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Tim-3 Directly Enhances CD8 T Cell Responses to Acute Listeria monocytogenes Infection

Jacob V. Gorman,* Gabriel Starbeck-Miller,* Nhat-Long L. Pham,* Geri L. Traver,† Paul B. Rothman,*‡ John T. Harty,*‡§ and John D. Colgan*,*¶

T cell Ig and mucin domain (Tim) 3 is a surface molecule expressed throughout the immune system that can mediate both stimulatory and inhibitory effects. Previous studies have provided evidence that Tim-3 functions to enforce CD8 T cell exhaustion, a dysfunctional state associated with chronic stimulation. In contrast, the role of Tim-3 in the regulation of CD8 T cell responses to acute and transient stimulation remains undefined. To address this knowledge gap, we examined how Tim-3 affects CD8 T cell responses to acute Listeria monocytogenes infection. Analysis of wild-type (WT) mice infected with L. monocytogenes revealed that Tim-3 was transiently expressed by activated CD8 T cells and was associated primarily with acquisition of an effector phenotype. Comparison of responses to L. monocytogenes by WT and Tim-3 knockout (KO) mice showed that the absence of Tim-3 significantly reduced the magnitudes of both primary and secondary CD8 T cell responses, which correlated with decreased INF-γ production and degranulation by Tim-3 KO cells stimulated with peptide Ag ex vivo. To address the T cell–intrinsic role of Tim-3, we analyzed responses to L. monocytogenes infection by WT and Tim-3 KO TCR-transgenic CD8 T cells following adoptive transfer into a shared WT host. In this setting, the accumulation of CD8 T cells and the generation of cytokine-producing cells were significantly reduced by the lack of Tim-3, demonstrating that this molecule has a direct effect on CD8 T cell function. Combined, our results suggest that Tim-3 can mediate a stimulatory effect on CD8 T cell responses to an acute infection. The Journal of Immunology, 2014, 192: 3133–3142.

The generation of effective CD8 T cell responses to infection requires TCR engagement by peptide–MHC I complexes, costimulation, and inflammation, each of which activates intracellular signaling pathways that drive cell proliferation and differentiation. The combined influence of these signaling pathways is a major determinant of the magnitude and quality of primary CD8 T cell responses and also dictates the functional properties acquired by memory CD8 T cells. With respect to costimulation, the emerging model is that this process involves the function of coinhibitory as well as costimulatory receptors and that the balance struck between these opposing factors has a dominant role in the outcome of primary CD8 T cell responses (for review see Ref. 1). Costimulatory receptors expressed by CD8 T cells include CD28, OX-40, and 4-1BB, whereas examples of coinhibitory receptors include CTLA-4, PD-1, LAG-3, and T cell Ig and mucin domain (Tim)-3.

Tim-3 is a member of the Tim family, which encompasses a group of type I transmembrane proteins expressed throughout the immune system (2–4). The Tim family in humans consists of three proteins (Tim-1, -3, and -4), whereas mice express four Tim proteins (Tim-1, -2, -3, and -4). Each of the Tim proteins contains an extracellular domain consisting of an N-terminal Ig V region–like (IgV) domain and mucin-like region that is subject to glycosylation. Published reports have shown that the IgV domains of Tim-1, -3, and -4 bind phosphatidylserine and that these proteins can function to promote the clearance of apoptotic cells (5–8). All Tim molecules also contain a C-terminal cytoplasmic tail that varies in length between family members. Although no function has been ascribed to the cytoplasmic tail of Tim-4, biochemical analysis has demonstrated that tyrosine residues within the tails of Tim-1 and Tim-3 can be phosphorylated by Src family kinases (9–13). These and other studies (14–17) have suggested that Tim-1 and Tim-3 influence signal transduction pathways known to regulate immune cell function. Studies published thus far have indicated that Tim-3 has a widespread and complex role in immune system regulation. Tim-3 was originally identified as a surface molecule expressed on the surface of Th1-type CD4 T (Th1) effector cells (18). This report and others that followed (7, 15, 19–23) demonstrated that Tim-3 is also expressed by dendritic cells, microglia, macrophages, mast cells, NK cells, and activated CD8 T cells. Regarding cells within the innate system, several studies have indicated that Tim-3 can promote activation and inflammatory cytokine production (15, 18, 24–26). However, other studies have shown that Tim-3 can suppress certain aspects of innate cell function, including IL-12 secretion and TLR-mediated activation (27–30). Similarly, Tim-3 has been shown to mediate both positive and negative effects on NK cell activation and functionality (21, 22, 31).

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Abbreviations used in this article: Act-LM-OVA, attenuated (actA-)/L. monocytogenes strain engineered to express OVA; Gal-9, Galectin-9; IgV, Ig V region–like; KO, knockout; LM-OVA, L. monocytogenes engineered to express OVA; OVAp, peptide spanning OVA residues 257–264, p.i., postinfection; RT, room temperature; Tim, T cell Ig and mucin domain; virLM-OVA, virulent LM-OVA; WT, wild-type.

