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Tim-3 is not Required for Establishment of CD8+ T Cell Memory to LCMV

Priyanka Manandhar[*,†], **Andrea L. Szymczak-Workman**[*], **Lawrence P. Kane**[*,†,‡]

[*]Dept. of Immunology, University of Pittsburgh, Pittsburgh, PA 15213

[†]Graduate Program in Microbiology and Immunology, University of Pittsburgh, Pittsburgh, PA 15213

Abstract

Tim-3 is a transmembrane protein that is best known for being highly expressed on terminally exhausted $CD8^+$ T cells associated with chronic infection and tumors, although its expression is not limited to those settings. Tim-3 is also expressed by $CD8^+$ T cells during acute infection and by multiple other immune cell types, including $CD4^+$ type 1 helper and regulatory T cells, dendritic cells, and mast cells. Here we investigate the role of Tim-3 signaling on $CD8^+$ T cell memory using a Tim-3 conditional knockout mouse model and mice lacking the signaling portion of the Tim-3 cytoplasmic domain. Together, our results indicate that Tim-3 has at most a modest effect on the formation and function of $CD8^+$ memory T cells.

Introduction

$CD8^+$ T cells undergo massive clonal expansion upon recognition of their cognate antigens and contract rapidly when the antigen is cleared. Although most of the effector $CD8^+$ T cells eventually undergo apoptosis, a small percentage of these cells survive in an antigen-independent and cytokine-dependent manner (1–4). $CD8^+$ T cells that respond to antigens generate effector cells that migrate to peripheral tissues and undergo a bifurcated response, forming either terminally differentiated short-lived effector cells (SLECs) or memory precursor cells (MPECs) (5). Memory precursors that form memory T cells provide long-lived immunity and have been shown to be protective for extended periods of time (up to decades in humans) (1, 2, 6–10). However, the developmental and maintenance requirements of $CD8^+$ memory T cells remain incompletely understood. TCR signal strength and duration contribute to $CD8^+$ T cell memory formation, among other factors including inhibitory receptors, cytokines, transcription factors, $CD4^+$ T cell help, and signals received during synapse formation (2, 3, 6, 11–16). Recent studies have shown that repeated stimuli with cognate antigens strengthen the memory population and these cells have been known to outlive the host organism (17). Additionally, co-regulatory molecules such as OX40 and PD-1 have also been shown to influence the formation of $CD8^+$ T cell memory (12, 14, 16).

[‡]To whom correspondence should be addressed: lkane@pitt.edu.

Memory T cells are broadly divided into two major subsets: central memory T cells (Tcm), effector memory T cells (Tem) and tissue resident memory cells (Trm). While Tem subset remained poised to provide immediate effector functions, Tcm were restricted to the lymphoid tissues and had greater self-renewing capacity (5, 18). Trm cells consist of a pool of $CD8^+$ memory T cells that permanently reside in non-lymphoid tissue and monitor distinct anatomical compartments (19). Several factors have now been recognized as being essential for the survival of memory T cells. In the absence of cognate antigens, memory T cells are maintained by signals through the common gamma chain-containing cytokine receptors (3, 5, 20, 21). Despite differences in location and functions, memory T cells are dependent on IL-7 and IL-15 for survival in the absence of their cognate antigens (2, 9, 18). IL-7 and IL-15 deliver survival and proliferative signals, respectively, as basal proliferation contributes to the maintenance of a stable pool of memory T cells (3, 21, 22). Expression of the IL-7 receptor CD127 has been speculated to provide a survival advantage for MPECs, although it is not sufficient for the formation of memory $CD8^+$ T cells (22). Transcription factors such as Eomes, Tcf-1 and Foxo1 have also been shown to support the formation and survival of memory T cells (2, 13, 23–25). Studies suggesting epigenetic modifications which control the access of transcription factors to the genome have suggested epigenetic modifications as a central mechanism for preserving the effector properties of memory T cells (5). Thus, understanding the process of T cell memory formation would lead to a robust memory T cell pool, which would lead to improvements in vaccination, including tumor vaccines (3, 5).

Tim-3 is an IgV like surface molecule shown to be highly expressed on terminally exhausted T cells, macrophages, Tregs and myeloid cells (26–33). In chronic infections, expression of Tim-3 has been linked to poor prognosis in patients infected with HIV or HCV (34–36). However, $Tim\text{-}3^+$ cells have also been shown to be the most responsive cells in tuberculosis infections in humans (37). Multiple ligands of Tim-3 have been identified, including galectin-9, HMGB1, phosphatidylserine (PS) and CEACAM1, which have been shown to have both positive and negative effects on T cells (38–44). In *in vitro* studies, ectopic expression of Tim-3 on Jurkat T cells has been shown to increase activation under conditions of acute stimulation, while deletion of tyrosine residues from the Tim-3 intracellular tail abrogated this co-stimulatory function (45, 46).

In addition to its expression during chronic infection, Tim-3 is also expressed on $CD8^+$ T cells during acute infection, including acute infection with *Listeria* (47, 48). In addition, Tim-3 germline knockout mice had a defective $CD8^+$ T cell response during the effector stage of *Listeria* infection (47). The role of Tim-3 in acute LCMV infection was previously investigated by our lab using germline Tim-3 KO and CD8-specific enforced Tim-3 expression mouse models. While germline deletion of Tim-3 skewed the $CD8^+$ T cell response towards a memory precursor phenotype, ectopic expression of Tim-3 on $CD8^+$ T cells conversely enhanced the number of short-lived effector cells (49). However, we do not know whether Tim-3 plays a cell-intrinsic role in the development of $CD8^+$ T cell memory, nor whether Tim-3 is required for a memory recall response. To better understand the role of Tim-3 expression and signaling on $CD8^+$ T cell memory development and function, we used a CD8-specific Tim-3 KO model, as well as a novel mouse model expressing a truncated, non-signaling, allele of Tim-3. Our findings show that Tim-3 has a limited effect on $CD8^+$

T cell memory and recall response, in the context of either a native (polyclonal) T cell repertoire or a TCR transgenic GP33-specific T cell clone.

