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Bat3 Protects T cell Responses by Repressing Tim-3-Mediated Exhaustion and Death

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Abstract

T cell immunoglobulin and mucin domain-containing-3 (Tim-3) is an inhibitory receptor expressed on exhausted T cells during HIV-1 and HCV infection. By contrast, Tim-3 expression and function are defective in multiple human autoimmune diseases. However, the molecular mechanisms governing Tim-3 function remain poorly understood. Here we show that HLA-B-associated transcript 3 (Bat3) binds to, and represses the function of Tim-3. Bat3-deficient T cells display elevated expression of exhaustion markers, and knocking down Bat3 in myelin antigen-specific CD4⁺ T cells dramatically inhibits the development of experimental autoimmune encephalomyelitis while promoting the expansion of a dysfunctional Tim-3^{hi}IFN γ ^{lo} CD4⁺ cell population. Furthermore, exhausted Tim-3⁺ T cells from murine tumors and HIV-1-infected individuals display substantially reduced Bat3 expression and targeted deletion of Bat3 induces an exhausted phenotype in T cells. These data indicate that Bat3 acts as a molecular safety catch that inhibits Tim-3-dependent cell death/exhaustion, suggesting that Bat3 may represent a viable therapeutic target in autoimmune disorders, chronic infections and cancers.

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Interferon (IFN) γ -secreting Th1 CD4⁺ cells and Tc1 CD8⁺ cells play an essential role in protection against intracellular pathogens and viruses, and IFN γ production from lymphocytes is a critical component of tumor immunosurveillance¹ and resistance to chronic viral infections^{2,3}. Exacerbated Th1 responses to self-antigens are, however, implicated in murine and human autoimmune diseases^{3,4}. Thus, IFN γ -producing T cells both induce human autoimmune diseases as well as protect against chronic viral infections and cancers.

T cell immunoglobulin and mucin domain-containing-3 (Tim-3) is an inhibitory receptor specifically expressed on terminally differentiated Th1 and Tc1 cells⁵. Tim-3 is critical for the induction of T cell tolerance *in vivo*^{6,7}, and defective Tim-3 expression and signaling have been observed in CD4⁺ T cells isolated from MS^{8,9} and colitis¹⁰ patients. Furthermore, provocative recent findings show that Tim-3 plays a critical role in the development of T cell exhaustion in chronic viral infections such as HIV¹¹, HCV¹², LCMV¹³ and Friend virus¹⁴ as well as in tumor-bearing individuals^{15–17}. The interaction of Tim-3 with its ligand galectin-9 triggers cell death and *in vivo* blockade of galectin-9 substantially enhances the severity of murine experimental autoimmune encephalomyelitis (EAE)¹⁸.

The galectin-9/Tim-3 axis is thus an important negative regulator of Th1 and Tc1 cell function that could be exploited therapeutically. While boosting Tim-3 signals could dampen autoimmunity, repressing Tim-3 function could augment immune responses to viral infections and cancers. Developing such strategies requires greater insight into the molecular mechanisms of Tim-3-mediated T cell regulation. Here we identify HLA-B-associated transcript 3 (Bat3) as a binding partner to the Tim-3 intracellular tail. Bat3 protects Th1 cells from galectin-9 mediated cell death, and promotes both proliferation and pro-inflammatory cytokine production. In contrast, knockdown of Bat3 in myelin antigen-specific Th1 cells ablates their function and pathogenicity, and promotes T cell exhaustion *in vivo*. Further, Bat3 expression is specifically reduced in exhausted Tim-3⁺ CD4⁺ T cells isolated from murine tumors and also from HIV-1-infected individuals. Bat3 may thus represent an important novel repressor of Tim-3 signaling that protects Th1 responses from Tim-3-mediated inhibition.

Results

Tim-3 binds to Bat3

To identify putative intracellular binding partners for Tim-3, we used a yeast two-hybrid approach to screen a mouse spleen cDNA library using the intracellular portion of Tim-3 as bait (Figure 1A). 11 clones corresponded to Bat3, which is a proline-rich 121 kDa cytoplasmic protein that regulates mammalian cell proliferation and death^{19,20}. To confirm the Bat3-Tim-3 interaction, we overexpressed tagged Bat3 and Tim-3 constructs in 293T cells (Figure 1A) and found that Tim-3 could be co-immunoprecipitated with Bat3 in the over-expression system (Figure 1B). In addition, endogenous Tim-3 and Bat3 could be co-immunoprecipitated in both EL4 mouse T lymphoma cells as well as normal T cells²¹ (Supplementary Figure 1), further demonstrating that the two proteins do associate even when they are not overexpressed by transfections. To identify the Bat3 binding domain on Tim-3, we generated Tim-3 deletion constructs (Figure 1A) that lack residues 252–281 (Tim-3 Δ _{252–281}) or 271–281 (Tim-3 Δ _{271–281}), and co-expressed them with Bat3 in 293T cells. While Tim-3 Δ _{271–281} was able to interact with Bat3, Tim-3 Δ _{252–281} was not, demonstrating that residues 252–270 of Tim-3 are critical for mediating binding to Bat3 (Figure 1C). Two tyrosines (Y256 and Y263) are contained within this Bat3-binding domain (Figure 1A). To assess the possible contributions of these two residues to the binding of Tim-3 to Bat3, we generated Tim-3 mutants in which tyrosine residues 256 or 263 were substituted with phenylalanine, thus abrogating the possibility of phosphorylation while preserving protein topography. We also generated Tim-3 mutants in which Y256 or Y263