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Most published reports that have addressed how Tim-3 affects T cell responses have provided evidence for an inhibitory role. For example, injection of Abs specific for Tim-3 (18, 32) or Tim-3–Fc fusion proteins (19, 33) was shown to exacerbate Th1-type responses by CD4 T cells in various disease models, suggesting that Tim-3–ligand interactions function to restrain Th1 responses. Studies involving Tim-3–deficient mice also provided support for this conclusion (19, 33, 34). Efforts to identify Tim-3 ligands led to the discovery that the glycan-binding protein Galectin-9 (Gal-9) has specificity for carbohydrate moieties attached to Tim-3 and showed that Th1 cells undergo apoptosis in response to the binding of Gal-9 to Tim-3 (35). In addition, several studies have demonstrated that Tim-3 is expressed by functionally impaired or “exhausted” CD8 T cells (36–43), which have been found to be generated as a consequence of immune responses to chronic infections or tumors (44). Further, combined blockade of ligand–receptor interactions by Tim-3 and PD-1 was shown to restore function to exhausted CD8 T cells (38, 39), suggesting that Tim-3 has a role in pathways that bring about CD8 T cell exhaustion.

As outlined above, a majority of published studies examined how Tim-3 influences T cell responses in the contexts of immune-mediated disease or chronic stimulation, which are conditions associated with immune system dysfunction. In contrast, the role of Tim-3 in T cell responses to acute stimulation has not been determined. To address this knowledge gap, we generated Tim-3–deficient mice and analyzed CD8 T cell responses in these animals to acute infection by strains of the facultative intracellular bacterium Listeria monocytogenes engineered to express OVA (LM-OVA). We found that the absence of Tim-3 impaired both primary and secondary CD8 T cell responses to LM-OVA infection. To determine whether this phenotype involved defects intrinsic to CD8 T cells, we used a coadaptor transfer system that allowed us to analyze responses to LM-OVA infection by wild-type (WT) and Tim-3–deficient CD8 T cells within the same host. In this context, the lack of Tim-3 expression by CD8 T cells resulted in impaired effector responses by both naive and memory cells concomitant with reductions in the number of cells that were generated. Combined, our data indicate that Tim-3 can function to promote CD8 T cell responses to acute infection through a cell-intrinsic mechanism.

Materials and Methods

Mice

Naive mice were housed in specific pathogen-free animal facilities and transferred to biosafety level 2 conditions for infection studies. WT, Thy1.1 (Thy1.1) congenic, and OT-1 TCR transgenic (OT-1) mice (45) of the C57BL/6J genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-1 mice generate CD8 T cells specific for a peptide spanning OVA residues 257–264 bound to the MHC I protein H-2Kd. Mice lacking Haver2, the gene encoding Tim-3, were generated in collaboration with Regeneron (Tarrytown, NY). Mouse embryonic stem cells of the 129 genetic background were electroporated with a targeting vector that confers neomycin resistance. Drug-resistant embryonic stem clones containing a disrupted Haver2 allele were identified and used to generate chimeric mice that transmitted the mutant allele to offspring. The disrupted Haver2 allele was transferred into the C57BL/6J background by performing 10 serial backcrosses. The resulting strain was used to generate Tim-3 knockout (KO) and Tim-3 KO OT-1 mice. Thy1.1/Thy1.2 (Thy1.1/Thy1.2) OT-1 mice were generated in-house. All animal procedures were performed according to guidelines established by the University of Iowa Institutional Animal Care and Use Committee.

L. monocytogenes infections

Generation and growth of virulent and attenuated (actA-deficient) strains of LM-OVA have been described previously (46, 47). Mice were infected by i.v. injecting 1 × 10⁷ CFU of actA-deficient LM-OVA or 1 × 10⁸ CFU of virulent LM-OVA (virLM-OVA).

Coadaptive transfer of OT-1 CD8 T cells

Peripheral blood samples were collected from WT (Thy1.1/Thy1.2) and Tim-3 KO (Thy1.2/Thy1.2) OT-I mice and depleted of B cells using anti-Vβ5 and anti-Vβ12 antibodies, as described for flow cytometric analysis. WT and Tim-3 KO samples were diluted in saline and mixed to generate a 1:1 ratio between the different OT-I cells, which was confirmed by subsequent flow cytometric analysis. A total of 2000 OT-I cells (1000 WT cells and 1000 Tim-3 KO cells) were injected i.v. into naive Thy1.1/Thy1.1 hosts, which were infected with actA-deficient LM-OVA on the next day. To analyze responses by memory cells, 10,000 WT (Thy1.1/Thy1.2) or Tim-3 KO OT-I (Thy1.2/Thy1.2) clones were transferred into multiple (six or seven) separate naive WT (Thy1.1/Thy1.1) hosts, which were infected with actA-deficient LM-OVA on the next day. At 43 d later, splenocytes were harvested and stained with Thy1.2-PE. OT-I cells were then isolated by positive selection, using PE-specific magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Recovered cells were diluted in saline and mixed to generate a 1:1 ratio between WT and KO OT-I cells. A total of 140,000 OT-I cells (4000 per cell population) were injected i.v. into naive WT Thy1.1/Thy1.1 hosts, which were infected with actA-deficient LM-OVA on the next day.

Flow cytometric analysis

Peripheral blood samples were collected from the retro-orbital plexus. Spleen cell suspensions were generated by pushing the organ through a 70-µm wire mesh. Cell suspensions were treated with Pharm Lyse (BD Biosciences, San Jose, CA) to deplete RBCs. Samples were resuspended in staining buffer (PBS containing 0.5% FBS) and incubated with anti-mouse CD16/32 (eBioscience, San Diego, CA) to prevent nonspecific Ab binding. Cells were incubated with fluorochrome-conjugated Abs for 30 min on ice, washed twice with stain buffer, and then fixed using Cytofix buffer (BD Biosciences). Flow cytometric analysis was performed using an LSR II (BD Biosciences), and collected data were analyzed using Flowjo (TreeStar, Ashland, OR). For all data analysis, debris and dead cells were excluded by gates drawn on plots of forward scatter area versus side scatter, and cell doublets were excluded by gates drawn on plots of forward scatter area versus forward scatter width. Fluorochrome-conjugated Tim-3 Abs (clone 215008) was purchased from R&D Systems. All other fluorochrome-conjugated Abs were purchased from BioLegend (San Diego, CA); BD Biosciences, or eBioscience. Abs clones used are as follows: CD8 (56.7), CD11a (M17/4), CD107a (H4A3), CD127 (LG.3A10), IFN-γ (XMIG1.2), KLRL1 (2F1), Thy1.1 (OX-7), Thy1.2 (53-2.1), and TNF (MP6-XT22). MHC I H-2Kd–OVA257–264 peptide tetramers were generated as previously described (48, 49).