Materials and Methods

Mice

E8i-cre and P14 TCR transgenic mice were obtained from Jackson Laboratories and bred at the University of Pittsburgh. Tim-3 T2 mutant and *Havcr2*$^{fl/fl}$ mice were generated at the University of Pittsburgh Department of Immunology Transgenic and Gene Targeting (TGT) core (Supplemental Fig. 1). *Havcr2*$^{fl/fl}$ mice were generated by using Crispr/Cas9 to insert LoxP sites on either side of exon 4 of the *Havcr2* gene in C57BL/6J zygotes. Disruption of this exon was predicted to result in a premature stop codon 20bp into exon 5, before the TM domain, predicted to lead to nonsense-mediated decay. Insertion of the LoxP sites was initially confirmed by PCR and restriction digest, followed by sequencing of the targeted region. Deletion of the *Havcr2* gene was confirmed at the RNA level. *Havcr2*$^{fl/fl}$ mice were genotyped using the primer sequences: For: 5'- AGAGAACCAAGAGGCCAGGA-3', Rev: 5'- CATCCCAGCTCTGTGGAGGT-3'. The WT allele is detected at 441 base pairs and the floxed allele is detected at 591 base pairs using this primer pair.

T2 mutant mice were generated by using Crispr/Cas9 to change the codon for tyrosine 256 in the *Havcr2* gene of C57BL/6J zygotes to a stop codon, resulting in a truncated mutant of Tim-3. Restriction sites were introduced to facilitate genotyping. Mice were genotyped using the primer sequences: For: 5'-GAGCATGTAGTGAAGGGCCA-3', Rev: 5'-TCAGTGGCTGTGGTCAGAAC-3', followed by digesting with HindIII. The WT allele is detected at 250 base pairs and the mutant allele is detected at 225 base pairs using this primer pair after the restriction enzyme digest. Expression of the truncated protein was confirmed by western blotting and flow cytometry. When analyzed at baseline, no differences in T cell development were observed in either the thymus or secondary lymphoid organs (Supplemental Fig. 2).

Adoptive transfers of naïve P14 cells were performed by injecting 10^3 CD44^{lo}CD62L^{hi} cells retro-orbitally. Both male and female mice 6–8 weeks of age were used for all experiments. Experiments were performed in accordance with protocols approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

LCMV and Listeria infections

LCMV Armstrong was obtained from Judong Lee and Rafi Ahmed and propagated and titered as previously described (50, 51). Mice were infected with 2×10^5 PFU i.p. infections occurred at day 0 and peripheral blood and spleens were analyzed by flow cytometry 14 days or 35+ days after infection in age- and sex-matched groups. Mice were cheek bled using animal lancets. LCMV tetramers were obtained from the NIH tetramer core (contract number 75N93020D00005) and stained in RPMI medium with 3% BSA at room temperature for one hour. *Ex vivo* peptide restimulation was performed by incubating 2×10^6 splenocytes in RPMI medium supplemented with 10% BSA, 1% penicillin-streptomycin, 1% L-glutamine, 1% HEPES, 1% non-essential amino acids, 1% sodium pyruvate and

0.05 mM 2-mercaptoethanol for five hours in standard TC culture conditions. A 1:1000 final concentration of Brefeldin-A containing GolgiPlug™ was added to the re-stimulation solution (BD 55029). GP33, NP396 and GP276 peptides were obtained from Anaspec and 100 ng/ml of pooled peptides were used for *ex vivo* restimulation. For Trm experiments, mice were retro-orbitally injected with 15ug of biotinylated antiCD8α antibody and sacrificed five minutes later. Lungs were harvested, weighed and processed for flow cytometry. *L. monocytogenes* expressing the GP33 epitope was obtained from Sue Kaech (Salk Institute) and propagated in brain heart infusion broth as previously described (52). Mice were rechallenged with 15×10^3 or 2×10^6 CFU of Listeria-GP33 i.v. retro-orbitally and harvested four days after re-challenge.

Antibodies

The following antibodies were used: CD8 alpha (BioLegend 563786), TCRβ (BD 748406), CD4 (BioLegend 564298), CD45.2 (BD 564616), CD45.1 (BioLegend 110724), CD44 (BD 612799), CD62L (BioLegend 104438), KLRG1 (Invitrogen 46-5893-80), CD127 (Tonbo 60-1271-U100), Tim-3 (BioLegend 119721), T-bet (BioLegend 644806), Eomes (Invitrogen 12-4875-80), Tcf-1 (Cell signaling 9066S), TCR Vα2 (BioLegend 127809), TCR Vβ8.1 (BioLegend 140103), TNF (BioLegend 506308), IFNγ (BioLegend 505829), biotinylated CD8α (BioLegend 139360), CD103 (BioLegend 121417), CD69 (BioLegend 104536), Ly6C (BioLegend 128024), CD8β (BD 740278), CD49a (BioLegend 564863).

Tissue processing for flow cytometry

Spleens or peripheral blood were harvested and single cell suspensions in PBS were produced by crushing spleens using histological slides. RBC lysis was performed by suspending single cell suspensions in RBC lysis buffer (eBioscience 00-4333-57) for two minutes on ice. Collected splenocytes were filtered through a 70 μm filter. Lungs were processed using the Macs-Miltenyi tissue dissociator in a mixture of collagenase (2.7 units/ml final concentration), DNAse (53.3 μg/ml final concentration) and base RPMI (1.5 ml total). A percoll gradient was performed using 44% and 67% percoll to isolate lymphocytes and spinning at 900g without brake at room temp for 20 min. Dead cells were excluded using the Zombie™ NIR fixable viability kit (BioLegend 423105). 5×10^6 lymphocytes were stained for flow cytometry. Surface staining was performed in PBS on ice followed by one hour fixation using a transcription factor staining set (eBioscience 00-5523-00). Cells were acquired on a 5-laser Cytek Aurora and analyzed using FlowJo version 10. Gating strategies for flow cytometry analysis of spleen and lungs are shown in Supplemental Figure 3.

Statistical analyses

Data were analyzed with GraphPad Prism. Each symbol represents one mouse. One way ANOVA and paired student's t-test were used where appropriate. Significance was defined as $p<0.05$.

Results

Impact of Tim-3 KO on a CD8$^+$ T cell response after LCMV Armstrong infection

Tim-3 is expressed as early as 72 hours after infection with LCMV Armstrong and is highly expressed on CD8$^+$ T cells at the effector stage of the infection (53). To elucidate the role of Tim-3 signaling on memory, CD8$^+$ T cell-specific conditional knockout (KO) mice were generated by crossing previously described, by crossing Tim-3 (gene name *Havcr2*) floxed mice to CD8 T cell-specific E8i-cre mice (28). Mice carrying E8i-Cre alone were used as controls in all experiments with Tim-3 floxed mice. Peripheral blood from Armstrong infected mice were analyzed by flow cytometry fourteen days after infection (Fig. 1A). No significant differences were observed in absolute levels of CD44 (by MFI), nor in the proportions of CD44^{hi}CD62L^{lo} and CD44^{hi}CD62L^{hi} cells, among CD8$^+$ T cells (Fig. 1B–D). About 20–30% of WT effector CD8$^+$ T cells were found to express Tim-3 at day fourteen after infection (Fig. 1E). As shown, no significant differences were seen in the KLRG1$^+$CD127$^-$ short lived effectors (SLECs) and KLRG1$^-$CD127$^+$ memory precursors (MPECs) (Fig. 1F–G).