were substituted with alanine, which disrupts both phosphorylation and topography (Figure 1A). While Tim-3^{Y256F} and Tim-3^{Y263F} could bind Bat3, Tim3^{Y256A} and Tim3^{Y263A} could not. This indicated that binding of Bat3 to residues 251–269 of Tim3 was not dependent on phosphorylation, but that it required intact domain structure (Figure 1C). To study a potential role for the Tim-3 ligand galectin-9 in regulating the binding of Bat3 to the Tim-3 tail, we co-expressed galectin-9 with Bat3 and Tim-3 in 293T cells. Intriguingly, co-expression of galectin-9 together with Bat3 and Tim-3 abolished Bat3/Tim-3 binding (Figure 1D). Similar results were obtained when galectin-9 was added exogenously to 293T cells co-transfected with Bat3 and Tim-3 (not shown). Addition of galectin-9 did not reduce cytoplasmic levels of Bat3 in Th1 cells (Supplementary Figure 2), suggesting that galectin-9-mediated release of Bat3 from the Tim-3 tail does not result in the reduction of Bat3 levels. As galectin-9 can induce phosphorylation of the Tim-3 tail²², we tested whether phosphorylation of residues Y256 and Y263 is required for galectin-9-mediated release of Bat3 from Tim-3 using a construct in which the two critical tyrosines were replaced with phenylalanine residues (Tim-3_{2YF}; Figure 1A). Tim-3_{2YF} could still bind Bat3 when these two molecules were co-expressed. However, in contrast to wildtype Tim-3, galectin-9 co-expression could not abrogate the binding of Tim-3_{2YF} to Bat3 (Figure 1D). Taken together, our data show that Bat3 binds to residues 252–270 of Tim-3, and that galectin-9 abrogates this interaction by inducing the phosphorylation of 2 tyrosine residues (Y256 and Y263) in the Tim-3 tail.

Bat3 protects Th1 responses from Tim-3 mediated inhibition

In mouse models, Bat3 can have contrasting, context-dependent effects on cell proliferation and survival^{19,20}. Using an antisera generated against the N-terminus of Bat3 (Supplementary Figure 3), we found that Bat3 is enriched in Th1 cells, yet not in Th0, Th17 (Figure 2A) or Th2 cells (Supplementary Figure 4). These data, taken together with our finding that galectin-9 abrogates the Tim-3/Bat3 interaction (Figure 1D), suggested that Bat3 can regulate the Tim-3 signaling pathway. Interestingly, we found that Bat3 expression in Th1 cells is lower in cells that do not express Tim-3, suggesting that Tim-3 expression may be required for optimal Bat3 expression in activated Th1 cells. (Supplementary Figure 5).

To elucidate whether Bat3 repressed or promoted Tim-3-mediated inhibition, we retrovirally overexpressed Bat3 (Supplementary Figure 6) in transgenic 2D2 Th1 cells bearing a T cell receptor (TcR) specificity for the encephalitogenic myelin oligodendrocyte glycoprotein (MOG)_{35–55} peptide²³. Bat3- and control- retroviral constructs contained a bicistronic GFP reporter that permitted us to faithfully track transduced cells. We sorted and transferred GFP⁺ RV-infected 2D2 Th1 cells to *Rag*^{−/−} mice, allowed the transferred cells to fill the immune compartment, and assessed the function of GFP⁺ T cells (Supplementary Figure 7) *ex vivo* 28 days post-transfer. Bat3 overexpressing 2D2 Th1 cells produced greater amounts of IFN γ and IL-2 than control cells after *in vivo* expansion (Figure 2B). Consistent with our intracellular staining data, Bat3 RV-2D2 cells produced significantly more IFN γ when restimulated with MOG_{35–55} (Figure 2C). As expected, neither control nor Bat3-overexpressing Th1 cells made significant amounts of IL-17; however, Bat3 overexpression resulted in a reduction in the percentage of Th1 cells expressing the immunosuppressive cytokine IL-10 (Figure 2B). Interestingly, Bat3-overexpressing 2D2 Th1 recipient mice developed EAE of earlier onset and severity when compared to control 2D2 Th1 recipients (Supplementary Figure 8). Thus, rather than inhibiting Th1 responses, Bat3 overexpression promotes them. Further, binding of Bat3 to Tim-3 may protect Th1 cells from Tim-3-dependent inhibitory signals.

To assess the effect of Bat3 overexpression on galectin-9/Tim-3-mediated signaling directly, Th1 cells transduced with either Bat3- or control-RV were treated with galectin-9 *in vitro*

and cell death was assessed by 7-AAD staining. A far lower percentage of Bat3-transduced Th1 cells underwent cell death than control vector-transduced Th1 cells (Figure 2D). Bat3 thus protects Th1 cells from Tim-3 mediated negative regulation both *in vitro* and *in vivo*.

Loss of Bat3 reduces EAE severity

As Bat3 appeared to protect Th1 cells from the inhibitory functions of Tim-3, loss of Bat3 *in vivo* could dampen autoimmunity by leaving the inhibitory functions of Tim-3 unopposed. We therefore tested the consequences of Bat3 ablation in murine EAE, an autoimmune disease driven primarily by inflammatory CD4⁺ reactivity against myelin antigens²⁴ which can be induced by Th1 cells independently of Th17 cells²⁵.

Bat3 deficiency results in embryonic lethality on the C57BL/6J strain^{19,20}. We therefore generated chimeric mice by transferring *Bat3*^{+/-} or *Bat3*^{-/-} fetal liver cells into *Rag*^{-/-} animals. These chimeras were immunized with MOG₃₅₋₅₅ in complete Freund's adjuvant (CFA), and monitored for the development of EAE. *Bat3*^{-/-} → *Rag*^{-/-} mice developed disease of significantly decreased severity compared to *Bat3*^{+/-} → *Rag*^{-/-} counterparts, suggesting that Bat3 plays an important role in promoting T cell-driven autoimmunity (Figure 3A, B). Further, we observed a decrease in IFN γ but there was no significant decrease in the frequency of IL-17 or IL-2 in the peripheral repertoire of the chimeric mice that were immunized with MOG in CFA (Figure 3C).

Loss of Bat3 induces an exhaustion-like phenotype in CD4⁺ T cells

To gain a better understanding of the consequences of Bat3 loss-of-function on the generation of effector T cell responses, we isolated CD4⁺ T cells from *Bat3*^{+/-} → *Rag*^{-/-} and *Bat3*^{-/-} → *Rag*^{-/-} mice in which lymphocytes had expanded homeostatically in a lymphopenic environment. We noted a profound decrease in the frequency of IFN γ and IL-2 producing cells in the absence of Bat3 expression (Figure 4A). In addition, this reduction in pro-IFN γ and IL-2 production was accompanied by a massive increase in expression of Tim-3 on the Bat-3 deficient T cells, with an increase in IL-10 production also observed from these cells (Figure 4A). This phenotype of reduction in IFN γ and IL-2 production and increase in Tim-3 and IL-10 is reminiscent of T cell exhaustion.