Ex vivo peptide stimulation and intracellular cytokine staining

Medium and additives were obtained from Gibco–Life Technologies (Carlsbad, CA). Spleen cells were resuspended in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 µM 2-ME. OV A 257–264 peptide (1 µM) and GolgiPlug or GolgiStop (BD Biosciences) were added to aliquots of cells, followed by incubation for 5 h at 37°C in a 5% CO2 incubator. Cells were washed with stain buffer and incubated with Abs for cell surface markers. After washing, cells were resuspended in Cytofix/Cytoperm (BD Biosciences) and collected data were analyzed using Flowjo. Spleen cell suspensions were generated by pushing the organ through a 70-µm wire mesh. Cell suspensions were treated with Pharm Lyse (BD Biosciences, San Jose, CA) to deplete RBCs. Samples were resuspended in staining buffer (PBS containing 0.5% FBS) and incubated with anti-mouse CD16/32 (eBioscience, San Diego, CA) to prevent nonspecific Ab binding. Cells were incubated with fluorochrome-conjugated Abs for 30 min on ice, washed twice with stain buffer, and then fixed using Cytofix buffer (BD Biosciences). Flow cytometric analysis was performed using an LSR II (BD Biosciences), and collected data were analyzed using Flowjo (TreeStar, Ashland, OR). For all data analysis, debris and dead cells were excluded by gates drawn on plots of forward scatter area versus side scatter, and cell doublets were excluded by gates drawn on plots of forward scatter area versus forward scatter width. Fluorochrome-conjugated Tim-3 Abs (clone 215008) was purchased from R&D Systems. All other fluorochrome-conjugated Abs were purchased from BioLegend (San Diego, CA); BD Biosciences, or eBioscience. Abs clones used are as follows: CD8 (56.7), CD11a (M17/4), CD107a (H4A3), CD127 (LG.3A10), IFN-γ (XMIG1.2), KLRL1 (2F1), Thy1.1 (OX-7), Thy1.2 (53-2.1), and TNF (MP6-XT22). MHC I H-2Kd–OVA257–264 peptide tetramers were generated as previously described (48, 49).

Intracellular staining for Bim and Bcl-xL

Spleen cells were stained for cell surface markers and resuspended in Cytofix (BD Biosciences). After 15 min at 4°C, cells were pelleted by centrifugation and resuspended in Perm Buffer III (BD Biosciences). After 30 min at 4°C, cells were washed and then incubated in stain buffer containing 50 µM 2-ME, OVA257-264 peptide (1 µM) and GolgiPlug or GolgiStop (BD Biosciences) were added to aliquots of cells, followed by incubation for 5 h at 37°C in a 5% CO2 incubator. Cells were washed with stain buffer and incubated with Abs for cell surface markers. After washing, cells were resuspended in Cytofix/Cytoperm (BD Biosciences). After 15 min at room temperature (RT), cells were washed with Perm/Wash (BD Biosciences), stained with Abs specific for CD107a, IFN-γ, or TNF, and processed for flow cytometric analysis.

Analysis of caspase activity

Spleen cells were resuspended in RPMI 1640 supplemented with 10% FBS, 1% L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 µM 2-ME. FLICA reagent (Life Technologies–Invitrogen, Carlsbad, CA) was added, and cells were incubated for 1.5 h. Cells were washed, incubated with fluorochrome-conjugated anti-rabbit secondary Abs, and then processed for flow cytometric analysis. Rabbit Abs specific for Bim and Bcl-xL were obtained from Cell Signaling Technology/Millipore (Billerica, MA).

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incubated with Abs for cell surface markers, and processed for flow cytometric analysis.

### Analysis of cell proliferation by BrdU incorporation

All fixation and permeabilization reagents were obtained from BD Biosciences. Mice were injected i.p. with 2 mg BrdU (Sigma-Aldrich, St Louis, MO) dissolved in saline. At 15 h later, spleens were harvested and single-cell suspensions prepared. Cells were incubated with Abs specific for cell surface markers, washed, and resuspended in Cytofix/Cytoperm. After 20 min at RT, cells were washed with Perm/Wash, incubated in Cytoperm Plus for 10 min at RT, washed again with Perm/Wash, and resuspended in Cytofix/Cytoperm. After 5 min at RT, cells were washed with Perm/Wash, resuspended in PBS containing 300 μg/ml DNase I (Sigma-Aldrich), and incubated for 1.5 h at 37°C in a 5% CO2 incubator. After washing with Perm/Wash, cells were stained with anti-BrdU (eBioscience; clone BU20A) and processed for flow cytometric analysis.

### Statistical analysis

Data were analyzed using the unpaired, two-tailed Student t test within the Prism software program (GraphPad Software, La Jolla, CA).

