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Review

Tim-3: A co-receptor with diverse roles in T cell exhaustion and tolerance

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ABSTRACT

T cell inhibitory co-receptors play a crucial role in maintaining the balance between physiologic immune responses and maladaptive ones. T cell immunoglobulin and mucin domain-containing-3 (Tim-3) is a unique inhibitory co-receptor in that its expression is chiefly restricted to interferon (IFN)γ-producing $CD4^+$ and $CD8^+$ T cells. Early reports firmly established its importance in maintaining peripheral tolerance in transplantation and autoimmunity. However, it has become increasingly clear that Tim-3 expression on T cells, together with other check-point molecules, in chronic infections and cancers can hinder productive immune responses. In this review, we outline what is currently known about the regulation of Tim-3 expression, its ligands and signaling. We discuss both its salutary and deleterious function in immune disorders, as well as the T cell-extrinsic and -intrinsic factors that regulate its function.

1. Introduction

T lymphocytes of the adaptive immune system coordinate the immune response to pathogens by recognizing, and proliferating in response to, specific cognate antigens. Given their ability to expand enormously, as well as their ability to direct and induce inflammatory responses, T cells must be placed under tight control to avoid attack against, and damage to, self-tissues. They must, therefore, maintain a precarious balance between initiating and promoting robust responses towards tumors and pathogens on the one hand and avoiding inappropriate inflammation directed towards the body's tissues on the other. Multiple mechanisms of immune control have thus evolved to keep this balance, one of which is the expression of negative regulatory surface receptors, or "immune checkpoints", on the surface of activated T cells. Signaling through these receptors suppresses T cell proliferation, decreases pro-inflammatory cytokine production, and upregulates immunomodulatory cytokine secretion. Two of the earliest, and best, described immune checkpoints are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death-1 (PD-1), both of which are relatively rapidly expressed on T cell activation and play crucial roles in suppressing T cell-mediated tissue destruction [1–3]. These molecules have gained recent prominence as they are targets for the treatment of aggressive, metastatic, cancers such as melanoma, non-small cell lung carcinoma (NSCLC), bladder cancer and renal cell carcinoma [4–9]. Abrogating the function of CTLA-4 (ipilimumab) or PD-1 (pembrolizumab, nivolumab), by antibody mediated blockade, reverses T cell dysregulation and augments adaptive immunity against the tumor resulting in tumor control.

In addition to CTLA-4 and PD-1, there are a number of other co-inhibitory molecules that have been identified which include T cell immunoglobulin and mucin-domain-containing-3 (Tim-3), lymphocyte activation gene 3 (LAG3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT), among others. In the past two decades, there has been a growing focus on the biology of the immune checkpoint molecule Tim-3. The function of Tim-3 is of great interest because, unlike the broadly expressed CTLA-4 and PD-1, its expression in the T cell compartment is largely restricted to inflammatory IFNγ-producing "type-1" $CD4^+$ T cells, $CD8^+$ T cells, NK cells and tissue resident $FoxP3^+$ T_{reg} [10]. Further, Tim-3 is also highly expressed on dysfunctional, exhausted, T cells [11,12], which display hierarchical and progressive loss of effector function [13]. Blockade of Tim-3 signaling *in vivo* can reverse T cell exhaustion [14] indicating that Tim-3 may act as a molecular switch that controls whether a T cell generates an inflammatory or a dysfunctional response, and that manipulation of its signaling pathway

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(s) can control which of these fates a T cell adopts. CTLA-4 [15] and PD-1 [16] signaling are well described; in recent years, a number of groups have begun to address the molecular pathways downstream of Tim-3 as well. In this review, we will review what is known about Tim-3 structure, function and signaling, and their implication for human disease.

2. The molecular structure of Tim-3

Tim-3 belongs to the TIM family of Type-I cell-surface glycoproteins. The TIM gene cluster is located at mouse chromosome 11B1.1 and human chromosome 5q33.2, which are chromosomal regions that have been repeatedly reported to be associated with autoimmunity and susceptibility to allergy [17–19]. Eight TIM genes exist in mouse (Tim-1 to -8); however, only Tim-1, Tim-3, and Tim-4 are conserved between mouse and human [20]. There is substantial evidence that the TIM gene family plays diverse roles in the regulation of immune responses in autoimmune disease, infectious disease, and tumor immunosurveillance and immunoevasion. Tim-3, in particular, is an inhibitory molecule in adaptive immunity but its role in innate immunity is beginning to be elucidated.

Tim-3 is characterized by 1) a conserved extracellular IgV domain with N-linked glycosylation sites, and a mucin-like stalk that contains O-linked glycosylation sites; 2) a transmembrane domain; and 3) an intracellular cytoplasmic tail with 5 tyrosines. The IgV domain serves as a binding recognition site for galectin-9, mediated by carbohydrate-modified moieties in IgV domain [21]. Importantly, a recent study identified mutations (Y82C, I97 M) in the Gal-9-binding domain of Tim-3 that are highly associated with the incidence of subcutaneous panniculitis T cell lymphoma (SPTCL) in humans. This finding indicates that Tim-3 structural variants may have profound effects on the binding and signaling downstream of its ligands [22]. It is important to note, however, that Cao et al. [23] showed that the IgV domain of Tim-3 possesses two unique disulfide bonds that bring its CC' and FG loops close to one another, thus creating a unique putative ligand-binding cleft that is dispensable for the interaction with galectin-9. This was the first evidence that Tim-3 may have ligands other than galectin-9. It was later shown that Ceacam1 and PtdSer bind within the vicinity of the CC' and FG loops [24].

All functional TIM family members, with the exception of Tim-4, have a 42–77 amino acid cytoplasmic domain. Tim-3 contains five conserved tyrosine residues in its cytoplasmic tail that are potential sites of phosphorylation and that play important roles in its function. The Src family kinase Fyn, and phosphoinositide 3′ kinase (PI3K) subunit p85, bind to this region in a phosphotyrosine-dependent manner [25], while inducible T cell kinase (Itk) phosphorylates Tyr 265 in human Tim-3 [26]. Further, we showed that HLA-B-associated transcript 3 (Bat3) binds to the unphosphorylated cytoplasmic tail of Tim-3 and represses its function [27,28]. Notably, Tim-3 additionally exists as a soluble form (sTim-3) that lacks the mucin and transmembrane domains [29]. Administration of sTim-3 augments tumor growth in the ectopic B16F1 melanoma model by inhibiting T cell function [30], suggesting that, rather than acting as a decoy that can reverse the T cell inhibition of membrane-bound Tim-3, soluble Tim-3 itself also acts as a negative regulator of T immunity.