A number of groups including ours have identified Tim-3 as a functional marker of exhausted T cells¹⁰⁻¹⁷. Further, T cells with an exhausted-like phenotype have been observed in *RasGRP1*^{-/-} mice, which suffer from lymphopenia²⁶. This raised the possibility that homeostatic expansion of *Bat3*^{-/-} T cells in *Rag*^{-/-} mice might favor the generation of an exhaustion-like phenotype in these cells, thus explaining the anti-inflammatory cytokine profile observed upon *ex vivo* analysis. In addition to the increase in Tim-3 expression, we found that Bat-3-deficient CD4⁺ T cells upregulated a number of exhaustion-associated markers such as the transcription factors Prdm1²⁷ and Pbx3²⁸, as well as the T cell exhaustion surface receptor Lag3²⁹ (Figure 4B). These data suggest that in the absence of Bat3, homeostatic expansion of CD4⁺ T cells in a lymphocyte-deficient environment can result in the development of an exhaustion-like T cell phenotype.

We next wanted to explore the potential mechanism(s) of Bat3 function in promoting Th1 responses and in suppressing the generation of T cell exhaustion. We have found that Tim-3 is incorporated into supramolecular activation clusters (SMACs) upon T cell activation (data not shown) and that application of an agonistic antibody against Tim-3 can reduce Th1 cell proliferation (Supplementary Figure 9) and IFN γ production³⁰. Further, it has been reported that *RasGRP1*^{-/-} T cells, which display profound defects in T cell receptor (TcR) signaling, spontaneously develop an exhausted phenotype *in vivo*²⁶. We therefore explored the possibility that loss of Bat3, and the resulting upregulation of Tim-3, could result in the

modulation of TcR-associated molecules. Intriguingly, when we examined CD3 ϵ^{lo} CD4 $^{+}$ T cells, we found that Tim-3 was upregulated on Bat3-deficient CD3 ϵ^{lo} cells. By contrast, no differences in Tim-3 expression were noted between Bat3-deficient and Bat3-sufficient cells in the CD3 ϵ^{hi} pool of CD4 $^{+}$ T cells (Figure 4C). These data suggest that loss of Bat3 function may favor the generation of a Tim-3 $^{+}$ CD3 ϵ^{lo} CD4 $^{+}$ T cell population.

Tim-3 has recently been described as binding to T cell receptor-associated Src family kinases that are required for the transduction of TcR-dependent signals³⁰. We found that Tim-3 could bind to the SH3 domain-containing TcR associated intracellular kinase Lck in a manner dependent on its intracellular tail, in that while full-length Tim-3 could bind Lck, a Tim-3 construct that lack its intracellular tail could not (Figure 4D). We therefore asked the question of whether Bat3, a proline-rich molecule, could interact with SH3 domain-containing Lck in a Tim-3-dependent manner and thereby modulate the downstream effects of Tim-3. Indeed, we found that Bat3 and Lck could interact physically when both molecules were expressed in 293T cells; the binding was detected regardless of whether it was Bat3 or Lck that was immunoprecipitated (Supplementary Figure 10). To assess whether the interaction of Bat3 and Lck was dependent on the function of Tim-3, we stimulated primary Th1 cells with anti-CD3+anti-CD28, transduced them with Bat3-RV and treated them with either an agonistic anti-Tim-3 antibody or with isotype control. We used anti-Tim-3 antibody rather than galectin-9 because of the increased specificity of the antibody for Tim-3 and because of the rapid cell death induced by galectin-9. We found that while Bat3 from control antibody-treated cells could pull down with Lck, Bat3 from anti-Tim-3-treated cells could not be immunoprecipitated (Figure 4E). When phosphorylated at Y394, Lck becomes catalytically active and is able to itself phosphorylate downstream substrates critical to optimal T cell activation³¹. By contrast, phosphorylation of Lck at Y505 renders Lck catalytically inactive, and it has been proposed that T cell activation is at least partially regulated by the balance of active and inactive Lck within a cell³². As our data indicated that Bat3 expression promotes T cell responses *in vivo* and enhances Th1 cell-driven autoimmunity, we asked whether Bat3 could bind to either catalytically active or inactive Lck. Intriguingly, Bat3 from control-treated, but not anti-Tim-3-treated, Th1 cells could pull down the catalytically active form of Lck (phospho-Y394). By contrast, Bat3 could not pull down catalytically inactive Lck under either condition. Notably, neither total levels of Lck nor levels of phosphorylated Lck were perturbed by anti-Tim-3 antibody treatment (Figure 4E). These data indicate that Bat3, which binds to the Tim-3 tail, can actively bind and bring in the catalytically active form of pLck to the TcR complex and that receptor ligation of Tim-3 triggers the release of catalytically active form of Lck from Bat3.

Our data show that Bat3 promotes T cell signaling and suppresses the induction of T cell exhaustion, perhaps by positively regulating Lck activity. We therefore asked whether loss of Bat3 could negatively regulate TcR mediated signaling by increasing the proportion of catalytically inactive Lck in T cells. While we found little to no Lck/pY505 in anti-CD3+anti-CD28-stimulated Bat3-sufficient T cells, a significant amount of catalytically inactive Lck accumulated in Bat3-deficient T cells (Figure 4F). Therefore, Tim-3, which is recruited to the SMAC during T cell activation, intracellularly forms a molecular complex with Bat3 and the catalytically active form of Lck to promote T cell signaling, activation and expansion. However, upon Tim-3 crosslinking, the catalytically active form of Lck is released from Bat3, resulting in the accumulation of the catalytically inactive form of Lck.

Bat3 is critical for the induction of Th1-driven autoimmune inflammation

To study the consequences of loss of Bat3 function exclusively in Th1-driven autoimmunity, we devised a system of ablating Bat3 expression specifically in myelin antigen-specific T cells. We generated an shRNA (Bat3KD1) that efficiently knocked down Bat3 expression in T cells (Supplementary Figure 11). We then retrovirally transduced 2D2 CD4 $^{+}$ T cells under

Th1 conditions with either Bat3KD1- or control RV. Knockdown of Bat3 had no apparent effect on T cell viability (Supplementary Figure 12). As the RV constructs contained a bicistronic GFP reporter, we sorted GFP⁺ cells and transferred them to *Rag*^{-/-} recipient mice. All control-2D2 Th1 cell recipients developed EAE, whereas mice receiving Bat3KD1-2D2 Th1 cells developed EAE at greatly reduced incidence (Figure 5A, Supplementary Table 1). Furthermore, there were 5-fold fewer GFP⁺CD4⁺ cells (Figure 5B), and significantly fewer inflammatory foci in the meninges and CNS parenchyma (Supplementary Table 1), of Bat3KD1-2D2 recipients. These findings demonstrate that Bat3 is crucial for pathogenicity and/or survival of Th1 cells, and they support a role for Bat3 in preserving Th1 cell function *in vivo*.