### Results

#### Effector CD8 T cells generated by L. monocytogenes infection express Tim-3

Initially, we analyzed Tim-3 expression by CD8 T cells in WT mice infected with L. monocytogenes. Mice were injected with an attenuated (actA-) L. monocytogenes strain engineered to express OVA (attLM-OVA). Afterward, longitudinal analysis of splenocytes was performed to monitor CD8 T cell responses. To assess Tim-3 expression across polyclonal CD8 T cell populations, previously described surrogate activation markers (50) were used to identify CD8 T cells responding to the infection; these were classified as Thy1.2+ cells expressing low levels of CD8α and high levels of CD11a (CD8αloCD11ahi; see Supplemental Fig. 1A). Analysis of naive mice showed that only a small fraction (∼2%) of CD8 T cells expressed Tim-3 in the absence of infection. In LM-OVA–infected mice, the majority (∼75%) of CD8αloCD11ahi cells were Tim-3+ on day 7 postinfection (p.i.), whereas at later time points the frequencies of CD8αloCD11ahi cells expressing Tim-3 were progressively smaller (Fig. 1A). We also analyzed Tim-3 expression by CD8 T cells that responded to a secondary LM infection, which was initiated by injecting virLM-OVA into mice that had been infected with attLM-OVA 50 d earlier. Similar to that observed on day 7 of the primary response, most (∼71%) of the CD8αloCD11ahi cells present on day 6 of the secondary response were Tim-3+ (Fig. 1A).

Prior studies have demonstrated that microbial infections generate effector and memory precursor CD8 T cells, which can be defined as KLRG1−CD127+ and KLRG1+CD127+ cells, respectively (51–53). Therefore, we performed longitudinal analysis of Tim-3 expression by these two subsets (Fig. 1B, 1C). Analysis of CD8αloCD11ahi populations confirmed that KLRG1−CD127+ and KLRG1+CD127+ cells were generated in response to attLM-OVA infection. On day 7 p.i., nearly all (∼92%) of KLRG1+CD127+ cells were Tim-3+, whereas about one third (∼34%) of the KLRG1−CD127+ cells were Tim-3+ at the same time point. On days 14 and 50 p.i., ∼30% KLRG1+CD127+ cells were Tim-3+, indicating that a subset of these cells maintained Tim-3 expression long-term. In contrast, the frequencies of Tim-3–expressing KLRG1+CD127+ cells detected on days 14 and 50 p.i. were substantially lower (14% and 9%, respectively), indicating that frequency of Tim-3+ cells in this fraction decreased with time. We also analyzed Tim-3 expression by KLRG1−CD127− and KLRG1−CD127− generated in response to secondary LM-OVA infection (Fig. 1B, 1C). Similar to that seen on day 7 after a primary infection, almost all of the KLRG1−CD127− cells (∼93%) present 6 d after secondary infection were Tim-3−, whereas a smaller percentage (∼26%) of KLRG1−CD127+ cells present at this time point expressed Tim-3.

These data demonstrate that Tim-3 expression is associated with acquisition of effector phenotypes by activated CD8 T cells.

LM-OVA infection generates CD8 T cells specific for a peptide spanning OVA residues 257–264 (OVAp), providing a means to evaluate Ag-specific responses. To assess Tim-3 expression by stimulated OVAp-specific CD8 T cells, splenocytes were isolated on day 7 p.i. and pulsed with OVAp. As identified by CD107a and IFN-γ expression, essentially all of the cells in the peptide-responsive subset were Tim-3+ (Fig. 1D). These data indicate that CD8 T cells elaborating Ag-induced effector function express Tim-3. In
addition, these data add support to the conclusion that Tim-3 is expressed by CD8 T cells with an effector phenotype.

The absence of Tim-3 expression impairs CD8 T cell responses to L. monocytogenes

The data described above show that a large fraction of effector CD8 T cells responding to LM-OVA infection express Tim-3. Therefore, we sought to determine whether the absence of Tim-3 had any impact on CD8 T cell responses to LM-OVA. Cohorts of WT and Tim-3 KO mice were infected with attLM-OVA, and longitudinal analysis of CD8 T cell responses was carried out. To assess polyclonal responses, we tracked CD8α^loCD11ahi populations in peripheral blood and spleen. On days 6, 7, and 8 p.i., the frequencies of CD8α^loCD11ahi cells in peripheral blood from Tim-3 KO mice were significantly lower relative to those in WT mice (Fig. 2A, Supplemental Fig. 1A). Likewise, the total numbers of CD8α^loCD11ahi cells in splenocytes from Tim-3 KO mice were significantly decreased on days 7 and 14 p.i. (Fig. 2B, Supplemental Fig. 1B).

To assess OVA-specific CD8 T cell responses to attLM-OVA infection, spleen samples were taken on days 7, 15, and 40 p.i. and stained with MHC I tetramers loaded with OVA257–264 peptide (OVA tetramers). Consistent with the results from analysis of polyclonal responses, samples from Tim-3 KO mice contained significantly fewer OVA tetramer^+ CD8 T cells on days 7 and 15 p.i. (Fig. 2C, 2D). To further assess OVA-specific CD8 T cell responses, splenocytes were isolated from WT and Tim-3 KO mice on days 7, 15, and 40 p.i. and pulsed with OVAp to elicit IFN-γ production and degranulation (Fig. 2E–G, Supplemental Fig. 1C–E). This analysis showed that, on days 7 and 15 p.i., the frequencies and numbers of IFN-γ–producing or CD107a^+ CD8 T cells in samples from Tim-3 KO mice were significantly decreased relative to those from WT mice, confirming that OVA-specific responses to the infection were decreased in the mutant mice. These data indicate that primary CD8 T cell responses to LM-OVA infection are impaired by the absence of Tim-3.

