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The TCF1-Bcl6 axis counteracts type I interferon to repress exhaustion and maintain T cell stemness

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Abstract

During chronic viral infections and in cancer, T cells become dysfunctional, a state known as T cell exhaustion. Although it is well recognized that memory CD8 T cells account for the persistence of CD8 T cell immunity after acute infection, how exhausted T cells persist remains less clear. Using chronic infection with lymphocytic choriomeningitis virus clone 13 and tumor samples, we demonstrate that CD8 T cells differentiate into a less exhausted TCF1^{high} and a more exhausted TCF1^{low} population. Virus-specific TCF1^{high} CD8 T cells, which resemble T follicular helper (T_{FH}) cells, persist and recall better than do TCF1^{low} cells and act as progenitor cells to

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SUPPLEMENTARY MATERIALS

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replenish TCF1^{low} cells. We show that TCF1 is both necessary and sufficient to support this progenitor-like CD8 subset, whereas cell-intrinsic type I interferon signaling suppresses their differentiation. Accordingly, cell-intrinsic TCF1 deficiency led to a loss of these progenitor CD8 T cells, sharp contraction of virus-specific T cells, and uncontrolled viremia. Mechanistically, TCF1 repressed several pro-exhaustion factors and induced Bcl6 in CD8 T cells, which promoted the progenitor fate. We propose that the TCF1-Bcl6 axis counteracts type I interferon to repress T cell exhaustion and maintain T cell stemness, which is critical for persistent antiviral CD8 T cell responses in chronic infection. These findings provide insight into the requirements for persistence of T cell immune responses in the face of exhaustion and suggest mechanisms by which effective T cell–mediated immunity may be enhanced during chronic infections and cancer.

INTRODUCTION

In response to immunization or acute infection, T lymphocytes differentiate into functional effector cells and develop immunological memory (1, 2). However, during cancer and chronic viral infections such as HIV, prolonged antigen exposure and immunosuppression undermine the efficacy of T cell responses, leading to a state called T cell exhaustion (3). T cell exhaustion is characterized by progressive loss of effector functions, reduced proliferative capacity, and failure of memory differentiation (4). This process is progressive: CD8 T cells early after chronic viral infection can still develop into memory cells, whereas those from the chronic phase of infection cannot (5). Exhausted T cells express a range of inhibitory receptors, including PD1, CTLA4, Tim3, CD244, and LAG3, which mediate intracellular signals contributing to poor T cell responsiveness (3, 6–10). Antibody blockade targeting inhibitory receptors, most prominently PD1 and CTLA4, has achieved major success in reversing T cell exhaustion in patients (11)—combined blockade of PD1 and receptors, such as Tim3, further improves therapeutic benefits (12, 13). Cytokines, such as interleukin-10 (IL-10) (14) and type I interferon (IFN) (15, 16), also affect T cell exhaustion. A recent study showed that type I IFN represses de novo generation of T helper 1 (T_H1) cells during chronic viremia (17). However, the effects of type I IFN on CD8 T cells during chronic infection remain unclear.

How exhausted T cells are maintained, particularly whether exhausted T cells are capable of self-renewal or whether a stem cell–like T cell population repopulates exhausted cells, is not well understood. Although CD8 T cells responding to acute infections are known to be diverse, containing memory precursors and terminal effectors (2), the heterogeneity of exhausted CD8 T cells is less well appreciated. During chronic infection by lymphocytic choriomeningitis virus (LCMV) clone 13, blocking PD1 selectively expands a PD1^{int} CD8 subset, which is less exhausted than PD1^{high} counterparts (18). Other studies have shown that exhausted CD8 T cells can be separated into a T-bet^{high} progenitor population and an Eomes^{high} terminal population (19). However, these studies mostly examined the chronic phase of viral infection. Whether there is earlier bifurcation of progenitor-like and more terminally differentiated CD8 subsets remains unclear.

The transcription factor TCF1 is crucial for the differentiation of various mature T cell subsets, including T central memory (T_{CM}) (20) and T follicular helper (T_{FH}) cells (21–23).