Tim-3 signaling does not appear to affect CD8$^+$ T cell memory in a polyclonal response.

Among other factors that influence CD8 T cell fate, co-regulatory markers have previously been shown to influence CD8$^+$ T cell memory (12, 14, 16, 54). The absence of PD-1 has been shown to accelerate viral clearance in the influenza virus infection, but memory cells formed in the absence of PD-1 have been shown to be poorly functional (16). Similarly, germline deletion of Tim-3 was shown to skew CD8$^+$ T cells towards a memory precursor phenotype (49). To assess the role of Tim-3 on CD8$^+$ T cells, CD8-specific Tim-3 KO mice and Tim-3 mutant mice lacking the tyrosine domains (Fig. 2B) in the intracellular tail were infected with LCMV Armstrong and CD8$^+$ T cell response was assessed 35 days later (Fig. 2A). Memory CD8 cells in the spleen and tissue resident memory cells in the lung were analyzed. The T2 mutant had significantly lower CD8$^+$ T cells in the spleen. However, total CD8$^+$ T cells were not significantly different between the WT and KO. (Fig. 2C). The number of CD8$^+$ T cells binding major LCMV Class I epitopes (GP33, NP396 and GP276) and the proportion of putative CD8$^+$ memory T cells (CD44$^+$KLRG1$^-$CD127$^+$) were not significantly different in the three backgrounds (Fig. 2D–E, I–J). Additionally, no observable differences were seen in the mean fluorescence intensities of Tcf-1, T-bet and Eomes (Fig. 2F–G, K), nor was the ratio of T-bet:Eomes significantly different (Fig. 2H). Memory CD8$^+$ T cells were also equally efficient at producing cytokines after five hours of *ex vivo* peptide restimulation (Fig. 2L).

Tissue resident memory cells appear to be independent of Tim-3 signaling.

Tissue resident memory (Trm,) cells serve as an integral part of the immune sensing network within tissues (55). Trm can be distinguished from Tem and Tcm by expression of surface phenotype and transcription factors (56). Multiple studies have illustrated that Trm differentiation occurs during the early stages of a T cell response, and that environmental conditions also contribute to Trm development (19). To investigate the role of Tim-3 on Trm formation in the lungs, LCMV Armstrong-infected mice were analyzed 40 days after infection, for the presence of tissue resident CD8$^+$ T cells. Anti-CD8α mAb was injected i.v.

into each mouse to label circulating (i.e. non-Trm) $CD8^+$ T cells and lungs were harvested five minutes later. There were no significant differences in the percent or total numbers of antigen-experienced $CD8^+$ Trm in the lungs (Fig. 2B and C). No significant differences were seen in $CD69^+CD103^+$ Trm T cells among the antigen-experiences $CD8^+$ T cells (Fig. 3D). Similarly, no observable differences were seen in the CD49a MFI or Ly6C positive cells in the lungs (Fig 3E and F).

P14 $CD8^+$ T cell memory is apparently independent of Tim-3 signaling

In order to study the effects of Tim-3 signaling in a monoclonal response, LCMV GP33 specific P14 mice were crossed to the T2 germline mutant or to *Havcr2*$^{fl/fl}$E8i-Cre mice. Naïve P14 CD8 T cells were then adoptively transferred into congenically mismatched (CD45.1) mice and infected with LCMV Armstrong (Fig. 4A). At 35 days after infection, there were no significant differences in the total memory P14 cells recovered from the spleen (Fig. 4B). No differences were seen in the $CD44^{hi}CD62L^{hi}$ effector memory (Tem) or $CD44^+CD62L^{lo}$ central memory (Tcm) populations (Fig. 4D–E). No significant differences were seen in the $KLRG1^+CD127^-$ long lived effector population (LLECs) or the $KLRG1^-CD127^+$ memory population (Fig. 4F–G). Mean fluorescence intensities of Tcf-1, T-bet and Eomes are indicated (Fig. 4C, H–I). Although there were no differences in the levels of these transcription factors by MFI, the T-bet: Eomes ratio was significantly lower in the T2 mutant and the KO (Fig. 4J). Eomes is a survival-associated TF and a lower T-bet:Eomes ratio is associated with increased memory formation (6, 13). Hence in this defined TCR transgenic system, there is a slight effect of Tim-3 deletion on this measure in the memory $CD8^+$ T cells. Nonetheless, WT, T2 and KO memory P14 cells were all able to robustly produce cytokines after peptide restimulation (Fig. 4K).

GP33 specific memory recall response is apparently unaffected by Tim-3 signaling

Memory $CD8^+$ T cells have the capacity to respond rapidly and robustly to their cognate antigens (1, 10, 57). To study the recall response of GP33 specific memory $CD8^+$ T cells in a polyclonal background, mice that had been previously infected with LCMV Armstrong were challenged with either a low dose (15×10^3) or high dose (2×10^6) of Listeria-GP33 (Fig. 5A). Mice infected with a low dose of Listeria did not show a significant difference in the total number of $CD8^+$ T cells or of $GP33^+$ cells, four days after infection (Fig. 5 B, D). No significant differences were seen in the levels (by MFI) of T-bet or Tcf-1, in antigen-experienced ($CD44^+CD62L^-$) cells (Fig. 5C and E). Similarly, after a high-dose infection with Listeria-GP33, there were no observable differences in the number of total $CD8^+$ T cells or GP33 tetramer$^+$ cells, at four days of infection (Fig. 5F and G). There were also no significant differences in the level of T-bet (by MFI) or cytokine production after peptide restimulation (Fig. 5I). The level of Tcf-1 (by MFI) was slightly higher in Tim-3 KO $CD8^+$ T cells, although this was not statistically significant (Fig. 5J).

P14 memory recall response appears to be unaffected by Tim-3 signaling

To assess the quality of the memory recall response in a defined TCR transgenic system, naïve P14 (WT, T2 or *Havcr2*$^{fl/fl}$E8i-cre) cells were transferred into congenically marked mice and infected with LCMV Armstrong. At 35 days after infection, memory P14 cells were sorted and adoptively transferred into naïve congenically marked mice which then got

infected with LCMV Armstrong. Eight days after infection, spleens were harvested and P14 cells were analyzed by flow cytometry (Fig. 6A). No significant differences were observed in total numbers of P14 cells from the spleen (Fig. 6B). There were also no significant differences in the expression (MFI) CD44 or T-bet of P14 cells at eight days after infection (Fig. 6C–D). Cytokine production by the T2 and Tim-3 KO P14 cells was slightly lower after peptide restimulation, although this did not reach statistical significance (Fig. 6E).