To assess the functional consequences of Bat3 knockdown on cytokine expression *in vivo*, we analyzed the surface phenotype and cytokine expression in immune and diseased tissues of the transferred 2D2 T cells in *Rag*^{-/-} recipients. Control-2D2 mice displayed large numbers of GFP⁺IFN γ ^{hi} expressing CD4⁺ T cells in lymph nodes. By contrast, Bat3KD1-2D2 transfer mice displayed very few GFP⁺IFN γ ^{hi} cells (Figure 5C). These data suggested that loss of Bat3 expression is incompatible with an IFN γ ^{hi} CD4⁺ T cell phenotype. Importantly, sorted 2D2 T cells were >98% GFP⁺ at the time of transfer. Notably, when we analyzed GFP⁺ T cells (Supplementary Figure 13) for cytokine production, we found that while Bat3KD1-2D2 T cells produced less IFN γ than controls, they produced substantially more IL-10 (Figure 5D), a phenomenon that has been observed in exhausted T cells.

Our data indicated that Bat3 protects Th1 cells from cell death induced by the Tim-3 ligand galectin-9, and that Tim-3 receptor ligation abrogates the interaction of Bat3 with catalytically active Lck. Further, we showed that knockdown of Bat3 in myelin antigen-specific Th1 cells dramatically reduced the incidence of EAE. To further establish that the observed effects are entirely due to Bat3 binding to Tim-3, we generated a retroviral gene expression construct (Tim-3_{ICΔBat3BD}) that consisted of the intracellular region of Tim-3 lacking the Bat3 binding domain (aa 252–281) (Figure 1A, C). We expressed Tim-3_{ICΔBat3BD} in Th1 cells and compared their production of IFN γ to Th1 cells expressing the full intracellular domain of Tim-3 (Tim-3_{IC}). Cells expressing Tim-3_{ICΔBat3BD} displayed an impaired capacity to generate IFN γ relative to Th1 cells expressing the intracellular domain of wildtype Tim-3 (Tim-3_{IC}), suggesting that loss of the Bat3/Tim-3 interaction allows Tim-3 to negatively regulate Th1 responses (Figure 5E). Taken together, these data suggested that the interaction of Bat3 with Tim-3 protects Th1 responses. To formally demonstrate that the defects in T cell function observed upon Bat3 diminution were Tim-3 dependent, we knocked down Bat3 expression in both *Tim-3*^{+/+} and *Tim-3*^{-/-} Th1 cells. We reasoned that if the effects of Bat3 reduction were Tim-3-dependent, then Bat3 knockdown would have little effect on *Tim-3*^{-/-} T cell function. Further, as we had shown that Bat3 represses the accumulation of the catalytically inactive form of Lck in T cells (Figure 4F), we hypothesized that reducing expression of Bat3 would reduce the proliferative capacity of T cells in a Tim-3-dependent manner. We found that *Tim-3*^{+/+} Th1 cells transduced with two different Bat3 specific shRNAs, Bat3KD1 or Bat3KD2 (Supplementary Figure 11), proliferated poorly in comparison with control-transduced cells upon restimulation. By contrast, *Tim-3*^{-/-} Th1 cells suffered no significant loss in proliferative capacity when Bat3 was downregulated with either Bat3KD1 or Bat3KD2 (Figure 5F). Further, knockdown of Bat3 resulted in a reduction in the frequency of IFN γ ⁺IL-2⁺ double producers among *Tim-3*^{+/+}, but not *Tim-3*^{-/-}, Th1 cells (Figure 5G). These data indicate that the loss of T cell function that accompanies reduction of Bat3 expression is dependent on Tim-3 when assessed by proliferative capacity and production of IFN γ and IL-2.

Tim-3⁺ exhausted T cells have lower Bat3 expression

Given the established role of Tim-3 in inducing/maintaining T cell exhaustion^{11–17} and our findings that Bat3 repressed the inhibitory functions of Tim-3, we asked whether Bat3 was differentially regulated in exhausted Tim-3⁺ T cells generated *in vivo*. We recently reported that Tim-3 expression characterizes a subpopulation of PD-1⁺ tumor infiltrating lymphocytes (TILs) that are deeply exhausted and display highly impaired effector functions^{15,17}. Tim-3⁺PD-1⁺ double-positive TILs produce greatly reduced amounts of IFN γ and TNF α relative to Tim-3[–]PD-1⁺ TILs in both the 4T1 mammary adenocarcinoma (Figure 6A) and CT26 colorectal carcinoma¹⁵ models. Further, we found that combined *in vivo* blockade of Tim-3 and PD-1 signaling increased the production of IFN γ from *ex vivo* isolated TILs relative to cells isolated from mice that had received PD-1 blockade alone (Figure 6B). These data suggested that Tim-3 signaling can enforce the maintenance of a deeply exhausted state, and led us to consider whether Bat3 expression was modulated in Tim-3⁺ TILs. Indeed, analysis of mRNA expression revealed that Tim-3⁺PD-1⁺ TILs displayed a greater than 50% reduction in Bat3 mRNA levels relative to Tim-3[–]PD-1⁺ cells (Figure 6C). To extend our findings to both chronic viral infections and to human disease, we asked whether Bat3 was differentially expressed in Tim-3⁺ exhausted T cells isolated from untreated HIV-1-infected individuals¹¹. We sorted Tim-3[–]PD-1⁺ and Tim-3⁺PD-1⁺ CD4⁺ T cell populations from the blood of three individuals with chronic untreated HIV-1 infection (median HIV-1 viral load 79200 RNA copies/mL, IQR 69630–169000). Bat3 mRNA expression was significantly reduced in Tim-3⁺ exhausted cells isolated from all three individuals (Figure 6D), indicating that reduced Bat3 expression in T cells correlates with Tim-3 expression *in vivo* and development of functional exhaustion in both mouse and man.