This review will focus on the role(s) of Tim-3 in regulating T cell function. Nevertheless, it is essential to recognize that Tim-3 is additionally expressed on, and regulates the function of, other immune cells such as myeloid cells, dendritic cells and NK cells (summarized in Table 2). Notably, Tim-3 is also expressed on CD11b$^+$ dendritic cells in an inactivated state. During EAE, besides T cells, Tim-3 is upregulated in microglia in white matter and infiltrating monocytes. Immunization in the presence of anti–Tim-3 led to disease with greater severity in EAE with massive activation of myeloid cells [31]. Although we initially thought that Tim-3 may be a positive regulator of DC function, accumulating data suggests that Tim-3 may also inhibit DC functions. Consistent with this data, a study showed that Tim-3 could be a negative regulator in DCs in breast cancer [32]. These data demonstrate that Tim-3 is an inhibitory molecule that suppresses response of multiple cell types in a context-dependent manner.

Table 1
Expression and function of Tim-3 on T cells.

T cells	Expression and Function	Reference
Th1	Expressed on mouse Th1	[10]
	Expressed on human Th1	[33]
	Inhibits Th1 function and important for tolerance	[29,42]
	Inhibits Th1/Tc1 function in humans infected with hepatitis C virus	[92]
	Tim-3$^+$ Th1 undergo gal-9-induced cell death	[44]
	T-bet drives Tim-3 expression in Th1	[36]
	Tim-3 inhibitory function in Th1 is repressed by Bat3	[75]
	Tim-3 represses Th1 responses in EAE	[27]
Th17	Expressed on mouse Th17	[111]
	Expressed on human Th17	[33,112]
	Tim-3 blockade enhances Th17 polarization *in vivo*	[105,112]
Treg	Tim-3$^+$PD-1$^+$ T_{reg} suppress allograft rejection	[34]
	Tim-3$^+$ T_{reg} infiltrate the tumor nest in multiple human cancers	[35]
	Tim-3 signaling is essential for Treg-mediated suppression of allograft rejection	[35,42,113]
CD8 + T	Tim-3 blockade exacerbates CD8 + T cell-mediated EAE	[75]
	Tim-3 marks exhausted CD8 + T cells upon clone 13 LCMV infection	[91]
	Tim-3 marks deeply exhausted CD8+ tumor-infiltrating lymphocytes	[12,91]

3. Expression and regulation of Tim-3 in immune cells

3.1. Expression

Tim-3 was initially identified as a T cell marker (summarized in Table 1) specific to terminally differentiated T helper 1 (Th1) cells and IFN-γ producing CD8$^+$ T cells and NK cells, with limited to no expression on naïve T cells and Th2 cells [10]. It was later shown that Tim-3 could additionally mark human Th17 cells, albeit at lower levels than on Th1 cells [33]. In addition to its role as a negative regulator of inflammatory T cell function, Tim-3 is also expressed on T_{reg} which play an important role in the suppression of autoimmune responses [34] as well as contributing to an immunosuppressive tumor milieu [35].

3.2. Regulation

As Tim-3 is upregulated so strongly on differentiated Th1 cells, it was logical to ask whether canonical Th1 transcription factors are required for its expression. Indeed, the expression of Tim-3 in Th1 cells depends on the master lineage transcription factor T-bet (Fig. 1). Tim-3 expression is significantly impaired in *T-bet*$^{-/-}$ T cells and is modestly downregulated in T cells lacking the IL-12-inducible transcription Stat4 in Tim-3. These data suggest a dominant role for T-bet in Tim-3 regulation: indeed, T-bet can directly bind the Tim-3 promoter [36]. Further, interferon-beta upregulates Tim-3 and inhibits IFN-γ from Th1 cells while simultaneously reducing phosphorylation of Stat4 [37].

The immunosuppressive cytokine IL-27 has become increasingly appreciated as a potent global regulator of T cell exhaustion. It induces an inhibitory gene module that incorporates Tim-3 as well as other checkpoint receptors such as PD-1, Lag3, and TIGIT [38]. IL-27 also drives Tim-3 expression directly (Fig. 1) by upregulating the transcription factor NFIL3, which in turn cooperates with T-bet to remodel the chromatin structure of the Tim-3 promoter, thus rendering it permissive to transcription [39]. Curiously, the CD8$^+$ T cell growth factors IL-2 and/or IL-15 may play a crucial role in regulating Tim-3 expression on exhausted cells *ex vivo*. Mice deficient in CD122, a common

Table 2
Expression and function of Tim-3 on innate immune cells.

Cell types	Expression and Function	Reference
Macrophages	Tim-3 was up-regulated on microglia upon stimulation Tim-3 promotes expression of TGF-β, TNF-α and IL-1β in microglia. Tim-3 promotes phagocytosis by microglia. Tim-3 expressed on peripheral blood monocytes and TAMs in patients with HCC. Enhancing TGF-β-mediated alternative activation of macrophages.	[114] [115]
Dendritic cells	Tim-3 constitutively expressed on dendritic cells Tim-3 mediates phagocytosis of apoptotic cells by CD8+ DCs and involving in cross presentation Depletion of Tim-3+ DCs of Gal-9-treated tumor-bearing mice decreased the number of IFN-γamma-producing CD8 + T cells Tim-3 suppressed PRR-mediated innate immune responses to nucleic acids through compete for binding to HMGB1 at the same domain. Tim-3 expression in pDCs inversely correlated with CD4 T cell counts and positively with HIV viral loads.	[116] [31,32,117] [67] [118]
NK	Tim-3 expressed on activated CD56 + NK cells act an activating coreceptor on NK cells and promotes IFN-γ production.	[119,120]
Monocytes	TLR activation facilitates Gal-9/Tim-3 cis association within the same monocytes to regulate Tim-3 negatively regulates IL-12 Tim-3 activation increase frequency of CD11b(+) Ly-6 G(+) cells Tim-3+ monocytes suppressed interferon gamma production by activated CD8 + T cells.	[121] [122] [56] [123]
mast cells	Tim-3 constitutively expressed on mouse mast cells Tim-3 expression was up-regulated upon IgE + Ag stimulation. Tim-3 blockade promotes IL-4, IL-6, and IL-13 production and suppressed mast-cell apoptosis	[124] [125]

