

ARTICLE

TGF- β promotes stem-like T cells via enforcing their lymphoid tissue retention

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Stem-like CD8⁺ T cells sustain the antigen-specific CD8⁺ T cell response during chronic antigen exposure. However, the signals that control the maintenance and differentiation of these cells are largely unknown. Here, we demonstrated that TGF- β was essential for the optimal maintenance of these cells and inhibited their differentiation into migratory effectors during chronic viral infection. Mechanistically, stem-like CD8⁺ T cells carried a unique expression pattern of $\alpha 4$ integrins (i.e., $\alpha 4\beta 1^{\text{hi}}$ and $\alpha 4\beta 7^{\text{lo}}$) controlled by TGF- β . In the absence of TGF- β signaling, greatly enhanced expression of migration-related markers, including altered expression of $\alpha 4$ integrins, led to enhanced egress of stem-like CD8⁺ T cells into circulation accompanied by further differentiation into transitional states. Blocking $\alpha 4$ integrin significantly promoted their lymphoid tissue retention and therefore partially rescued the defective maintenance of Tcf-1⁺ subset in the absence of TGF- β signaling. Thus, TGF- β promotes the maintenance and inhibits the further differentiation of stem-like T cells at least partially via enforcing their lymphoid tissue residency.

Introduction

During chronic antigen exposure, effector T cells enter an exhausted state (Hashimoto et al., 2018; McLane et al., 2019). A subset of less exhausted CD8⁺ T cells carry unique markers including chemokine receptor CXCR5 and transcription factor Tcf-1 and behave like stem cells or progenitors to sustain T cell response (He et al., 2016; Im et al., 2016; Leong et al., 2016; Utzschneider et al., 2016; Wu et al., 2016). Tcf-1⁺ stem-like CD8⁺ T cells are largely confined to the secondary lymphoid tissues (He et al., 2016; Im et al., 2016) and do not enter the circulation, similar to tissue-resident cells (Im et al., 2020). This unique location is conserved between mouse and human (Miron et al., 2018). Even though stem-like CD8⁺ T cells have been identified among tumor-infiltrating T cells (Brummelman et al., 2018; Kurtulus et al., 2019; Miller et al., 2019; Sade-Feldman et al., 2018; Siddiqui et al., 2019), which by definition is outside a secondary lymphoid tissue, a T cell zone-like local environment exists inside solid tumors to host these stem-like CD8⁺ T cells

(Jansen et al., 2019). Thus, the retention inside secondary lymphoid organs or similar microenvironment may be essential for stem-like CD8⁺ T cells. Leaving such environment may be associated with further differentiation accompanied by the loss of stemness. However, the signals or mechanisms controlling the migration or retention of stem-like CD8⁺ T cells are largely unknown.

TGF- β is generally considered a negative regulator of immune responses during chronic infection or anti-tumor immunity (Giordano et al., 2015; Park et al., 2016; Tinoco et al., 2009). TGF- β inhibitor has been successfully combined with checkpoint blockade therapy to improve tumor control (Mariathasan et al., 2018; Tauriello et al., 2018). Intriguingly, TGF- β has been identified as a potent inducer of CXCR5 expression on effector CD8⁺ T cells during in vitro culture (Mylvaganam et al., 2017), suggesting a possible role of TGF- β in stem-like CD8⁺ T cells. Recently, it has been demonstrated that TGF- β promotes Tcf-1⁺ stem-like T cells via controlling mTOR activity and preserves

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mitochondrial capacity during chronic viral infection (Gabriel et al., 2021). In addition to metabolic regulation, whether TGF- β controls the migration and localization of stem-like T cells remains unknown. Here, using TGF- β receptor-deficient CD8⁺ T cells, we show that TGF- β promotes stem-like CD8⁺ T cells, especially during the late stages of chronic viral infection. The defect in TGF- β receptor-deficient cells is partially due to dramatically altered expression of $\alpha 4$ integrins, which leads to enhanced egress of stem-like CD8⁺ T cells into circulation accompanied by subsequent differentiation into transitional effector T cells.

Results

TGF- β promotes stem-like CD8⁺ T cells

To determine the role of TGF- β in antigen-specific stem-like CD8⁺ T cells, we employed LCMV (lymphocytic choriomeningitis virus) Clone 13 infection model and TGF- β receptor II conditional knockout (*Tgfb2^{fl/fl}* distal *Lck-cre*, hereafter referred to as *Tgfb2^{-/-}*) P14 TCR transgenic mice (Ma et al., 2017) that carried CD8⁺ T cells specific for LCMV epitope H-2D^b-GP₃₃₋₄₁. As illustrated in Fig. 1 A, naive P14 T cells carrying distinct congenic markers were isolated from WT control and *Tgfb2^{-/-}* mice, mixed at a 1:1 ratio, and adoptively co-transferred into unmanipulated sex-matched C57BL/6 (B6) recipient mice followed by LCMV Cl13 infection. In this system, WT control P14 T cells and *Tgfb2^{-/-}* ones are compared side-by-side in the same WT environment. Percentage-wise, early after infection, there were no significant changes in the induction of CXCR5 (Fig. S1, A and B) or Tcf-1 (Fig. 1, B and C) in donor P14 T cells lacking TGF- β receptor. However, as the infection progressed, a dramatic reduction of CXCR5⁺ and Tcf-1⁺ percentage was observed in *Tgfb2^{-/-}* cells (Fig. 1, B and C; and Fig. S1, A and B). As we have reported before, there was a decrease in the expansion of *Tgfb2^{-/-}* CD8⁺ T cells during both acute and chronic infections (Ma and Zhang, 2015; Zhang and Bevan, 2013). Thus, when focusing on the total number of Tcf-1⁺ vs. Tcf-1⁻ subsets, *Tgfb2^{-/-}* cells exhibited a two- to threefold reduction for both subsets at early stages. In contrast, at day 22 after infection, Tcf-1⁺ subset was specifically reduced while Tcf-1⁻ one was apparently normal in the absence of TGF- β receptor (Fig. 1 D). CD39 has been identified as an exhaustion marker while CD73 has been associated with CXCR5⁺ subset (Gupta et al., 2015; Im et al., 2016). CD39^{lo}CD73^{hi} cells were almost exclusively limited to Tcf-1⁺ subset (Fig. S1 C). In the absence of TGF- β receptor, CD39^{lo}CD73^{hi} cells were dramatically reduced (Fig. S1 D). Interestingly, the defects of CD73 induction occurred from the early stages of the infection, presumably due to the fact that CD73 was a direct target of TGF- β signaling in T cells (Chen et al., 2019; Takimoto et al., 2010). In addition, several other surface markers associated with CXCR5⁺Tcf-1⁺ subset (e.g., Ly6C, CXCR3, and CD62L) were similarly defective in *Tgfb2^{-/-}* cells at later stages of the infection (Fig. S1, E and F). Because stem-like CD8⁺ T cells are associated with poly-functionality, consistent with defective maintenance of stem-like subset, IL-2 production was greatly reduced at late, but not early stage of Cl13 infection in the absence of TGF- β signaling (Fig. 1 E).

TGF- β is critical for Tcf-1⁺ stem-like subset. We wondered whether Tcf-1⁺ and Tcf-1⁻ cells received differential TGF- β

signaling in vivo. We did detect a slight trend of higher pSmad2/3 signal in Tcf-1⁺ cells although it did not reach statistical significance (Fig. S1 G). In addition, when WT and *Tgfb2^{-/-}* P14 T cells were separately transferred into different B6 recipients, we did not observe a dramatic difference in viral clearance or tissue pathology (Fig. S2, C and E). Transient depletion of CD4⁺ T cells prolonged the viremia. Side-by-side comparison demonstrated that the maintenance of stem-like CD8⁺ T cells required TGF- β both in the presence and the absence of CD4 help (Fig. S2, A and B). In the following experiments, we chose to use LCMV Cl13 infection without CD4 depletion. Together, TGF- β promotes stem-like CD8⁺ T cells during chronic viral infection.

TGF- β controls $\alpha 4$ integrins

We have previously discovered that TGF- β inhibits integrin $\alpha 4\beta 7$ expression on effector CD8⁺ T cells during chronic LCMV infection (Zhang and Bevan, 2013). Thus, using the same experimental system, we have identified two seemingly distinct defects in *Tgfb2^{-/-}* cells, which are (1) enhanced $\alpha 4\beta 7$ integrin expression and (2) defective stem-like CD8⁺ T cells. We wondered whether these two phenotypes were related or independent. We first examined the expression of $\alpha 4$ integrins on Tcf-1⁺ vs. Tcf-1⁻ CD8⁺ T cell subsets. Interestingly, we found that on WT cells, the expression of integrin $\alpha 4\beta 7$ was almost exclusively restricted to Tcf-1⁻ subset (Fig. 1 F). However, in the absence of TGF- β receptor, the expression of $\alpha 4\beta 7$ integrin was significantly increased, especially on Tcf-1⁺ subset (Fig. 1, F and H). There are two $\alpha 4$ -containing integrin heterodimers on T cells, namely, integrin $\alpha 4\beta 1$ and $\alpha 4\beta 7$. Consistent with the restricted expression of $\alpha 4\beta 7$ on WT Tcf-1⁻ cells, $\beta 1$ integrin was dominant on stem-like Tcf-1⁺ subset while $\beta 1$ and $\beta 7$ co-expressed on the majority of Tcf-1⁻ subset (Fig. 1 G). On *Tgfb2^{-/-}* cells, the expression of $\beta 7$ integrin was substantially increased on Tcf-1⁺ cells accompanied by reduced expression of $\beta 1$ integrin (Fig. 1, G–I). $\beta 1$ and $\beta 7$ co-expressing cells were significantly enhanced in *Tgfb2^{-/-}* cells, especially in Tcf-1⁺ stem-like subset (Fig. 1 G right panel). To be noted, altered expression of $\alpha 4$ -containing integrins on *Tgfb2^{-/-}* cells occurred as early as day 7–10 after infection (Fig. 1, F–I). To further validate these findings, we measured integrin ligand binding. As shown in Fig. S3, compared with WT counterparts, *Tgfb2^{-/-}* Tcf-1⁺ cells exhibited significantly higher $\alpha 4\beta 7$ ligand (i.e., mucosal vascular addressin cell adhesion molecule-1 [MAdCAM-1]) binding and slightly reduced $\alpha 4\beta 1$ ligand (i.e., vascular cell adhesion molecule-1 [VCAM-1]) binding. Importantly, the altered integrin ligand binding could be detected as early as day 10 (Fig. S3). Thus, we have shown that integrin $\beta 1$ and $\beta 7$ exhibits distinct expression pattern on Tcf-1⁺ vs. Tcf-1⁻ CD8⁺ T cells. The vast majority of WT Tcf-1⁺ cells only express $\beta 1$ integrin while a significant portion of *Tgfb2^{-/-}* Tcf-1⁺ ones carry slightly reduced $\beta 1$ and dramatically increased $\beta 7$ integrins, resembling WT Tcf-1⁻ effectors.

