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TIM-1 and TIM-3 Proteins in Immune Regulation

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Abstract

Over the last several years, there has been increasing interest in the role of proteins of the TIM (T cell immunoglobulin and mucin domain) family in regulating immune responses. Despite what the name suggests, proteins of this family function in a much more widespread manner than just on T cells, as we will discuss in this review. We therefore propose that the definition of TIM be adjusted to “*transmembrane* immunoglobulin and mucin.”

Keywords

T cells; macrophages; apoptosis; activation; tolerance

Introduction

Here we will focus mainly on findings on TIM-1 and TIM-3 over the last several years. Other recent review articles have covered in depth the earlier work in this field [1–6]. Throughout this review, the term TIM is used to refer either to the proteins of this family in a general sense or to the human proteins, while Tim is used to refer to the rodent proteins.

TIM-1 and disease

Tim-1 has been linked to a wide variety of physiologic and pathologic functions. Also known as kidney injury molecule (KIM-1), Tim-1 was first identified as a marker of acute kidney injury [7]. While there are low levels in normal kidneys, Tim-1 mRNA and protein dramatically increase after ischemic kidney injury. Tim-1 may also have utility as a cancer marker, since it is up-regulated in urine and tissue samples of patients with renal cell carcinoma [8,9].

In addition to cancer and kidney injury, extracellular polymorphisms in TIM-1 have been associated with immune dysfunction. It was first described as HAVCR1, a receptor for the Hepatitis A virus (HAV) [10,11]. In this capacity, understanding the function and regulation of TIM-1 may provide mechanistic support for the hygiene hypothesis, as it provides a potential link between infection and asthma susceptibility. Being both sero-positive for HAV and having a six amino acid insertion in the mucin domain of TIM-1 are associated with protection from atopic diseases [12]. In addition, positional cloning identified murine Tim-1 as co-segregating with *Tapr* (T cell and airway phenotype regulator) [13]. Thus, allelic variant of *Tim1* encode for differences between DBA/2 mice and BALB/c, which may help account for their

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differences in asthma susceptibility. Human TIM-1 has also been associated with other types of immune dysfunction, such as atopic dermatitis, allergy, rheumatoid arthritis, asthma and systemic lupus erythematosus [14–18]. However, the mechanisms underlying these effects are not known.

TIM-1 structure and function

TIM-1 is a type I membrane protein with an IgV domain followed by a heavily glycosylated mucin domain, a transmembrane domain and an intracellular cytoplasmic tail with one tyrosine phosphorylation motif [4]. The known protein ligands are HAV, which binds to the IgV domain, and Tim-4, binding of which appears to require both the Ig and mucin domains [19–22]. In addition, there is evidence for homophilic binding [19]. Crystal structure analysis of the immunoglobulin domain identified two antiparallel β sheets (BED and GFC) with four cysteine residues joining a CC' loop to the GFC β sheet. A Tim-1 IgV domain from the opposite cell surface is thought to interact through the upper portion of the BED sheet. Two residues, His 64 and Glu 67, of the TIM-1 DE loop are thought to be important for this *trans*-Tim-1 binding, since a point mutation of histidine at position 64 to glutamic acid led to significant reductions in homophilic Tim-1:Tim-1 binding as well as partially decreased levels of Tim-1:Tim-4 binding [19]. This could be of potential biologic relevance because the homophilic binding is conserved in humans. Interestingly, the authors demonstrated that Tim-1 clusters mostly in the cytoplasm but localizes to the cell surface upon ionomycin or phorbol ester stimulation, at least in some cell types. More recently a metal ion dependent ligand binding site of the IgV domain has been identified. Mutations of residues in the metal ion binding site also appear to increase localization of Tim-1 to the cell surface [23].

TIM-1 and T cell activation

TIM-1 can function as a co-stimulatory molecule for T cell activation. Cross-linking Tim-1 with antibodies, in conjunction with TCR and CD28 stimulation, enhances the proliferation of CD4⁺ T cells [24]. The administration of anti-Tim-1 Ab *in vivo* was shown to increase levels of IL-4 and IFN- γ but *in vitro* could only enhance IL-4. These effects are strong enough to abrogate induction of respiratory tolerance [24]. Selective induction of IL-4 production may be due to the association of Tim-1 expression with increased levels of the Th2 transcription factor Gata-3, as seen in a mouse model of asthma [25]. However, a direct relationship between Tim-1 and Gata-3 has not been well defined.