component of the IL-2 and IL-15 cell surface receptors, displayed an almost complete lack of Tim-3$^+$ exhausted cells upon infection with the chronic clone-13 strain of lymphocytic choriomeningitis virus (LCMV) [40]. These findings support earlier observations that Tim-3 expression can be induced in an antigen-independent manner by multiple common γ-chain cytokines that include IL-2 and IL-15 [41]. It remains to be determined whether IL-27 synergizes with IL-2 and/or IL-15 to augment Tim-3 expression on dysfunctional exhausted T cells.

4. Ligands of Tim-3

Several early lines of evidence demonstrated that Tim-3 negatively regulates Th1 function. A fusion protein, comprising the Tim-3 extracellular domain linked to Fc (Tim-3-Ig), abrogated *in vivo* tolerance to soluble antigen while augmenting the production of Th1 cytokines from CD4$^+$ T cells [29]. Further, Tim-3-deficient mice rapidly reject allograft transplantation [42]. These findings led to intense interest in identifying Tim-3 ligands that could inhibit Th1 responses. Since that time, multiple such ligands have been discovered, shedding light on the role of the Tim-3 signaling pathway in regulating T cell inflammation and exhaustion.

4.1. Galectin-9

Galectin-9 (gal-9) was the first Tim-3 ligand to be described. It belongs to the highly conserved galectin family of lectins and contains two carbohydrate binding domains connected by a link peptide [43]. Zhu et al. showed that gal-9 specifically recognizes a carbohydrate motif on Tim-3 IgV in Th1 cells and induces apoptosis. Gal-9 treatment *in vivo* reduced the production of IFN-γ from CD4$^+$ T cells, and *in vivo* application of a gal-9 siRNA worsened the severity of EAE [44]. These data were the first direct evidence that the Tim-3 pathway negatively regulates Th1 cell function.

Galectins are soluble proteins that are widely expressed in various cell types and mediate their functions both intracellularly and extracellularly. Gal-9 has been detected in tissues and different cell types in autoimmune diseases, tumor microenvironment and chronic infections [45–48]. At the cellular level, Gal-9 was found to be highly expressed on thymic epithelial cells in mice and is linked to the clonal deletion of T-cells [49]. Gal-9 is also expressed on endothelial cells, tumor cells, stromal cells, and myeloid cells and can be secreted into the extracellular space [46,50,51].

Gal-9/Tim-3 interactions regulate T cell functions in multiple inflammatory models. Gal-9 treatment helped achieve prolonged survival of fully allogeneic cardiac allografts, which was accompanied by suppression of Th1/Th17 responses in dLN as well as in peripheral blood [52]. Further, gal-9 expression is critical to the ability of T_{reg} to suppress Tim-3$^+$ effector T cell function, and Gal-9 expression on T_{reg} is reduced in autoimmune hepatitis [53]. Intriguingly, gal-9 is released from damaged hepatocytes and suppresses pro-inflammatory cytokine production from T cells in the liver [54]. Thus, the gal-9/Tim-3 pathway can play a crucial role in resolving inflammation in the target organ. On the other hand, there is evidence that tumors and pathogens can exploit gal-9/Tim-3 interactions in order to subvert beneficial immune responses. For example, nasopharyngeal carcinoma cells release galectin-9 exosomes that induce apoptosis of antigen- specific CD4$^+$ T cells; this effect can be blocked by the presence of antibodies against gal-9 or Tim-3 [55].

While they lose effector functions, exhausted T cells can persist long-term. As gal-9 induces apoptosis through Tim-3, one might therefore conclude that it plays a limited role in promoting exhaustion. However, gal-9-transgenic (Tg) mice display increased numbers of CD11b$^+$Ly6G$^+$ myeloid-derived suppressor cells [56] that positively regulate T cell exhaustion. The expansion of these cells was absent in gal-9-Tg x *Tim3*$^{-/-}$ mice [56], indicating that the interaction of gal-9 and Tim-3 is crucial for the generation of this suppressive population. How gal-9 signals through Tim-3 to promote exhaustion rather than death is not fully understood, though one possibility is that it uncouples Tim-3 from T cell receptor-proximal positive regulatory signaling molecules [27].

4.2. CEACAM-1

Carcinoembryonic-antigen-related cell-adhesion molecule 1 (CEACAM1) is a type-1 transmembrane protein containing an extracellular N-terminal domain which is homologous to Ig variable domain followed by up to three constant C2-like immunoglobulin domains [57]. We have shown that CEACAM1 is expressed on T cells and engages Tim-3 *via*interaction of the IgV domains on both molecules [24]. Indeed, the existence of a T cell-expressed ligand for Tim-3 had been predicted from Tim-3-Ig binding assays [29,44]. Tim-3 cell surface expression is augmented in the presence of CEACAM1, which appears critical for Tim-3 function in a number of contexts. While Tim-3-Tg mice are relatively protected from dextran sodium sulfate-induced

