apoptosis (Figure 4C).

Tim-3 is upregulated in vitro with repeated rounds of Th1 polarization and in vivo under chronic inflammatory conditions [95, 149]. Tim-3 expression on CD4+ and CD8+ T cells in chronic viral infections is associated with immune "exhaustion" [69, 149]. In such settings, Tim-3+ cells only respond to TCR ligation when the interaction between Tim-3 and its ligand(s) is disrupted. This suggests that when Tim-3 is occupied by a ligand, it can antagonize signals emanating from the T-cell receptor without causing cell death. The ligand(s) that elicit this effect from Tim-3 remains unknown but gal-9 is an unlikely candidate at this point, as it has not been shown to negatively regulate T cell effector function in a manner that is independent of cell death or apoptosis.

Our findings raise the question of how gal-9 is able to induce the bimodal effects that we have observed. We propose that for the induction of apoptosis, sufficient gal-9/receptor interactions must be formed on the surface of effector T cells, to reach a certain threshold for signaling. This could be achieved through the formation of high order lattice structures that allow gal-9 to crosslink multiple receptors in close proximity. Another possibility is that gal-9 binds to multiple distinct receptors with varying affinities to induce apoptosis or cytokine production. Thus, the bimodal effects we observe in Th1 cells may be the result of gal-9 having a greater affinity for the receptor required for cytokine production than the receptor required for apoptosis. While biochemical studies have validated the gal-9:Tim-3 interaction, the exact biological effects of this ligand/receptor pair have not been firmly established [57]. One of the main challenges stems from the fact that gal-9 can have a wide range of effects on immune cells [139]. In addition, both T cells and antigen presenting cells can upregulate expression of gal-9 and Tim-3

under similar conditions [56, 66, 136]. The ability of galectins to bind to more than one cell surface receptor to modulate T helper cells is not unusual [150, 151]. Galectin-1, a prototype member of the galectin family can induce apoptosis of T cells through CD7 and CD45 [152, 153].

Therefore, when gal-9 is administered to wild type mice undergoing chronic inflammation, it is difficult to distinguish between the Tim-3 dependent and Tim-3 independent effects of gal-9 on T cells and antigen presenting cells. Further in vivo validation of our findings will require mice deficient for both Tim-3 and gal-9, or a panel of blocking antibodies to both Tim-3 and gal-9 with well-characterized epitopes.

4.0 PHOSPHOTYROSINE-DEPENDENT COUPLING OF TIM-3 TO TCR SIGNALING PATHWAYS

4.1 INTRODUCTION

During the expansion phase of an acute infection, newly activated viral antigen-specific CD8+T cells expand rapidly and acquire effector functions. This is then followed by a period of contraction, where all but 5% to 10% of these CD8+T cells succumb to apoptosis[154]. The remaining CD8+ T cells constitute the memory pool – multifunctional T cells that persist in the host in an antigen-independent manner with the ability to respond quickly upon re-exposure to viral antigen[155]. However, during chronic viral infection, effector CD8+ T cells generated during the expansion phase fail to develop into memory CD8+ T cells[156]. Instead, these effector CD8+ T cells appear to be exhausted[99].

T cell exhaustion is characterized as the progressive and stepwise loss in the ability to secrete IL-2, TNF-a, and IFN-g in response to antigenic stimulation, culminating in the most extreme cases in apoptosis[157]. This system of clonal deletion has been documented in conditions of persistent antigen stimulation such as high grade chronic viral infections in both mouse and human and, most recently, in patients with advanced melanoma[158, 159]. Exhausted CD8+T cells have a distinct molecular signature that resembles effector T cells more than memory T cells[160]. Of the 338 genes differentially upregulated in exhausted CD8+ T cells,

some are inhibitory receptors such as CTLA-4, LAG-3 and PD-1. Several studies have confirmed that PD-1 is upregulated on exhausted CD4+ and CD8+ T cells. However, blocking the interaction between PD-1 and its ligand does not always completely restore the effector functions to exhausted T cells, which suggests the involvement of other receptors [14]. Recently, it was shown that blocking Tim-3/Tim-3L interactions, along with PD-1/PD-1L, has an additive and sometimes synergistic effect on the invigoration of exhausted T cells [13, 70].

Tim-3 is a type-I glycoprotein receptor whose expression often parallels that of PD-1 in conditions of chronic inflammation [13]. Prior to its implication in T cell exhaustion, Tim-3 was shown to be important in the induction of tolerance and suppression of effector T cell function [63, 64]. Recent studies show that Tim-3 promotes the inhibition of T cells by expanding myeloid-derived suppressor cells (MDSC)[66]. However, this effect is dependent on the interaction between gal-9 expressed by MDSC and Tim-3 on CD4+ T cells. MDSC inhibit T cell proliferation by producing arginase II, an enzyme that catalyzes arginine metabolism, which removes this semi-essential amino acid from the microenvironment[83]. However, there is insufficient data to determine if T cells suppressed by MDSC are exhausted or whether Tim-3 is even activated during this process.

The cooperation between Tim-3 and PD-1 in maintaining T cell exhaustion indicates that these two receptors either employ the same signaling pathway (quantitative effect) or distinct signaling pathways (qualitative effect) when ligated [13]. Despite the wealth of literature on the *in vivo* effects of these receptors, little is known of their signaling mechanisms. The PD-1 cytoplasmic tail contains ITIM and ITSM motifs. Ligation of either CD3 alone or with CD28 and PD-1 leads to the recruitment of the tyrosine phosphatase SHP-2[161]. However, PD-1 can only inhibit T cell function when it is ligated along with CD3 and CD28. Single point mutations

disrupting either motif show that, in T cells activated with anti-CD3 and CD28, SHP-2 is recruited predominantly to the ITSM motif of PD-1. SHP-1 can also bind to the ITSM motif, although this has only been shown in pervanadate stimulated T cells[161]. The Tim-3 cytoplasmic has six well-conserved tyrosines, although there are no obvious signaling motifs (Figure 18). One of these tyrosine residues has been shown to be phosphorylated in HEK293 cells stimulated with pervanadate. However, the role of the cytoplasmic tail tyrosines in the downstream signaling of Tim-3 in T cells has not yet been determined.

In this paper we explored the role of these tyrosines using a Jurkat cell system that we have used in the past to show that Tim-1 is a co-stimulatory molecule, while Tim-2 is inhibitory - observations that have since been validated by other groups. Interestingly, overexpression of Tim-3 enhances NFAT/AP-1- and NF-kB-driven luciferase reporters in T cells activated anti- through TCR/CD3 and CD28. We also show that two or more tyrosines in Tim-3 can be phosphorylated and that the enhancement of CD3/CD28 signals is largely dependent on Y256 and Y263. Although these tyrosines can be phosphorylated by both Lck and Fyn, Lck appears to be the more efficient kinase. A phosphopeptide containing Y256 and Y263 binds to the SH2 domains of Lck and Fyn, although their interaction in vitro has yet to be established. Lastly, we show that the signaling pathway downstream of Tim-3 intersects with that of the TCR and CD28.