While T cell exhaustion is detrimental in chronic viral infections and cancers, it may have evolved as a mechanism of peripheral tolerance to autoantigens and could thus be highly desirable in the context of autoimmune disorders such as MS. Both the greatly reduced encephalitogenic potential of Bat3KD1 Th1 cells (Figure 5A, Supplementary Table 1) and the decreased Bat3 expression observed in Tim-3⁺ TILs (Figure 6C) and Tim-3⁺ exhausted T cells from HIV-1-infected individuals (Figure 6D) led us to consider the possibility active reduction of Bat3 levels could promote an exhaustion-like phenotype in Th1 cells. We therefore tested the effect of Bat3 downregulation on the development of effector T cell responses in 2D2 MOG_{35–55}-specific transgenic T cells following adoptive transfer *in vivo*. We analyzed Tim-3 and IFN γ co-expression on *ex vivo* isolated 2D2 Th1 cells in which Bat3 expression had been knocked down. The majority of peripheral CD4⁺ T cells from control-2D2 recipients produced high levels of IFN γ with no Tim-3 expression. In contrast, T cells from 5 of 11 Bat3KD1-2D2 recipients displayed a high proportion of Tim-3^{hi}IFN γ ^{lo} cells in their peripheral lymphoid compartments, with the small number of IFN γ ^{hi} T cells isolated from Bat3KD1-2D2 transferred mice all being Tim-3-negative (Figure 6E). Interestingly, these Tim-3⁺ T cells were PD-1-negative (Supplementary Figure 14). Bat3-depleted Th1 cells thus resemble the recently described Tim-3⁺ exhausted T lymphocyte population observed in chronic infections caused by HIV¹¹ or HCV¹² and tumors^{15,17} in many ways, including reduced proliferation (Figure 5F), high Tim-3 expression coupled with low IFN γ production (Figure 4A, 6E), and increased IL-10 production (Figure 5D). Thus, Bat3 may be a regulator of both T cell death and exhaustion in Th1 cells.

Discussion

While galectin-9 has been identified as a Tim-3 ligand that induces rapid cell death and downregulation of Th1 responses¹⁸, there have been no reports of intracellular molecules that interact with and mediate the function of Tim-3. Identification of such interactions will be essential if we are to devise therapeutic strategies that target the Tim-3 pathway in order

to prevent development of exhaustion in chronic viral infections or promote tolerance during autoimmune reactions. Here we show that the molecular adaptor Bat3 binds to the intracellular tail of Tim-3 and protects Th1 cells from Tim-3-mediated cell death or exhaustion. Bat3, the mammalian homologue of the evolutionarily conserved protein Scythe, contains lengthy proline-rich tracts, making it an attractive molecular scaffold that can mediate multiple protein-protein interactions^{33–37}. Interestingly, polymorphisms in the Bat3 locus have been linked to increased incidence of type I diabetes³⁸, suggesting that regulation of Bat3 expression can affect the prevalence of human autoimmune disorders.

Intriguingly, galectin-9 mediates the release of Bat3 from Tim-3, via a mechanism dependent on phosphorylation of residues Y256 and Y263 in the Tim-3 tail. We propose a model in which Bat3 binds to Tim-3 under steady state conditions, repressing the inhibitory function of Tim-3 while promoting T cell expansion. Galectin-9 mediated phosphorylation of the Tim-3 tail causes Bat3 to be released, thus permitting the transduction of inhibitory signals by Tim-3.

Our findings indicate that Bat3 can both promote IFN γ production *in vivo* and render Th1 cells resistant to galectin-9 mediated cell death. This suggests that physical interaction of Bat3 with Tim-3 provides a means by which IFN γ ^{hi} Th1 cells can be protected from Tim-3-dependent negative regulation. Furthermore, shRNA-mediated ablation of a single transcript, Bat3, in myelin antigen-specific Th1 cells resulted in decreased IFN γ production as well as a dramatic loss in their ability to induce EAE. These data are consistent with a model in which Tim-3⁺ IFN γ -producing cells are protected from death by the expression of Bat3. Notably, however, the percentage of IL-10-producing GFP⁺ cells was increased in Bat3 knockdown-2D2 *Rag*^{−/−} recipients. These findings imply that Bat3 may be critical not only for the survival of Th1 effector T cells *in vivo* but may either directly or indirectly repress the production of the immunosuppressive cytokine IL-10, which is highly induced in exhausted T cells¹³.

We found that loss of Bat3 resulted in the generation of peripheral CD4⁺ populations that were Tim-3^{high}IFN γ ^{lo}, a phenotype characteristic of exhausted T cells in multiple disease settings^{11–13,15,17}. We also find, in agreement with previous reports^{11,15}, that exhaustion is a potentially reversible phenomenon that can be at least partially modulated by Tim-3 signaling blockade. Importantly, we observe decreased Bat3 expression in both murine Tim-3⁺ TILs and in exhausted Tim-3⁺ CD4⁺ T cells isolated from untreated HIV-1-infected individuals. Furthermore, active downregulation of Bat3 in Th1 cells induces an “exhaustion-like” phenotype. Loss or downregulation of Bat3 in T cells not only decreases effector functions (proliferation, IFN γ and IL-2) but also increases the expression of multiple molecules involved in T cell exhaustion including Tim-3, LAG-3, Prdm3, Pbx3 and IL-10. It is thus tempting to speculate that promoting the interaction of Bat3 and Tim-3 in exhausted virus- or tumor-specific T cells could reverse their dysfunction. Further, our data point towards a novel and possibly promising strategy in autoimmune therapy. By augmenting Tim-3 signaling in autoaggressive T cells, one could potentially shift an inflammatory response characterized by tissue infiltration towards an exhausted response characterized by loss of T cell effector functions.

We report that Bat3 binds to catalytically active Lck, that Tim-3 receptor ligation abrogates this interaction, and that ablation of Bat3 expression in CD4⁺ T cells results in an accumulation of catalytically inactive Lck. In the absence of Bat3, CD3e^{lo} T cells upregulate their expression of Tim-3. In addition, we demonstrate that while Bat3 overexpression promotes IL-2 production, Bat3 loss of function leads to a downregulation of IL-2 production from T cells and a corresponding defect in T cell proliferation upon stimulation. Taken together with our preliminary findings that Tim-3 localizes to

supramolecular activation clusters (SMACs) upon T cell stimulation, our data suggest that in addition to its role in protecting Th1 cells from galectin-9-mediated cell death, Bat3 can repress the development of T cell exhaustion by positively regulating the function of signaling components downstream of the TcR-CD3 complex, thus promoting the ability of differentiated effector T cells to produce IL-2 and to proliferate in response to TcR driven signals. Interestingly, loss of IL-2 production and proliferative capacity are among the earliest indicators of T cell exhaustion³⁹, and we show that deeply exhausted Tim-3⁺ TILs, which display a defect in their ability to proliferate and to produce effector cytokines¹⁵, have reduced Bat3 expression. Further work is required to fully characterize the role of the Bat3/Tim-3 steady state interaction in inhibiting T cell exhaustion.