Recent work has begun to identify the signaling pathways triggered downstream of Tim-1 engagement, using either Tim-1 antibodies or Tim-4 as ligands. Reporter assays demonstrate that over-expression of Tim-1 leads to NFAT/AP-1 transcriptional activation, dependent on Y276 in the cytoplasmic tail [26]. In addition, capping experiments suggest that TIM-1 associates with CD3 and is recruited to the TCR signaling complex in human T cells [27]. In this same study, the use of agonistic anti-Tim-1 antibodies led to phosphorylation of phospho-Zap-70 and IL-2-inducible T cell kinase (ITK), and recruitment of an ITK and PI3K complex to the TCR signaling complex. Our own work has demonstrated that the p85 subunit of PI3K is recruited directly to tyrosine 276 in Tim-1 after Lck-dependent phosphorylation of the cytoplasmic tail [28]. Studies using a Tim-4-Ig fusion protein have shown that Tim-4 engagement on CD3⁺ T cells also leads to phosphorylation of LAT, Akt and ERK1/2, perhaps through Tim-1, although other ligands for Tim-4 cannot be ruled out [29]. In this same study, evidence was also provided that Tim-4 treatment might promote T cell survival through the induction of Bcl-2.

Though most studies thus far indicate that Tim-1 usually positively regulates T cell activation, this may not always be the case. The effects of agonistic (3B3) and antagonistic (RMT1-10) monoclonal antibodies to Tim-1 suggest both possible positive and negative co-stimulatory

roles [30]. The agonistic antibody was found to increase secretion of IFN- γ and IL-17 and to increase the severity of EAE, while the antagonistic antibody promoted a Th2 type response *in vivo*. To further determine the underlying mechanism, it was found that while the agonistic and antagonistic antibody both bound to the IgV domain of Tim-1 and induced CD3 capping, the agonistic antibody had much greater affinity and caused cytoskeletal reorganization [30].

TIM-1 and regulatory T cells in allograft rejection

While the majority of studies have focused on the role of Tim-1 in effector T helper subsets, Tim-1 also appears to be important in modulating the function of regulatory T cells. Taking advantage of the different Tim-1 antibodies, two studies demonstrated that Tim-1 engagement can promote allograft acceptance or rejection, effects that are dependent on regulatory T cells. *In vitro* T cell stimulation with an agonistic anti-Tim-1 Ab increased the number of IL-17- and IFN- γ -producing cells, but decreased the mRNA expression of FoxP3, GITR, and other markers of regulatory T cells [31]. Anti-Tim-1 antibody also negated the protective effects of anti-CD154 treatment and resulted in allograft rejection. In contrast, the antagonistic anti-Tim-1 Ab was able to inhibit the Th1 cytokine IFN- γ and to promote Th2 cytokines, such as IL-5 [32]. By contrast, antagonist Tim-1 antibody treatment appeared to *prevent* chronic allograft rejection by a mechanism dependent on regulatory T cells. Interestingly, antagonistic anti-Tim-1 antibody treatment did not convert naïve CD4⁺ T cells into regulatory T cells, but rather appeared to inhibit the proliferation of allogeneic effector T cells. The mechanisms underlying the effects of Tim-1 antibodies on regulatory T cells still remain to be discovered.

TIM-1 and TIM-4 Function on mast cells and macrophages

Although it has been best studied in T cells, Tim-1 also appears to influence the function of other cells of the immune system, particularly mast cells and macrophages. Tim-1 can be detected on the surface of peritoneal mast cells and bone marrow-derived cultured mast cells. Expression is down-regulated after IgE and antigen stimulation. Tim-4 addition promotes production of Th2 cytokines, such as IL-4, IL-6 and IL-13, without affecting degranulation [33]. Macrophages also appear to be influenced by Tim-1, through its interaction with Tim-4 on the surface of the macrophages. The addition of Tim-1 to a macrophage cell line resulted in increased cytokine production of TNF- α , IL-6 and IL-10, as well as increased levels of the co-stimulatory molecules B7-1, B7-H1, and PD-L2 [34]. At least in the case of PD-L2, the level of expression after Tim-1 treatment was significantly higher than with LPS stimulation.