Co-inhibitory receptors

```
------------VLILKWYSCKK-KKLSSLSLITLANLPPGGLANAGAV 247 mTim-3
------------VFCSTSMSEARGAGSKDDTLKEEPSAAPVPSVAYEEL 228 mPD-1
VS------------LGLFFYSFLVTAVSLSKMLKKRSPLTTGVYVKMPPT 207 mCTLA-4

RIRSE-------ENIYTIEENVYEVENSN--------------------- 269 mTim-3
DFQGR-------EKTPELPTACVHTEYAT--------------------- 250 mPD-1
EPECE----------KQFQPYFIPIN------------------------ 223 mCTLA-4

---------------------------EYYCYVNSQQPS----------- 281 mTim-3
-----------------------------IVFTEGLGASAMGR------R 265 mPD-1
-------------------------------------------------     mCTLA-4

-------------------------     mTim-3
GSADGLQGPRPPRHEDGHCSWPL-- 288 mPD-1
-------------------------     mCTLA-4
```

Co-stimulatory receptors

```
GVGVSAGLTLALIIGVLILKWYSCKKKKLSSLSLITLANLPPGGLANAGA 246 mTim-3
----VLFCYGLLVTVALCVIWTNSRRNRLLQSDYMNMTPRRPG-LTRKPY 204 mCD28
----CAAFVVVLLFGCILIIWFSKKKYGSSVHDPNSEYMFMAA-VNTNKK 192 mICOS

VRIRSEENIYTIEENVYEVENSNEYYCYVNSQQPS 281 mTim-3
QPYAPARDFAAYRP--------------------  218 mCD28
SRLAGVTS--------------------------  200 mICOS
```

Figure 18: Alignment of cytoplasmic tail sequences of Tim-3 and known T-cell co- receptors

Tyrosines in the Tim-3 cytoplasmic tail is in boldface.

Signaling Motifs:

SH2 binding (YxxM)

SH3 binding (Pxxp)

GRB2 binding (YxN)

ITIM (V/I/LxYxxL)

ITSM (TxYxxl)

4.2 MATERIALS AND METHODS

4.2.1 Cell Lines

Parental Jurkat T cells and variants were cultured in RPMI-1640 media, supplemented with 5% bovine growth serum (BGS; Hyclone). Mutant Jurkat lines were cultured in RPMI-1640 supplemented with 10% BGS. AD10 cells were maintained in RPMI supplemented with 10% BGS, and re-stimulated every two weeks with mitomycin-C treated splenocytes from B10.A or B10.BR mice in the presence of pigeon cytochrome C (30 ug/mL; Sigma). A fast-growing variant of the D10.G41 Th2 T cell clone was maintained in RPMI supplemented with 10% BGS.

4.2.2 Reagents and Antibodies

Anti-Flag mAb was obtained from Sigma. Polyclonal anti-Tim-3 antibody used for western blotting was obtained from R&D. Monoclonal anti-Tim-3 antibody 5D12 was used for flow cytometry. Anti-phosphotyrosine antibody was from Millipore. HRP-conjugated anti-mouse-HRP and Protein-A-HRP were from Pierce and GE Healthcare, respectively. Monoclonal antibody specific for the Jurkat TCR (C305.2) was obtained from Arthur Weiss. Anti-human CD28 was from Caltag/Invitrogen. Anti-mouse CD3, CD4 and CD28 were from BD Biosciences. Anti-IFNγ antibody conjugated to PE, Annexin V-FITC and 7-AAD were obtained from BD Bioscience.

4.2.3 DNA Constructs

Murine Tim-3 carrying an extracellular Flag tag was generated by PCR and cloned into the pCDEF3 vector. Truncation mutants were also generated by PCR. Point mutants were generated using the QuikChange mutagenesis kit from Stratagene. Lck and Fyn constructs were obtained from Tom Smithgall, University of Pittsburgh. All constructs were verified by automated sequencing.

4.2.4 Transfections

Jurkat T cells were transfected by electroporation as described previously [162]. HEK293FT cells were obtained from Invitrogen and transfected using the calcium phosphate method, as described previously [163].

4.2.5 Luciferase Assays

The day after transfection, cells were stimulated in round bottom 96-well plates for six hours and kept frozen at -80ºC until analyzed. Luciferase assays were conducted as described previously [162, 163].

4.2.6 Immunoprecipitation / SDS-PAGE / Western Blotting.

Cells transfected with Flag-Tim-3 constructs were stimulated the next day with pervanadate [164], then lysed with NP-40 lysis buffer, as described previously [162]. IPs were carried out with anti-Flag agarose beads, then washed three times with NP-40 lysis buffer, followed by addition of reducing 2x SDS sample buffer. IPs and cell lysates (10% of total lysate before IP) were separated on 10% Laemmli gels and blotted to PVDF (Millipore). Blots were blocked with 4% BSA in TBS-Tween for 1 hour at room temperature, then probed with anti-Tim-3 antibody and HRP-conjugated secondary antibody. After imaging, blots were stripped and re-probed with anti-phosphotyrosine antibody. Blots were imaged on a Kodak ImageStation 4000R. Images were exported in JPG format and assembled into final figures in Canvas 8.

4.2.7 Intracellular Cytokine and Viability Staining.

For intracellular staining of IFN-γ, cells were cultured in the presence of GolgiPlug (BD Biosciences) for the last four hours of a six hour stimulation. Cells were then fixed in 4% paraformaldehyde. Staining was performed with PE-conjugated anti-mouse-IFN□ (BD Biosciences) in the presence of 0.1% saponin. Viability staining was performed with Annexin V-FITC and 7-AAD, according to the manufacturer's intstructions.

4.2.8 Flow Cytometry

Data were collected on a Becton-Dickinson LSR II flow cytometer, and analyzed using FlowJo. Figures were exported and assembled in Canvas 8.

4.3 RESULTS

4.3.1 The Tim-3 cytoplasmic tail couples to signaling pathways associated with T cell activation

We previously reported that ligation of Tim-3 can augment the activation of dendritic cells, in contrast to its previously described ability to induce apoptosis of Th1 T cells [57, 65]. Paradoxically, we also found that Tim-3 ligation was capable of inducing NF-κB activation in both DCs and T cells [65]. We were intrigued by the fact that the Tim-3 cytoplasmic tail contains six conserved tyrosine residues, which might couple to intracellular signaling pathways that regulate T cell activation (either positively or negatively). We therefore expressed murine and human Tim-3 in T cell lines, to study the signals responsible for such effects, similar to previous work from our group on Tim-1 and Tim-2[32, 51]. As shown in
Figure 19A (left panel), expression of full-length Tim-3 in Jurkat T cells which lack endogenous Tim-3, enhanced NF-κB and NFAT/AP-1 reporter activation, both in the basal state (i.e. with no further stimulation) and in conjunction with TCR/CD28 crosslinking (
Figure **19**B).

To determine whether signaling to NF-κB requires the cytoplasmic tail of Tim-3, we generated two truncation mutations. Truncation 1 (T1) contains all but the three most C-terminal tyrosines, while truncation 2 (T2) lacks all but one tyrosine, which is predicted to reside very close to the membrane (Figure 20).The T1 construct functioned at least as well as wild-type Tim-3 to augment NF-κB reporter activity (Figure 21B). Conversely, the shorter T2 truncation lost all ability to enhance NF-κB activity. All Flag-tagged Tim-3 constructs were expressed at equivalent levels (Figure 21A, right panel). The ability to enhance activation of NFAT/AP-1 and NF-κB is consistent with a role for Tim-3 in positively regulating T cell activation.