TGF- β is essential for lymphoid tissue retention of Tcf-1⁺ cells

Because stem-like CD8⁺ T cells are commonly restricted to lymphoid tissues (He et al., 2016; Im et al., 2016; Im et al., 2020) and integrins are often associated with cell migration, we wondered whether TGF- β controlled the retention of Tcf-1⁺ cells

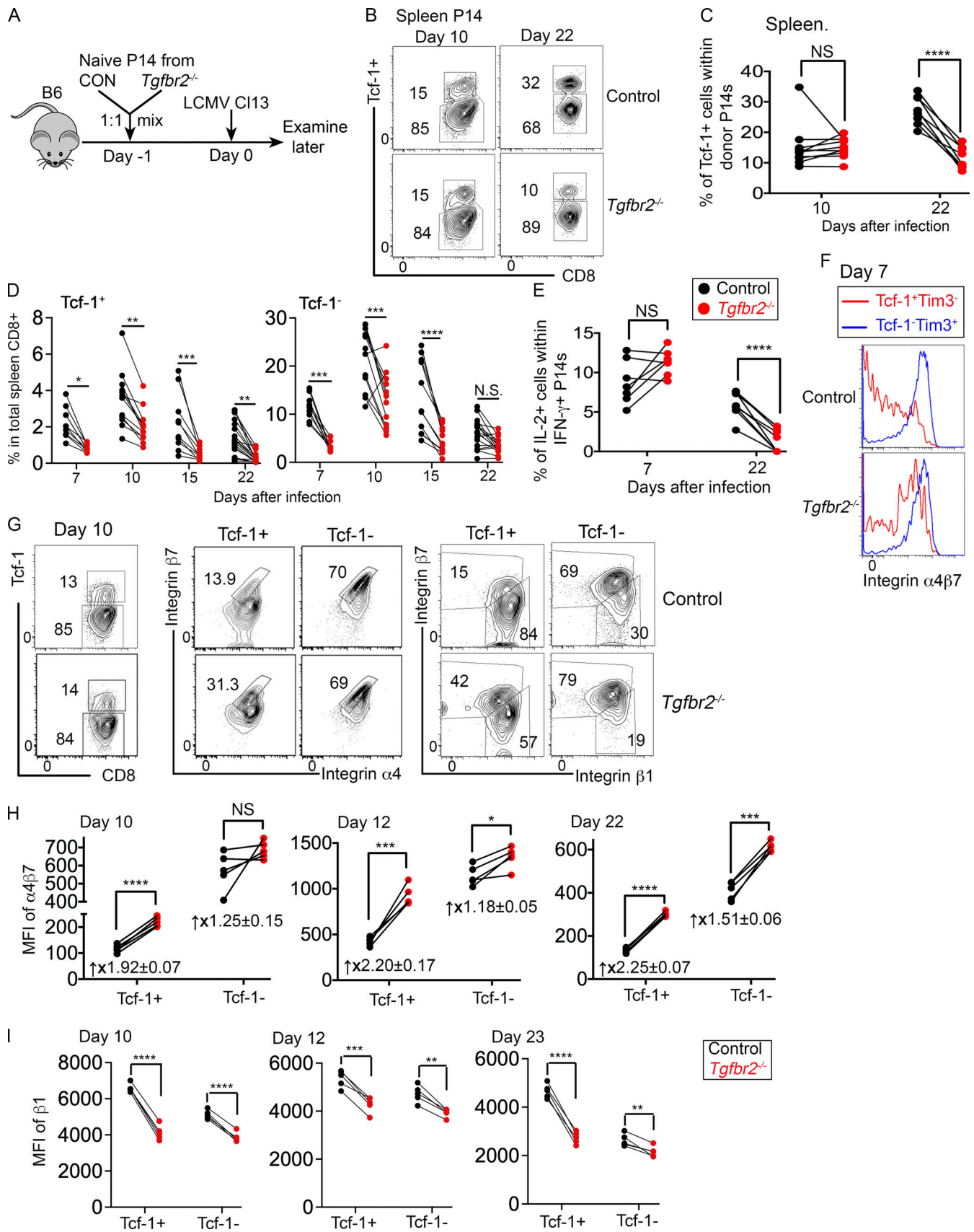


Figure 1. **TGF- β promotes stem-like CD8⁺ T cells during chronic viral infection.** (A) Experimental design. (B) At indicated days after infection, representative FACS profiles of pre-gated donor P14 T cells are shown (six repeats, $n = 4-5$ /each). (C) The percentage of Tcf-1⁺ cells in P14 T cells is shown. (D) The

percentage of Tcf-1⁺ (left) and Tcf-1⁻ (right) donor P14 T cells in total splenic CD8⁺ T cells is shown. **(E)** The percentage of IL-2 producing cells in IFN- γ ⁺ P14 T cells is shown. **(F and G)** Day 7 (F) and day 10 (G) after infection, representative FACS profiles of pre-gated Tcf-1⁺ and Tcf-1⁻ P14 T cells are shown (three repeats, $n = 4-5$ /each). **(H and I)** Mean fluorescence intensity (MFI) of integrin $\alpha 4\beta 7$ (H) and MFI of integrin $\beta 1$ (I) on Tcf-1⁺ and Tcf-1⁻ P14 cells are shown. The number in H was calculated as the ratio of MFI $\alpha 4\beta 7$ on *Tgfb2*^{-/-} over MFI $\alpha 4\beta 7$ on co-transferred control P14s in each recipient mouse. Each pair of symbols in C–E, H, and I represents the results from an individual recipient. Combined results from three (C, total $n = 8-10$ /time point), six (D, $n = 10-18$ /time point), three (E, $n = 7-9$ /time point), and three (H and I, $n = 5$ /time point) independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by paired Student's *t* test for each time point.

inside lymphoid tissues via manipulating the expression of integrins. To this end, we compared Tcf-1⁺ P14 donor T cells isolated from the spleen and peripheral blood of the same animals. At early stage after LCMV Cl13 infection, although highly enriched in lymphoid follicles, a clear Tcf-1⁺ stem-like P14 subset was detected in the peripheral blood (Fig. 2 A). Interestingly, we observed a stepwise reduction of Tcf-1⁺ subset in the blood as infection progressed (Fig. 2 B). Blood Tcf-1⁺ subset was composed of a much higher portion of *Tgfb2*^{-/-} cells compared with both spleen red pulp and spleen follicles (Fig. 2 C), demonstrating that Tcf-1⁺ cells were more likely to enter the circulation in the absence of TGF- β signaling. Although we observed a subtle increase of *Tgfb2*^{-/-} Tcf-1⁺ cells in spleen red pulp compared with lymphoid follicles, the difference did not reach statistical significance (Fig. 2 C). Furthermore, circulating WT Tcf-1⁺ subset carried significantly higher portion of $\beta 1 \beta 7$ double positive cells than their splenic counterparts (Fig. 2, D–E). *Tgfb2*^{-/-} Tcf-1⁺ subset carried increased population of $\beta 1 \beta 7$ double-positive cells in both blood and spleen (Fig. 2, D and E). Together, consistent

with altered integrin expression pattern, TGF- β promotes the retention of stem-like CD8⁺ T cells inside lymphoid tissues and limits their egress into the circulation.

To further evaluate the role of TGF- β during chronic LCMV infection, we examined the distribution of TGF- β -producing cells in the spleen by immunohistochemistry (Fig. S2 F). We could detect TGF- β -producing cells both inside and outside lymphoid follicles. Together with the recent findings that stem-like CD8⁺ T cells carried higher levels of TGF- β -activating integrin $\alpha \nu \beta 8$ (Gabriel et al., 2021), it is likely that there is a TGF- β -rich microenvironment for stem-like T cells inside lymphoid organs during chronic viral infection.

Enhanced differentiation of transitional subsets in the absence of TGF- β

It has been demonstrated that both stem-like and terminally exhausted CD8⁺ T cells carry tissue-resident T cell marker CD69 and are not circulating. Only transitional subsets are migratory (Beltra et al., 2020). Given our findings that *Tgfb2*^{-/-} stem-like

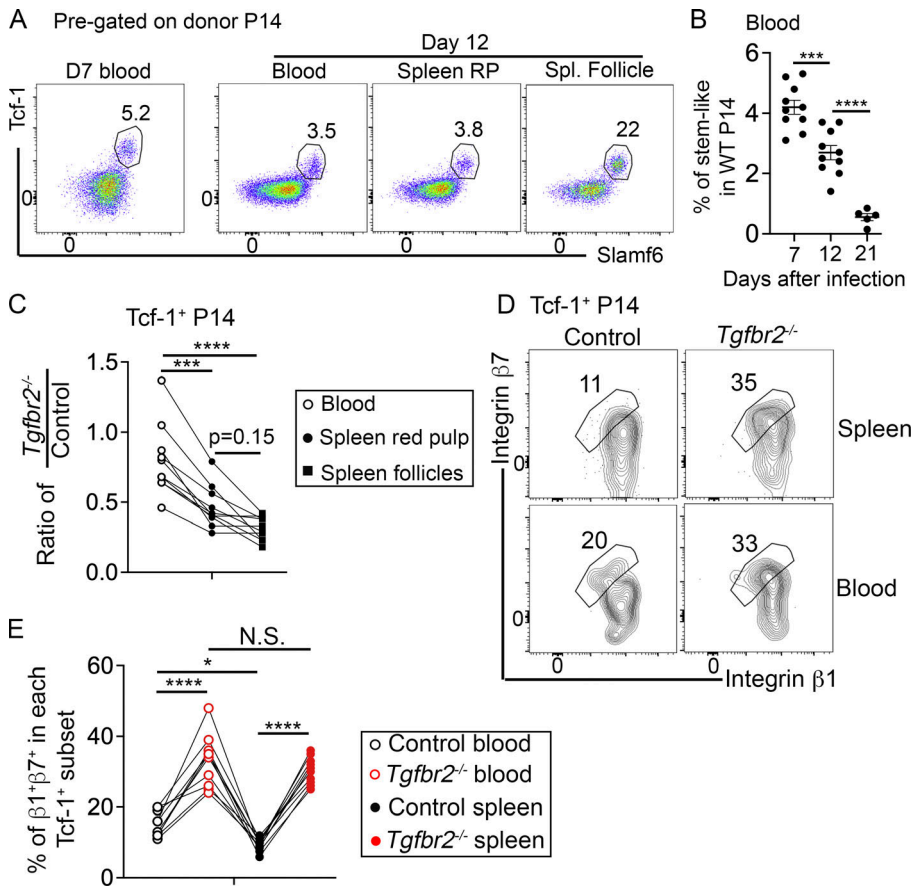


Figure 2. TGF- β controls the lymphoid tissue retention of Tcf-1⁺ CD8⁺ T cell subset. Same experimental setup as in Fig. 1. **(A)** Day 7 and day 12 after infection, representative FACS profiles of pre-gated donor WT P14 T cells are shown (two independent repeats, $n = 4-5$ /each). **(B)** The percentage of Tcf-1⁺Slamf6⁺ subset in WT P14 T cells from peripheral blood is shown. **(C)** Day 12 after infection, the ratio of *Tgfb2*^{-/-} Tcf-1⁺ over control Tcf-1⁺ P14 T cells from different tissues is shown. **(D)** Representative FACS profiles of pre-gated Tcf-1⁺ P14 T cells are shown (two independent repeats). **(E)** The percentage of $\beta 1^+ \beta 7^+$ cells in each Tcf-1⁺ subset is shown. Each symbol in B and each group of connected symbols in C and E represents the results from an individual recipient mouse. Pooled results from five (B, total $n = 5-10$) or two (C and E, total $n = 10$) independent experiments are shown. *, $P < 0.05$; ***, $P < 0.001$; and ****, $P < 0.0001$ by ordinary one-way ANOVA.

subset exhibited defective maintenance and enhanced circulation, we wondered whether TGF- β suppressed the differentiation of transitional CD8⁺ subsets during chronic viral infection. To this end, we examined the four subsets of exhausted CD8⁺ T cells defined by the expression of Tcf-1 and CD69, i.e., Tcf-1⁺CD69⁺ resident stem, Tcf-1⁺CD69⁻ transitional stem, Tcf-1⁻CD69⁻ migratory effector, and Tcf-1⁻CD69⁺ terminal effector (red pulp resident). As shown in Fig. 3, A and B, while both Tcf-1⁺CD69⁺ tissue-resident stem-like and terminally exhausted Tcf-1⁻CD69⁺ subsets were significantly reduced, Tcf-1⁻CD69⁻ migratory effectors were dramatically enhanced in the absence of TGF- β signaling. Transitional stem-like Tcf-1⁺CD69⁻ subset was slightly reduced in *Tgfb2*^{-/-} cells. Intravascular anti-CD8 labeling separates splenic CD8⁺ T cells into red pulp and lymphoid follicle compartments. We found that Tcf-1⁺ subsets, especially transitional Tcf-1⁺CD69⁻ subset exhibited enhanced access to intravascular antibody labeling without TGF- β signaling (Fig. 3, C and D), suggesting that *Tgfb2*^{-/-} Tcf-1⁺ T cells were more likely to localize outside lymphoid follicles. As expected, both Tcf-1⁻CD69⁻ and Tcf-1⁻CD69⁺ were largely confined to the red pulp in the presence and absence of TGF- β signaling (Fig. 3, C and D). Consistent with the results from early time points (Figs. 1 and 2), *Tgfb2*^{-/-} cells carried significantly increased populations of β 1 β 7 double positive cells at day 22 after infection (Fig. 3 E). Importantly, the impacts of TGF- β signaling on β 1 β 7-co-expressing population were much more dramatic for migratory subsets (CD69⁻ cells, including both Tcf-1⁺CD69⁻ and Tcf-1⁻CD69⁻) compared with resident counterparts (CD69⁺ cells, including both Tcf-1⁺CD69⁺ and Tcf-1⁻CD69⁺; Fig. 3 E).