TIM-1 and TIM-4 as PS receptors

Perhaps the most exciting recent revelation regarding the TIM's is the identification of both Tim-1 and Tim-4 as phosphatidylserine (PS) receptors. Initially, Tim-4 was identified as a ligand for Kat5-18, a monoclonal antibody that could inhibit macrophage phagocytosis of apoptotic cells. Surprisingly, fusion proteins of Tim-4 and Tim-1 bound to PS via the IgV domain, and Tim-4 transfection of fibroblasts enabled engulfment of apoptotic cells [35]. In support of this idea is functional evidence of Tim-1 and Tim-4 uptake of apoptotic cells from several other groups. Endogenous Tim-4 on macrophages and Tim-1 on kidney cells, as well as transfected Tim-1 or Tim-4 of NIH 3T3 fibroblasts, are all capable of phagocytosing apoptotic cells [36]. In addition, pre-incubation of cells with Tim-1 and Tim-4 antibodies can block uptake of apoptotic cells [36]. In another model, kidney epithelial cells also become phagocytic after injury. Confocal imaging studies demonstrate that Tim-1 co-localizes around the sites of phagocytosed cells and can mediate internalization of the injured tubule cells by PS and oxidized lipoprotein recognition [37]. Tim-1 and Tim-4 binding to PS appears to be dependent on the presence of divalent cations, since Tim-1 binding is abolished by the addition of EDTA [23,37]. It is interesting that kidney epithelial cells recognize both PS and

phosphatidylethanolamine (PE), since the above studies with other cell types did not report interaction of Tim-1 or Tim-4 with PE [35,36].

The Tim-1 and Tim-4 crystal structures suggest that a metal-ion-dependent ligand binding cavity built by CC' and FG loops in the IgV domain is responsible for the recognition of PS. The hydrophilic phosphate head of PS can enter the cavity and interact with the metal ion while the fatty acid tail can interact with the aromatic residues of the FG loop [23]. Single mutations of the metal ion residues decreased Tim-1 and Tim-4 binding to liposomes containing PS, while a double mutation completely abolished PS binding. Individual mutations of the four amino acids of the CC' and FG loops dramatically decreased the ability of Tim-4 to mediate uptake of apoptotic red blood cells [23,36]. More work is still needed to better understand the physiologic role and mechanism of Tim-1 and Tim-4 in mediating uptake of apoptotic cells.

TIM-3

TIM-3 was originally identified through a screen for T_H1-specific markers, but since then it has also been found on cytotoxic CD8⁺ T cells, T_H17, T_{reg}, monocytes, dendritic cells, mast cells and microglia [38–42]. 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. Although the soluble form (a splice variant) of Tim-3 lacks both the mucin and transmembrane domains, it still appears to possess the binding specificity of the membrane-bound form [43]. This is consistent with structural studies, which suggest so far that only the IgV domain is required for ligand binding [44]. The function of the other domain of Tim-3 has yet to be explored. We are currently trying to determine the importance of the tyrosine residues present in the cytoplasmic domain for downstream signaling by Tim-3. These tyrosines are well conserved in both mouse and human homologues of Tim-3 and at least one may be inducibly phosphorylated, at least in fibroblasts [45]. We recently reported evidence that Tim-3 signals differently in T cells versus myeloid cells, since its ligation results in different patterns of tyrosine phosphorylation in the two cell types [41]. This finding may lead to a better understanding of the basis for the reported differences in the effects of Tim-3 ligation on different cell types.

Thus far, only galectin-9 has been identified as a ligand for Tim-3 [46]. However, several papers have shown that as-yet-unidentified ligand(s) for Tim-3 exist and can be detected on naive, effector, memory, regulatory T cells and dendritic cells, with Tim-3-Ig fusion proteins [43]. Interestingly, a putative ligand for Tim-3 is down-regulated following activation in CD4⁺CD25[−] T cells but maintained on CD4⁺CD25⁺ T cells [47]. Preliminary studies suggest that as Tim-3 expression is up-regulated (along with its ligand) during T cell activation, the two interact in *cis* and suppress the ability of T cells to secrete cytokines and proliferate [48]. According to this model, by disrupting this interaction with a blocking antibody or Tim-3-Ig fusion protein, Tim-3 signaling would be terminated and T cell effector function restored. However, because antigen presenting cells also express both Tim-3 and ligand(s), the effects of Tim-3 activation on both T cell and antigen presenting cells need to be dissected before the outcome of Tim-3 studies performed *in vivo* can be fully understood.