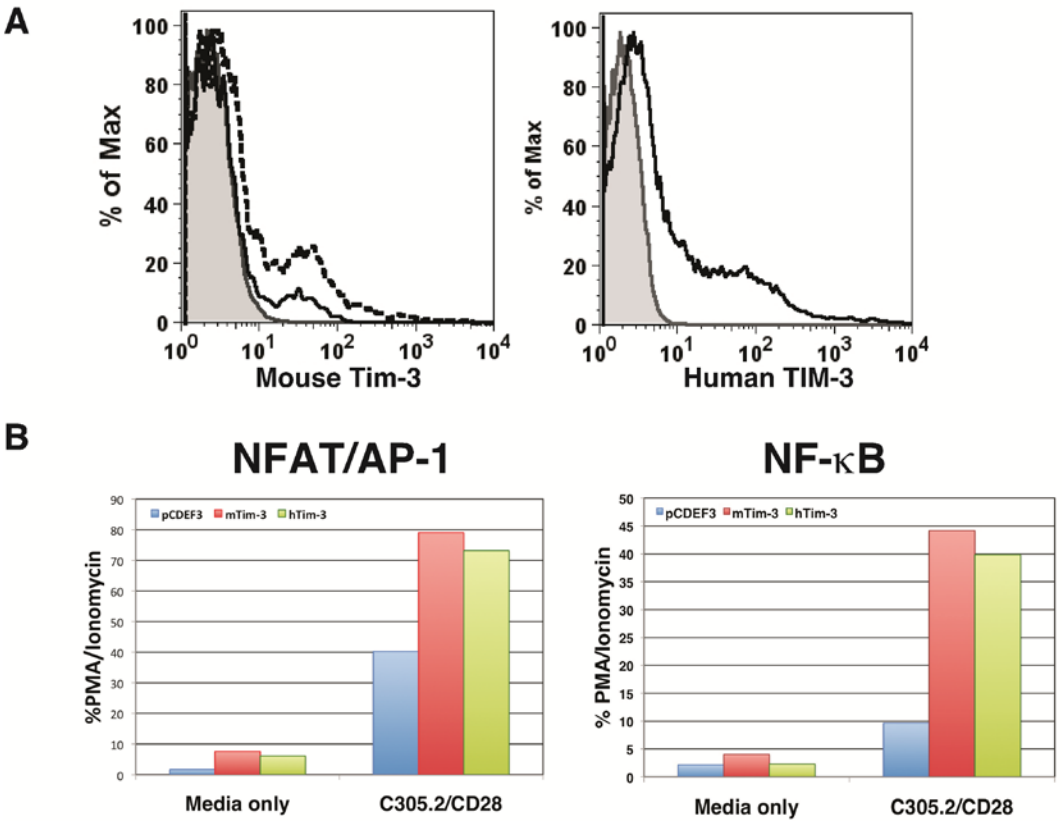

Figure 19: Mouse and human TIM-3 enhance TCR/CD28 activation.

A) Left panel: Jurkat T cells transfected with either vector (solid line) or mouse Tim-3 (dotted line) were then stained with biotinylated anti-FLAG and/or stained with streptavidin-APC (solid histogram). Right panel: Jurkat T cells transfected with vector (solid histogram) or human TIM-3 (solid line) were stained with anti-TIM-3 PE. B) NFAT/AP-1 and NF-κB-luciferase reporter activity in anti-TCR/CD28 stimulated Jurkat T cells transfected with vector and or TIM-3.

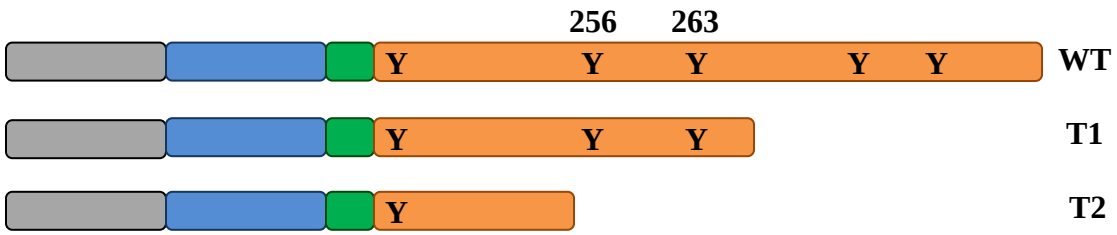

Figure 20: Schematic diagram of wild-type and mutant constructs of Tim-3

The IgV, mucin and transmembrane domains are highlighted in grey, blue and green respectively. The cytoplasmic tail is highlighted in orange. The approximate points of truncation and the positions of the six tyrosines in Tim-3 truncation mutants are shown.

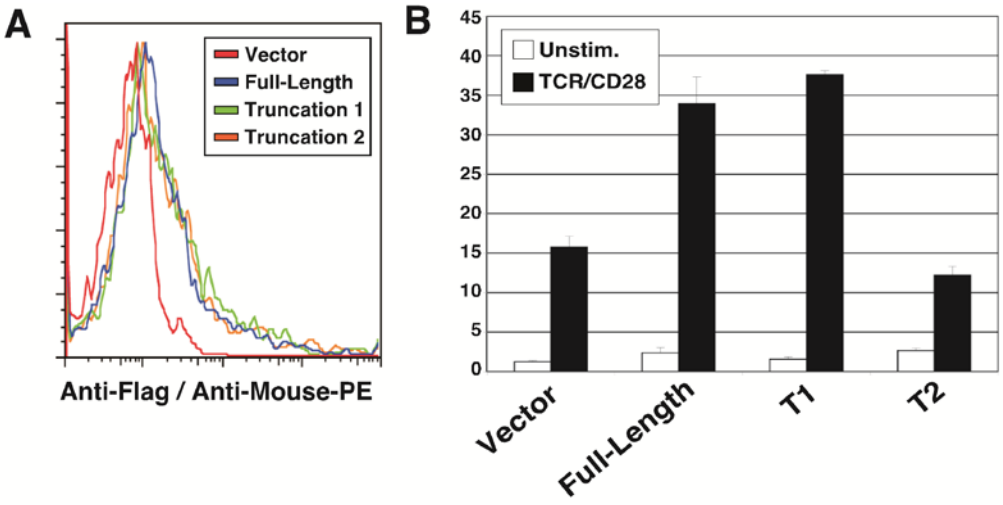

Figure 21: Examining the ability for Tim-3 truncation mutants to modulate T cell activation

(A) Jurkat T cell transfected with both wild-type and Tim-3 truncation mutants were stained with anti-FLAG antibody (M2) and detected with streptavidin PE. (B) NF-κB luciferase reporter activity in Jurkat T cells transfected with wild-type and Tim-3 truncation mutants following stimulation with anti-TCR/CD28.

4.3.2 Tyrosines 256 and 263 are required to mediate downstream signaling of Tim-3

To investigate the role of individual tyrosine residues in Tim-3 signaling, we focused on two tyrosines retained in the T1, but not T2, Tim-3 construct. Therefore, we mutated tyrosines at position 256 and 263 to phenylalanine, both individually and together. Representative flow cytometry for expression of some of these constructs is shown in Figure 22A. By impairing the ability of these tyrosines to be phosphorylated, we hoped to abolish binding of any downstream mediators that may interact directly and indirectly with the Tim-3 cytoplasmic tail when it is phosphorylated. Interestingly, we observed that full-length Tim-3 carrying the Y256/263F double mutation loses some, but not all, of its ability to enhance NFAT/AP-1 activation by anti-TCR/CD28 stimulation (Figure 22B). However, the Y256/263F double mutation on the T1 background led to a much more dramatic loss of NFAT/AP-1 activation (Figure 22B). Mutating either Y256 or Y263 individually to phenylalanine did not affect the ability of Tim-3 to enhance NFAT/AP-1 activation (data not shown). We observed a partial effect of the Y256/263F mutant, with more of an effect on the T1 background, in cells transfected with an NF-κB luciferase reporter (Figure 22C), suggesting that common receptor-proximal signaling proteins couple Tim-3 to both NFAT and NF-κB induction. Importantly, the same effects observed in Jurkat T cells with each Tim-3 construct described above were also obtained in a non-transformed T cell line - the TH2 clone D10.G41 - using the NFAT/AP-1 luciferase reporter as a readout (Figure 22D). Thus, Y256 and Y263 may be able to compensate for each other's loss, while the more C-terminal residues are also required for Tim-3 to signal optimally.