In contrast to what was observed on days 6 through 15 p.i., analysis of samples taken at later time points did not reveal significant differences between CD8α^loCD11ahi populations in WT and Tim-3 KO mice (Fig. 2, Supplemental Fig. 1). These data indicate that LM-induced CD8 T cell responses in Tim-3 KO mice normalize with time.

Responses by Tim-3 KO CD8 T cells are impaired following transfer to a normal host

The defects observed in Tim-3 KO mice support the hypothesis that Tim-3 has a direct role in promoting CD8 T cell responses to LM infection. To test this hypothesis, we used a adoptive transfer system in which responses by WT and Tim-3 KO CD8 T cells within the same WT host could be monitored (Fig. 3A). WT (Thy1.1/Thy1.2) or Tim-3 KO (Thy1.2/Thy1.2) OT-I CD8 T cells were mixed 1:1, and a total of 2000 OT-I cells (1000 of each population) were transferred into WT C57BL/6J (Thy1.1/Thy1.1) hosts. Cell mixtures were analyzed immediately prior to transfer to confirm that hosts received a 1:1 ratio of WT and Tim-3 KO cells (Fig. 3B). Hosts were infected with attLM-OVA, and responses by the transferred cells were analyzed at time points thereafter. As part of these studies, longitudinal analysis of splenocytes was performed to assess Tim-3 expression by OT-I cells following injection of attLM-OVA (Fig. 3C, 3D). On days 6 and 7 p.i., ~70% of WT OT-I cells were Tim-3^−, whereas at later time points, the frequencies of WT OT-I cells that expressed Tim-3 were lower and became progressively smaller with time. These data confirmed that WT OT-I cells expressed Tim-3 in response to LM-OVA infection and that the temporal pattern of Tim-3 expression by these cells was similar to that displayed by polyclonal CD8 T cell populations generated in response to LM-OVA infection (Fig. 1A).

We also analyzed surface expression of KLRG1 and CD127 by the transferred cells on day 6 p.i. (Fig. 3E, Supplemental Fig. 2A); this revealed that, relative to WT cells, the frequency of KLRG1^+ CD127^− cells in the KO cell fraction was modestly but significantly increased concomitant with a decrease in the frequency of KLRG1^− CD127^+ cells.

To assess effector responses by the transferred cells, splenocytes were isolated from hosts on day 7 p.i. and pulsed with OVAp ex vivo. Of note, the frequencies of Tim-3 KO OT-I cells expressing IFN-γ or TNF were significantly reduced relative to WT (Fig. 3F–H). Thus, similar to what was observed in mice lacking Tim-3 entirely, Tim-3 KO CD8 T cells within a normal host generate fewer cells with the ability to produce effector cytokines, indicating that Tim-3 has a cell-intrinsic effect on CD8 T cell responses.

We also performed longitudinal analysis of the transferred OT-I cells by taking peripheral blood and splenocyte samples at progressively later time points p.i. Notably, analysis of peripheral blood samples obtained on days 8 and 20 p.i. showed that the ratio of WT and Tim-3 KO cells increased with time (Fig. 4A–C). Similar
results were obtained when WT and Tim-3 KO OT-I cells in spleen were analyzed at different time points up to day 68 p.i. (Fig. 4D, 4E). Moreover, this analysis showed that, between days 5 and 11 p.i., the ratio between WT and Tim-3 KO cells in spleen progressively increased, reaching a maximum of ∼4:1 that was maintained up to day 68 p.i. (Fig. 4E). These data indicate that the absence of Tim-3 expression by CD8 T cells directly affects the ability of these cells to persist during the contraction phase of the response. Further, on the basis of the analysis of splenic OT-I cells present on day 7 p.i., the lack of Tim-3 affected the persistence of both KLRG1+CD127− and KLRG1−CD127+ cells (Supplemental Fig. 2B).

**Lack of Tim-3 reduces proliferation, but not survival, by adoptively transferred CD8 T cells**

To determine how persistence of Tim-3 KO CD8 T cells might be impaired, we performed studies that analyzed markers for cell death and proliferation at different time points after LM-OVA infection. We found that the proapoptotic factor Bim and the antiapoptotic factor Bcl-xL were both expressed at similar levels in WT and Tim-3 KO OT-I cells (Fig. 5A, 5B, Supplemental Fig. 3). In addition, similar levels of activated caspases 3 and 7 were detected in WT and Tim-3 KO cells (Fig. 5C, 5D). To assess cell proliferation, the nucleotide analog BrdU was injected into LM-infected mice on different days p.i., and BrdU incorporation into the DNA of OT-I cells was analyzed 15 h later. This approach showed that, relative to WT cells, BrdU incorporation by Tim-3 KO CD8 T cells was significantly reduced when assessed on days 6 and 7 p.i. (Fig. 5E, 5F), indicating that the KO cells underwent less proliferation during this period. Furthermore, subdividing CD8 T cells into KLRG1+CD127− and KLRG1−CD127+ showed that proliferation by both subsets was impaired (Fig. 5G).