TIM-3 and EAE/MS

The role of Tim-3 *in vivo* has been most rigorously tested in EAE, a mouse model of MS. The antigen used to induce EAE in these studies was PLP (139–151), administered in combination with pertussis toxin. Co-administration of Tim-3 antibody did not alter the onset of EAE but accelerated disease progression [39]. In addition, the demyelinating lesions of anti-Tim-3 treated mice were filled with macrophages containing myelin fragments. As evidence that this hyper-acute phenotype is driven by activated macrophages, splenocytes were harvested after the onset of disease and examined for a variety of markers. As expected, there was a marked

increase in the percentage of CD11b⁺ cells. These splenocytes had a high basal rate of proliferation, an effect that can be partially recapitulated only by co-culturing CD3⁺, CD11b⁺ and B220⁺ cells from anti-Tim-3 treated EAE mice. When separated by a permeable membrane, the effect is only additive and if cultured independently these cells exhibit little basal proliferation [39]. Altogether, this implies that the total effect of TIM-3 blockade requires 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. Thus, siRNA knockdown of TIM-3 in peripheral blood CD4⁺ T cells from healthy subjects increased their ability to proliferate and secrete IFN- γ in response to anti-CD3 and anti-CD28 stimulation [49]. CD4⁺ T cells isolated from peripheral blood mononuclear cells (PBMCs) of MS patients also have lower levels of TIM-3 transcripts [48]. Interestingly, blocking TIM-3 on MS CD4⁺ T cells did not enhance the secretion of IFN- γ in response to CD3 stimulation, in contrast to what was observed with cells from control subjects. These studies suggest that CD4⁺ T cells in MS patients are dysregulated, due to their lack of TIM-3 expression. Whether these cells are also deficient in the expression of the putative ligand(s) of TIM-3 has not yet been explored.

Analysis of CNS tissue has revealed that TIM-3 mRNA levels are much higher in the border of MS lesions in comparison to adjacent regions and non-inflamed human CNS tissue. The expression of the TIM-3 ligand galectin-9 was also found to be higher in MS lesions than normal human CNS tissue [50]. In this same paper, it was shown that treating Tim-3⁺ CD11b⁺CD11c⁺ mouse splenocytes with galectin-9 induces the secretion of TNF- α . Because it has been reported that peripheral monocytes and resident microglia promote inflammation of the central nervous system, the relationship between Tim-3 and CD11b⁺CD11c⁺ cells was further explored in mouse EAE. Thus, Tim-3 expression was only upregulated in CD11b⁺ cells infiltrating the CNS (microglia and monocytes) and not activated macrophages cells present in the periphery [50]. Mice that received myelin PLP (139–151) in incomplete Freund's adjuvant with Tim-3 antibody developed more severe EAE than their counterparts that were treated with isotype antibody instead.

While human monocytes were shown to secrete TNF- α in response to galectin-9 treatment [50], it will be important to determine if activation of Tim-3 also modulates other aspects of monocyte function such as survival, expression of co-stimulatory molecules, secretion of other inflammatory cytokines such as IL-1 β and IL-6, as well as expression of chemokine and adhesion receptors that may enhance their infiltration of the CNS from the periphery. With this knowledge we may then be better able to determine how monocytes promote inflammation in the CNS and to what extent. Another question that needs to be addressed is whether the TIM3-expressing cells in the border of MS lesions receive a specific cue to upregulate TIM-3 during the progression of MS or whether these cells migrate from the white matter, which has been shown to contain TIM-3 expressing cells in normal human tissue.

Recently, it was suggested that the pathogenesis associated with EAE is mediated not by T_H1 but rather by T_H17 cells [40]. Removal of IFN- γ or IFN- γ -secreting cells can actually accelerate disease progression. Therefore, if activation of Tim-3 selectively induces the death of T_H1 cells [46], does that allow for the expansion of T_H17 cells? This hypothesis has a caveat, being that T_H17 cells also express Tim-3, albeit at lower levels than T_H1 cells [51]. Thus far, only one group has shown that CD4⁺ T cells from MS patients secrete higher levels of T_H17 than CD4⁺ T cells from control subjects when stimulated with anti-CD3 [48]. T_H17 cells are expanded by IL-23, a cytokine secreted by both macrophages and dendritic cells. Because depletion of activated macrophages or IL-23 has been shown to inhibit development of EAE

[52], it will be important to determine if engagement of Tim-3 on macrophages induces their production of IL-23.