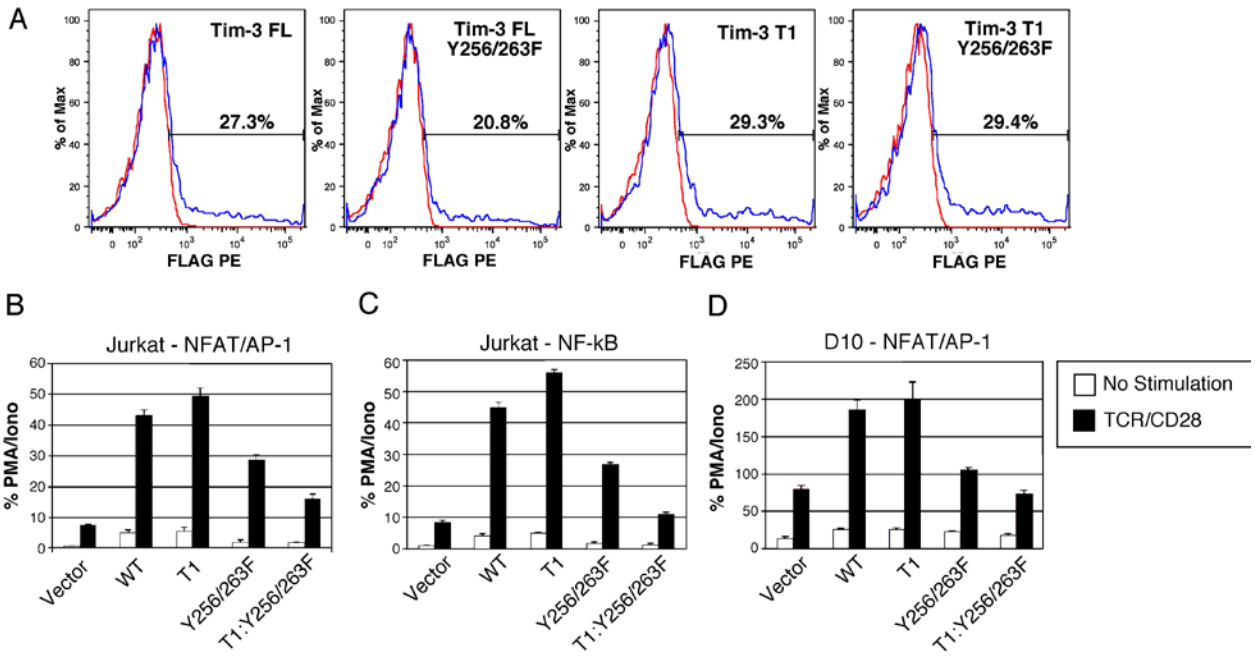

Figure 22: Stimulation of signaling pathways associated with T cell activation by Tim-3.

(A) Jurkat T cells transfected with wild-type and mutant constructs were stained with secondary antibody only (red line) and anti-FLAG antibody (blue line) (B) Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated Flag-Tim-3 constructs. The next day, cells were cultured in media alone ("No Stimulation," white bars) or with anti-TCR/CD28 antibodies (black bars) for six hours. Luciferase activity was then determined. (C) Jurkat T cells were transfected with an NF-κB luciferase reporter and the indicated Flag-Tim-3 constructs and assayed as above. (D) D10 T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated Flag-Tim-3 constructs and assayed as above. Results shown are the average of triplicate samples from a single experiment, representative of at least five experiments in each case.

Inducible tyrosine phosphorylation of Tim-3

We then proceeded to determine more directly whether the tyrosine residues in Tim-3 examined above could indeed become phosphorylated. We transfected the constructs discussed in Figure 21 into cells and treated the cells with pervanadate, a protein tyrosine phosphatase inhibitor and

potent inducer of tyrosine phosphorylation. Flag-tagged Tim-3 constructs were immunoprecipitated from cell lysates and then probed with antibody to phosphotyrosine (4G10). As shown in Figure 23A, wild-type Tim-3 was efficiently phosphorylated, with a partial loss of phosphorylation of the T1 construct (compare anti-phosphotyrosine signals in the upper panel to total Tim-3 in the lower panel). However, the T2 construct was incapable of being phosphorylated, even by pervanadate, consistent with its one remaining tyrosine being closely juxtaposed to the predicted transmembrane domain (Figure 20).

Next, we examined tyrosine phosphorylation of the Tim-3 constructs with individual tyrosine point mutations. Mutation of both Y256 and Y263 ("2YF") abolished the phosphorylation of Tim-3 by pervanadate, with the mutation of Y256 having a partial effect (Figure 23B). By contrast, we observed no detectable effect of the Y263F mutation on overall levels of tyrosine phosphorylation, suggesting either that it is not recognized by the anti-phosphotyrosine antibody or that it is phosphorylated at low stoichiometry. These findings are largely in agreement with our luciferase data and provide further support for the model that phosphorylation of the Tim-3 cytoplasmic tail is important for its function.

Although a previous report suggested that tyrosine 256 in the cytoplasmic tail of Tim-3 could be phosphorylated by the Tec family kinase Itk [165], our analysis of the sequence around tyrosines 256 and 263 with the ScanSite algorithm revealed that it was likely that a src family kinase could phosphorylate one or both of these sites. Thus, we expressed wild-type Tim-3, either alone or together with the Src family tyrosine kinases Lck or Fyn, in 293 cells, then stimulated the cells with pervanadate. As shown in Figure 24A, when Tim-3 was expressed alone, stimulation with pervanadate induced significant, although transient, tyrosine phosphorylation. However, when Lck was co-expressed with Tim-3, stimulation led to more

robust and sustained phosphorylation (Figure 24A). Similar results were obtained when the Fyn tyrosine kinase was co-expressed with Tim-3, although in this case, the basal level of Tim-3 phosphorylation was consistently higher than when Lck was expressed with Tim-3. These results demonstrate that both Lck and Fyn can mediate phosphorylation of Tim-3.

To understand how tyrosine phosphorylation of Tim-3 contributes to downstream signaling, we focused further on Lck and Fyn. J.CaM.1 is an Lck-deficient Jurkat T cell line with low endogenous levels of Fyn [166]. To determine if Tim-3 requires Lck and/or Fyn to enhance TCR/CD28 signaling, we transfected J.CaM.1 cells with wild-type Tim-3 and either Lck or Fyn, then assayed for modulation of NFAT/AP-1 activity in the presence or absence of signals from TCR/CD28. Transfection of J.CaM.1 cells with Tim-3 alone led to a small but reproducible increase in NFAT/AP-1 reporter activity (Figure 24B). Co-expression of Lck restored the ability of Tim-3 to activate NFAT/AP-1, either alone or together with anti-TCR/CD28, to levels similar to what we observed in parental Jurkat T cells (Figure 19). J.CaM.1 cells co-transfected with Fyn and Tim-3 also demonstrated enhanced NFAT/AP-1 activity (Figure 24B), although to a lesser extent than in the presence of Lck. Similar results were obtained in J.CaM.1 cells transfected with an NF-κB reporter (data not shown). These results suggest that while either Lck or Fyn can phosphorylate Tim-3, Lck is more efficient at mediating downstream signaling by Tim-3.