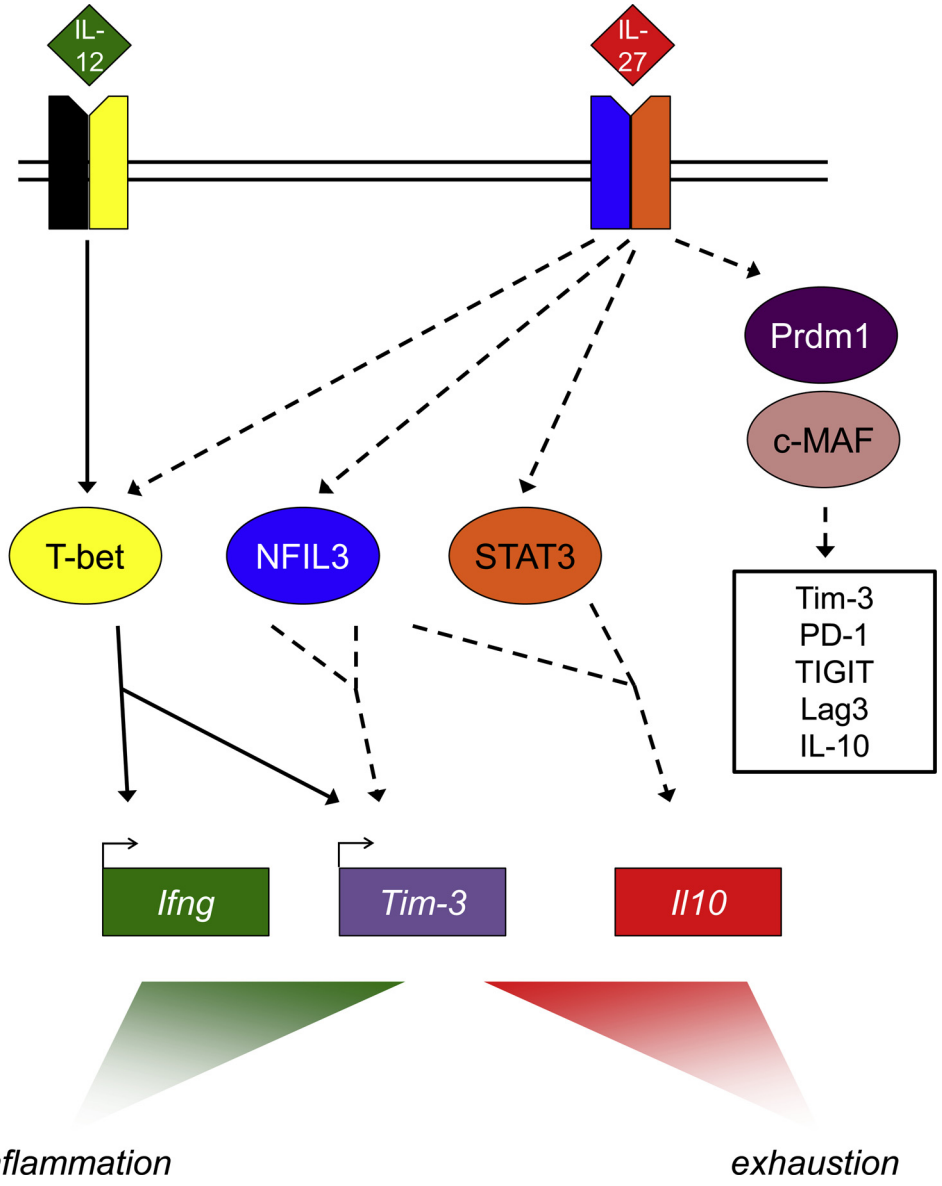

Fig. 1. Context-dependent Tim-3 transcription is driven by extracellular cues. Under inflammatory conditions (solid lines), the Th1 differentiation cytokine IL-12 induces transcription of Tim-3 and IFN-γ *via*the master Th1 transcription factor T-bet [36]. Under immunomodulatory or exhausted conditions (dashed lines), IL-27 signals through T-bet, NFIL3 and Stat3. T-bet and NFIL3 co-operate to upregulate Tim-3, while NFIL3 and Stat3 collaboratively induce IL-10 [39]. IL-27 additionally induces a T cell inhibitory gene module *via*PRDM1 and c-MAF [38], both these factors cooperatively transactivate Tim-3.

colitis, this effect is lost when CEACAM1 is absent. Crucially, CEACAM1 is enforcing Tim-3-dependent suppression of anti-tumor T cell responses, and CEACAM1$^+$Tim-3$^+$ T cells from HIV-infected individuals show profound defects in their capacity to produce IFN-γ [24]. Thus, CEACAM1 binds to, and regulates the function of, Tim-3 in the contexts of both inflammation and exhaustion.

4.3. PtdSer

Phosphatidylserine (PtdSer) is a phospholipid component located in the inner lipid leaflet of cell membranes, which becomes rapidly exposed to cell surface during apoptosis [58,59], cell fusion [60] and other processes [61,62]. The exposure of PS on the outer leaflet is a key "eat-me" marker that can initiate recognition by engulfment receptors on phagocytes [63]. Both human and mouse Tim-3 can serve as a PtdSer receptor *via*the unique binding cleft in the IgV domain described above [23,64] and promote the engulfment processes of PtdSer$^+$ apoptotic cells [65]. However, Tim-3 lacks two of the four conserved residues required for tight binding to PtdSer; therefore, binding of Tim-3 to apoptotic bodies may not be as strong as that of other Tim-family members like Tim-1 or Tim-4. PtdSer may thus be a key ligand for Tim-3 when it is expressed on macrophages or DCs, and the interaction of Tim-3 and PtdSer may be critical for initiating an inhibitory signal without inducing engulfment of apoptotic cells.

4.4. HMGB1

High mobility group box 1 (HMGB1) ubiquitous nuclear protein which plays a critical role as a damage-associated molecular pattern (DAMP). Therefore, it is considered an "alarmin", reflecting its function as a factor secreted by cells stressed by stimuli [66]. Chiba et al. provided evidence that HMGB1 binds to Tim-3 expressed on DCs, and suggested that this interaction suppresses innate immune responses to nucleic acids [67]. More recently, it has been posited that HMGB1/Tim-3 may downregulate T cell responses as well: binding of HMGB1 to Tim-3 on CD8$^+$ T_{reg} can license them to effectively suppress the

proliferation of effector T cells [68]. Thus, HMGB1 may trigger Tim-3 dependent pathways in both T cells and innate immune cells.