**FIGURE 3.** Decreased responses to LM by Tim-3 KO CD8 T cells within a WT host. (A) Outline of the coadaptive transfer system used to assess responses by WT and Tim-3 KO OT-I CD8 T cells within a shared WT host. Samples containing 1000 WT and 1000 KO OT-I cells of the indicated Thy1 allotypes were prepared and injected into Thy1.1/1.1 hosts. The next day, hosts were infected with attLM-OVA. (B) Frequencies of WT and Tim-3 KO OT-I cells in cell mixtures immediately prior to injection into hosts. (C) Tim-3 expression by splenic OT-I cells, as determined on the indicated days p.i. (D) Longitudinal analysis of Tim-3 expression by WT OT-I cells in spleen. Each symbol represents the mean and SE of between 4 and 20 data points. (E) Expression of KLRG1 and CD127 by OT-I cells in splenocytes obtained on day 7 p.i. (F and G) Expression of IFN-γ (F) or TNF (G) by OT-I cells in splenocytes following ex vivo stimulation with OVA p. Data were obtained from spleens isolated on day 6 p.i. (H) Frequencies of IFN-γ+ and TNF+ cells within the fractions of WT and Tim-3 KO OT-I cells detected in splenocytes. Frequencies were calculated from data represented in (E) and (F). All data shown are representative of results from at least two independent experiments. Each set of box and whiskers was generated from 12 data points, **p ≤ 0.01.

**FIGURE 4.** The ability of Tim-3 KO CD8 T cells to persist after LM infection is impaired. WT and Tim-3 KO OT-I cells (1000 each) were injected into WT hosts. The next day, hosts were infected with attLM-OVA. OT-I cells were analyzed as indicated. (A and B) Frequencies of WT and Tim-3 KO OT-I cells in peripheral blood on day 8 (A) or day 20 (B) p.i. with attLM-OVA. (C) Ratios between WT and Tim-3 KO OT-I cells in peripheral blood samples taken on the indicated days p.i. (D) Frequencies of WT and Tim-3 KO OT-I cells in splenocytes obtained on the indicated days p.i. (E) Ratios between WT and Tim-3 KO OT-I cells in splenocytes obtained on the indicated days p.i. Filled circles in (C) and (E) represent the mean and SE of values from 4 to 16 independent samples. Flow cytometric data shown are representative of results from at least three independent experiments.
Tim-3 deficiency impairs secondary CD8 T cell responses to L. monocytogenes

Given the deficits in the primary CD8 T cell response to L. monocytogenes, we sought to determine whether the absence of Tim-3 affected CD8 T cell responses to a secondary LM-OVA infection. WT and Tim-3 KO mice given attLM-OVA 50 d earlier were infected with virLM-OVA, and longitudinal analysis of OVAp-specific CD8 T cell responses was performed by OVA tetramer staining. Immediately prior to secondary challenge, the frequencies of OVAp-specific CD8 T cells in peripheral blood from WT and Tim-3 KO mice appeared similar (~0.3% of the CD8 T cell pool). However, following virLM-OVA infection, the frequencies of these cells in peripheral blood from Tim-3 KO mice were significantly reduced relative to those observed in WT samples at all time points evaluated (Fig. 6A). Similarly, splenocytes from Tim-3 KO mice obtained 6 or 13 d after secondary infection contained significantly fewer OVAp-specific CD8 T cells relative to those from WT, as assessed by OVA tetramer staining (Fig. 6B). We also analyzed functional responses by OVAp-specific CD8 T cells, as elicited by pulsing splenocytes isolated on day 6 or day 13 after secondary infection with OVAp. This analysis showed that the frequencies and numbers of IFN-γ–producing cells in splenocytes from Tim-3 KO mice were significantly lower relative to those in WT samples (Fig. 6C, 6D). These data indicate that CD8 T cell responses to secondary LM-OVA infection are impaired by the absence of Tim-3.

The analysis we performed following attLM-OVA infection indicated that WT and Tim-3 KO mice generate similar numbers of OVAp-specific memory CD8 T cells (Fig. 2, Supplemental Fig. 1), suggesting that the decreased secondary responses by Tim-3 KO mice were not due to the presence of fewer memory cells. Nonetheless, we sought to rule out this possibility. In addition, we sought to determine whether the reduced secondary responses reflected a defect intrinsic to memory CD8 T cells. Therefore, we analyzed responses by WT and Tim-3 KO memory OT-I cells following coadaptive transfer into naive WT hosts. The experimental design used for these studies was similar to that shown in Fig. 2A. To generate memory OT-I cells, naive WT and Tim-3 KO cells (10,000 of each) were transferred into separate WT hosts that were infected with attLM-OVA on the next day. After 43 d, memory cells present in spleens were isolated, and WT and Tim-3 KO cells were mixed to create a 1:1 ratio between the two populations, as confirmed by flow cytometric analysis (Fig. 7A). WT and Tim-3 KO cells showed similar expression patterns for KLRG1 and CD127 following isolation and mixing (Fig. 7B). A total of 140,000 OT-I cells (70,000 of each population) were injected into WT hosts, which were infected with attLM-OVA on the following day.

Analysis of surface marker expression by OT-I cells in splenocytes isolated on day 6 p.i. showed that essentially all WT cells were expressing Tim-3 (Fig. 7C) and that the WT and Tim-3 KO cell
populations contained similar frequencies of KLRG1+CD127− and KLRG1+CD127+ cells (Fig. 7D, Supplemental Fig. 4A). To assess functional responses by the transferred cells, splenocytes isolated on day 6 p.i. were pulsed with OVA p ex vivo. Similar to that observed when primary responses were analyzed, the frequencies of Tim-3 KO OT-I cells expressing IFN-γ or TNF were significantly reduced relative to WT (Fig. 7E–G). We also analyzed the ratios between WT and Tim-3 KO OT-I cells present in splenocytes isolated on day 6 p.i. (Fig. 7H, 7I). Strikingly this ratio was on average ~10:1, indicating that the absence of Tim-3 expression reduced the accumulation or persistence of the mutant OT-I cells. Further, this defect appeared to affect both KLRG1+CD127− and KLRG1+CD127+ cells (Supplemental Fig. 4B). Combined, these data demonstrate that the inability to express Tim-3 profoundly impairs secondary CD8 T cell responses to LM-OVA in a cell-intrinsic manner.