To identify downstream mediators that could bind to the Tim-3 cytoplasmic tail when phosphorylated, we probed an SH2 domain array with a recombinant peptide that contained phosphorylated Y256 and Y253 with flanking Tim-3 sequences (Figure 25). This array showed that the SH2 domains of Fyn and the regulatory subunit of phosphatidylinositol 3 kinase (PI3K), p85α and p85β had the strongest binding to the Tim-3 phosphopeptide. Other SH2 domains that interacted with the phosphopeptide include RasGAP, PLC-γ1, Lck and Yes. Addition of a

phosphotyrosine analogue, phenyl phosphate, competed with the Tim-3 phosphopeptide for binding to the SH2 domain of most of these proteins except for Fyn, and to a much lesser extent, p85. This suggests that Fyn can either bind to the Tim-3 cytoplasmic tail independently of phosphorylated tyrosines or binds to the Tim-3 cytoplasmic tail with very high affinity such that it cannot be competed off by phenyl phosphate. Further work is required to establish these interaction within a cell and their role in mediating the enhancement of TCR/CD28 signals by Tim-3.

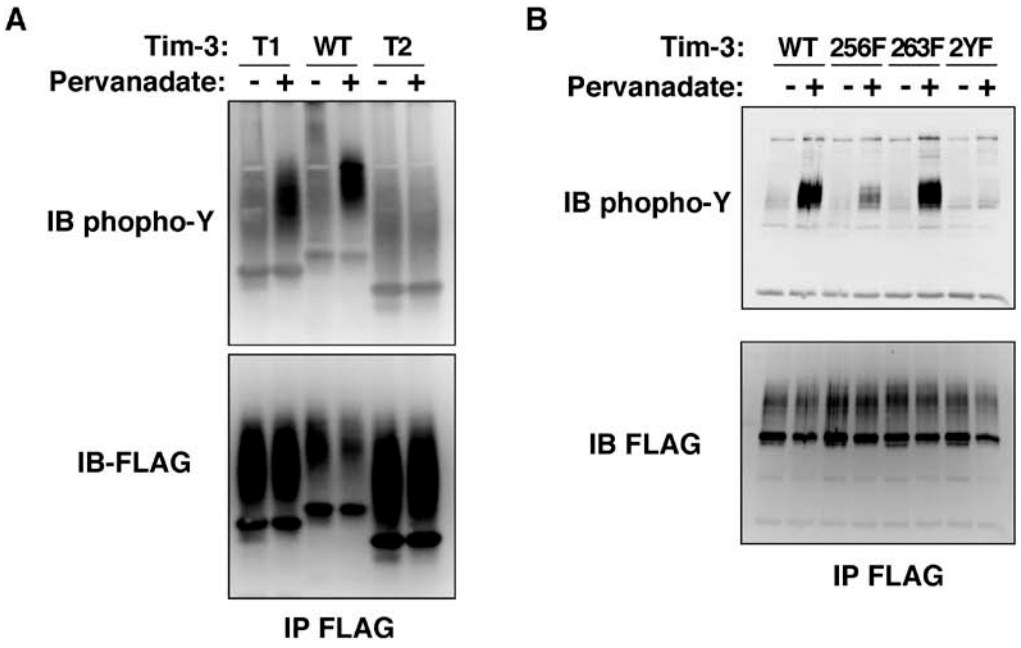

Figure 23: Tyrosine phosphorylation of the Tim-3 cytoplasmic tail.

(A) 293FT cells were transfected with wild-type Tim-3 or the T1 or T2 truncations. The next day, cells were stimulated with pervanadate and lysed. Lysates were subjected to IP with anti-Flag antibody and blotted with anti-phosphotyrosine antibody. (B) 293FT cells were transfected with wild-type Tim-3 or the indicated tyrosine mutants. The next day, cells were stimulated with pervanadate and analyzed for tyrosine phosphorylation as above. Results shown are representative of at least three experiments in each panel.

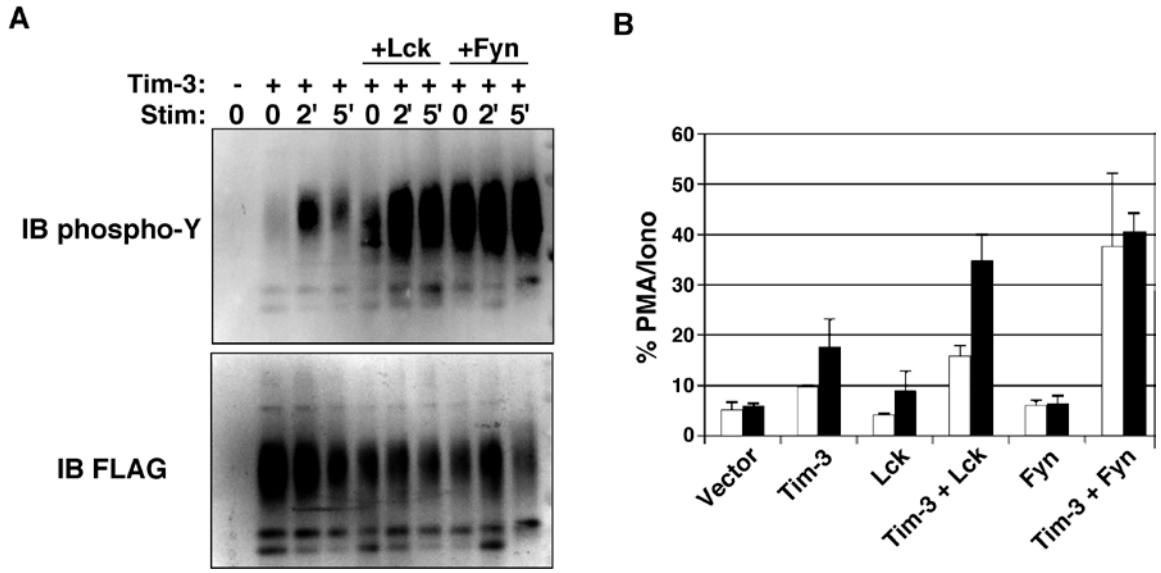

Figure 24: Role of src family tyrosine kinases in Tim-3 signaling.

(A) 293FT cells were transfected with wild-type Tim-3, either alone or together with lck or fyn. The next day, cells were stimulated with pervanadate for the indicated times, and analyzed for tyrosine phosphorylation as above. (B) Lck-deficient J.CaM.1 cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs. The next day, cells were left unstimulated (white bars) or stimulated with anti-TCR/CD28 antibodies for six hours, followed by determination of luciferase activity. Results shown are the average of triplicate samples from a single experiment, representative of five that were performed.