5. Signaling downstream of Tim-3

The Tim-3 cytoplasmic tail contains five conserved tyrosine residues that are potential sites of phosphorylation; a substantial body of evidence now indicates that it interacts with, and modulates the function of, multiple molecules that are proximal to the T cell receptor (TcR). The importance of the Tim-3 tail was first shown by van de Weyer and colleagues, who found that gal-9 ligation resulted in phosphorylation of Y265 of hTim-3 [26] and that inducible T cell kinase (Itk) phosphorylates the same residue. A later study showed that phosphorylation of the murine Tim-3 tail required residues Y256 (homolog of human Y265) and 263. SH2 domain screening revealed that both the p85 subunit of phosphoinositide 3' kinase (PI3K) and the Src family kinase Fyn can bind to the Tim-3 tail, though the latter may do so in a phosphorylation-independent manner. Further, Fyn and the related Src kinase Lck could themselves phosphorylate Tim-3 [25]. Taken together, these findings provided strong evidence that phosphorylation of the Tim-3 tail is important for signal transduction.

5.1. Bat3: a repressor of Tim-3 inhibition

Intriguingly, evidence suggests that Tim-3 may act as a constitutive positive regulator of T cell function, at least under conditions of acute stimulation. $CD8^+$ T cells from *Tim-3*$^{-/-}$ mice showed impaired recall responses upon infection with the acute Armstrong strain of lymphocytic choriomeningitis virus (LCMV), and enforced expression of Tim-3 *in vivo* drove the generation of short-lived effector $CD8^+$ T cells in an mTOR-dependent manner [69].

How can we reconcile these findings with the well-established role as an inhibitory receptor? An important early clue came from the observation that while ectopic expression of Tim-3 in Tim-3^{neg} cells could upregulate NFAT and NF-kB activity, this phenotype could be abolished in the presence of an agonistic Tim-3 antibody [25]. The pro-inflammatory profile of T cells expressing ectopic Tim-3 was abolished in the presence of an agonistic Tim-3 antibody [25]. This suggested that any costimulatory function of Tim-3 could be reversed in the presence of its ligand(s).

Reasoning that Tim-3-mediated signaling pathways must therefore be more complex than previously thought, we conducted a yeast two-hybrid screen to identify molecules that bind to the Tim-3 intracellular domain. The "hit" that arose with the greatest frequency was HLA-B associated transcript 3 (Bat3), a highly conserved and proline-rich adaptor molecule involved in numerous protein-protein interactions [70–72] that we found to be highly enriched in differentiated Th1 cells [27]. Bat3 could bind to both wildtype Tim-3 as well as a Tim-$3_{256YF263YF}$ mutant, in which residues 256 and 263 were refractory to phosphorylation yet maintained their native topography. Intriguingly, the presence of gal-9 abrogated the interaction of Bat3 to WT Tim-3 but not to Tim-$3_{256YF263YF}$, indicating that phosphorylation of these critical residues in the Tim-3 tail inhibited, rather than promoted, Tim-3/Bat3 interaction in the presence of ligand. Importantly, Bat3 co-immunoprecipitated with a catalytically active form of Lck (pY394), and this interaction was also abolished by the presence of agonistic anti-Tim-3. Further, in the absence of Bat3, T cells lost the ability to produce large amounts of IFN-γ and IL-2, and accumulated a catalytically inactive form of Lck (pY505). Importantly, knocking down Bat3 in myelin antigen-specific transgenic 2D2 Th1 cells profoundly reduced their ability to transfer EAE and generated an exhausted Tim-$3^{hi}IFN\gamma^{lo}$ phenotype. Our data thus indicated that by recruiting active Lck to the Tim-3 tail, Bat3 might be a critical mediator of early, activating signal. The absence of Bat3, however, might shift the balance of Tim-3-driven signals towards an inhibitory profile (Fig. 2). It has been shown that while Tim-3 localizes to lipid rafts and binds to Lck protein, it specifically excludes catalytically active pY394 [73]. We also showed that by ectopically expressing a Tim-3 mutant that lacked a Bat3-binding motif, we could actively reduce the production of IFN-γ from Th1 cells [27]. More recently, it was demonstrated that a Tim-3 long non-coding RNA (lnc-Tim-3) blocks the interaction of Tim-3 and Bat3 in the cytoplasm, resulting in Bat3 sequestration in the nucleus and in T cell exhaustion [74]. These findings lend credence to a model in which Bat3 promotes a positive signal downstream of Tim-3 during the early stages of Th1 differentiation *via*the recruitment of activated Lck. In the presence of Tim-3 ligand, and/or in exhausted cells, Bat3 may detach from the phosphorylated Tim-3 tail, thus resulting in the transduction of an inhibitory signal. However, the mechanisms by which this occurs remain to be fully elucidated. Kane and colleagues have also proposed a different, though not mutually exclusive, model, by which sustained positive signals *via*Tim-3 eventually promote exhaustion of Tim-3^+ T cells at the expense of memory cell generation [69], though how these exhausted, ex-effector Tim-3^+ T cells avoid galectin-9-mediated cell death is not yet understood.

6. Role of Tim-3 in autoimmunity

As might be expected for an inhibitor of inflammatory Th1 cells, Tim-3 has been reported as a negative regulator of disease in a number of murine models of autoimmune disease such as EAE [10,56,75], type I diabetes [42,47,76], autoimmune heart disease [77] and hepatitis [53]. Both functional blockade [10,42] and genetic ablation [42] of Tim-3 has been shown to worsen the disease, while overexpression of Tim-3 and/or gal-9 ameliorates it [56]. Notably, Goverman and colleagues [75] showed that absence of Tim-3 did not improve outcomes in the C3HeB/FeJ model of EAE, but rather shifted the balance of effector T cells to one favoring Th1 cells as opposed to Th17. Further, *Tim-3*$^{-/-}$ mice tended to develop "classic" EAE, characterized by spinal cord lesions, rather than a Th17-driven "atypical" form that affects balance and predominates in WT C3HeB/FeJ mice. Autoimmune inflammation is generally thought to result from the co-operation of both Th1 and Th17 responses to self-antigen. Therefore, it is probable that Tim-3 collaborates with other negative regulatory receptors to modulate autoimmune inflammation.