Discussion

We used a L. monocytogenes infection model and Tim-3 KO mice to assess the role of Tim-3 in the context of an acute immune challenge. We focused our analysis on CD8 T cells because these cells are mobilized by LM infection and express Tim-3 as a consequence. Our data demonstrate that the absence of Tim-3 attenuates primary CD8 T cell responses to LM, as manifested by reduced accumulation of activated cells and blunted functional responses. Our data also show that secondary CD8 T cell responses to LM infection were impaired by the absence of Tim-3, indicating a role in the mobilization of memory cells. Although not examined in this article, it has been suggested by others that the lack of Tim-3 has impact on multiple pathways that can influence CD8 T cell function. Nonetheless, our studies examining LM-induced activation of WT and Tim-3 KO OT-I cells within a common host demonstrate that Tim-3 can enhance CD8 T cell responses via a cell-intrinsic mechanism. In addition, this approach provided evidence that Tim-3 promotes the proliferation of Ag-stimulated CD8 T cells. On the basis of our findings, we conclude that, under some circumstances, Tim-3 can function to positively regulate CD8 T cell responses.

Our data show that Tim-3 is expressed on the majority of activated CD8 T cells present on day 7 following LM infection. We also found that Tim-3 expression within this compartment is tightly associated with an effector CD8 T cell phenotype. These findings are consistent with data from other studies that used mouse models of viral infections, which all showed that some fraction of virus-specific effector CD8 T cells express Tim-3 (23, 38, 54–56). Our analysis also shows that Tim-3 expression by activated CD8 T cells is largely transient, which is similar to what was observed in studies of CD8 T responses to acute lymphocytic choriomeningitis virus infection (38). In addition, our data indicate that a substantial fraction of effector-phenotype CD8 T cells maintain Tim-3 expression for an extended period. Together, these results support the conclusion that Tim-3 marks functionally competent effector CD8 T cells in addition to exhausted CD8 T cells, as reported previously (36, 38). Of note in this regard, reports by others have shown that, in certain settings, Tim-3–expressing T cell fractions contain higher frequencies of IFN-γ–producing cells relative to their Tim−3− counterparts (24, 57).

Overall, our data demonstrate that Tim-3 expression by LM-specific CD8 T cells is necessary for optimal CD8 T cell expansion and acquisition of effector function. This conclusion is supported by data from our studies of Tim-3 KO mice as well as those from the coadoptive transfer system. These latter studies also provided evidence that Tim-3 helps to sustain CD8 T cell numbers as the response to LM infection resolves. Further, our results suggest that Tim-3 augments CD8 T cell proliferation from days 6 to 7 following infection, which coincides with maximal expression of Tim-3 by activated CD8 T cells. Of note, we did not observe any abnormalities in expression of apoptotic markers by Tim-3 KO CD8 T cells, suggesting that Tim-3 does not impinge on pathways that regulate cell survival.

Although we clearly observed a defect in the persistence of Tim-3 KO CD8 T cells in the coadoptive transfer model, this effect...
was less apparent in mice lacking Tim-3 entirely. Several potential explanations for this difference can be imagined based on previously published data. As an example, studies using agents designed to block interaction between Tim-3 and Gal-9 provided evidence that Tim-3 has an important role in regulating Th1 cell effector function (19, 32, 33, 35, 42). In these settings Tim-3 appeared to directly inhibit Th1 responses and also to promote regulatory T cell function. These observations suggest that the complete absence of Tim-3 in mice will disrupt inhibitory pathways that likely impinge on CD8 T cell function. In contrast, these inhibitory mechanisms would remain operative in the coadaptive transfer model we used. Regardless of these potential complications, our data provide strong support for the conclusion that Tim-3 can function to augment CD8 T cell responses and that this involves a cell-intrinsic mechanism.

Activated CD8 T cells express an array of costimulatory receptors that, when engaged by cognate ligand or counterreceptor, function to augment CD8 T cell expansion and functionality (1). Our data suggest that Tim-3 has a similar role in the context of LM infection. Among costimulatory receptors, the molecular functions of the TNFR superfamily members 4-1BB and OX40 have been characterized in particular detail (58). This work has revealed that 4-1BB and OX40 activate pathways that not only synergize with TCR signaling to boost proliferation but also promote survival by regulating the expression of proteins within the Bcl2 family (58). Our data suggest that the mechanism by which Tim-3 augments CD8 T cell responses is somewhat distinct from that of 4-1BB and OX40 in that we observed effects on proliferation, but not apoptosis.

The molecular mechanisms by which Tim-3 influences CD8 T cell function are not well understood. However, studies using transformed cell lines have shown that tyrosine residues in the cytoplasmic tail of Tim-3 are subject to phosphorylation (11, 12) and that this domain can interact with both the Src-family kinase Fyn and the p85 subunits of phosphoinositide-3 kinase (12). Moreover, ectopic expression of Tim-3 in transformed human Jurkat T cells was shown to augment TCR-induced activation of the transcription factors NFAT and NF-κB (12), which are both critical for optimal proliferation and cytokine production by T cells. These findings suggest a model consistent with our data in which Tim-3 functions to boost signaling downstream of the TCR, thereby increasing cell expansion and effector function by stimulated CD8 T cells.