Recently, a mechanism was described by which Bat3 release in exosomes (from tumor cells) can engage Nkp30 on NK cells, thus mediating NK cell cytotoxicity and cytokine release⁴⁰. While highly novel, it is unlikely to account for the pro-inflammatory function of Bat3 that we see in our EAE models, as both the MOG_{35–55}+CFA model and the 2D2 cell transfer model are T-cell-driven models of central nervous system autoimmunity. Ex vivo analysis of T cells from *Bat3*^{-/-} → *Rag*^{-/-} MOG_{35–55}-immunized mice, as well as T cells from *Bat3*KD-2D2 → *Rag*^{-/-} transfer mice, indicated a defect in IFN γ production from these cells. This points strongly towards a T-cell intrinsic defect upon loss or reduction of Bat3 levels. Further, NK cells have been described as having an immunomodulatory rather than immunostimulatory role in the pathogenesis of MOG_{35–55}-induced EAE⁴¹.

Taken together, our findings suggest that inflammatory Th1 responses must operate under multiple levels of regulation. While Tim-3 inhibits Th1 function, Bat3 acts as a molecular safety catch that accumulates and binds to Tim-3 in Th1 cells. Interaction of Tim-3 with its ligand galectin-9 abrogates the Bat3/Tim-3 binding activity, allowing Tim-3 to mediate downregulatory signals that inhibit Th1 responses. Active downregulation of Bat3 expression induces an exhausted phenotype in Th1 cells that would otherwise be encephalitogenic, and Bat3 expression is lowered in Tim-3⁺ exhausted TILs and CD4⁺ T cells from individuals with chronic untreated HIV-1 viral infection. Manipulation of the interaction between Bat3 and Tim-3 could present a promising molecular target for therapeutic intervention in autoimmune disease, cancer and chronic viral infections.

Materials and Methods

Primary T cell culture, differentiation and functional analysis

CD4⁺, CD8⁺, CD19⁺ or CD11b⁺ cells were enriched from spleen/lymph node single cell suspensions using monoclonal Ab-labeled magnetic beads (Miltenyi). For some experiments, cells were labeled with fluorescent markers and sorted using a FACS Aria digital cell sorter (Becton Dickinson). Cells were cultured in DMEM (Invitrogen), supplemented as described⁶. For Th differentiation, CD4⁺ T cells were stimulated with plate-bound anti-CD3+anti-CD28 under Th0, Th1 (IL-12+anti-IL-4), Th2 (IL-4+anti-IFN γ) conditions. For experiments requiring Th17 (TGF β +IL-6) polarization, CD4⁺CD62L^{hi}CD25⁻ cells were purified and stimulated with anti-CD3 + anti-CD28, and the required cytokines and/or blocking antibodies were added. After 2 days, cells were transferred to uncoated plates with IL-2 (Th0, Th1 and Th2) or IL-23 (Th17). For additional rounds of polarization, cells were restimulated with platebound antibodies and the appropriate cytokines/blocking antibodies 5 days after the initial stimulation. For antigen-specific assays, T cells were stimulated with MOG_{35–55} (1 μ g/mL) or tumor antigen AHI1 (20 μ g/mL) and γ -irradiated splenocytes at a ratio of 1 T cell: 5 splenocytes. Supernatant for IFN γ ELISA (R&D Biosystems) was collected at 48 hrs, and if necessary, cells were then pulsed for 16 hours with 1.25 μ Ci/well [³H]-thymidine (Perkin-Elmer) for proliferation assays.

Real time PCR

Total RNA was extracted from target cells and tissues using RNEasy reagents (Qiagen) and reverse transcription was done with iScript Reverse Transcriptase (BioRad). Real-time PCR was done using validated primer-probe sets to murine *Bat3*, *Lag3*, *Prdm1* or *Pbx3*, human *Bat3*, the appropriate endogenous control β -actin (mouse) or GAPDH (human) primer-probe sets, Taqman FAST mix and a 7500 RT PCR cyclor (all Applied Biosystems) as described⁵.

Surface marker and intracellular cytokine staining analysis

Cells were labeled with fluorochrome-labeled antibodies against surface markers at 4°C. For intracellular cytokine staining, cells restimulated for 4h with 50 ng/mL PMA and 1 μ M ionomycin (both Sigma). They were fixed, permeabilized and stained for intracellular markers using the Fixation/Permeabilization Kit (BD). After >2 washes, cells were analyzed using a BD LSRII digital flow cytometer. Negative staining was determined using the appropriate isotype control antibody. Cell death was measured by 7-AAD (BD) positivity. All flow cytometric data were analyzed using FlowJo software (Treestar).

Retroviral transduction

Retroviral expression constructs were co-transfected into 293T cells along with *eco* and *gag/pol* viral envelope constructs. Viral supernatant was collected 48 hours post-transfection, and added to primary T cells that had been activated with platebound anti-CD3 + anti-CD28 for 24 hours. Cells were spun at 2000 rpm in the presence of polybrene (Sigma; 8 μ g/mL) for 45 minutes at room temperature and were then returned to 37°C. GFP⁺ cells could be detected 2–3 days post-infection.