CX3CR1 has been established as a convenient marker to define migratory effector CD8⁺ T cells with superior cytotoxic activity (Zander et al., 2019). Consistently, in WT cells, CX3CR1 expression was largely restricted to Tcf-1⁻ CD69⁻ cells (Fig. 3, F and G). In *Tgfb2*^{-/-} cells, the expression of CX3CR1 was further enhanced on Tcf-1⁻ CD69⁻ subset and significantly increased on terminally exhausted Tcf-1⁻ CD69⁺ subset (Fig. 3, F and G). In Fig. 1 E, we have demonstrated altered cytokine production in *Tgfb2*^{-/-} P14 T cells. Further, it is generally accepted that TGF- β signaling directly suppresses CD8⁺ effector functions. To accurately access the role of TGF- β in CD8⁺ effector function during chronic viral infections, we incorporated Tcf-1 staining to separate stem-like vs. non-stem P14 T cells. Interestingly, when separating Tcf-1⁺ vs. Tcf-1⁻ subsets, at day 22 after infection, we did not detect any significant changes in both cytokine production and granzyme expression in the presence vs. absence of TGF- β receptor (Fig. 4, A–C). These results suggest that TGF- β does not directly control CD8 effector function at late stages during chronic viral infection, but instead regulates Tcf-1⁺→Tcf-1⁻ cell differentiation. Consistent with this notion, when examining exhaustion markers (Tox and Tim-3) on Tcf-1⁺ cells, *Tgfb2*^{-/-} cells exhibited comparable expression levels as WT controls (Fig. 4, D–F). We did detect a reduction of Tox expression in *Tgfb2*^{-/-} Tcf-1⁻ subset (Fig. 4, D and E), presumably due to decreased Tcf-1⁻CD69⁺ terminally exhausted subset in the absence of TGF- β receptor (Fig. 3, A and B).

To directly test whether TGF- β inhibits the differentiation from Tcf-1⁺ to Tcf-1⁻ CD8⁺ T cells, we performed a stem-like CD8⁺ adoptive transfer assay. Briefly, congenically marked WT

and *Tgfb2*^{-/-} mice were infected by LCMV Cl13. 2 wk later, stem-like CD8⁺ T cells were FACS sorted and adoptively transferred into infection-matched WT mice (Fig. 4 G). 7 d after transfer, we did not detect significant difference in the population size between WT and *Tgfb2*^{-/-} donor cells (Fig. 4 H). In contrast, significantly increased CX3CR1⁺ cells were observed in *Tgfb2*^{-/-} donors (Fig. 4, I and J). A consistent trend of reduction was detected for Tcf-1⁺ subset (Fig. 4, I and J).

Together, in *Tgfb2*^{-/-} CD8⁺ T cells, reduced Tcf-1⁺ stem-like population is associated with enhanced differentiation into transitional subsets and elevated circulation.

α 4 integrin blocking promotes Tcf-1⁺ maintenance

To test whether there was a causal link between altered integrin expression, defective Tcf-1⁺ cells and enhanced differentiation into transitional effectors in *Tgfb2*^{-/-} CD8⁺ T cells, we blocked α 4 integrins starting after the early stages of the infection (Fig. 5 A). Percentage-wise, 12 d of blocking significantly rescued *Tgfb2*^{-/-} Tcf-1⁺ subset (Fig. 5, C–E; and Fig. S4 A). Further, blockade of α 4 integrins rapidly boosted Tcf-1⁺ cells even in WT cells (Fig. 5, C and D). To be noted, at the dose of blocking antibody we were using, the blocking was not complete as we could still detect slightly enhanced expression of integrin α 4 β 7 on *Tgfb2*^{-/-} P14 T cells (Fig. S4 B). In addition, α 4 integrin blocking did not significantly interfere with viral clearance (Fig. 5 B), which allowed us to avoid confounding factors when examining the differentiation of P14 T cells. Consistent with the percentage, when total population size was considered, α 4 integrin blocking did not impact Tcf-1⁻ subset while it significantly boosted Tcf-1⁺ subset, especially for *Tgfb2*^{-/-}Tcf-1⁺ cells (Fig. 5 F). One step further, we asked whether α 4 integrin blocking could boost Tcf-1⁺ cells when given at a later stage. Indeed, when blocking was initiated on day 25 after infection, we could detect a similar and significant enhancement of Tcf-1⁺ subsets 5 d later (Fig. 5, G and H). Together, we have demonstrated that altered expression of α 4 integrins was at least partially responsible for the defective maintenance of Tcf-1⁺ stem-like cells in the absence of TGF- β signaling.

α 4 Integrin blocking leads to accumulation of migratory effectors

So far, we have demonstrated that TGF- β controls α 4 integrin expression and boosts Tcf-1⁺ subset during chronic viral infection possible via enforcing lymphoid tissue retention of stem-like subset. In other words, the differentiation of Tcf-1⁺ stem-like subset is tightly associated with the appearance of migratory effectors, which require α 4 integrin to leave lymphoid tissues. If this was true, we expected that α 4 integrin blocking would not only boost Tcf-1⁺ stem-like T cells but would also trap more migratory effectors inside the spleen. As shown in Fig. 6 A, when examining CX3CR1 and CD69 together, we could identify two subsets of P14 T cells, i.e., CX3CR1⁺CD69^{lo} migratory and CX3CR1^{lo}CD69⁺ resident ones. For WT Tcf-1⁺ cells, the vast majority was CX3CR1^{lo}CD69⁺ resident cells while for WT Tcf-1⁻ ones, both CX3CR1⁺CD69^{lo} migratory and CX3CR1^{lo}CD69⁺ resident cells were readily detectable (Fig. 6 A, left column). For *Tgfb2*^{-/-} P14 T cells, there was a significant increase in CX3CR1⁺CD69^{lo} migratory subset, especially for Tcf-1⁻ subset

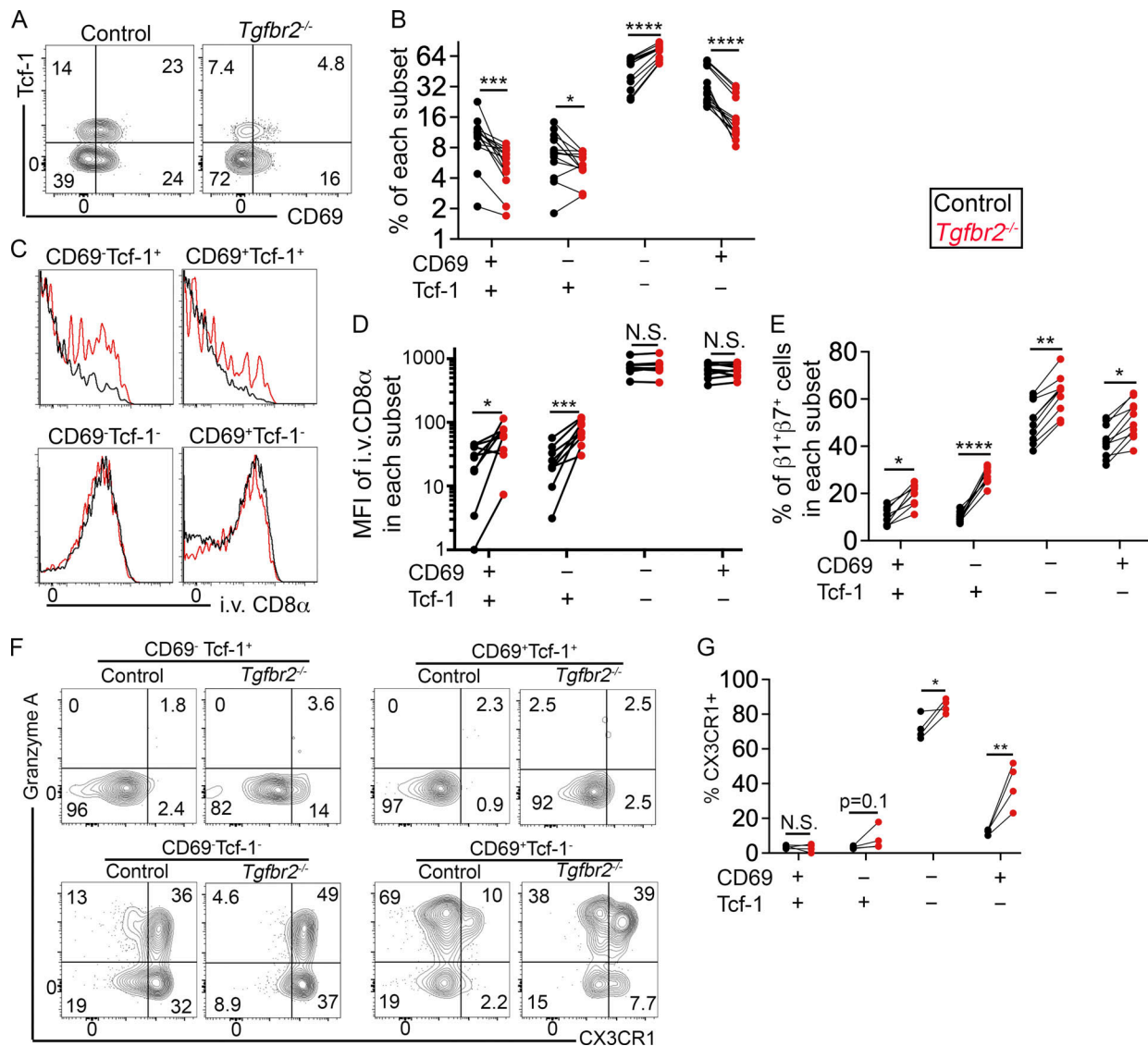


Figure 3. TGF- β suppresses the differentiation of transitional subsets of CD8⁺ T cells. (A) Same experimental setup as in Fig. 1. Day 21–23 after infection, representative FACS profiles of pre-gated donor P14 T cells isolated from the spleen (three independent repeats). (B–C) Characterization of the four P14 subsets defined by the expression of Tcf-1 and CD69. The percentage of each subset in total P14 T cells (B), intravascular anti-CD8 α labeling in each subset (C and D), and the percentage of β 1⁺ β 7⁺ cells in each subset (E) are shown. (F) Representative FACS profiles to show the expression of CX3CR1 and Granzyme A in each subset of P14 T cells. (G) The percentage of CX3CR1⁺ cells in each subset is shown. Each pair of symbols in B, D, E, and G represents the results from an individual recipient mouse. Pooled or representative results from two (C–E, $n = 10$; F and G, $n = 4$) or three (A and B, $n = 14$) independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by ordinary one-way ANOVA or Student’s t test.

(Fig. 6 A). In the presence of α 4 integrin blocking, we detected a slight, but often significant increase of CX3CR1⁺CD69^{lo} migratory cells and a consistent reduction of CD69⁺ resident cells (Fig. 6, A–D). Importantly, α 4 blocking had minimal impacts on the effector functions of P14 T cells when Tcf-1⁺ and Tcf-1⁻ subsets were separately examined (Fig. 6, E–G).

To further strengthen this conclusion that α 4 integrin blocking boosts stem-like T cells via suppressing T cell migration, we FACS-sorted Slamf6⁺ vs. Slamf6⁻ P14 T cells and cultured sorted cells with GP₃₃₋₄₁ peptide-pulsed splenocytes in the presence or absence of α 4 blocking antibody (Fig. S4 C). 4 d after culture, as expected, Slamf6⁻ input cells remained Slamf6⁻Tcf-1⁻ while Slamf6⁺ input ones differentiated into both Tcf-1⁺ and

Tcf-1⁻ cells. Importantly, α 4 integrin blocking did not impact Tcf-1⁺→Tcf-1⁻ differentiation in this in vitro setting when cell migration was not involved (Fig. S4 D).

Together, these results strongly support the conclusion that α 4 integrin blocking promotes Tcf-1⁺ stem-like subset via suppressing T cell migration.

α 4 Integrin blocking promotes the retention of stem-like T cells inside lymphoid follicles

To test whether α 4 integrin blocking directly impacts lymphoid tissue retention of stem-like CD8⁺ T cells, we shortened the duration of the treatment. As illustrated in Fig. 7 A, mice were examined after two injections of α 4 blocking antibody.