The hypothesis that Tim-3 functions to activate intracellular signaling pathways suggests a need for interaction with specific ligands or counterreceptors. One candidate molecule is Gal-9, which interacts with Tim-3 by recognizing carbohydrate moieties attached to the mucin stalk of Tim-3 (35). Although Gal-9 induces T cells to undergo apoptosis in vitro (35, 56), Tim-3–expressing T cells have been shown to accumulate in vivo in several different experimental contexts, arguing that Gal-9–induced apoptosis is constrained by other factors. Moreover, settings in which Gal-9 can promote cell activation have been identified. For example, Tim-3–mediated ligation of Gal-9 expressed on macrophages was shown to boost IL-1 production and bactericidal activity (59). Another study showed that infusion of Gal-9 prolongs the survival of tumor-bearing mice, which correlated with increased numbers of activated CD8 T cells and enhanced cytokine responses (24). Aside from Gal-9, the alarmin HMGB1 and phosphatidylserine, as displayed by apoptotic cells, have been identified as physiologically relevant ligands for Tim-3 (7, 8, 27). Finally, studies using recombinant forms of Tim-3 as probes provided evidence that the IgV domain of Tim-3 can recognize specific carbohydrate molecules as well as ligands of unknown identity (60, 61). For future studies, it would be of interest to further explore how these candidate molecules affect the function of Tim-3–expressing CD8 T cells.

Beginning with a seminal report by Jones et al. (36), several studies have demonstrated that Tim-3 is expressed by exhausted CD8 T cells (44), which arise under conditions involving chronic Ag stimulation (38-40, 43, 55, 62-66). Further, combinatorial targeting of Tim-3– and PD-1–mediated signaling was shown to counteract CD8 T cell exhaustion (38, 39). These findings are consistent with a model in which both Tim-3 and PD-1 provide inhibitory signals that result in exhaustion. Such a role for Tim-3 seems in contrast with our findings, which suggest that Tim-3 promotes CD8 T cell responses. One potential explanation for this contradiction is that the function of Tim-3 varies depending on the context and the type of infection involved. For example, responses by the innate system and the inflammatory environment are likely to be very different under conditions of transient versus chronic stimulation, which will have impact on the functionality and responsiveness of CD8 T cells. Moreover, RNA expression profiling demonstrated that CD8 T cell exhaustion is associated with gene expression signatures that are distinct from those established by functionally competent CD8 T cells (67, 68). Thus, factors intrinsic to CD8 T cells may also contribute to potential context-dependent function by Tim-3.

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Disclosures

The authors have no financial conflicts of interest.

References


**SUPPLEMENTAL FIGURE 1.** Analysis of CD8 T cell responses to LM infection in wild-type (WT) and Tim-3 KO (KO) mice. Mice were infected with attLM-OVA and longitudinal analysis of CD8 T cell responses was performed as described in the text. CD8 T cells activated by attLM-OVA infection were defined as Thy1.2^+^CD8α^+^CD11a^hi^.

(A) Analysis of peripheral blood samples taken from WT and Tim-3 KO mice on day 7 postinfection. (B) Analysis of splenocytes isolated from WT and KO mice on day 7 postinfection. Data are representative of results from 5 independent experiments. (C) Analysis of OVA tetramer^+^CD8 T cells in splenocytes obtained on the indicated days postinfection. (D, E) IFN-γ and CD107a expression by CD8 T cells following ex vivo stimulation with OVAp. Assays were performed using splenocytes obtained on the indicated days postinfection. Data shown are representative of results obtained from at least 2 independent experiments.
SUPPLEMENTAL FIGURE 2. (A) Frequencies of KLRG1^+^CD127^-^ and KLRG1^+^CD127^+^ cells within the splenic pools of WT and Tim-3 KO OT-I cells. Spleens were obtained and analyzed on day 6 postinfection. (B) Frequencies of KLRG1^+^CD127^-^ and KLRG1^+^CD127^+^ cells within the total splenic pool of OT-I cells. Spleens were obtained and analyzed on day 6 postinfection. Each symbol represents data obtained from an individual host mouse that received a 1:1 mix of WT and Tim-3 KO OT-I cells and was infected with attLM-OVA on the following day. Data were generated from 3 independent experiments; from these a total of 17 host mice were analyzed. *p<0.01; ***p<0.0001.
SUPPLEMENTAL FIGURE 3. Normal expression of the anti-apoptotic factor Bcl-xL by Tim-3 KO OT-I cells. Wild-type (WT) and Tim-3 KO (KO) OT-I cells (1000 each) were injected into WT hosts. The next day, hosts were infected with attLM-OVA. (A) Expression of Bcl-xL by splenic WT and Tim-3 KO OT-I cells as assessed on day 8 postinfection. (B) Mean fluorescence intensities (MFI) for Bcl-xL levels as determined on the indicated days postinfection. Bcl-xL expression was analyzed as shown in panel A. Each symbol represents the mean and standard error of 4 data points. Data are representative of results from 2 independent experiments.
SUPPLEMENTAL FIGURE 4. (A) Frequencies of KLRG1+CD127− and KLRG1−CD127+ cells within the splenic pools of WT and Tim-3 KO OT-I cells. Splenocytes were obtained and analyzed on day 6 postinfection. (B) Frequencies of KLRG1+CD127− and KLRG1−CD127+ cells within the total splenic pool of OT-I cells. Splenocytes were obtained and analyzed on day 6 postinfection. Each symbol represents data obtained from an individual host mouse that received a 1:1 mix of memory WT and Tim-3 KO OT-I cells and was infected with attLM-OVA on the following day. Data were obtained from one independent experiment in which a total of 6 host mice were analyzed. ***p<0.0001.