T_{FH} cells persist better than do T_H1 cells during chronic viral infection (24), suggesting a potential role of TCF1 in sustaining antiviral T cell responses during persistent infection. We demonstrate here that CD8 T cells differentiate into $TCF1^{high}$ and $TCF1^{low}$ subsets during both chronic viral infection and cancer. Virus-specific $TCF1^{high}$ CD8 T cells, which transcriptionally resemble T_{FH} cells, express lower levels of exhaustion markers such as Tim3, persist better, and mount a stronger recall response than do $TCF1^{low}$ CD8 T cells. $TCF1^{high}Tim3^{low}$ cells act as progenitor cells that either remain as progenitors or terminally differentiate into $TCF1^{low}Tim3^{high}$ cells. We further demonstrate that differentiation of this progenitor-like CD8 subset is driven by TCF1 and repressed by type I IFN in a cell-autonomous manner. Mechanistically, TCF1 represses expression of Blimp1, Tim3, and Cish while promoting expression of Bcl6, which increases the progenitor-like CD8 T cell population. Thus, we have identified a $TCF1^{high}$ progenitor CD8 subset, which is critical for sustained antiviral T cell responses and is programmed by an interplay between type I IFN and the TCF1-Bcl6 axis early after chronic viral infection.

RESULTS

$TCF1^{high}Tim3^{low}$ and $TCF1^{low}Tim3^{high}$ CD8 T cells in chronic LCMV infection resemble T_{FH} and T_H1 cells

During acute LCMV infection, virus-specific CD4 T cells can be separated into $CXCR5^{high}TCF1^{high}$ T_{FH} and $CXCR5^{low}TCF1^{low}$ T_H1 subsets, which express reciprocal levels of Blimp1 (21–23). Similarly, during chronic infection with LCMV clone 13 (25), we found that virus-specific CD4 T cells, identified by I-Ab GP66 tetramer staining, segregated into $CXCR5^{high}TCF1^{high}Blimp1^{low}$ and $CXCR5^{low}TCF1^{low}Blimp1^{high}$ subsets (fig. S1, A and B).

TCF1 promotes the differentiation of memory CD8 T cells (20, 26, 27), whereas Blimp1 drives CD8 T cell exhaustion during chronic viral infection (28). Like CD4 T cells, we found that virus-specific CD8 T cells (identified by either H-2Db GP276 tetramer or H-2Db GP33 tetramer staining) could be divided into two distinct subsets on the basis of expression of TCF1, on day 7 of chronic LCMV infection (Fig. 1A and fig. S1C). $TCF1^{low}$ CD8 T cells were more abundant and expressed higher levels of both Blimp1 and Tim3. In contrast, $TCF1^{high}$ cells constituted less than 20% of virus-specific CD8 T cells and expressed much less Tim3 and Blimp1. Four weeks after infection, $TCF1^{low}Tim3^{high}Blimp1^{high}$ CD8 T cells still made up the majority of virus-specific CD8 T cells (Fig. 1B and fig. S1D) and had further down-regulated TCF1 to levels similar to those in B cells, which do not express TCF1. Thus, virus-specific CD8 T cells differentiate into $TCF1^{high}Tim3^{low}Blimp1^{low}$ and $TCF1^{low}Tim3^{high}Blimp1^{high}$ subsets early after infection with LCMV clone 13, even before T cell exhaustion has been fully established (5).

To further characterize the $TCF1^{high}$ and $TCF1^{low}$ CD8 T cells generated after chronic LCMV infection, we used Tim3 and Blimp1 as surrogate markers to sort $Tim3^{low}Blimp1^{low}$ and $Tim3^{high}Blimp1^{high}$ virus-specific CD8 T cells on day 7 after infection and profiled their transcriptomes (fig. S1E). *Tcf7*, which encodes TCF1, was one of the most up-regulated genes in $Tim3^{low}Blimp1^{low}$ CD8 T cells (Fig. 1C and table S1), validating this method to distinguish $TCF1^{high}$ and $TCF1^{low}$ populations. $Tim3^{low}Blimp1^{low}$ CD8 T cells also

expressed higher levels of genes encoding transcription factors (such as *Aff3, Bcl6, Id3, Bach2*, and *Ikzf2*), cytokine receptors (such as *Il23r* and *Il7r*), and migration and adhesion receptors (such as *Cxcr5, Sell*, and *Ccr7*) (Fig. 1C and table S1). In contrast, Tim3^{high}Blimp1^{high} CD8 T cells expressed more transcripts encoding inflammatory chemokine receptors such as *Ccr5, Ccr2*, and *Cxcr6* (table S1).