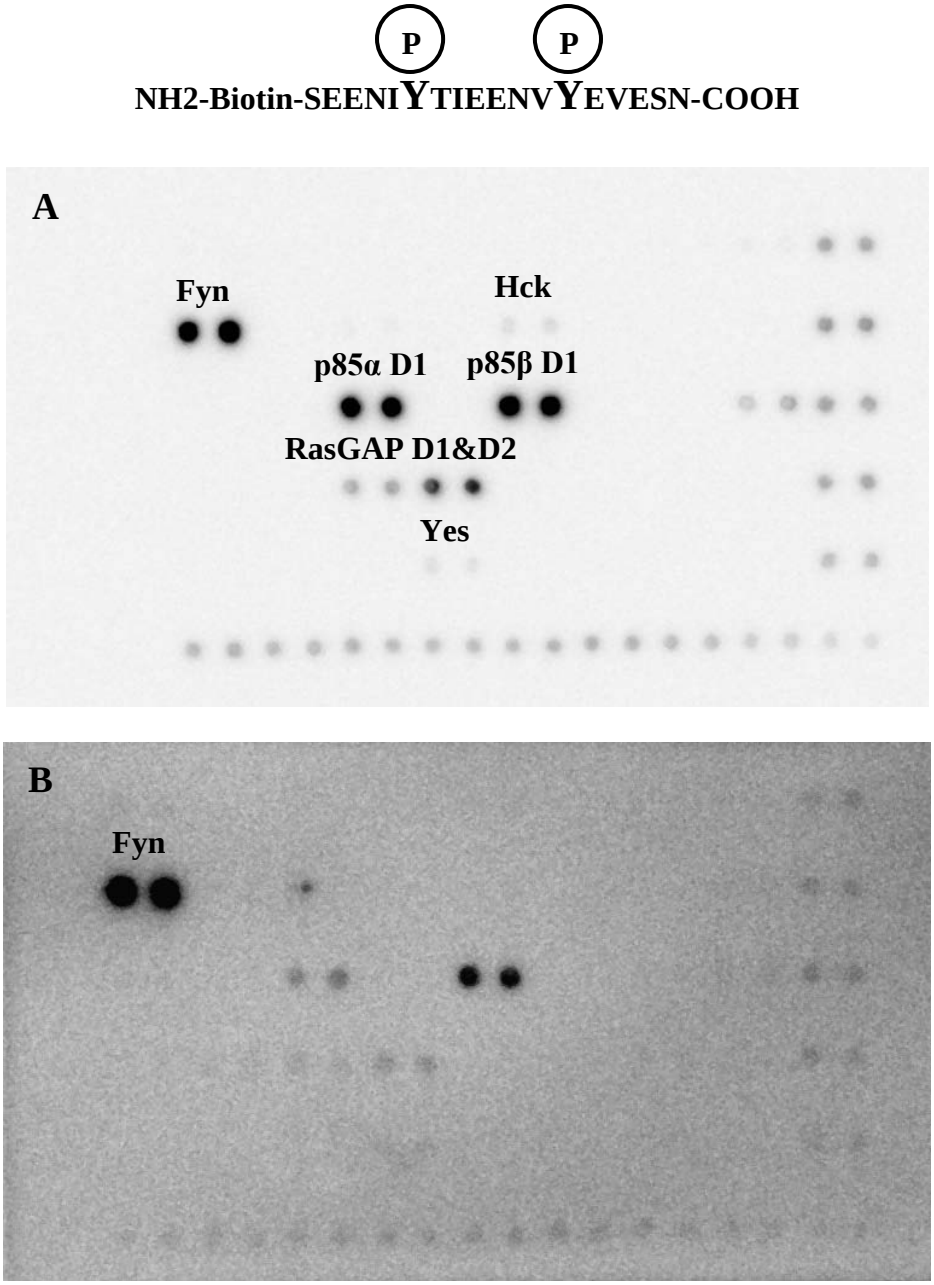

Figure 25: SH2-domain array probed with a Tim-3 phosphopeptide

An array containing the conserved binding sites of individual SH2 domains was probed only with biotinylated peptides consisting of phosphorylated Y256 and 263 with flanking Tim-3 sequences (A) or in the presence of phenyl phosphate (B). Bound peptides were then detected with streptavidin horseradish peroxidase and chemiluminescent substrate.

Intersection of Tim-3 signaling with TCR/CD3 signaling pathways

Next we focused further downstream in the TCR signaling pathway to better define the requirements for Tim-3 stimulatory and co-stimulatory signaling. We took advantage of the existence of mutant Jurkat T cell lines lacking expression of ZAP-70 and SLP-76, a critical tyrosine kinase and adaptor, respectively. As shown in Figure 26A, expression of wild-type Tim-3 in ZAP70-deficient Jurkat T cells led to a modest, although reproducible, increase in basal NFAT/AP-1 reporter activity. This level of reporter activity was not enhanced any further with TCR/CD28 stimulation, consistent with the strict requirement for ZAP-70 in the TCR signaling pathway. However, in ZAP-70 mutant cells stably reconstituted with wild-type ZAP-70, we observed full basal and co-stimulatory Tim-3 activity, very similar to what was observed in parental Jurkat cells (Figure 19). Very similar results were obtained in a Jurkat mutant line lacking expression of the adaptor protein SLP-76, which is critical for nucleation of signaling complexes downstream of the TCR/CD3 complex [121]. Thus, expression of Tim-3 in Jurkat T cells lacking SLP-76 led to a small increase in NFAT/AP-1 reporter activity that was not enhanced by TCR/CD28 stimulation (Figure 26B). These results indicate that Tim3-mediated T cell activation shares signaling pathways employed by TCR/CD3.

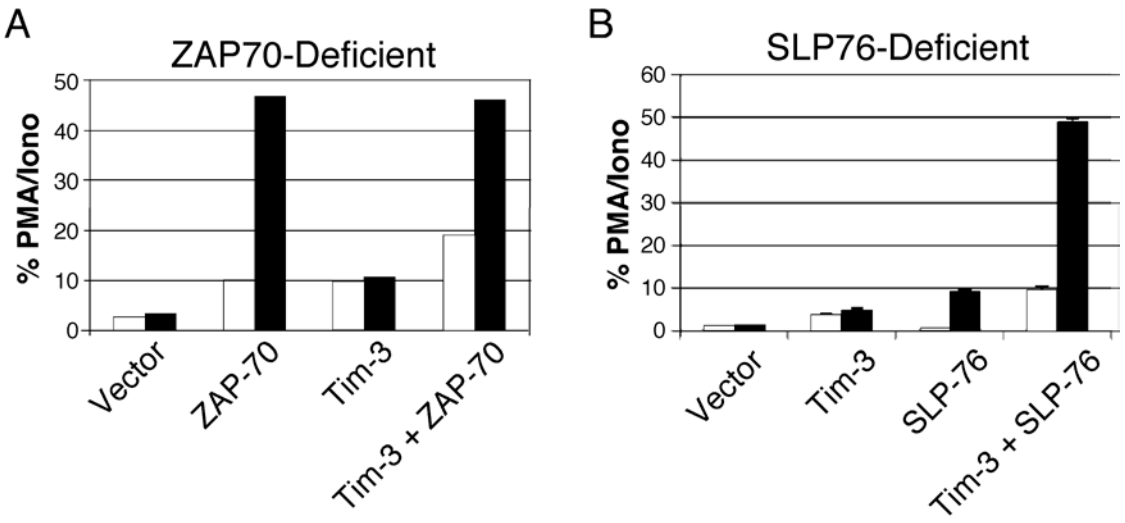

Figure 26: Role of downstream TCR signaling molecules ZAP-70 and SLP-76 in Tim-3 signaling.