Much of what we know about Tim-3 dysregulation in human autoimmune disease comes from the MS literature. T cell clones generated from the cerebrospinal fluid of MS patients were highly IFNγ-positive yet Tim-3^{lo}, and these cells were refractory to the induction of tolerance *in vitro* [78]. This suggested that downregulation of Tim-3 may be a mechanism for the persistence of inflammatory autoreactive T cells. Tim-3 inhibitory signaling is functionally dysregulated during MS, as T cells from healthy controls, but not MS patients, produce more IFN-γ upon blockade of Tim-3 *in vitro*. Intriguingly, the Tim-3-dependent upregulation of IFN-γ from T cells during MS was partially rescued when taken from patients undergoing immunotherapy [14], indicating that the Tim-3 pathway is plastic and may be a potential target for therapy in autoimmune disease. In addition to MS, Tim-3 expressing T cells are decreased in other human autoimmune diseases including psoriasis and ulcerative colitis [79,80].

7. Role of Tim-3 in Exhaustion (infection and cancer)

T cell exhaustion is formally defined as the hierarchical and progressive loss of effector functions from antigen-experienced T cells [81]. T cell exhaustion often occurs in the context of chronic viral infection or cancer setting, when T cells are exposed to persistent antigens. This can lead to a defect in controlling chronic infections and tumors. Blockade of coinhibitory molecules such as PD-1 can partially rescue T cell "exhaustion" and restore antigen-specific T cell responses in various disease settings [82–88]. Given the recent success of immune checkpoint therapy in cancer [89], there has been increasing interest in the role of Tim-3 as a possible target.

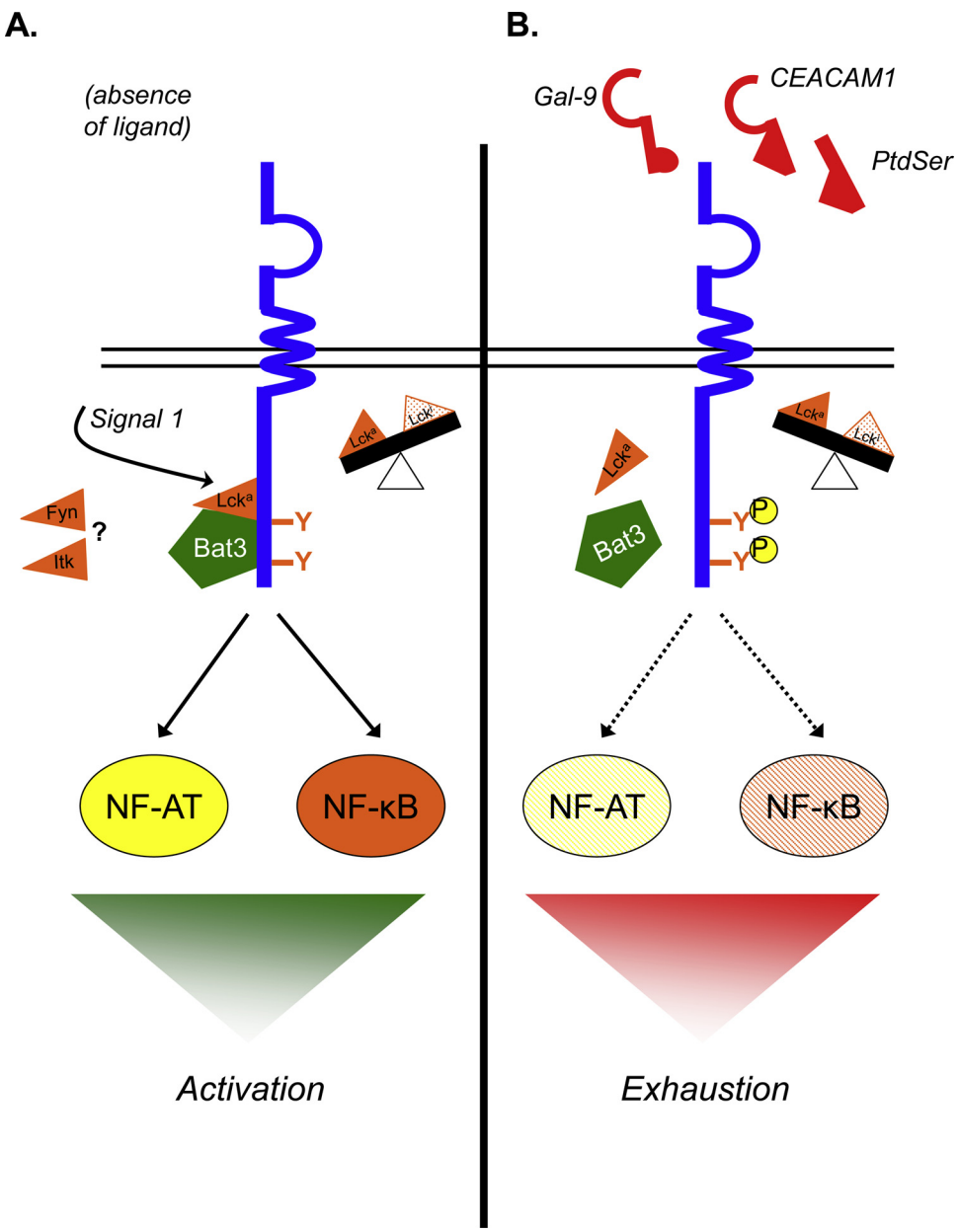

Fig. 2. A model of Tim-3 signaling. A. The molecular adaptor Bat3 binds to the Tim-3 tail, and additionally binds to the active form (pY394; Lck^a) of the the TcR-proximal Src kinase Lck [27]. The Src kinases Itk [26] and Fyn [25] have additionally been implicated in Tim-3-mediated positive regulation. Signaling through Tim-3 activates NF-AT and NF-κB pathways [25], thus promoting T cell activation and inflammation, though it has also been suggested that sustained stimulation through Tim-3 ultimately induces exhaustion [69]. **B.** Ligation of Tim-3 by its ligands can phosphorylate critical tyrosine residues in the Tim-3 tail [26,27], thereby disrupting the Tim-3/Bat3/Lck^a complex and causing a shift towards inactive Lck (Lck^i) in the cell. Loss of Bat3 induces T cell exhaustion [27].