Discussion

$CD8^+$ memory T cells provide immunological protection against pathogens; however, the process of memory formation remains incompletely understood (2, 11, 57–61). Co-inhibitory and co-stimulatory molecules have been shown to influence T cell activation and subsequently influence $CD8^+$ memory and have been shown to have a significant role in the formation of robust $CD8^+$ T cell memory (12, 61–63). Here, we have investigated the role of Tim-3 on $CD8^+$ memory T cell responses to LCMV Armstrong. Complete deletion of Tim-3 from $CD8^+$ T cells did not appear to affect the endogenous, polyclonal, $CD8^+$ T cell memory response after LCMV Armstrong infection. However, we did observe a lower number of total, polyclonal, Tim-3 T2 mutant $CD8^+$ T cells at the memory timepoint. We also noted a statistically significant decrease in the T-bet:Eomes ratio of both T2 and Tim-3 KO $CD8^+$ memory T cells in the P14 adoptive transfer system. Nonetheless, recall responses to LCMV Armstrong or *Listeria*-GP33 appeared to be unaffected by loss of Tim-3. Finally, there were no apparent differences between WT and KO mice in the formation of endogenous, polyclonal, Trm in the lungs. Taken together, these data suggest that Tim-3 plays, as most, a minor role in the establishment of $CD8^+$ T cell memory to LCMV Armstrong.

While Tim-3 has been used as a marker of exhaustion, it is expressed on multiple cells with different functions in each cell type (26, 28, 30, 64). Tim-3 has been shown to be co-stimulatory on NK cells and it has been shown to increase proximal FcεRI signaling in mast cells, increasing degranulation and cytokine release upon activation (30, 33). In T cells, the function of Tim-3 has been described in the context of an acute *Listeria* infection where total germline Tim-3 KO mice had an impaired $CD8^+$ T cell response during the effector stage of the infection (47, 48). While in the memory phase, germline Tim-3 KO mice also had increased $CD8^+$ T cell memory after LCMV Armstrong infection and ectopic expression of Tim-3 on $CD8^+$ T cells resulted in an increase in long-lived effector $CD8^+$ T cells, at the expense of memory precursor cells (49). During acute infection, $Tim\text{-}3^+$ primary T cells showed higher expression of the Nur-77 GFP reporter and had increased pAkt and pS6 and, in addition, Tim-3 is recruited to the immunological synapse during T cell activation (46). These studies established Tim-3 as a co-stimulator under conditions of acute stimulation/infection and suggested that germline deletion of Tim-3 has effects on $CD8^+$ T cell memory (45–49). Tim-3 was also shown to signal through the Akt-mTOR pathway, which was abrogated when tyrosine residues in the cytoplasmic domain were truncated (45, 49).

Using mice with a floxed fourth exon of Tim-3 and crossed to mice expressing CD8-specific Cre, we investigated $CD8^+$ T cell memory formation after LCMV infection. We also used P14 TCR Tg T cells, specific for LCMV GP33 peptide presented by $H\text{-}2D^b$, to study

the antigen-specific effects of deleting Tim-3 or its cytoplasmic domain on the formation of $CD8^+$ T cell memory. Based on our previous findings (49), we hypothesized that the deletion of Tim-3 or truncation of its cytoplasmic domain would result in more efficient differentiation of $CD8^+$ memory T cells, at the cost of long-lived effector $CD8^+$ T cells. Thus, Tim-3 deletion in the total $CD8^+$ T cell population had a modest effect on these cells. In the P14 system, surface markers of activation/differentiation or cytokine production after peptide restimulation were not statistically different. However, the T2 mutant and Tim-3 KO $CD8^+$ T cells had slightly increased Eomes expression and a significantly lower T-bet:Eomes ratio, suggesting that Tim-3 signaling has some influence on $CD8^+$ memory T cell formation.

Similarly, Tim-3 KO germline mice were also shown to have a defective memory recall response when mice that had cleared an LCMV Armstrong infection were challenged with Listeria-GP33 at 35 days after primary infection (49). We also probed the effects of Tim-3 deletion on $CD8^+$ T cells in both polyclonal and antigen receptor-restricted (P14 transfer) settings. CD8-specific Tim-3 conditional KO mice were infected with *Listeria*-GP33 35 days after LCMV Armstrong infection, and both low-dose and high-dose *Listeria* infection were used to probe the $CD8^+$ T cell recall response. Total and GP33-specific $CD8^+$ T cells were not found to be statistically significant. Expression of Tcf-1 was slightly higher in Tim-3 KO T cells while, conversely, T-bet expression and cytokine production were slightly lower. Memory recall response was also assessed in the P14 T cell transfer system. Thus, P14-T2 and P14-KO $CD8^+$ T cells had slightly lower cytokine production after restimulation, compared with WT P14 T cells.

In the context of previous studies performed with germline Tim-3 KO, deletion of Tim-3 on $CD8^+$ T cells showed a mild effect on the $CD8^+$ memory T cell population and subsequent memory recall experiments. In line with Tim-3 being expressed on multiple cell types with varying functions, the effects seen on $CD8^+$ T cell memory in the germline Tim-3 KO mice might have been a combined effect of Tim-3 signaling in different cell types, while Tim-3 deletion on $CD8^+$ T cells alone had a limited effect on CD8 memory and recall response. Tim3-expressing $CD8^+$ T cells have increased activation and increased cytotoxicity (unpublished data) at the effector stage of Armstrong infection. It is possible that the effects of Tim-3 are most evident during the effector stage, when it is robustly expressed, but gradually diminishes as its expression declines during the memory stage.

Finally, although LCMV Armstrong is a widely used model for study of acute infection and T cell memory (2, 65), a limitation of our study is that only one infection model was used to probe the impact of Tim-3 deletion on $CD8^+$ T cell memory. Thus, it remains to be determined whether the loss (or blockade) of Tim-3 would impact the formation of CD8+ T cell memory in response to other infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points:

- Tim-3 has a minimal role in CD8 memory T cell formation after acute LCMV infection.
- Tim-3 has a small impact on expression of memory-associated transcription factors.
- No observable effects of Tim-3 deletion in the CD8 memory recall response.