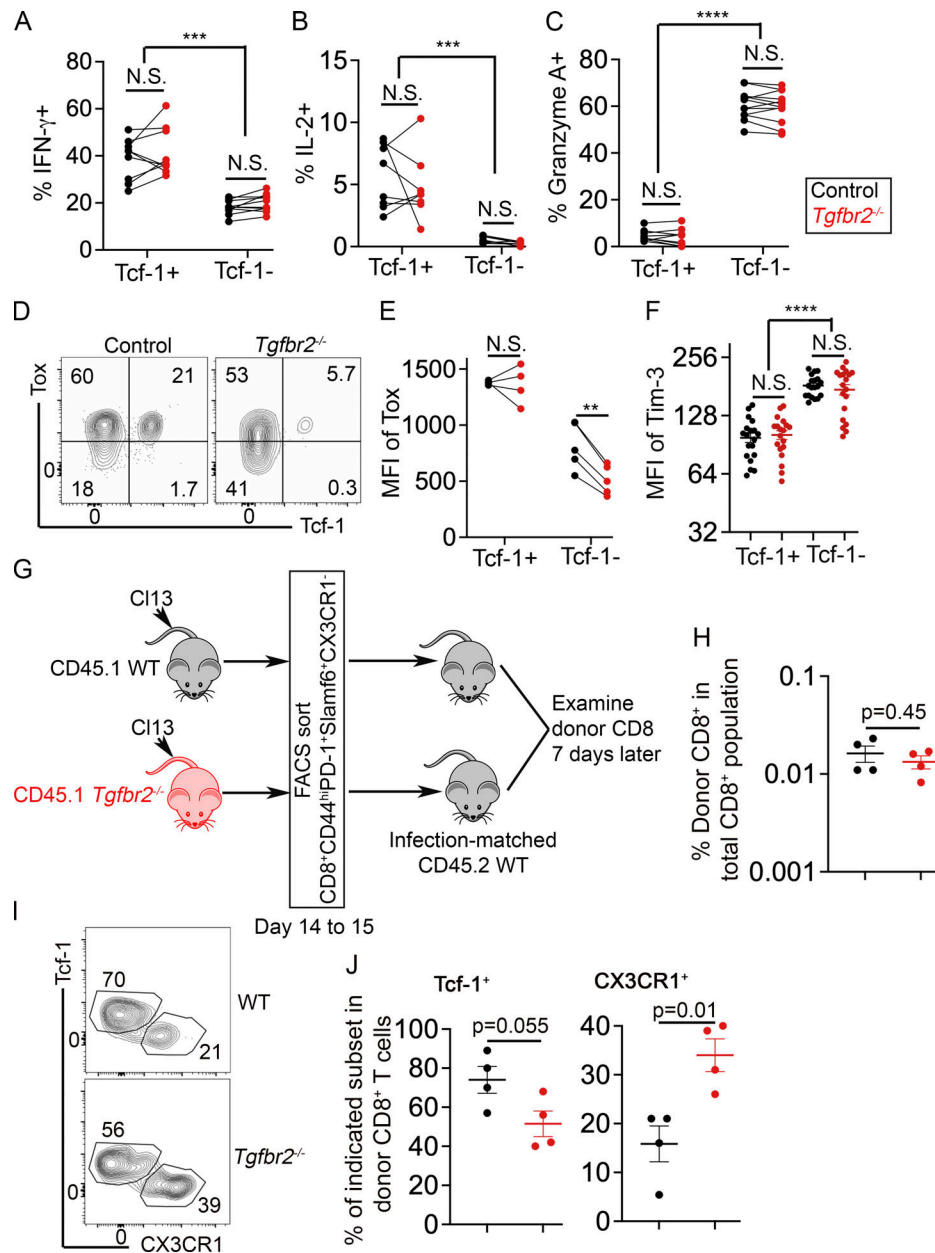


Figure 4. TGF- β suppressed the differentiation, but not the effector functions of Tcf-1⁺ stem-like T cells. Similar experimental setup to Fig. 1 (A–C) Day 22 after infection, the percentage of IFN- γ ⁺ (A), IL-2⁺ (B), and granzyme A⁺ (C) cells in Tcf-1⁺ and Tcf-1⁻ subsets of donor P14 T cells are shown. (D) Representative FACS profiles to show the expression of Tox. (E) MFI of Tox in Tcf-1⁺ and Tcf-1⁻ subsets are shown. (F) MFI of Tim-3 is shown. (G) Experimental setup for H–J. (H) The percentage of donor CD8⁺ T cells in the total CD8⁺ population is shown. (I) Representative FACS profiles of pre-gated donor CD8⁺ T cells are shown. (J) The percentage of Tcf-1⁺ (left) and CX3CR1⁺ (right) cells in donor CD8⁺ T cells are shown. Each pair of symbols in A–D, and each symbol in E, H, and J represents the results from an individual recipient mouse. Pooled results from two (A–C, $n = 8$ –12; D and E, $n = 5$; H–J, $n = 4$ /each) and five (F, $n = 19$) independent experiments are shown. **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by ordinary one-way ANOVA (A–F) or Student’s t test (H–J).

Consistent with previous findings (Im et al., 2016), stem-like CD8⁺ T cells were highly enriched in the lymphoid follicles (Fig. 7, B and C). Briefly, $\alpha 4$ integrin blockade dramatically enhanced stem-like CD8⁺ T cells, especially in the lymphoid follicle compartment of the spleen (Fig. 7, B and C). When total cell population was examined, shortened $\alpha 4$ blocking significantly promoted *Tgfb2*^{-/-} stem-like subset, but not Tcf-1⁻ subsets (Fig. 7 D). We could detect reduced CD69 expression after $\alpha 4$ blocking (Fig. 7 E), suggesting the trapping of CD69⁻ migratory

cells. Interestingly, at this early time point, we did observe increased effector function for *Tgfb2*^{-/-} cells (i.e., granzyme A and IFN- γ). But CD8⁺ effector functions were not affected by $\alpha 4$ integrin blocking (Fig. 7, F–H). Together, blocking $\alpha 4$ integrins significantly improves the lymphoid tissue retention and maintenance of stem-like CD8⁺ T cells in the absence of TGF- β signaling.

To further test the possibility that promoting lymphoid tissue retention would promote stem-like T cells, infected mice were

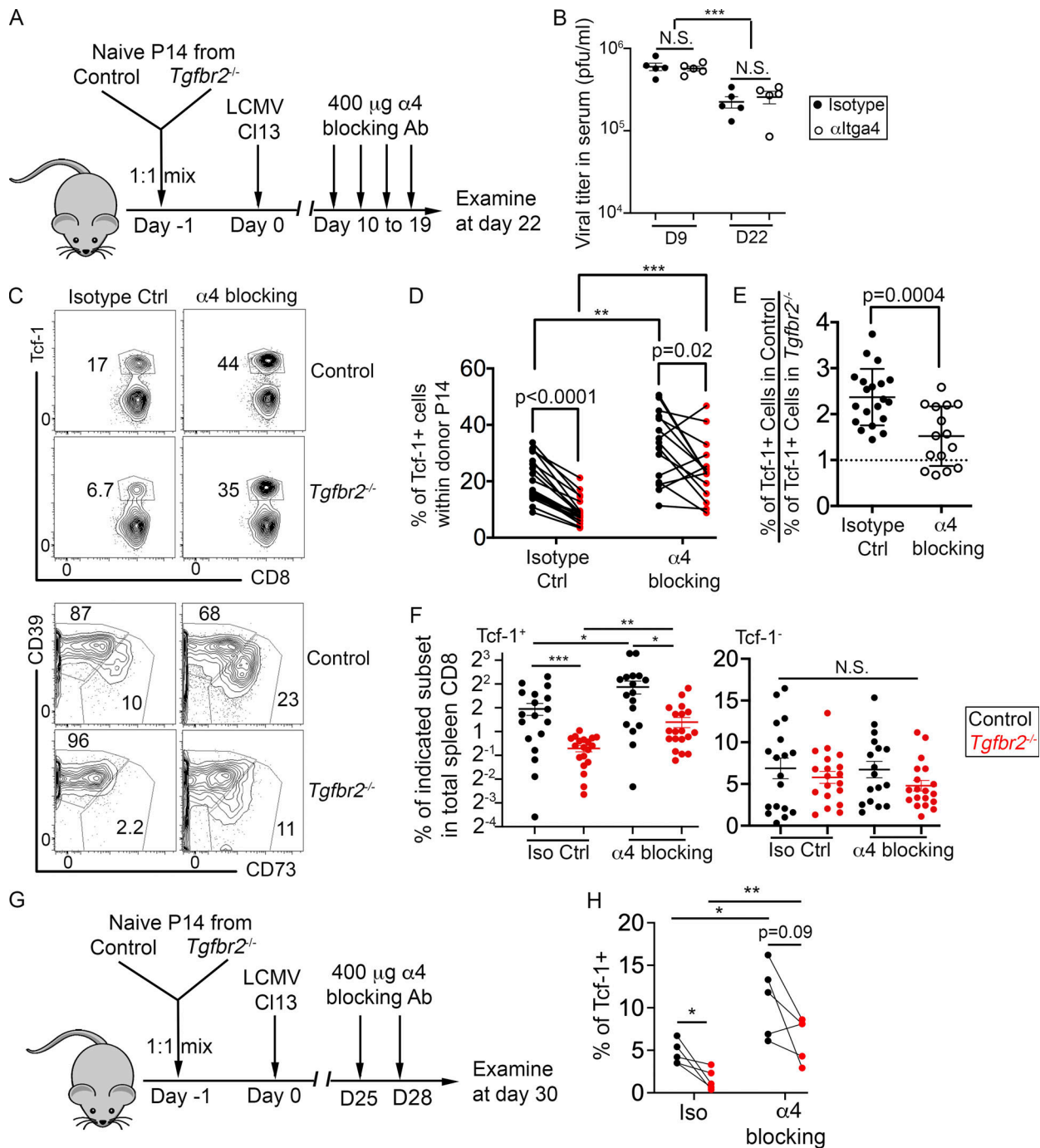


Figure 5. Blocking integrin $\alpha 4$ partially corrects the defective maintenance of *Tgfr2*^{-/-} Tcf-1⁺ CD8⁺ T cells. (A) Experimental design. Same P14 co-transfer and LCMV infection setup as in Fig. 1. Starting from day 10 after infection, integrin $\alpha 4$ blocking antibody was administrated every 3 d and examined on day 22. (B) The virus titer in the serum before (d9) and after (d22) *altga4* blocking are shown. (C) Representative FACS profiles of pre-gated donor splenic P14 T cells are shown. (D) The percentage of Tcf-1⁺ cells among P14 T cells is shown. (E) The ratios of Tcf-1⁺ percent in control P14s over Tcf-1⁺ percent in *Tgfr2*^{-/-} P14s are shown. (F) The percentage of Tcf-1⁺ (left) and Tcf-1⁻ (right) donor P14 T cells in total splenic CD8⁺ population are shown. (G) Experimental design for delayed integrin $\alpha 4$ blocking. (H) The percentage of Tcf-1⁺ subset in donor P14 T cells at day 30 after infection in the presence or absence of delayed $\alpha 4$ blocking. Each pair of symbols in D and H, and each symbol in B, E, and F represent the results from an individual mouse. Representative or pooled results from five independent experiments are shown in (C-F; $n = 15-20$). Pooled results from two independent experiments are shown in B ($n = 5$ /each) and H ($n = 5$ /each). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ by Student's t test in D, E, and H, or by ordinary one-way ANOVA in B and F.

treated with FTY720, which is a potent inhibitor of lymphocyte egress (illustrated in Fig. S5 A). FTY720 treatment leads to substantial improvement of Tcf-1⁺ population in both WT and *Tgfr2*^{-/-} cells (Fig. S5 B). Thus, lymphoid tissue retention

promotes the maintenance of stem-like CD8⁺ T cells. To further validate our findings that TGF- β promotes stem-like CD8⁺ T cells via enhancing their lymphoid tissue retention and residency, we examined the transcriptional profiles of WT