7.1. Infection

The initial evidence for Tim-3 as an exhaustion marker came from studies of HIV-1-infected individuals. Tim-3 is up-regulated on both $CD4^+$ and $CD8^+$ T cells during both acute and chronic human HIV-1 infection, and its expression correlates positively with HIV-1 viral load and negatively with the absolute number of T cells. In contrast to Tim-3^- HIV-specific T cells, Tim-$3^{lo/hi}$ T cells do not produce IFN-γ [11] and have dysfunctional perforin and granzyme release [90]; notably, defects in cytotoxic capacity can be reversed *in vitro* by Tim-3 blockade, again suggesting that the pathway is therapeutically tractable. Similar findings have been observed in the murine clone-13 LCMV model of chronic viral infection, where in collaboration with Rafi Ahmed, we showed that Tim-3 is co-expressed with PD-1 on exhausted T cells. LCMV-specific $CD8^+$ T cells are profoundly defective in their ability to make IL-2, IFN-γ and TNF-α. However, they show substantially increased IL-10 production [91]. Thus, T cell exhaustion is defined not only by the loss of inflammatory capacity, but also by the upregulation of immunoregulatory pathways. In hepatitis C (HCV) infection, Tim-3 is a marker of exhausted T cells [92], while gal-9 is expressed at high levels on macrophage-like Kupffer cells in the liver [93]. These data further demonstrate the importance of the Tim-3 pathway in chronic viral infection.

An open question is whether Tim-3 expression is accompanied by co-expression of other inhibitory molecules. Kassu and colleagues [94] found that, on average, greater than 30% of viral-specific $CD4^+$ T cells from HIV-1 infected individuals were triply-positive for CTLA-4, Tim-3 and PD-1. In mouse studies the majority of LCMV-specific $CD8^+$ T cells are Tim-3^+PD-1^+, 30 days after inoculation with clone 13 [91]. By contrast, others have shown that Tim-3 and PD-1 expression are largely divergent on HIV-1-specific T cells [11]. Resolving the potential for co-expression of multiple checkpoint markers on virus-specific T cells will be a key question if Tim-3 is to be considered a target for therapy. Indeed in HCV infections, Tim-3 is highly co-expressed on HCV specific $CD4^+$ and $CD8^+$ T cells and antibody- mediated blockade of Tim-3 resulted in enhanced proliferation and IFN-γ production from responding T cells [92].

While T cell exhaustion has typically been thought to manifest during chronic viral [81] rather than bacterial infection, there is evidence that Tim-3^+ exhausted T cells arise in response to *M. tuberculosis*

(TB) infection as well. With Sam Behar, we recently showed that bacterial antigen-specific CD4$^+$ and CD8$^+$ T cells from TB-infected mice show phenotypic exhaustion. Further, T cells expressed increased Tim-3 as a function of time [95]. Curiously, we observed two distinct Tim-3$^+$ phenotypes: Tim-3$^+$PD-1$^+$ T cells were deeply exhausted, as we have also seen in response to cancer [12], while Tim-3$^+$PD-1$^-$ cells strongly upregulated inflammatory effector transcripts. Thus, Tim-3 may have conflicting roles in TB: indeed, it was previously shown that Tim-3 interaction with gal-9 on TB-infected macrophages causes the latter to induce an antimicrobial response in the myeloid cells in Gal-9 dependent manner [96]. However, the Tim-3 exhausted phenotype appears to be dominant, as *in vivo* blockade of Tim-3 in TB-infected mice both improved disease outcomes and augmented production of IL-2, TNF-α and IFN-γ by T cells. Importantly, Tim-3 expression is also increased on T cells from human subjects infected with TB and these cells are functionally defective, most notably expressing lower levels of the cytotoxic marker CD107a [97].

7.2. Cancer

As is the case in chronic viral infections, Tim-3 is upregulated in various malignancies, in which it is associated with a T-cell exhaustion phenotype. Given the success of anti-PD-1 and anti-CTLA-4, it is perhaps not surprising that there has been intense interest in Tim-3's potential as a marker and/or target in cancer. Early evidence for Tim-3 being an inhibitory immune checkpoint came from follicular B cell non-Hodgkin lymphoma (FL), in which dysfunctional Tim-3$^+$ T cells were over-represented in the T cell pool. Curiously, Tim-3$^+$ T cell exhaustion was linked to chronic exposure to the Th1 differentiation factor IL-12 in these patients [98]. More recently, it has been shown that Tim3$^+$ T cells from FL patients are refractory to TCR stimulation as measured by cytokine production and activation of the lytic machinery, and that the increased presence of Tim-3$^+$ T cells in these individuals can predict a poor response to treatment [99]. Indeed, the accumulation of dysfunctional PD-1$^+$Tim-3$^+$ T cells can also predict the chances of leukemia relapse after stem cell transplantation [100]. Thus, Tim-3 is a potential marker of poor prognosis in multiple cancers.

Tim-3$^+$ T cells associate with gal-9$^+$ antigen presenting cells inside human liver carcinomas, suggesting that engagement of the Tim-3 pathway within the tumor milieu is essential to inhibiting anti-tumor immunity [101]. Using multiple murine models of ectopic tumor implantation, we showed that most tumor-infiltrating Tim-3$^+$ T cells are double positive for PD-1. Notably, Tim-3$^+$PD-1$^+$ tumor-infiltrating T cells showed greater defects in production of IFN-γ, TNFα and IL-2 than Tim-3$^-$PD-1$^+$ cells, and additionally were more likely to stall at the G_0 stage of the cell cycle [12]. Treatment with a neutralizing anti-Tim-3 antibody synergized with anti-PD-1 blockade to rescue T cell function and induced tumor regression. In a model of acute myelogenous leukemia, however, PD-1 and Tim-3 were co-expressed on the vast majority of exhausted T cells [102]. These findings indicate that expression of these two inhibitory markers is closely linked in tumor-infiltrating T cells and Tim-3 may be a marker of deeply exhausted T cells that are more functionally impaired than those that are singly positive for PD-1 alone. Indeed, our recent studies show that Tim-3 is part of co-inhibitory gene module consisting of PD-1, Tim-3, Lag-3 and TIGIT and the module is co-expressed and co-regulated in tumor infiltrating lymphocytes [38].