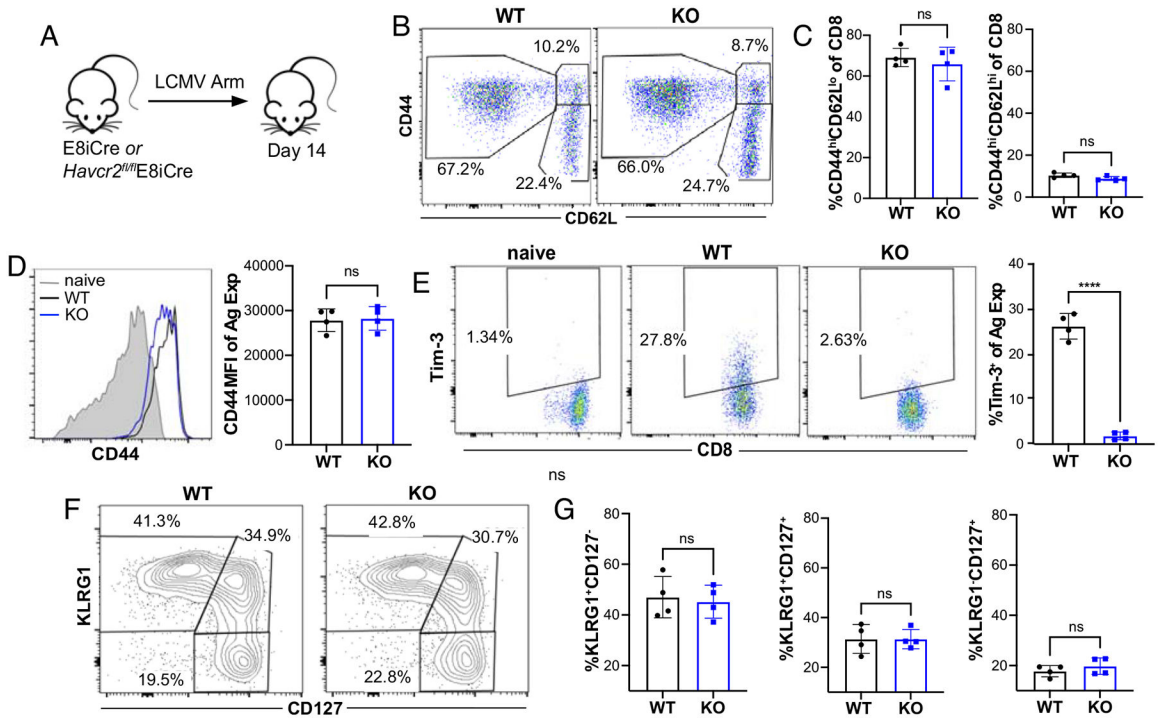

Figure 1: CD8+ T cell response at day fourteen after LCMV Armstrong infection.

Peripheral blood from mice infected with 2×10^5 pfu LCMV Armstrong were processed for flow cytometry on day 14 after infection. **(A)** Schematic representation of experimental set up. **(B)** CD44 and CD62L expression in $CD8^+$ T cells were analyzed. **(C)** $CD44^{hi}CD62L^{lo}$ population and $CD44^{hi}CD62L^{hi}$ population of $CD8^+$ cells. **(D)** CD44 MFI of antigen experienced $CD8^+$ T cells. **(E)** Tim-3 expression on antigen experienced $CD8^+$ T cells and **(F)** KLRG1 and CD127 expression on antigen experienced cells were analyzed. **(G)** $KLRG1^+CD127^-$, $KLRG1^+CD127^+$ SLECs and $KLRG1^-CD127^+$ MPECs. In the graphs, each point represents an individual mouse and bars indicate ±SD (WT, n=4; KO, n=4). The experiment was performed once. No significant differences between the groups were identified by a student's t-test.

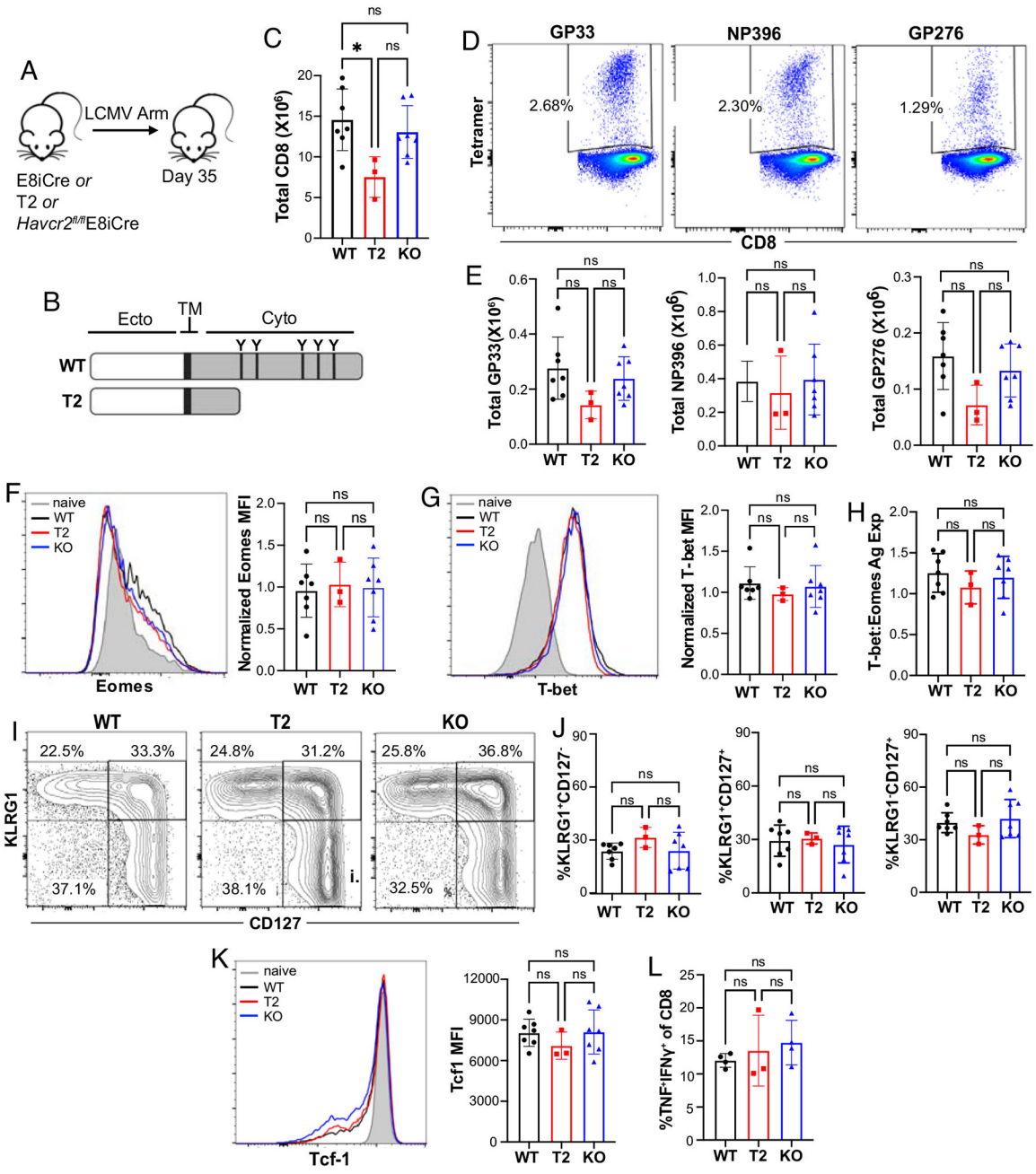

Figure 2: Tim-3 signaling does not appear to affect CD8 memory in a polyclonal response.