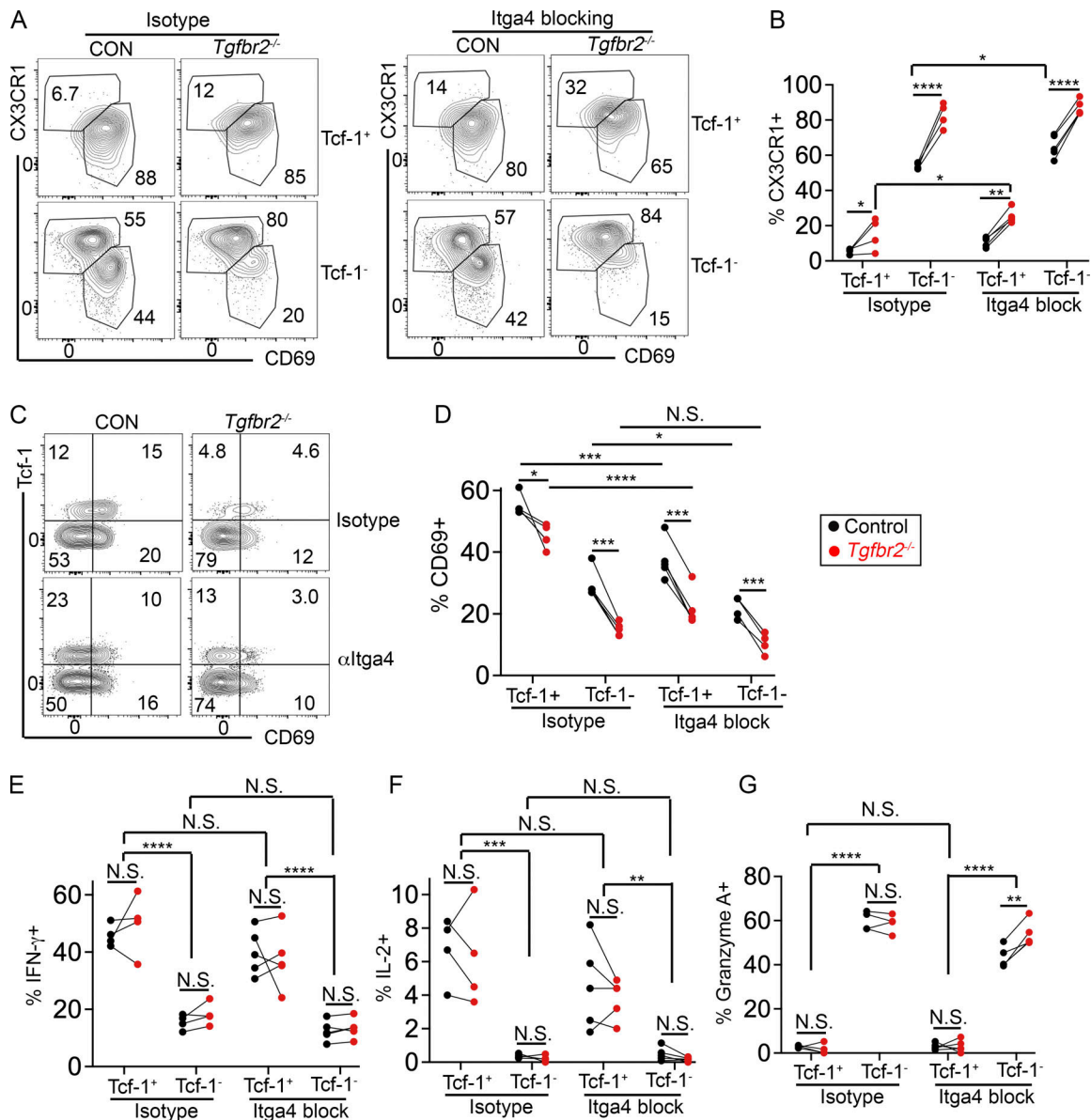


Figure 6. Integrin $\alpha 4$ blocking traps migratory cells and inhibits the differentiation of stem-like CD8⁺ T cells. Similar experimental setup to Fig. 5 A. **(A)** Representative FACS profiles of pre-gated Tcf-1⁺ (upper) and Tcf-1⁻ (lower) P14 T cells to show the expression of CX3CR1 and CD69. **(B)** The percentage of CX3CR1⁺ cells in Tcf-1⁺ and Tcf-1⁻ P14s are shown. **(C)** Representative FACS profiles of donor P14 T cells are shown. **(D)** The percentage of CD69⁺ in Tcf-1⁺ and Tcf-1⁻ P14s are shown. **(E–G)** The percentage of IFN- γ ⁺ (E), IL-2⁺ (F), and granzyme A⁺ (G) cells in Tcf-1⁺ and Tcf-1⁻ P14s are shown. Each pair of symbols in B, D–F, and G represents the results from an individual recipient. Representative or pooled results from two independent experiments are shown (n = 4–5/each). *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001 by ordinary one-way ANOVA.

and *Tgfb2*^{-/-} P14 T cells. Briefly, WT and *Tgfb2*^{-/-} donor P14 T cells were FACS sorted into four subsets based on the expression of Slmf6 and CD69. Taking advantage of established circulating vs. resident core signatures of memory T cells (Milner et al., 2017), we found that most *Tgfb2*^{-/-} subsets carried significantly defective resident signatures (Fig. S5 D). CD69⁺ subsets of exhausted CD8⁺ T cells have been demonstrated to carry tissue resident features (Beltra et al., 2020). Interestingly, in the absence of TGF- β receptor, both Slmf6⁺CD69⁺ and Slmf6⁻CD69⁺ resident subsets harbored significantly increased circulating T cell signature (Fig. S5 C). These results further strengthen our findings that TGF- β -dependent lymphoid

residency is an integrated component of CD8⁺ T cell biology during chronic viral infection.

Discussion

Together, we have demonstrated that TGF- β signaling suppresses the differentiation of stem-like CD8⁺ T cells into transitional subsets partially via controlling the expression of $\alpha 4$ integrins, limiting their egress into the circulation and enhancing their lymphoid tissue retention. Our results suggest a model that the migration out of lymphoid environment is coupled with further differentiation of stem-like CD8⁺ T cells. TGF-

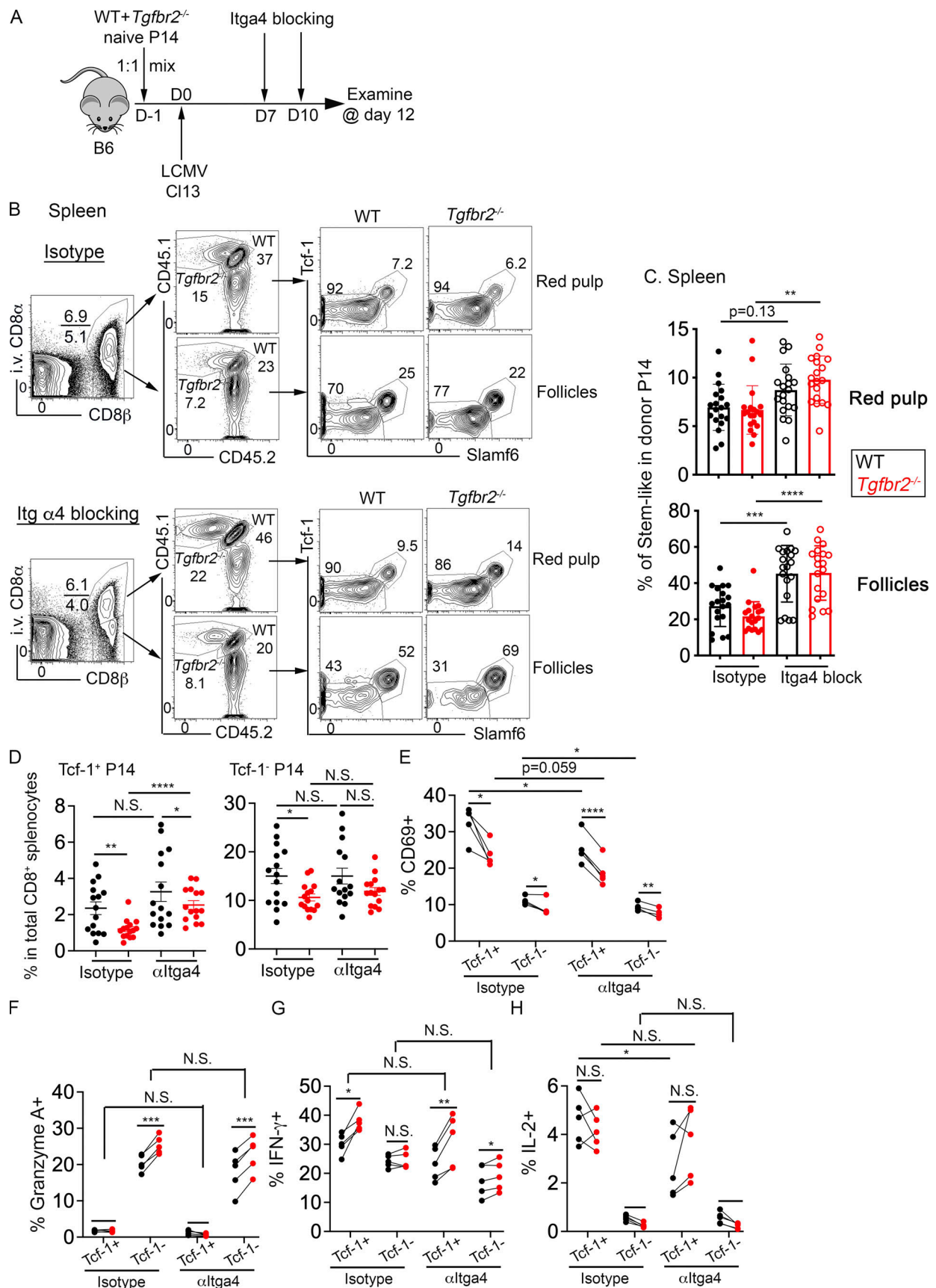


Figure 7. **Blocking α 4 integrins enhances lymphoid tissue retention of stem-like CD8⁺ T cells.** (A) Experimental design. Same P14 co-transfer and LCMV infection setup as in Fig. 1. On day 7 and 10 after infection, integrin α 4 blocking antibody was administered and examined on day 12. (B) Representative FACS

profiles of splenic lymphocytes are shown. **(C)** The percentage of stem-like subset in P14 T cells located in red pulp (upper) and follicles (lower) is shown. **(D)** The percentage of Tcf-1⁺ (left) and Tcf-1⁻ (right) donor P14 T cells in total splenic CD8⁺ population is shown. **(E)** The percentage of CD69⁺ in Tcf-1⁺ and Tcf-1⁻ P14s is shown. **(F-H)** The percentage of granzyme A⁺ (F), IFN- γ ⁺ (G), and IL-2⁺ (H) cells in Tcf-1⁺ and Tcf-1⁻ P14s is shown. Each pair of symbols in E-H, and each symbol in C and D represents the results from an individual recipient. Representative or pooled results from four (B-D, $n = 15-19$ /each) or two (E-H, $n = 5$) independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by ordinary one-way ANOVA.

β promotes lymphoid tissue retention and therefore inhibits the effector differentiation of stem-like CD8⁺ T cells. A recent publication has demonstrated that TGF- β signaling inhibits mTOR to preserve cellular metabolism and maintenance of stem-like T cells during chronic viral infection (Gabriel et al., 2021), which provides elegant evidence that the differentiation of stem-like T cells is controlled by a unique metabolic program. Our results provide another perspective linking TGF- β signaling with stem-like T cells via controlling the retention and localization of stem-like T cells inside lymphoid environment. Our results demonstrate that manipulating the migration and residency of stem-like T cells is sufficient to control their differentiation. Considering the recent evidence that TGF- β -controlled mitochondrial metabolism is required for tissue-resident memory T cell (T_{RM} cell) differentiation (Borges da Silva et al., 2020), it will be interesting to examine whether TGF- β -dependent metabolic program is directly connected with TGF- β -controlled resident program for stem-like T cells in the future.

TGF- β cytokines are secreted as inactive latent forms. Integrin $\alpha\beta 6$ and $\alpha\beta 8$ can activate TGF- β . Gabriel et al. have demonstrated that Tcf-1⁺ stem-like CD8⁺ T cells express higher levels of $\alpha\beta 8$ during chronic viral infection, which may partially explain how to maintain a TGF- β -rich microenvironment around stem-like T cells (Gabriel et al., 2021). Our findings demonstrate that TGF- β -controlled integrin $\alpha 4\beta 7$ and $\alpha 4\beta 1$ are essential components of a larger tissue resident program, which helps stem-like T cells to be maintained inside the lymphoid microenvironment. Lymphoid follicles may provide a critical shield from inflammatory insults. Further into the biology of integrins on T cells, it remains unknown whether integrin $\alpha\beta 8$ directly controls the migration of stem-like T cells in addition to TGF- β activation. It is interesting to note that both $\alpha\gamma$ and $\alpha 4$ integrin can form heterodimer with integrin $\beta 1$ (Hynes and Naba, 2012). Whether this potential competition has any functional consequence in stem-like T cells is unknown.