In addition to its expression on exhausted CD8$^+$ T cells, Tim-3 is upregulated on regulatory T cells (T_{reg}) in the tumor microenvironment. Tim-3$^+$ T_{reg} are potent suppressors of immune responses. It is expressed on around 60% of FoxP3$^+$ T_{reg} isolated from non-small cell lung cancer (NSCLC) tumor tissue, and the presence of such Tim-3$^+$ T_{reg} correlates with poor prognosis [103]. Similar findings have been observed in liver, colorectal and ovarian cancer [35]. The mechanisms of action of Tim-3$^+$ T_{reg} have thus become an area of interest. In the future, conditional deletion of Tim-3 on T_{reg} in mouse models will allow one to clearly study its role on Tregs. Human Tim-3$^+$ T_{reg} can suppress proliferative responses of both Th1 and Th17, while Tim-3$^-$ T_{reg} can suppress Th1 only [104]. Further, the bulk of IL-10 production from T_{reg} comes from the Tim-3$^+$ fraction [105]. Thus, in addition to limiting effector T cell function, Tim-3 may be an important positive regulatory of T_{reg}-mediated suppression *in vivo*.

Multiple clinical trials of Tim-3 inhibitors in cancer are currently underway (recently reviewed in [106]). It is unclear whether Tim-3 blockade will be most effective as a first-line therapy or whether it needs to be administered with other check-point molecules. Multiple lines of evidence, principally from animal models, favor the latter approach. In the CT26 colon adenocarcinoma model, anti-Tim-3 treatment had no positive effect on tumor regression [12], yet concomitant anti-Tim-3/anti-PD-1 was more effective than anti-PD-1 alone. Similarly, combined anti-Tim-3 + anti-CD137/4-1BB significantly inhibited the growth in mice with established ovarian tumors relative to monotherapy [107]. In a striking recent study, it was shown that Tim-3 is upregulated on PD-1$^+$ T cells from mice and humans who are refractory to PD-1-directed cancer immunotherapy [108], indicating that tumor-associated T cells may induce Tim-3 as an evasive mechanism in response to PD-1 blockade, and this that concomitant blockade may be required in some cases for full remission. However, PD-1$^-$Tim-3$^+$ T cells exceed PD-1 + Tim-3- in malignant Schwannoma, and show more profound defects in perforin/granzyme expression and cytotoxic function [109]. Thus, it is not obligatory that Tim-3 expression and function be secondary to PD-1 on exhausted T cells.

8. Concluding remarks

Fifteen years after its discovery, it is now known that Tim-3 has two very different faces. On the one hand, it acts as a physiological negative regulator of inflammatory T cell responses, such as in autoimmunity. On the other, it acts a maladaptive enforcer of T cell dysfunction and exhaustion in cancer and chronic viral infection. However, it is still an open question as to whether these distinct cell fates of Tim-3$^+$ cells are driven by T cell-extrinsic factors, or whether they result from differential signaling downstream of Tim-3.

Tim-3 was first identified, and as is arguably still best understood as, an inhibitory marker of Th1 and CD8$^+$ T cells. However, it is instructive that it is only expressed on terminally differentiated effector Th1, and requires multiple rounds of stimulation in order to be detected *in vitro*. By contrast, Tim-3 is rapidly induced by the immunomodulatory differentiation cytokine IL-27 [38,39,110]. It is thus possible that the fate of a given Tim-3$^+$ T cell *in vivo* depends on the relative balance of inflammatory and/or immunomodulatory cytokines in the local milieu. Support for such a model has come from analyses of transcriptional regulation of Tim-3. Tim-3 is induced in a T-bet-dependent manner by both IL-12 [36] and IL-27 [39]. However, while IL-12 induces Stat4 activation, which is partially responsible for Tim-3 activation in Th1 cells [36]. IL-27 additionally activates the transcription factor NFIL3, which requires T-bet coactivation to drive Tim-3 transcription in this context [39]. Recent studies show that Tim-3 expression may induced by the joint action of two transcription factors, c-Maf and Prdm-1 [38]. Thus, the number of transcription factors that regulate Tim-3 expression has increased to at least five that include T-bet, STAT-4, NFIL3, Prdm-1 and c-Maf. One must also keep in mind that while the characterization of Tim-3 expression pattern(s) has been largely conducted under defined conditions *in vitro*, the *in vivo* situation (in either lymphoid organs or sites of infection/inflammation) is likely to feature heterogeneous T cell differentiation cues and that multiple transcriptional modules may be operating to regulate Tim-3 expression.

While Tim-3-driven T cell exhaustion is typically considered deleterious to the organism, it is also likely that it evolved as a mechanism by which to control excessive responses to self-Ag. It is known that the Tim-3 tail is critical to both stimulatory and inhibitory signaling events. Notably, we found that Bat3 represses Tim-3-driven inhibition,

promoting T cell expansion and that this Bat-3 mediated repression of Tim-3 inhibitory function is abrogated by phosphorylation of critical tyrosine residues in the Tim-3 tail. Further, ablation of Bat3 drove a T cell exhaustion-like phenotype to self-Ag *in vivo*, even in the context of a Th1-driven response [27]. It is possible that Bat3 is an important T cell-intrinsic regulator of inflammatory *vs.* exhausted fate. In its presence, Th1 cells may be driven towards a classic inflammatory phenotype in which they are deleted by Tim-3/Tim-3 L interactions upon cessation of an immune response; by contrast, in the absence of Bat3, Tim-3^+ T cells may persist in the repertoire but progressively lose effector functions and become exhausted. Interestingly, ligation to, and repression of, Tim-3 by Bat3 was abolished by the presence of galectin-9; it will be interesting to examine whether the same holds true for other Tim-3 ligands such as CEACAM1.

The success of immune checkpoint therapies has raised the possibility that Tim-3 may itself be an attractive target in cancer therapy. Further, its expression on Th1 cells and also on some Th17 cells, raises the possibility that it may be therapeutically tractable in autoimmunity as well. It is clear that much still needs to be learned regarding the mechanisms by which Tim-3 regulates T cell dysfunction to regulate autoimmunity and tolerance.

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