The higher mRNA for two canonical T_{FH} markers, *Cxcr5* and *Bcl6*, in Tim3^{low}Blimp1^{low} (TCF1^{high}) CD8 T cells suggested a potential similarity with T_{FH} cells. TCF1^{high} CD8 T cells expressed more Bcl6 and CXCR5 protein than did their TCF1^{low} counterparts on day 7 after infection (fig. S1, F and G). This difference became more evident 4 weeks after infection (Fig. 1D and fig. S1H). To determine whether TCF1^{high} CD8 T cells resemble T_{FH} cells on a transcriptome level, we performed Gene Set Enrichment Analysis (GSEA) on our microarray data using previously described T_{FH} and T_H1 signature gene sets from LCMV-infected mice (21). The T_{FH} gene signature was strongly enriched in Tim3^{low}Blimp1^{low} (TCF1^{high}) CD8 T cells, whereas almost all T_H1 signature genes were up-regulated in Tim3^{high}Blimp1^{high} (TCF1^{low}) CD8 T cells (Fig. 1E). GSEA using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome curated pathway databases revealed that Tim3^{low}Blimp1^{low} CD8 T cells exhibited enrichment of genes related to branched-chain amino acid degradation (fig. S1I), similar to T_{FH} cells (21). Enrichments of ribosome and type I IFN signaling pathways were also observed (fig. S1I). Thus, the bifurcation of TCF1^{high} and TCF1^{low} virus-specific CD8 T cells after chronic LCMV infection shows notable similarities with T_{FH} and T_H1 CD4 T cells after acute viral infection.

TCF1^{high}Tim3^{low} marks a less exhausted T cell population in chronic viral infection and cancer

Tim3^{low}Blimp1^{low} CD8 T cells exhibited higher mRNA levels of *Tnfrsf4*, encoding OX40, a costimulatory receptor that enhances T cell survival during chronic viral infection (Fig. 1C) (29). In contrast, besides *Havcr2* (encoding Tim3) and *Prdm1* (encoding Blimp1), Tim3^{high}Blimp1^{high} CD8 T cells had higher expression of other genes associated with T cell exhaustion, including *Cd244, Il10*, and *Cish* (Fig. 1C and table S1) (30). Evaluation of protein confirmed that TCF1^{high} CD8 T cells expressed lower levels of surface 2B4 and LAG3, despite having slightly higher surface PD1, on day 7 after infection (Fig. 2A and fig. S2A). By 4 weeks after infection, PD1, 2B4, and LAG3 were all lower on TCF1^{high} CD8 T cells compared with their TCF1^{low} counterparts (Fig. 2B and fig. S2B). Thus, TCF1^{high} CD8 T cells appear less exhausted than the TCF1^{low} population, especially after infection progresses to chronic phase.

To address whether TCF1 expression inversely correlates with T cell exhaustion in other models, we implanted C57BL/6 mice with 3-methylcholanthrene–induced fibrosarcoma (MCA205) cells and examined tumor-infiltrating lymphocytes (TILs). CD8 TILs could be readily separated intoTCF1^{high} and TCF1^{low} populations (Fig. 2C), withTCF1^{low} CD8 T cells exhibiting higher PD1, Tim3, 2B4, and LAG3 (Fig. 2C and fig. S2C). Furthermore, most PD1^{high}Tim3^{low} cells expressed low levels of TCF1 (fig. S2D). However, most Tim3^{low} CD8 TILs did not express high CXCR5, unlike virus-specific CD8 T cells during chronic LCMV. Similarly, PD1 levels in CD8 TILs from melanoma patients also correlated

negatively with TCF1 expression and positively with Tim3 expression (Fig. 2D). Thus, reduced TCF1 expression appears to be a universal hallmark of T cell exhaustion during both chronic viral infection and cancer.

Because the proportion of the less exhausted TCF1^{high} CD8 T cells only moderately increased over time after infection, we hypothesized that TCF1^{high} CD8 T cells might function as a progenitor population that continuously differentiates into and replenishes the TCF1^{low} population. Using Tim3as a surrogate marker, we sorted Tim3^{low}PD1^{+}CD44^{high} and Tim3^{high}PD1^{+}CD44^{high} CD8 T cells from infected C57BL/6 mice on day 7 after infection and transferred either population into infection-matched CD45.1 mice. Seven days after transfer, we recovered greater than fivefold more total and tetramer^{+} donor CD8 T cells from mice that had received Tim3^{low} CD8T cells than from those that had received Tim3^{high} cells (fig. S2E). Whereas the vast majority of Tim3^{high} donor cells remained Tim3^{high}, more than half of Tim3^{low} donor cells became Tim3^{high} after 7 days (fig. S2F).