TIM-3 and Inflammatory Heart Disease

BALB/c mice infected with Coxsackievirus B3 (CVB3) develop chronic myocarditis and dilated cardiomyopathy. Interestingly, infection of BALB/c mice with this virus increases Tim-3 expression on macrophages from the heart, spleen and peritoneal lavage as soon as six hours post-infection [53]. Heart homogenates from infected mice also contained elevated levels of TNF- α , IL-1 β , IFN- γ , IL4 and IL-10. Because Tim-3 has been reported to modulate the function of macrophages and neutrophils [39], cells known to promote myocarditis and chronic heart failure, Frisancho-Kiss et al. concurrently administered blocking Tim-3 antibody and infected BALB/c mice with CVB3 to determine how Tim-3 modulates inflammatory heart disease. The authors found that blocking Tim-3 activity reduced the expression of CD80 but increased levels of CD86 on mast cells and macrophages from hearts and spleens of infected mice. Interestingly, they also found an increased expression of CD28 but lowered CTLA-4 expression on Tim3-positive T cells isolated from spleens of infected mice. Finally, the heart of infected mice had a larger proportion of macrophages/neutrophils but reduced proportion of T_{reg} cells. The net effect was that hearts of infected mice treated with blocking Tim-3 antibody displayed a higher degree of myocarditis.

A follow-up report investigated the reason why male BALB/c mice infected with CVB3 developed more severe myocarditis than their female counterparts, an observation that has also been made in humans. The authors compared the levels of Tim-3 on infected male and female BALB/c mice and found that there was significantly less Tim-3 on mast cells, macrophages and CD4⁺ T cells isolated from the hearts of CVB3-infected male mice [54]. Interestingly, macrophages and mast cells from TLR4-deficient, CVB3-infected male mice had higher than wild-type levels of Tim-3. Consistent with this, blocking Tim-3 also increased TLR4 expression on macrophages and mast cells isolated from the hearts of CVB3-infected male mice. Investigation of the CD4⁺ T cell compartment in TLR4-deficient CVB3-infected male mice revealed a significantly lower proportion of cells expressing CTLA-4. This work raises several questions. First, does the Tim-3 antibody induce a negative signal through Tim-3 or does it prevent the delivery of activating signals induced through interaction of Tim-3 with its ligands? In addition, do macrophages and mast cells secrete higher levels of inflammatory cytokines when they express higher expression of Tim-3, and by what mechanism? Are CTLA-4 and CD28 levels modulated on the same cell? Lastly, do these T cells have an enhanced or suppressed response when it engages a peptide-MHC complex?

TIM-3 and 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. 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), in which CD4⁺CD25⁺ T cells provide donor-specific allograft tolerance. Concurrent administration of Tim-3-Ig fusion protein precipitated rejection of donor islet allograft in DST/anti-CD154 treated mice and maintained the responsiveness of T cells in antigen-tolerized mice [47]. The authors showed that Tim-3-Ig could only prevent tolerance when present during, and not after, CD4⁺CD25⁺ T cells had acquired their suppressive activity. 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 PLP (139–151)-induced tolerance, it was observed that splenocytes from mice treated with Tim-3-Ig proliferated and secreted high levels of IL-2 and IFN- γ independently of antigenic stimulation [43]. Interestingly, the spontaneous proliferation and cytokine secretion by T cells can only be seen in mixed splenocyte cultures. 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 [43]. 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 galectin-9 to modulate Tim-3 activity, mice receiving galectin-9 were able to delay the rejection of fully allogeneic skin grafts for up to 6 days [55]. This delay was attributed to the ability of galectin-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 mice day 7 post-transplantation. The authors also noted a reduction in serum IFN- γ levels of galectin-9 treated mice, with a slight increase in both IL-2 and IL-4 levels. In an earlier report, the authors had shown that galectin-9 prolongs skin allograft survival, inducing apoptosis in CD8⁺CD44^{high}CD62L^{low} T cells and reducing the cytolytic activity of those cells that survive [42]. Thus, the effects of Tim-3 modulation on immune responses *in vivo* may be mediated through complex effects on multiple cell types. More work will be required to better define the nature of these effects.

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