Animals and induction of EAE

7 to 10 week old C57BL/6, SJL, BALB/cJ and *Rag*^{-/-} mice were purchased from the Jackson Laboratory. MOG_{35–55} antigen-specific 2D2 mice²³, *Tim*^{-3/-} mice⁷ and *Bat3*^{-/-} mice²⁰ have been described previously. *Rag*^{-/-} mice were sublethally irradiated and reconstituted with *Bat3*^{+/-} or *Bat3*^{-/-} fetal liver hematopoietic cells as described⁴². EAE was induced by subcutaneous injection of mice with 100 μ l of an emulsion containing CFA, 250 μ g Mycobacterium tuberculosis extract H37-Ra (Difco), and either 100 μ g MOG_{35–55} (MEVGWYRSPFSRVVHLYRNGK) or 100 μ g PLP_{139–151} peptide (HSLGKWLGHDPKF; QCB BioSource International). Mice received 200 ng pertussis toxin (List Biological Laboratories) intraperitoneally on days 0 and 2. For cell transfer experiments, 2D2 Th1 were retrovirally transduced and sorted on GFP⁺ 5 days after initial stimulation, 2 $\times$ 10⁶ GFP⁺ cells were transferred i.v. to each *Rag*^{-/-} recipient. All animals received 200 ng of pertussis toxin (List Biological Laboratories) i.p. at d0 and d2. Animals were monitored daily for development of EAE according to the following criteria: 0, no disease; 1, decreased tail tone; 2, hind limb weakness or partial paralysis; 3, complete hind limb paralysis; 4, front and hind limb paralysis; 5, moribund state. Mice were sacrificed at the indicated timepoints. All mice were housed in a specific pathogen-free animal facility, and all breeding and experiments were reviewed and approved by the appropriate institutional authorities.

Histopathological analysis

At sacrifice, brains and spinal cords were fixed in 10% neutral buffered formalin and processed routinely for paraffin embedment. Slides were stained with Luxol fast blue-H&E stains. Inflammatory foci (>10 mononuclear cells) were counted in leptomeninges and parenchyma in a blinded fashion.

Isolation of TILs

5×10^5 CT26 colorectal carcinoma cells, or 1×10^5 4T1 mammary adenocarcinoma cells, were implanted into the right flank of wild-type BALB/c mice. TILs were isolated by dissociating tumor tissue in the presence of 2.5 mg/ml collagenase D for 20 min before centrifugation on a discontinuous Percoll gradient (GE Healthcare). For some experiments, mice were treated with 100 μ g anti-Tim-3 blocking antibody (d0, 2, 4) and/or 200 μ g anti-PD-L1 blocking antibody (d0, 3, 6, 9, 12), or with the appropriate isotype control antibody.

Isolation of CD4⁺ T cells from HIV-infected individuals

PBMCs were isolated from leukapheresis specimens from three individuals with untreated chronic HIV-1 infection using Ficoll-Histopaque density centrifugation (Sigma-Aldrich). Viable Cells were stained for surface expression of CD3, CD4, Tim-3 and PD-1, and were sorted into CD3⁺CD4⁺Tim-3⁻PD-1⁺ and CD3⁺CD4⁺Tim-3⁺PD-1⁺ populations on a FACSaria cell sorter in a BL2 containment facility.

Statistical evaluation

A two-tailed, unpaired, Student's *t*-test was used for statistical evaluation for normally distributed data. Disease incidence data were analyzed using Fisher's exact test. Maximal disease scores were analyzed using the Mann-Whitney *U* test. To assess the differential onset of disease between *Bat3*^{+/-}→*Rag*^{-/-} and *Bat3*^{-/-}→*Rag*^{-/-} mice, linear regression analysis was performed to determine the slope of disease onset.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Bat3 binds to the Tim-3 tail in a galectin-9-dependent manner

a. Domain structure and location of tyrosine residues in the Tim-3 tail, and depiction of Tim-3 deletion and substitution constructs used for yeast two-hybrid screening and co-IP studies. **b.** Bat3-myc, Tim-3-HA and ICOS-HA fusion constructs were generated to confirm the specificity of the Bat3/Tim-3 interaction in mammalian cells. Bat3+Tim-3 or Bat3+ICOS were co-expressed in 293T cells and cell lysates were prepared for IP with anti-myc and blotting with anti-HA. Representative of eight experiments. **c.** Bat3-myc was co-expressed in 293T cells with HA-tagged Tim-3 deletion and substitution mutants. Lysates were prepared for IP with anti-myc and blotting with anti-HA. Representative of three experiments. **d.** Tim-3 and Bat3, or Tim-3_{2YF} and Bat3, were co-expressed in 293T with or without a galectin-9 expression plasmid. Cell lysates were prepared for IP with anti-myc and blotting with anti-HA. Representative of three experiments.

Figure 2. Bat3 promotes Th1 cell function and protects from galectin-9 - mediated cell death
a. B6 CD4⁺ T cells were polarized under Th0, Th1 or Th17 conditions for 4 days. Cell lysates were prepared and probed with Bat3 antisera (top) or anti- β -tubulin (bottom). Representative of three experiments. **b,c.** 2D2 CD4⁺ cells specific for myelin oligodendrocyte glycoprotein 35–55 (MOG_{35–55}) were polarized under Th1 conditions and transferred to *Rag*^{-/-} recipients. At 4 weeks post-transfer, splenocytes were recovered and analyzed for intracellular cytokine (ICC) expression on GFP⁺ cells by flow cytometry (**b**), or were restimulated with MOG_{35–55} peptide and assessed for presence of IFN γ in culture supernatant 48 hours later (**c**). **d.** B6 CD4⁺ cells were Th1 polarized and were infected with Bat3-RV or empty RV. GFP⁺ cells were sorted 2 days after 3rd round polarization and then cultured with or without galectin-9 for 3 hours. Cell death was assessed on the basis of 7AAD-positivity. Data in the bar graph (right) represent the % 7-AAD⁺ cells post-galectin-9 treatment divided by the % 7-AAD⁺ cells pre-treatment. Representative of three experiments.

Figure 3. Bat3 expression in the hematopoietic compartment promotes CNS autoimmunity

a. $Bat3^{+/-} \rightarrow Rag^{-/-}$ and $Bat3^{-/-} \rightarrow Rag^{-/-}$ fetal liver chimeras were immunized subcutaneously with 100 μ g MOG in complete Freund's adjuvant (CFA), and EAE development was monitored. The mean disease score of each group is shown. Representative of two experiments. 11 $Bat3^{+/-} \rightarrow Rag^{-/-}$ (■) and 8 $Bat3^{-/-} \rightarrow Rag^{-/-}$ (▲) mice were analyzed in total. **b.** Linear regression curves of the acute phase of the disease are shown for the experiment represented in (a). The slopes are significantly different between the two groups ($p < 0.0001$). The 95% confidence intervals for each curve are depicted with dashed lines. **c.** CD4 $^{+}$ cells from immunized $Bat3^{+/-} \rightarrow Rag^{-/-}$ (■; n=7) and $Bat3^{-/-} \rightarrow Rag^{-/-}$ (▲; n=5) mice were isolated 38 days after MOG immunization, and intracellular cytokine production was measured. Each data point represents an individual mouse.