(A) ZAP70-deficient Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs. The next day, cells were left unstimulated (white bars) or stimulated with anti-TCR/CD28 antibodies for six hours, followed by determination of luciferase activity. (B) SLP76-deficient Jurkat T cells were transfected with an NFAT/AP-1 luciferase reporter and the indicated constructs, and analyzed as above. Results in each panel are representative of at least five experiments that were performed.

4.3.3 Discussion

Here we have demonstrated for the first time that ligation of the transmembrane protein Tim-3 can enhance activation of T cells, which correlates with the induction of transcription factors important for T cell activation, i.e. NFAT, AP-1 and NF-kB. We have also shown that five tyrosine residues within the Tim-3 cytoplasmic tail regulate T cell signaling by Tim-3 in a complex fashion. Thus, the tyrosines at position 256 and 263 appear to be most critical for Tim-3 function. However, mutation of 256 and 263 to phenylalanine has the most dramatic effects when the more distal three tyrosines are also removed by truncation. While this finding suggests a role for phosphorylation of the distal tyrosines, mutation of tyrosines 256 and 263 was

apparently sufficient to abolish inducible tyrosine phosphorylation of Tim-3. Thus, it is still formally possible that the distal part of the Tim-3 cytoplasmic tail amplifies signaling via a mechanism that is independent of tyrosine phosphorylation. However, there are no obvious signaling motifs in this part of the protein.

A previous report showed that Y265 in human TIM-3 (corresponding to Y256 in the mouse Tim-3 analyzed here) is phosphorylated by the Tec family kinase Itk [62]. While our data do not rule out a role for Itk in phosphorylation of Tim-3, our results demonstrate that both Lck and Fyn are capable of mediating Tim-3 phosphorylation and NFAT activation, with Fyn possibly being the more efficient of the two. Currently, we are attempting to determine the role of Tim-3 tyrosine phosphorylation in coupling this protein to downstream signaling pathways leading to NFAT and NF-κB. The most likely possibility is that, upon phosphorylation, one or more of the tyrosines analyzed here mediate recruitment of SH2 domain-containing proteins. This might include a tyrosine kinase (e.g. Fyn or ZAP-70) or an adaptor protein (e.g. SLP-76). The requirements for Lck, ZAP-70 and SLP-76 in Tim3-mediated activation suggest that Tim-3 intersects closely with TCR signaling pathways. This finding is reminiscent of previous work on another Tim family member - Tim-1. For example, we showed that Tim-1 also requires proximal T cell receptor signaling machinery in order to activate NFAT/AP-1 [34]. In addition, it has been reported that in human T cells Tim-1 can be co-capped with the TCR/CD3 complex [33]. There may therefore be some similarities in the signaling pathways employed by Tim-1 and Tim-3. Nonetheless, while Tim-1 augments activation of NFAT/AP-1, but not NF-κB [32], we show here that Tim-3 can activate all these pathways; also, among the Tim proteins only Tim-3 has been shown to cause cell death. Thus, significant differences in the signaling pathways employed by Tim-1 and Tim-3 must also exist.

We do not believe that our results can be attributed merely to over-expression in a particular cell line. First, our previous studies on the Tim family proteins Tim-1 and Tim-2 with this system have yielded results largely consistent with those obtained with other approaches [32, 51, 167, 168]. Also, although many of our reporter activation studies were initially carried out in the Jurkat human leukemic line, we validated our findings in a non-transformed T cell clone (D10) that does not express endogenous Tim-3.

Our novel finding that Tim-3 ligation can augment T cell activation would appear to be at odds with published data that points towards Tim-3 as an inhibitory molecule. However, it is possible that Tim-3 can have both inhibitory and stimulatory abilities as proposed for another Tim family member, Tim-1. These opposing effects can be produced by ligating Tim-1 with two antibodies that target its IgV domain but with varying avidities. The “stimulatory” antibody, 3B3 binds Tim-1 with avidity 17 times higher than the “inhibitory” antibody RMT1-10[108]. It is postulated that 3B3 co-stimulates T cell activation by bringing Tim-1 closer to the TCR/CD-3 complex, stabilizing it and therefore, allowing the formation of large supra-molecular clusters. RMT1-10 however, does not engage Tim-1 long enough to have such stabilizing effects. Instead, Tim-1 crosslinking by RMT1-10 leads to partial T cell activation, akin to partial and antagonistic ligands[108]. Thus, it is possible that depending on the ligand it associates with, Tim-3 can either have an inhibitory or stimulatory effect.

In support of this hypothesis is the observation that although Tim-3 is upregulated on viral antigen-specific CD8+ T cells in both acutely and chronically infected mice, only viral antigen-specific Tim-3+ CD8+ T cells in chronically infected mice become exhausted[91, 95]. A largely unappreciated fact is that exhausted T cells are effector T cells that are unable to completely differentiate into memory T cells[169]. Receptors and transcription factors associated

with T cell exhaustion i.e. PD-1, Blimp-1, T-bet are also upregulated during acute infection but do not seem to drive exhaustion unless there is persistent antigen stimulation [91, 170-172]. This suggests that during chronic inflammation, global changes occur both intracellularly and consequently, extracellularly to induce and maintain exhaustion in antigen-specific T cells. Therefore, it is distinctly possible that the ligands available to Tim-3 on the surface of T cells can vary between the different stages of activation leading up to exhaustion.

How then does Tim-3 contribute towards T cell exhaustion when it enhances instead of inhibits TCR/CD28 signals? We propose that early during the induction of T cell exhaustion, Tim-3 enhances T cells activation, which leads to the overexpression of T-bet. Studies have showed that T-bet is enriched in exhausted viral specific CD8+ T cells and in chronically infected Tbx21-/- mice depleted of CD4, this exhaustion is prevented. Interestingly, conditions that induce high levels of T-bet expression in CD8+ T cells favor the development of effector cells above memory T cells[172, 173]. Gene array studies show that of the genes differentially upregulated in exhausted CD8+ T cells, a greater majority of these genes overlapped with effector T cells than memory T cells[160]. This suggests that exhausted T cells are effector T cells that have not completely differentiated into memory cells. This is consistent with the observation that central memory T cells (T_{CM}) are more superior in their ability to secrete IL-2 and proliferate upon secondary stimulation to effector memory T cells (T_{EM})[174]. Likewise, the inability to produce IL-2 in response to antigen stimulation is the first defect that is seen in exhausted CD8+ T cells[88, 175].

4.3.4 Acknowledgements

Judong Lee (Immunology Graduate Program, University of Pittsburgh School of Medicine), constructed the Y263/263F Tim-3 mutants and examined their function in both Jurkat and D10 T cells (Figure 22), determined the activity of Tim-3 in SLP-76 and ZAP-70 deficient Jurkat T cells (Figure 26), examined the phosphorylation status of the Tim-3 cytoplasmic tail in HEK293 cells (Figure 23) and determined the role of both Lck and Fyn in mediating the downstream signaling of Tim-3 (Figure 24). Sarah Hainline (SURP, University of Pittsburgh School of Medicine) assisted in the construction and testing of the Tim-3 truncation mutants.