Considering the well-established role of TGF- β in T_{RM} cell differentiation following acute infections (Casey et al., 2012; Liao et al., 2021; Mackay et al., 2013; Sheridan et al., 2014; Zhang and Bevan, 2013), our results provide evidence that there is a general requirement of TGF- β signaling to maintain tissue-resident phenotypes regardless of acute or chronic infection settings. Consistently, global transcriptional analysis from the accompanying publication by Hu et al. (2022) and our own RNA sequencing (RNA-seq) analysis has demonstrated that *Tgfb2*^{-/-} CD8⁺ T cells carry reduced residency signatures and enriched circulating signatures. Thus, in addition to $\alpha 4$ integrins, TGF- β may help stem-like CD8⁺ T cells establish tissue residency-related transcriptional programs.

Importantly, during chronic LCMV infection, stem-like T cells gradually establish T_{RM} cell program in lymphoid

tissues, consistent with stepwise reduction of circulating stem-like T cells (Fig. 2 B), which also explains why TGF- β is preferentially required for the later stages of LCMV infection. Thus, stem-like CD8⁺ T cells progress along the TGF- β -dependent T_{RM} cell pathway during chronic antigen exposure.

In spite of these similarities, it is noteworthy to point out that there are clear differences between stem-like CD8⁺ T cells in chronic infection versus T_{RM} cells generated following acute infection. For example, stem-like CD8⁺ T cells are Tcf-1⁺ while T_{RM} cells often downregulate Tcf-1 during differentiation (Liao et al., 2021; Wu et al., 2020). Following chronic viral infection, effector CD8⁺ T cells (including stem-like subset) do not express CD103, which is a commonly used marker for mucosal T_{RM} cells (Zhang and Bevan, 2013). Together, lymphoid tissue-residency is essential for stem-like CD8⁺ T cells, which share certain features with T_{RM} cells generated following acute infections. Common mechanisms, such as TGF- β signaling are involved in limiting T cell migration in both acute and chronic infections.

In the absence of TGF- β signaling, the percentage of Tcf-1⁺ stem-like cell population exhibited a two- to threefold reduction. The magnitude of the reduction was maintained and the total population of *Tgfb2*^{-/-} P14 T cells was not significantly reduced in our hands (Zhang and Bevan, 2013) or increased at later stages (accompanying publication by Hu et al., 2022). The difference may be due to the fact that we used LCMV Cl13 infection system with CD4 help and therefore viremia is largely controlled at a later stage.

Another key difference between our results and the ones from Hu et al. (2022) is the subset phenotype of Tcf-1⁺ stem-like T cells. In our resolving chronic infection model (i.e., with CD4 help), virus titer was dramatically reduced at later time points (i.e., around day 20–30) and Tcf-1⁺ cells can be further divided into CD69⁺ vs. CD69⁻ subsets. It is CD69⁻Tcf-1⁺ cells directly differentiating into CD69⁻Tcf-1⁻ migratory effectors (Beltra et al., 2020). TGF- β mainly controls $\alpha 4$ integrin expression on both CD69⁻Tcf-1⁺ and CD69⁻Tcf-1⁻ transitional cells and suppresses the migratory capacity of CD69⁻Tcf-1⁺ transitional stem subset. In contrast, in life-long viremia model, Tcf-1⁺ stem-like cells are often uniformly CD69⁺ and largely tissue-resident with little exchange with the circulation (Im et al., 2020). In this situation, CD69⁺Tcf-1⁺ stem-like cells downregulate both CD69 and Tcf-1 to differentiate into transitional and circulating effectors, which is inhibited by TGF- β (accompanying publication by Hu et al., 2022). Together, regardless of resolving vs. life-long viremia, we have demonstrated that TGF- β inhibits the differentiation of Tcf-1⁺ stem-like CD8⁺ T cells into transitional states during chronic infection, coinciding with the loss of tissue residency and enhanced migration.

Recently findings in tumor immunotherapies have demonstrated that TGF- β inhibitors are beneficial due to their effects

on tumor stromal compartments (Mariathasan et al., 2018; Tauriello et al., 2018) and CD4⁺ T cell-mediated blood vasculature remodeling (Li et al., 2020; Liu et al., 2020). Since it is the migratory effector CD8⁺ T cells, not the stem-like ones carrying most cytotoxic activity (Hudson et al., 2019; Zander et al., 2019), our results suggest that in addition to the stromal compartments and CD4⁺ T cells, direct targeting TGF- β signaling in CD8⁺ T cells may promote differentiation from stem-like cells into cytotoxic effectors and therefore contribute to the beneficial effects seen in combined tumor immunotherapies.

Materials and methods

Mice and virus

C57BL/6J (B6) mice were obtained from The Jackson Laboratory and a colony of D^b-GP₃₃₋₄₁ TCR transgenic (P14) mice was maintained at our specific pathogen-free animal facilities at the University of Texas Health Science Center at San Antonio (San Antonio, TX). All recipient mice were used at 6–10 wk of age and were directly ordered from The Jackson Laboratory. *Tgfb2^{fl/fl}* and *dLck-cre* mice were described before (Zhang and Bevan, 2012). All donor P14 mice have been backcrossed to C57BL/6 background for at least 12 generations. All mice were housed at our specific pathogen-free animal facilities at the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee guidelines. Mice were infected i.v. by 2×10^6 pfu LCMV Clone 13. Viruses were grown and quantified as described (Zhang and Bevan, 2013).

Antibodies and flow cytometry

For in vivo $\alpha 4$ integrin blocking, anti- $\alpha 4$ integrin (PS/2) antibody was from Bio X Cell. Fluorescent dye-labeled antibodies specific for PD-1 (J43), CXCR5 (SPRCL5), CD8 β (H35-17.2), CD45.1 (A20), CD45.2 (104), CD8 α (53-6.7), CD44 (IM7), CX3CR1 (SA011F11), Granzyme A (GzA-3G8.5), integrin $\alpha 4\beta 7$ (DAPK32), CXCR3 (CXCR3-173), CD39(24DMS1), CD73(TY/11.8), CD62L(MEL-14), Ly6C(HK1.4), CD49d (R1-2), integrin $\beta 1$ (HMB1-1), integrin $\beta 7$ (FIB504), IFN- γ (XMG1.2), IL-2 (JES6-5H4), Slamf6 (330-AJ), Tim-3 (RMT3-23), and Tox (TXRX10) were purchased from eBioscience, Biolegend, and Tonbo. Anti-CD16/32 (2.4G2) was produced in the lab and used in all FACS staining as FcR blocker. Anti-Tcf-1 (C63D9) and pSmad2/3 (EBF3R) were from Cell Signaling. Intranuclear staining for Tcf-1 and Tox was performed using a Foxp3 staining buffer set (Tonbo Bioscience). For intracellular cytokine staining, freshly isolated splenocytes were cultured with 0.1 μ M GP₃₃₋₄₁ peptide (AnaSpec) in the presence of Golgi Stop (BD) for 4–5 h at 37°C. After surface staining, IFN- γ and IL-2 were performed using permeabilization buffer (Invitrogen) following fixation. Ghost Dye Violet 510 (Tonbo Bioscience) was used to identify live cells. Washed and fixed samples were analyzed by BD LSRII or BD FACSCelesta and analyzed by FlowJo (TreeStar) software.

Naive T cell isolation and adoptive transfer

Naive CD8⁺ T cells were isolated from pooled spleen and lymph nodes using MojoSort mouse CD8 T cell isolation kit (Biolegend)

following the manufacturer's instructions. During the first step of biotin antibody cocktail incubation, biotin- α CD44 (IM7; Biolegend) was added to label and deplete effector and memory T cells. Isolated naive CD8⁺ T cells were enumerated, 1:1 mixed, 10^4 cells adoptively transferred into each sex-matched unmanipulated B6 recipient via an i.v. route.

Integrin ligand binding assay

As described before (Dimitrov et al., 2019), at indicated time after infection, splenocytes were incubated with 5 μ g/ml recombinant mouse ICAM-1-Fc (human IgG; Biolegend), rmMAdCAM-1-Fc (human IgG; R&D Systems), or rmVCAM-1-Fc (human IgG; R&D Systems) in the presence of 0.1 μ M GP₃₃₋₄₁ at 37°C for 5 min followed by surface staining including FITC-donkey anti-human IgG (Jackson ImmunoResearch) on ice and analyzed by flow cytometry.

FTY720 treatment

100 μ g/kg body weight FTY720 (Sigma-Aldrich) was injected peritoneally starting from day 10 after Cl13 infection on a daily basis. P14 T cell response was examined 12 d after the initial FTY720 injection.

Integrin $\alpha 4$ blocking

400 μ g/mouse anti-integrin $\alpha 4$ antibody (PS/2, Bio X cell) or isotype control antibody was injected peritoneally starting from day 7, 10, or 25 after LCMV Cl13 infection every 3 d. Mice were examined at indicated time points.

Intravascular labeling of CD8⁺ T cells

3 μ g biotin- α CD8 α (53-6.7; Tonbo Biosciences) was injected i.v. 5 min before euthanasia. After lymphocyte isolation, fluorescence-labeled streptavidin (Thermo Fisher Scientific) was used during surface staining to identify red pulp-located splenic CD8⁺ T cells.

H&E staining

At indicated time points after infection, various tissues were dissected and fixed in 10% neutral buffered formalin. All further procedures including paraffin-embedding, sectioning, and H&E staining were performed by UT Health San Antonio pathology core facility.

Immunohistochemistry

To detect LCMV antigen, at indicated time points after infection, livers were snap-frozen in OCT Compound (Thermo Fisher Scientific). At room temperature, 7 μ M sections were fixed with 4% paraformaldehyde for 30 min, permeabilized by 1% Triton for 20 min, and blocked with 4% FCS/PBS for 20 min. Slides were incubated with anti-LCMV (VL-4; Bio X Cell) followed by second antibody incubation (Peroxidase AffiniPure Goat anti-Rat IgG; Jackson Immuno). After washing, KPL TrueBlue Substrate (Seracare Life Sci.) was applied following the manufacturer's instruction. The slides were counterstained by KPL Contrast RED (Seracare Life Sci.).

To detect TGF- β , at indicated time points after infection, various tissues were dissected and fixed in 10% neutral buffered formalin. All further procedures including paraffin-embedding,

sectioning, immunohistochemistry, and H&E staining were performed by UT Health San Antonio pathology core facility. Briefly, sections were incubated with 5 $\mu\text{g}/\text{ml}$ $\alpha\text{TGF-}\beta\text{1}$ (#NBPI-80289; Novus Biologicals) as first antibody, an anti-rabbit IgG-HRP as secondary antibody, and developed with 3,3'-diaminobenzidine substrate.

Stem-like T cell sorting and transfer

WT and *Tgfb2*^{-/-} mice were infected by LCMV Cl13. Day 14–15 after infection, total CD8⁺ T cells were enriched from pooled spleen and lymph nodes by depleting CD4⁺ and CD19⁺ cells. CD44^{hi}PD-1^{hi}Slamf6⁺CX3CR1⁻CD8⁺ T cells were FACS sorted from enriched cells. 10⁵ sorted cells/recipient were adoptively transferred into infected match B6 recipients. Donor CD8⁺ T cells were examined 7 d later.

RNA-seq analysis

Day 15 after LCMV Cl13 infection, donor WT and *Tgfb2*^{-/-} P14 T cells were FACS sorted into four subsets based on the expression of Slamf6 and CD69. Total RNA was extracted from sorted cells using the Quick-RNA MiniPrep kit from Zymo Research. Biological independent duplicates were included. RNA-seq analysis was performed by Novogene, and the results can be accessed by GSE206646.

Stem-like T cell in vitro culture

WT P14 T cells were adoptively transferred into B6 mice followed by LCMV Cl13 infection. Day 14 after infection, Slamf6⁺ and Slamf6⁻ P14 T cells were FACS sorted from total splenocytes and lymph node cells. Meanwhile, CD8/NK-depleted splenocytes were purified from infection-matched B6 controls. 10⁴ sorted P14 T cells were co-cultured with 10⁵ CD8/NK-depleted splenocytes in the presence of 1 μM GP₃₃₋₄₁ + 10 ng/ml IL-2 (Biological) with or without 10 $\mu\text{g}/\text{ml}$ PS/2 antibody. Live P14 T cells were examined 4 d later by flow cytometry.