Mice were infected with 2×10^5 pfu of LCMV Arm. CD8 memory cells in spleen were analyzed 35 days later. **(A)** Schematic representation of experimental set up. **(B)** Domain structure of Tim-3 showing tyrosine residues in the intracellular domain. **(C)** Total $CD8^+$ cells T cells from the spleen **(D)** LCMV tetramer specific $CD8^+$ T cells (GP33, NP396 and GP276) **(E)** Total $GP33^+$, $NP396^+$ and $GP276^+$ cells. **(F)** Eomes MFI, **(G)** T-bet MFI and **(H)** T-bet:Eomes ratio of antigen experienced $CD8^+$ cells. **(I)** KLRG1 and CD127 were analyzed on antigen experienced $CD8^+$ cells. **(J)** $KLRG1^+CD127^-$, $KLRG1^+CD127^+$ long lived effector and $KLRG1^-CD127^+$ memory precursors of antigen experienced $CD8^+$ cells.

(K) Tcf-1 MFI of antigen experienced $CD8^+$ cells. **(L)** Cytokine production of $CD8^+$ T cells after *ex vivo* stimulation with pooled LCMV peptides for five hours. In the graphs, each point represents an individual mouse and bars indicate ±SD. Panels C-L represent a combination of two experiments (WT, n=7, T2, n-3, KO, n=7). No significant differences between the groups were identified by a one-way anova.

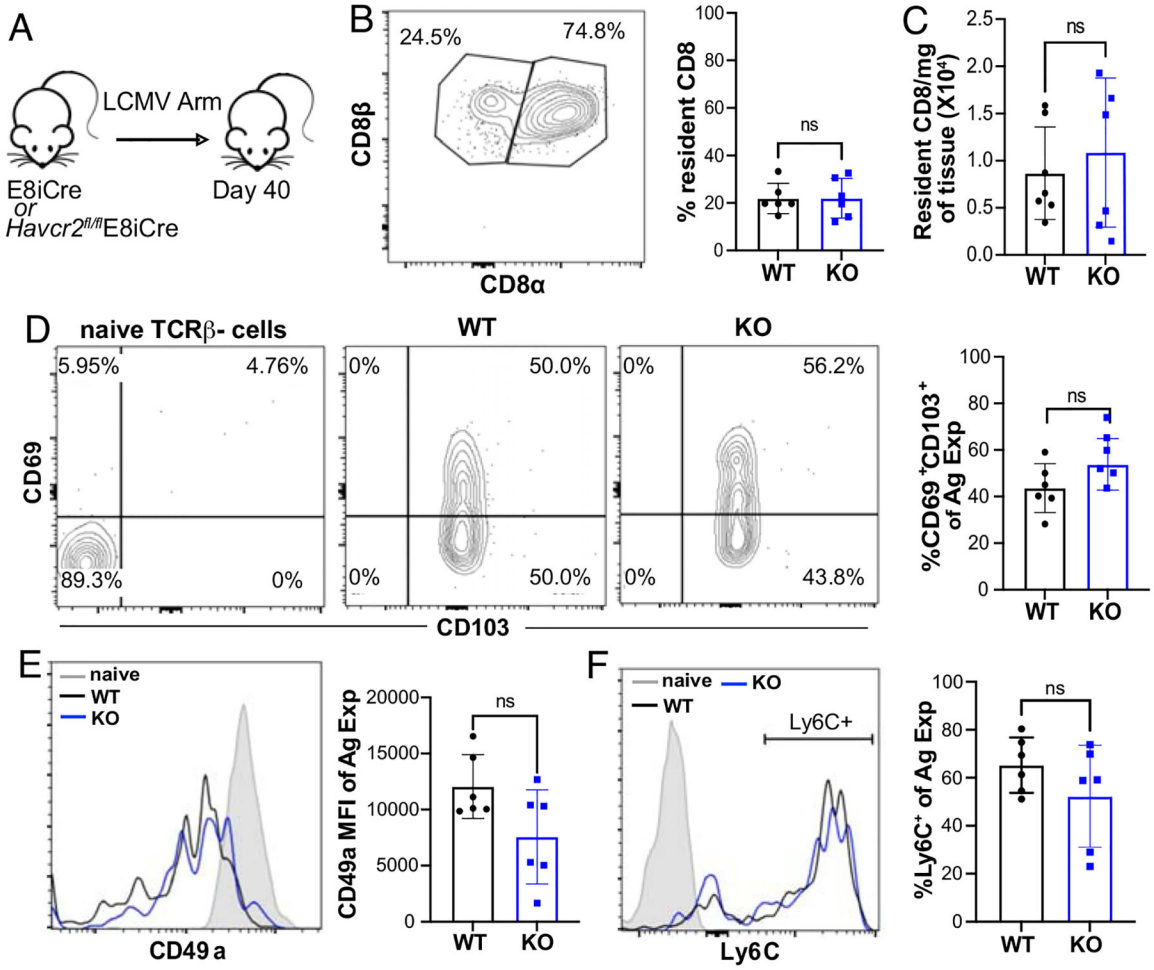

Figure 3: Tissue resident memory cells appear to be unaffected by Tim-3 deficiency.

Mice were infected with 2×10^5 pfu of LCMV Arm and lung tissue was analyzed 40 days later. Mice were injected i.v. with biotin-anti-CD8α antibody and sacrificed 5 minutes after injection. Lung tissue was harvested and processed for flow cytometry. Streptavidin AF488 was used to label circulating $CD8^+$ cells. **(A)** Schematic representation of experimental set up. **(B)** Resident $CD8^+$ T cells in the lungs. **(C)** Total number of CD8 Trm per mg of lung tissue. **(D)** CD69 and CD103 expression in $CD44^{hi}CD62L^{lo}$ antigen experienced CD8 TRM cells. **(E and F)** CD49a MFI and $\%Ly6C^+$ cells of antigen experienced Trm cells. In the graphs, each point represents an individual mouse and bars indicate ±SD. **(A-F)** represent one experiment (WT, n=6, KO, n=6). No significant differences between the groups were identified by a student's t-test.