5.0 SUMMARY

The work presented in this dissertation challenges the widely accepted hypothesis that TIM-3 is a negatively regulator of effector T cells. Central to this hypothesis is the interaction of TIM-3 and its ligand, galectin-9 (Gal-9), which has been shown to induce apoptosis in IFN-γ-secreting T cells [57]. Paradoxically, the gal-9/TIM-3 interaction does not appear to induce apoptosis in dendritic cells (DC). Instead, gal-9 induces their phenotypic and functional maturation [78]. Therefore, the gal-9/TIM-3 interaction appears to negatively regulate T cells but positively regulates DC. This led to my first hypothesis that the signaling pathway(s) downstream of TIM-3 differs in T cells and myeloid cells. In chapter 2, I tested this hypothesis by comparing the tyrosine phosphorylation patterns in a Th1 clone and a dendritic cell line incubated with a Tim-3-specific antibody. Using this approach, I was able to detect proteins that were differentially tyrosine phosphorylated in T cells and DC, supporting my hypothesis. However, attempts to identify these proteins by mass spectrometry were not fruitful. Since this work was performed, other groups have shown that gal-9 can also positively regulate Tim-3 expressing T cells. Gal-9 expands regulatory T cells (Tregs) and enhances the cytolytic function of CD8+ T cells without any apparent effect on their viability. Therefore, this raised the question of whether TIM-3 is a positive or negative regulator of effector T cells.

To establish the regulatory nature of TIM-3 on T cells, I examined the ability for gal-9 to modulate cytokine production and viability of Tim-3 expressing Th1 cells. Previous studies using TIM-3/TIM-3 ligand blocking reagents have shown that TIM-3 has the ability to suppress effector T cell function [64, 69]. Therefore, as a TIM-3 specific agonist, I hypothesized that gal-9 would possess the ability to induce apoptosis as well as inhibit the function of effector T cells. In chapter 3, I demonstrated that gal-9 induces the apoptosis of Tim-3 expressing Th1 cells, which is consistent with previous reports. Surprisingly, I found that gal-9 also induced the production of IFN-γ from fully polarized Tim-3+ Th1 cells. To ensure that these effects were Tim-3 specific, I treated Tim-3- Th2 cells with gal-9. Despite the lack of Tim-3 expression on these cells (confirmed by surface staining), gal-9 could still induce Th2 cells to undergo apoptosis and secrete cytokine, which suggests that Tim-3 is not required to mediate the effects of gal-9. I also showed that gal-9 can induce the production of IFN-γ and apoptosis in fully differentiated Th1 cells from both wild-type and Tim-3-/- mice. Therefore, my work shows that gal-9 can both negatively (apoptosis) and positively (cytokine secretion) regulate effector T cells. However, these gal-9 mediated effects do not require Tim-3 expression. These findings have important implications for the way in which we investigate the function of TIM-3 in immune responses in the future. Many biological effects elicited by gal-9 in the past have been attributed to TIM-3 based on the assumption that the interaction between gal-9 and TIM-3 is exclusive. Therefore, it is crucial that future studies take into consideration the possibility that gal-9 can bind to other cell-surface receptors apart from TIM-3 to mediate its effects.

Since gal-9 is not a TIM-3 specific agonist and thus, cannot be used to study the signaling pathways downstream of TIM-3 in T cells, I employed a luciferase-based reporter system that has been used in the past to characterize to regulatory role of other TIM family proteins. In

chapter 4, I showed that ectopic expression of Tim-3 enhances the activation of NFAT/AP-1 and NF-κB signaling pathways in T cells stimulated with anti-TCR and anti-CD28. This is at odds with published reports that point towards Tim-3 as a negative regulator of T cells. I then proceeded to demonstrate that of the 6 conserved tyrosines in the Tim-3 cytoplasmic tail, Y256 and Y263 are the most crucial for mediating the enhancement of TCR/CD28 signaling by Tim-3. Src kinases Lck and Fyn can both phosphorylate these tyrosines**,** although Fyn appears to be the more efficient kinase. Lastly, I show that the downstream signaling of Tim-3 couples to ZAP-70 and Slp-76 to enhance T cell activation. My findings here suggest that under certain conditions, TIM-3 has the potential to act as a co-stimulatory receptor. Future efforts will be focused on identifying the exact conditions that allow TIM-3 to act as either a positive or negative regulator of effector T cells.

Establishing the true nature of a co-receptor has always been complex. Initially, the co-inhibitory receptor cytotoxic lymphocyte antigen-4 (CTLA-4) was classified as co-stimulatory because CTLA-4 specific antibodies that were thought to be agonistic were in fact, blocking [176]. Engagement of PD-1 by either PD-L1 or PD-L2 has been shown to both inhibit and enhance T cell proliferation and cytokine production**,** despite the observation by several groups that administration of anti-PD-1 exacerbates EAE in mice [177-182]. These inconsistencies are postulated to arise from the preparation of PD-ligand fusion proteins as either agonists or antagonists. However today, it is well-established that both CTLA-4 and PD-1 are inhibitory, as mice deficient in either co-receptor develop autoimmune disease. Despite its reputation as a negative regulator of T cells, Tim-3-/- mice do not suffer the same fate as mice lacking the expression of certain co-inhibitory receptors [183, 184]. This suggests that the role of TIM-3 in immune responses is more complex than previously thought. To clarify the role of TIM-3 on T

cells, it is important to first establish the target epitopes of known TIM-3 blocking antibodies**.** This will then allow us to determine if any of the known TIM-3 ligand(s) is responsible for the suppression of T cell effector function, as often observed in the presence of APCs. Then, the effect of these ligand(s) on TIM-3 expressing T cells from wild-type and Tim-3-/- mice need to be compared in the absence of APCs. Finally, if Tim-3 can promote T cell exhaustion, CD8+ T cell responses should be comparable in acutely and chronically infected Tim-3-/- mice.

APPENDIX

Table 5: Comparison of the structure and function of TIM family proteins

	TIM-1	TIM-2	TIM-3
Expression	Primarily Th2 cells	Primarily Th2 cells	Th1, Th17, Tregs
Structure 1) MILIBS 2) Glycosylation 3) Number of tyrosines in the cytoplasmic tail	Yes Heavily glycosylated One	No Heavily glycosylated One	Yes Lightly glycosylated Six
Ligands	Phosphatidylserine, Tim-4 (indirect, mediated through exosomes)	Sema4A, H-ferritin	Galectin-9, Phosphatidylserine
Inhibitory/Stimulatory	Stimulatory (Agonistic Ab, ectopic expression in Jurkat T cells, Tim-4) Inhibitory (Antagonistic Ab)	Inhibitory (Agonistic Ab, ectopic expression in Jurkat T cells)	Inhibitory (Galectin-9 and Tim-3/Tim-3 ligand blocking reagents)
Disease Modulation **EAE**	Exacerbates (Agonistic Ab) Ameliorates (Antagonistic Ab)	Ameliorates (Sema4A Ig)	Exacerbates (Blocking Tim-3 Ab) Ameliorates (Galectin-9)
Tolerance	Abrogates airway tolerance (Agonistic Ab) Prolongs fully-MHC mismatched cardiac allograft (Antagonistic Ab)	N/A	Abrogates allograft tolerance (Tim-3 Ig)

APPENDIX A

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1. Su, E.W., Lin, J.Y., and Kane, L.P. TIM-1 and TIM-3 proteins in immune regulation. *Cytokine* 2008;44(1):9-13.
2. Su, E.W., Bi, S., and Kane, L.P. Galectin-9 regulates T-helper function independently of Tim-3. *Glycobiology*, ePub Dec 27 2010.

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