Statistical analysis

Ordinary one-way ANOVA or Student's *t* test from Prism 9 was used.

Online supplemental material

[Fig. S1](#) shows defective stem-like CD8⁺ T cells in the absence of TGF- β R, further supporting the results in [Fig. 1, A–E](#). [Fig. S2](#) shows stem-like T cells in the presence and absence of CD4 help, viral clearance, non-lymphoid tissue pathology, as well as the detection of TGF- β -producing cells during chronic viral infection. [Fig. S3](#) shows integrin ligand binding on donor P14 T cells. [Fig. S4](#) shows the results from lymph node P14 T cells after in vivo α4 integrin blocking as well as the results from in vitro α4 blocking. [Fig. S5](#) shows the results from in vivo FTY720 treatment experiments and bulk RNA-seq experiments.

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Author contributions: C. Ma and N. Zhang designed the experiments. C. Ma, W. Liao, Y. Liu, S. Mishra and G. Li performed the experiments. L. Wang performed the RNA-seq experiment. C. Ma, L. Wang, and N. Zhang analyzed the results. X. Zhang, Y. Qiu, and Q. Lu contributed to overall experimental design and analysis. C. Ma and N. Zhang wrote the manuscript.

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Supplemental material

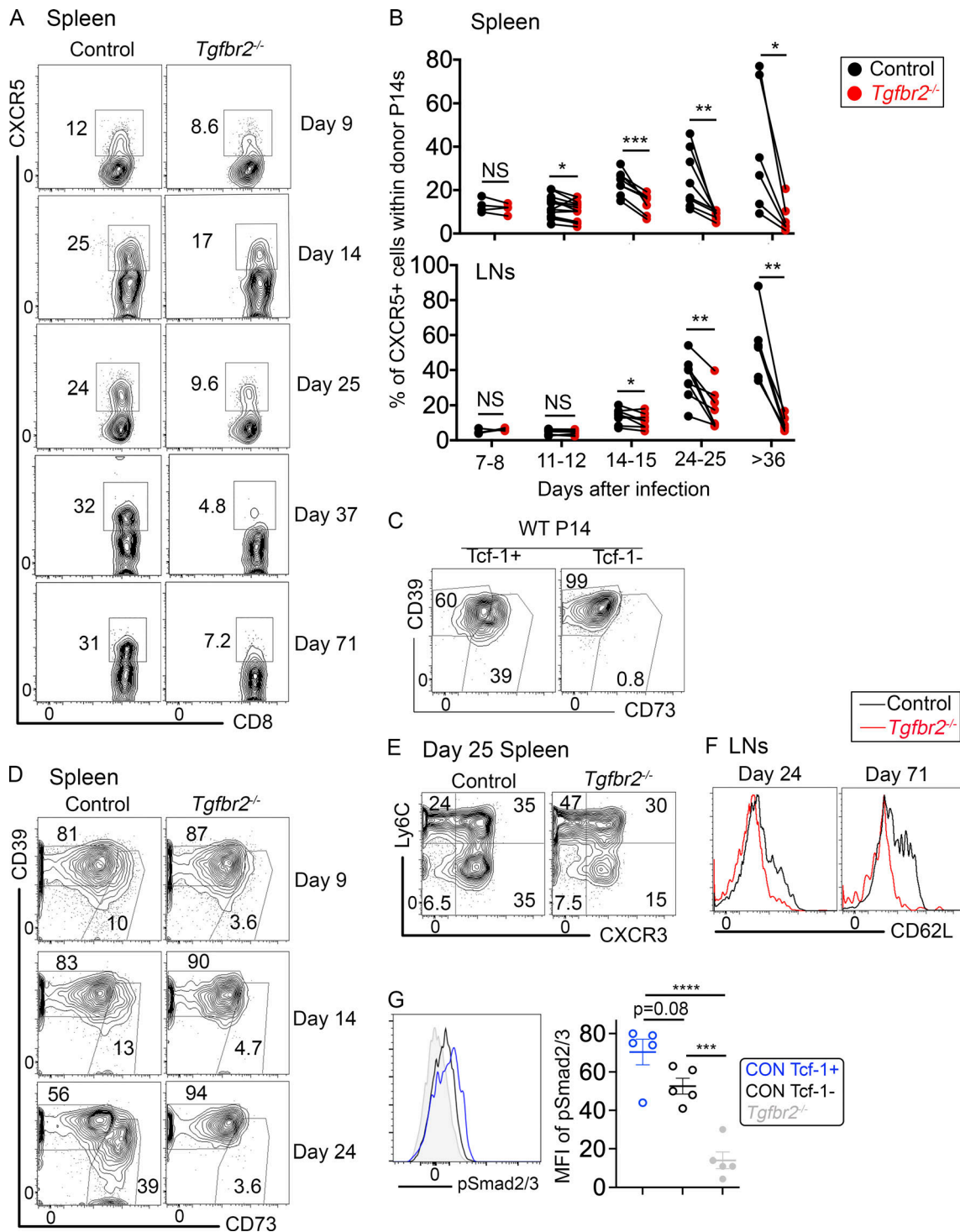


Figure S1. **Defective stem-like CD8⁺ T cell maintenance in the absence of TGF- β signaling.** Same experimental setup as in Fig. 1. **(A)** At indicated days after infection, the expression of CXCR5 on splenic P14 T cells was determined. **(B)** The percentage of CXCR5⁺ cells in donor P14 T cells is shown. Upper, spleen; lower, lymph nodes (LNs). Representative (A) or pooled (B) results from four independent experiments are shown ($n = 4\text{--}14/\text{time point}$). **(C)** Day 15–25 after infection, pre-gated Tcf-1⁺ and Tcf-1⁻ WT splenic P14 T cells were examined by flow cytometry. Representative FACS profiles from three independent experiments ($n = 5/\text{each}$) are shown. **(D–F)** Representative FACS profiles of pre-gated donor WT and *Tgfbr2^{-/-}* P14 T cells are shown (three repeats, $n = 10$). Representative results from three independent experiments are shown. **(G)** Phosphorylated Smad2/3 was measured by FACS. Each symbol represents the results from an individual mouse. Pooled results from two independent experiments are shown in G ($n = 5$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by paired Student's t test (B) or one-way ANOVA (G).

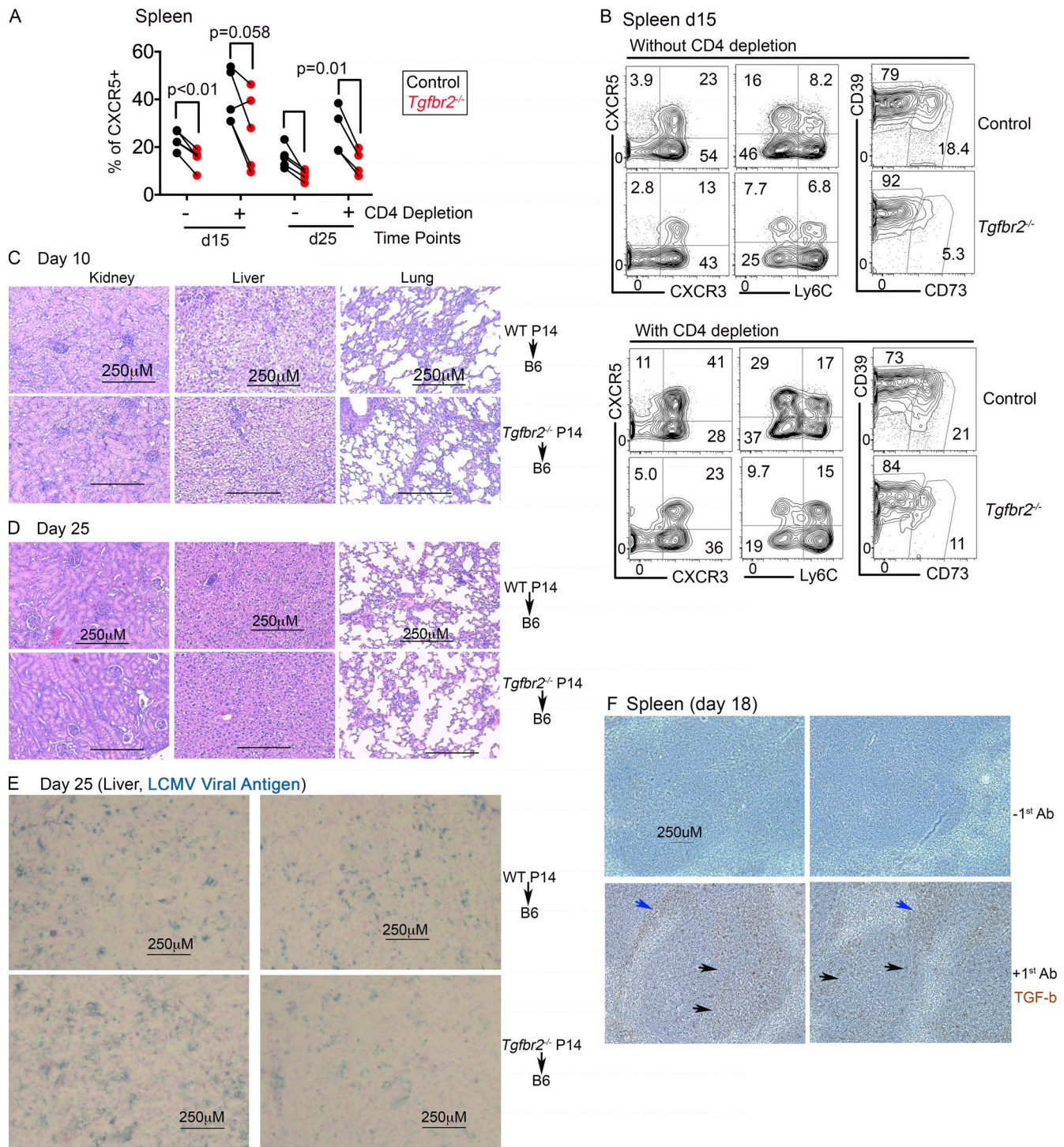


Figure S2. **Defective maintenance of CXCR5⁺ stem-like CD8⁺ T cells in the absence of TGF- β without apparent defects in viral clearance or pathology.** Similar experimental setup to Fig. 1. One group of mice received CD4 depleting antibody 1 d before and 1 d after infection. **(A)** The percentage of CXCR5⁺ cells in donor P14 T cells are shown at indicated time points. **(B)** Day 15 after infection, representative FACS profiles of pre-gated donor P14 T cells are shown (two independent repeats, $n = 5$ /each). Each pair of symbols in A represents the results from an individual recipient. Pooled results from two independent repeats are shown in A ($n = 5$ /each). P values were calculated by paired Student's t test. WT and *Tgfb2^{-/-}* P14 T cells were separately transferred into B6 recipients followed by LCMV Cl13 infection. **(C and D)** Day 10 (C) or day 25 (D) after infection, representative H&E staining of different tissues are shown ($n = 3$ /each, two repeats). **(E)** Day 25 after infection, immunohistochemistry of liver sections to show LCMV antigen (blue). $n = 3$ /each, two repeats. **(F)** Day 18 after LCMV Cl13 infection, spleen sections were examined by immunohistochemistry. Top, negative staining control without primary antibody; bottom, staining with primary antibody (two independent repeats, $n = 2$ /each). Black arrows indicate intra-follicle TGF- β producing foci, and blue ones indicate extra-follicle TGF- β producing foci.