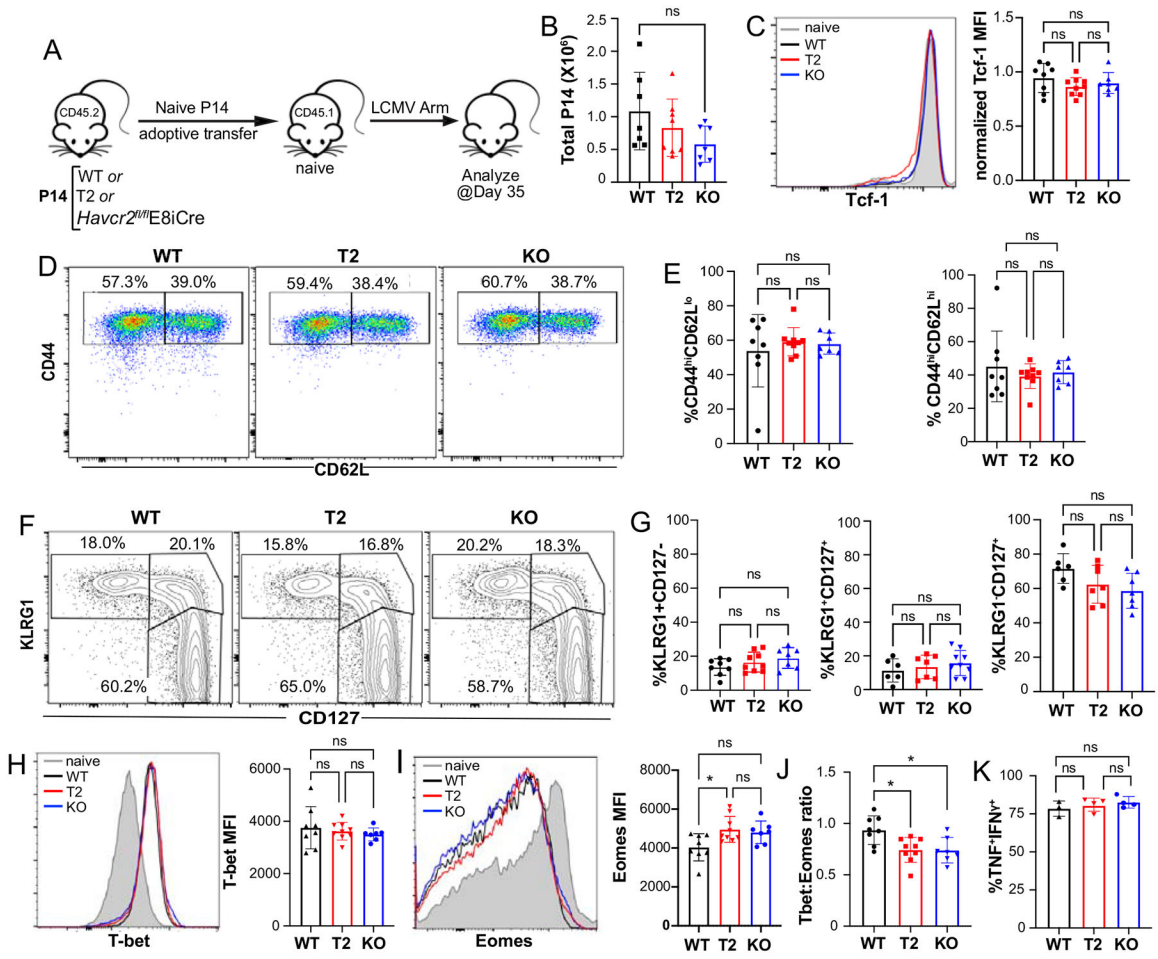

Figure 4: CD8 memory in a defined TCR transgenic system is apparently independent of Tim-3 signaling.

Naïve $CD8^+$ T cells (10^4) from P14, P14-T2 or P14-$Havcr2^{fl/fl}$E8icre mice were transferred into congenically marked naïve mice and infected with 2×10^5 pfu of LCMV Arm. Memory P14 cells from the spleen were analyzed 35 days later. **(A)** Schematic representation of experimental set up. **(B)** Total memory P14 cells from the spleen **(C)** Tcf-1 MFI of P14 cells. **(D)** CD44 and CD62L expression of P14 cells. **(E)** $CD44^{hi}CD62L^{lo}$ and $CD44^{hi}CD62L^{hi}$ population of P14 cells. **(F)** KLRG1 and CD127 expression of P14 cells. **(G)** $KLRG1^+CD127^-$, $KLRG1^+CD127^+$ and $KLRG1^-CD127^+$ populations of P14 cells. **(H and I)** T-bet and Eomes MFI; **(J)** T-bet:Eomes ratio of P14 cells. and **(K)** Cytokine production of P14 cells after *ex vivo* stimulation with gp33 peptide for five hours. In the graphs, each point represents an individual mouse and bars indicate ±SD. Data shown represent combined data from two experiments. A one-way anova was used to determine statistical significance.