Figure 4. Loss of Bat3 function induces an exhausted T cell phenotype and Bat3 regulates T cell receptor signaling

a,b,c,e. CD4 $^{+}$ T cells were isolated from $Bat3^{+/-} \rightarrow Rag^{-/-}$ and $Bat3^{-/-} \rightarrow Rag^{-/-}$ mice, and were stimulated with anti-CD3+anti-CD28 for 5 days. **a.** Cells were assessed for intracellular expression of IFN γ , IL-2 and IL-10, and for surface expression of Tim-3. **b.** mRNA were extracted, and *Lag3*, *Prdm1* and *Pbx3* message were assessed by quantitative PCR. **c.** Cells were gated on level of CD3 ϵ expression and were analyzed for Tim-3 surface levels by flow cytometry. **d.** 293T cells were transfected with Lck plus either HA-tagged wildtype Tim-3 or HA-tagged Tim-3 lacking the intracellular domain (Tim-3 ΔIC). Cell lysates were prepared, immunoprecipitated with anti-HA and blotted with anti-Lck. **e.** Th1 cells were transduced with a Bat3 RV construct containing a C-terminal myc tag and bicistronic GFP reporter. Cells were sorted for GFP positivity and were incubated for 20 minutes with 10 μ g/mL anti-Tim-3 or mIgG1. Lysates were prepared for IP with anti-myc and IB with anti-phospho-Src to detect pLck(Y394), pLck(Y505) or anti-Lck(total). Representative of three experiments. **f.** $Bat3^{+/-} \rightarrow Rag^{-/-}$ and $Bat3^{-/-} \rightarrow Rag^{-/-}$ CD4 $^{+}$ T cells were stimulated for 5 days with anti-CD3+anti-CD28. Cell lysates were prepared and blotted for anti-phospho-Lck(Y505). Representative of three experiments.

Figure 5. Bat3 expression in CD4⁺ T cells is critical for the induction of Th1-driven EAE
a. GFP⁺-sorted, control- or Bat3KD1-RV infected, 2D2 Th1 cells were transferred to *Rag*^{-/-} recipients, which were monitored for EAE development. Control 2D2 → *Rag*^{-/-} (■), Bat3KD1-2D2 → *Rag*^{-/-} (▲). Representative of 1 of 3 transfers. 13 control-2D2 and 14 Bat3KD1-2D2 recipients were analyzed in total. **b.** CNS-infiltrating mononuclear cells were collected at day 38 post-transfer from *Rag*^{-/-} mice that had received control- or Bat3KD1 RV-infected 2D2 Th1 cells, and were analyzed for CD4 expression by flow cytometry. Representative of 4 control-2D2 and 2 Bat3KD1-2D2 recipients. **c,d.** Lymph node cells were collected at day 38 post-transfer from *Rag*^{-/-} mice that had received control- or Bat3KD1 RV-infected 2D2 Th1 cells, and ICC staining was performed for IFN γ (**c**, **d**) and IL-10 (**d**). **c.** Plots gated on CD4⁺. Data are representative of 4 (control; ■) or 5 (Bat3KD1; ▲) mice. **d.** Data first gated on CD4⁺GFP⁺ cells. Each data point represents an individual mouse. **e.** Th1 cells were transduced with empty, Tim-3_{IC} or Tim-3_{IC}ΔBat3BD-expressing retrovirus. GFP⁺ cells were analyzed for intracellular expression of IFN γ 5 days after 2nd round stimulation. Representative of three experiments. **f.** *Tim-3*^{+/+} and *Tim-3*^{-/-} Th1 cells were infected with either control-, Bat3KD1- or Bat3KD2-expressing RV and cultured for 5 days. Cells were sorted for GFP positivity and were restimulated with 1 μ g/mL anti-CD3+anti-CD28 for 48 hours before [3H]-thymidine pulse for an additional 16 hours. Representative of three experiments. **g.** *Tim-3*^{+/+} and *Tim-3*^{-/-} Th1 cells were infected with either control- or Bat3KD1-expressing RV and cultured for 5 days. Cells were assessed for intracellular expression of IFN γ and IL-2 (gated on GFP⁺). Representative of three experiments.

Figure 6. Loss of Bat3 results in an exhausted-like phenotype in T cells

a. TILs were isolated from implanted 4T1 mammary adenocarcinomas 26 days post-implantation, and were analyzed for intracellular production of IFN γ and TNF α . Each data point represents an individual mouse. **b.** Lymphocytes in draining lymph nodes from CT26 tumor bearing mice treated with anti-Tim-3 or anti-PD-L1 antibody, or both, were stimulated in vitro with tumor antigen (AH1) for 48 hours and then measured for IFN γ in the supernatant. * p < 0.05, ** p < 0.01, *** p < 0.001. **c.** TILs were isolated from implanted CT26 colorectal carcinomas, 21 days post-injection. Cells were sorted into Tim-3⁺PD-1⁺ and Tim-3⁺PD-1⁻ subpopulations, mRNA were isolated and Bat3 message expression was assessed. Data were normalized to β -actin levels. Data represent pooled tumors from ten recipients. **d.** CD4⁺Tim-3⁺PD-1⁺ and CD4⁺Tim-3⁺PD-1⁻ populations were sorted from the peripheral blood of three antiretroviral therapy-naïve individuals with chronic progressive HIV-1 infection. mRNA were prepared from each subpopulation, and Bat3 message was assessed by quantitative PCR. Patient 1, p < 0.024; patient 2, p < 0.004; patient 3, p < 0.004. **e.** 2D2 Th1 cells infected with Bat3KD1 RV were transferred into *Rag*^{-/-} mice, and were analyzed 38 days later for IFN γ and Tim-3 co-expression. 5 of 11 Bat3KD1-2D2 recipients displayed GFP⁺CD4⁺Tim-3^{hi}IFN γ ^{lo} cells in peripheral lymphoid tissues (*right panel*). GFP⁺CD4⁺ cells from control-2D2 mice were Tim-3⁺IFN γ ^{hi} (*left panel*, representative of 9 mice).