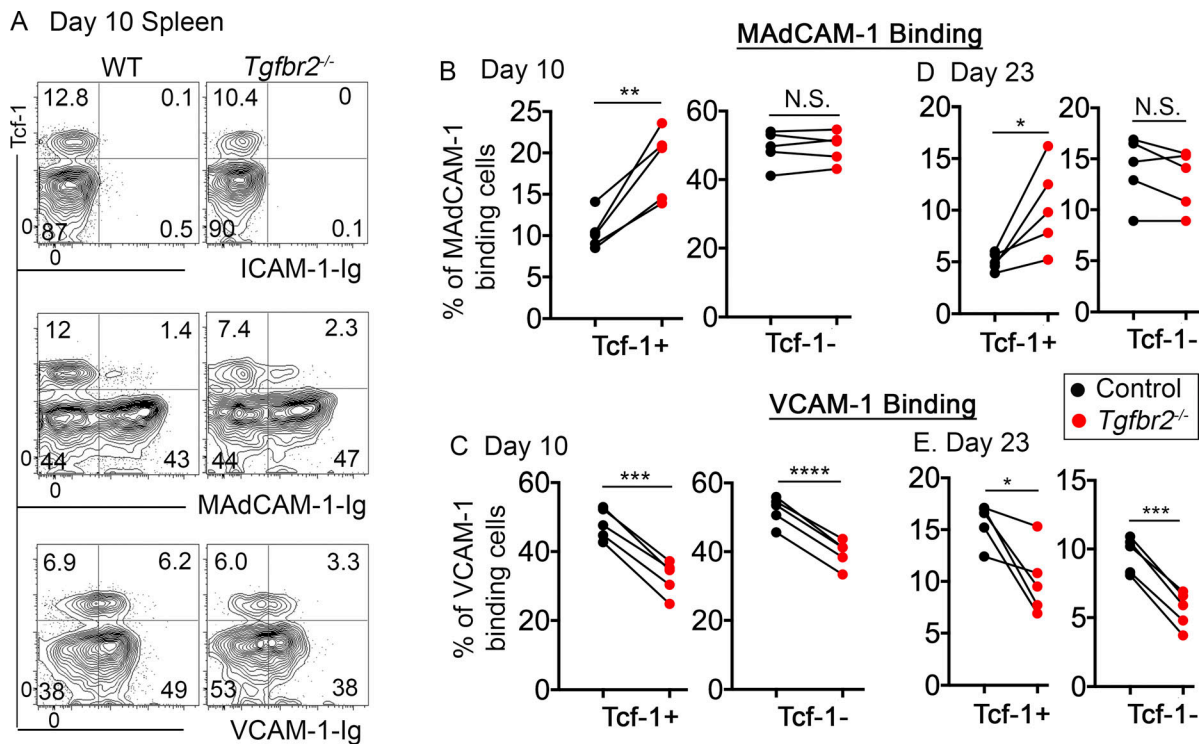


Figure S3. **Enhanced MAdCAM-1 and reduced VCAM-1 binding in the absence of TGF- β signaling.** ICAM-1 (intercellular adhesion molecule-1) mainly binds to β 2-containing integrins and serves as a negative control. MAdCAM-1 binds to α 4 β 7 while VCAM-1 binds to α 4 β 1. Same experimental setup as in Fig. 1. Splenic P14 T cells were incubated with either ICAM-1-Ig, MAdCAM-1-Ig, or VCAM-1-Ig in the presence of GP₃₃₋₄₁ at 37°C for 5 min followed by standard flow cytometry staining and analysis. **(A)** Representative FACS profiles of pre-gated WT control and *Tgfb β 2^{-/-}* P14 T cells are shown (three repeats, $n = 5$ /each). **(B-E)** The percentage of MAdCAM-1 binding (B and D) or VCAM-1 binding (C and E) at day 10 (B and C) and day 23 (D and E) in pre-gated Tcf-1⁺ and Tcf-1⁻ P14 T cells are shown. Each pair of symbols in B-E represents the results from an individual recipient (three repeats, $n = 5$ /each). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ by paired Student's t test.

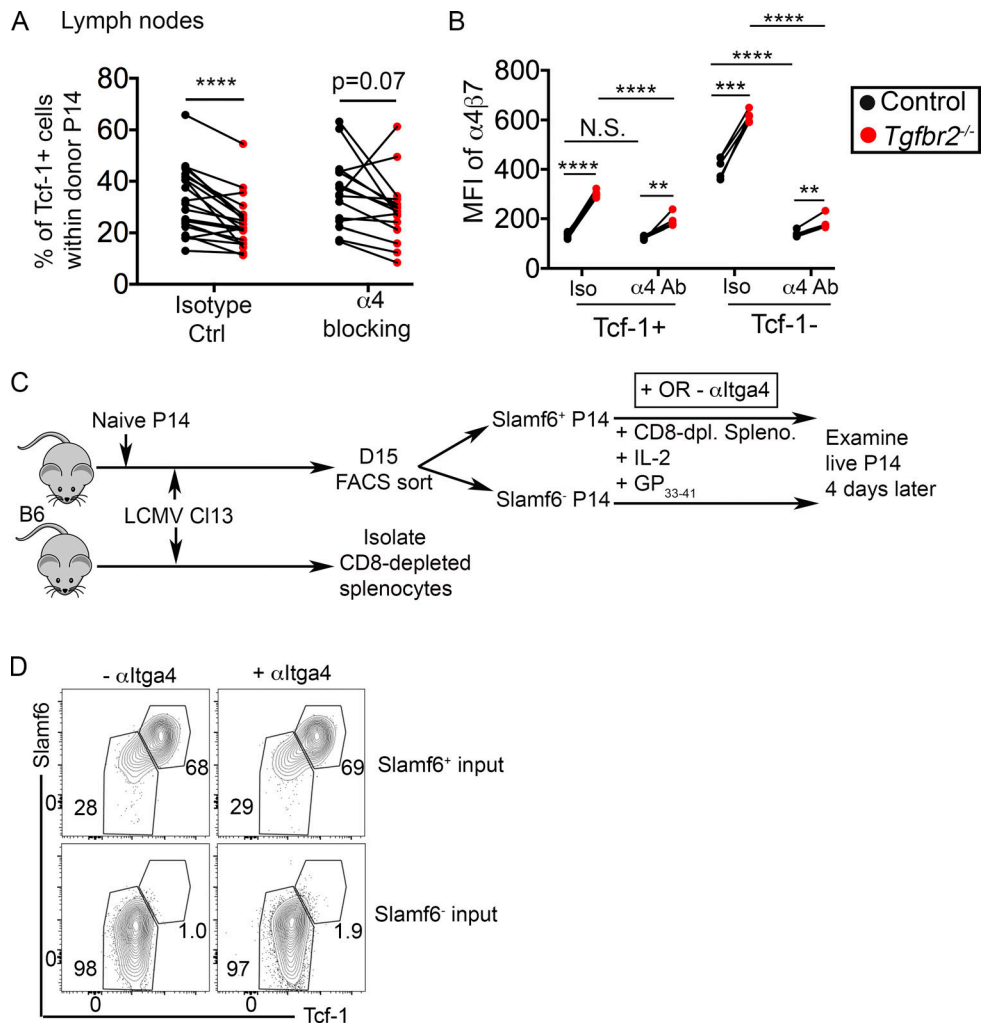


Figure S4. **Integrin α 4 blocking rescues the defective maintenance of Tcf-1⁺ *Tgfbeta2*^{-/-} in vivo without directly interfering with Tcf-1⁺ cell differentiation in vitro.** Same experimental setup as in Fig. 5. **(A)** The percentage of Tcf-1⁺ cells in donor P14 T cells isolated from the lymph nodes are shown. **(B)** MFI of α 4 β 7 on Tcf-1⁺ and Tcf-1⁻ donor P14s are shown. Each pair of symbols represents the results from an individual recipient. Pooled results from five independent experiments are shown in A ($n = 15$ –20). Representative results from two independent experiments are shown in B ($n = 5$). **(C)** Experimental design for in vitro integrin α 4 blocking. **(D)** Representative FACS profiles of pre-gated live P14 T cells after 4-d culture are shown. Representative results from two independent experiments (P14 T cells were pooled from five mice/each experiment as sorting input cells) are shown in D. **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$ calculated by paired Student's t test.

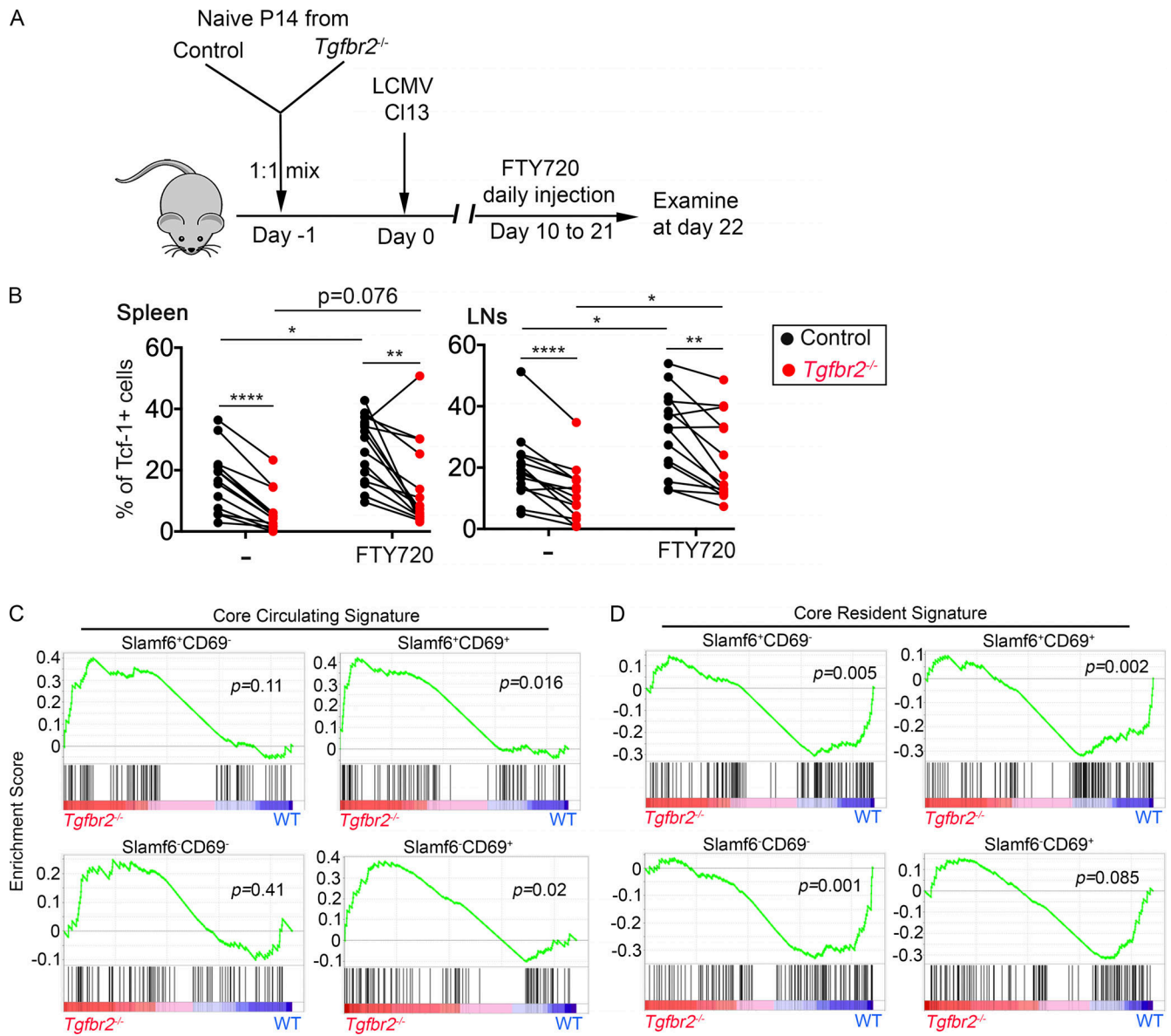


Figure S5

Figure S5. TGF- β -dependent resident program in exhausted CD8⁺ T cells during chronic viral infection. (A) Experimental setup for B. Briefly, naive P14 isolated from WT and *Tgfr2*^{-/-} mice were co-transferred into B6 recipients followed by LCMV CI13 infection. Daily injection of FTY720 was started 10 d later. (B) The percentage of Tcf-1⁺ cells was determined on day 22 after infection by flow cytometry. Each pair of symbols in B represents the results from an individual recipient. Pooled results from three independent experiments are shown (*n* = 15). *, *P* < 0.05; **, *P* < 0.01; and ****, *P* < 0.0001 by Student's *t* test. (C and D) Similar experimental setup as in Fig. 1 A. Day 15 after infection, different subsets of control and *Tgfr2*^{-/-} P14 T cells were FACS sorted from pooled spleen and lymph nodes and subjected to bulk RNA-seq analysis. Gene set enrichment analysis for core circulating memory T cells gene signature (C) and core resident memory T cell gene signature (D) are shown. For C and D, each group contain biological independent duplicates.