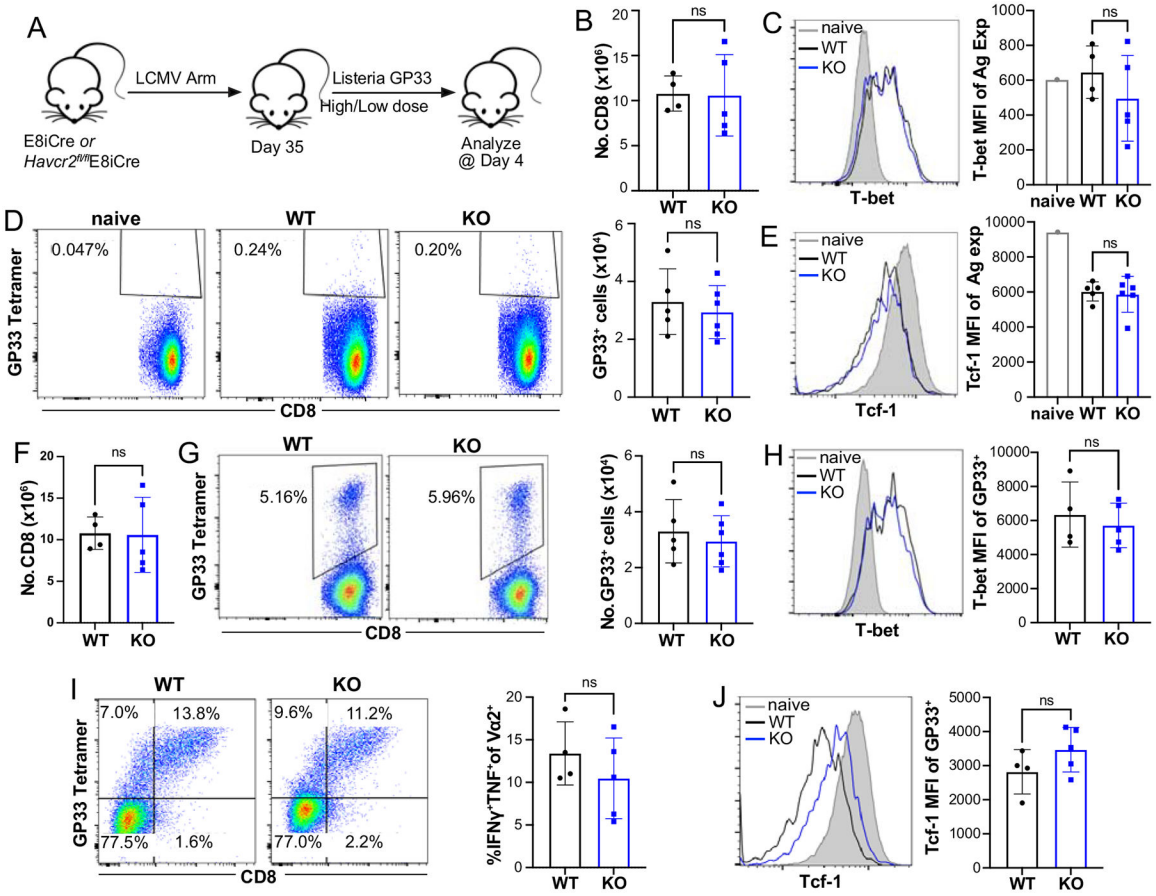

Figure 5: GP33 specific memory recall response is apparently unaffected by Tim-3 signaling.

Mice were infected with 2×10^5 pfu of LCMV arm and subsequently infected with either 15×10^3 or 2×10^6 cfu of Listeria-GP33 35 days later. Memory recall response was assessed four days after Listeria infection. Splenocytes were processed for flow-cytometry. Recall response after infection with 15×10^3 cfu **(B-E)** or 2×10^6 cfu **(F-J)** of Listeria. **(A)** Schematic representation of experimental set up. **(B)** Total $CD8^+$ T cells in spleen. **(C)** T-bet MFI and **(D)** Total $GP33^+$ $CD8^+$ cells. **(E)** Tcf-1 MFI of antigen experienced cells. **(F)** Total CD8 T cells from splenocytes. **(G)** Total $GP33^+$ tetramer specific cells. **(H)** T-bet MFI of $GP33^+$ specific cells. **(I)** Cytokine production after *ex vivo* stimulation with Gp33 peptide for five hours. **(J)** Tcf-1 MFI of GP-33^+ cells. In the graphs, each point represents an individual mouse and bars indicate ±SD (WT, n=4;, KO, n=6 or 4). The experiments were performed once each. No significant differences between the groups were identified by a student's t-test.

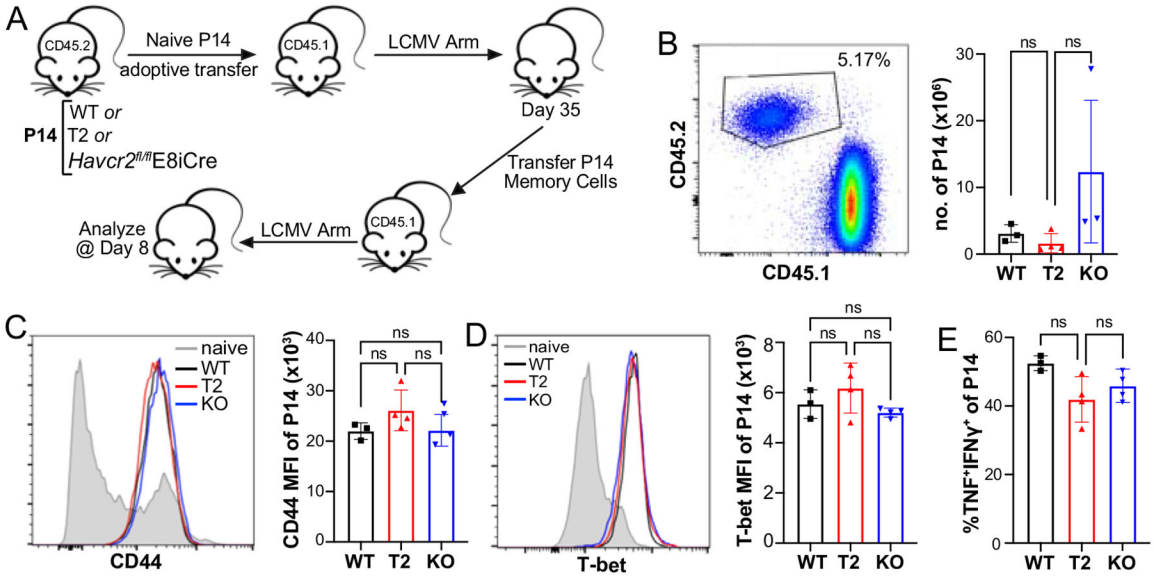

Figure 6: P14 memory recall response appears to be unaffected by Tim-3 signaling.

Naïve CD8 T cells (10^4) from P14, P14-T2 or P14-Havcr2$^{fl/fl}$E8icre mice were transferred into congenically marked naïve mice and infected with 2×10^5 pfu of LCMV arm. Memory P14 cells were sorted from the spleen on day 35 day after infection and 2×10^3 cells were adoptively transferred into naïve mice which were infected with 2×10^5 pfu of LCMV Arm. Splenocytes were harvested eight days after infection and processed for flow cytometry. **(A)** Schematic representation of experimental set up. **(B)** Total P14 cells in spleen. **(C)** CD44 MFI of P14 cells. **(D)** T-bet MFI of P14 cells. **(E)** Cytokine production of P14 cells after *ex-vivo* stimulation with GP33 peptide for five hours. In the graphs, each point represents an individual mouse and bars indicate ±SD (WT, n=3; T2,n=3, KO, n=3). The experiment was performed once. No significant differences between the groups were identified by a one-way anova.