To further evaluate functional differences between Tim3^{low} (TCF1^{high}) and Tim3^{high} (TCF1^{low}) cells, we transferred sorted Tim3^{low}PD1^{+}CD44^{high} or Tim3^{high}PD1^{+}CD44^{high} CD8 T cells from day 7 infected C57BL/6 mice into naïve CD45.1 mice that were then infected with LCMV clone 13. After 7 days, there were about sixfold more of both total and tetramer^{+} donor CD8 T cells in mice that had received Tim3^{low} CD8 T cells (Fig. 2E). Furthermore, >98%of tetramer^{+} Tim3^{high} donor CD8 T cells remained Tim3^{high} and TCF1^{low} (Fig. 2, F and G). In contrast, although the majority of tetramer^{+} Tim3^{low} donor CD8 T cells converted into Tim3^{high} cells and down-regulated TCF1, a small fraction of them maintained high TCF1 expression (Fig. 2, F and G). Thus, Tim3^{low} TCF1^{high} CD8 T cells both persist better and mount stronger recall responses than do Tim3^{high} TCF1^{low} CD8 T cells, and like stem cells, they can either maintain their phenotype or differentiate into and repopulate terminally differentiated Tim3^{high} TCF1^{low} cells.

TCF1 is essential for the early generation of Tim3^{low} CD8 progenitor cells and long-lasting CD8 responses

Our observations suggested a potentially important role of TCF1 in T cell responses during chronic viremia. To test this hypothesis, we infected control [wild-type (WT)] and previously described *Tcf7*$^{loxP/loxP}$CD4-Cre [*Tcf7* conditional knockout (cKO)] mice (21), in which TCF1 is efficiently deleted in mature T cells, with LCMV clone 13. On day 7 after infection, virus-specific CD4 T cell numbers were comparable between WT and cKO mice (fig. S3A), although loss of TCF1 reduced the frequency and number of virus-specific T_{FH} cells (fig. S3B). In the CD8 compartment, TCF1 deficiency mildly reduced numbers of virus-specific T cells (Fig. 3A and fig. S3C). However, the Tim3^{low} population virtually disappeared in tetramer^{+} CD8 T cells in TCF1-deficient mice (Fig. 3B and fig. S3D). Thus, TCF1 is required for the programming of Tim3^{low} virus-specific CD8 T cells early during LCMV clone 13 infection. In addition, TCF1-deficient virus-specific CD8 T cells expressed higher 2B4 (fig. S3, E and F), suggesting that these cells exhibited characteristics of T cell exhaustion. Nonetheless, viral titers in the blood and spleen of TCF1-deficient mice were not significantly higher than those of WT on day 7 after infection (fig. S3, G and H).

We then examined the effect of TCF1 deficiency during the chronic phase of LCMV clone 13 infection. The loss of TCF1 caused a severe reduction in the frequencies and numbers of both virus-specific CD4 (Fig. 3C) and CD8 T cells (Fig. 3D and fig. S3I). Thus, antiviral T cell responses during chronic viremia could not be sustained without TCF1. In addition, cKO CD8 T cells exhibited higher average levels of Tim3, PD1, and 2B4 (Fig. 3, E to G, and fig. S3J), suggesting that the remaining virus-specific CD8 T cells were more exhausted. Consistent with these observations, ~5-fold less CD4 and >20-fold less CD8 T cells in cKO mice produced IFN-γ after restimulation with class II– or class I–restricted LCMV-derived peptide epitopes (fig. S3K), respectively, with less IFN-γ produced per cell (fig. S3L). Moreover, cKO mice had ~10-fold higher viral titers in the blood and ~100-fold higher viral titers in the spleen than their WT counterparts (Fig. 3H).

By 3 months after infection, LCMV-specific CD8 T cells were almost undetectable in TCF1 cKO mice; LCMV-specific CD4 T cells in these mice were also greatly diminished (Fig. 3, I and J, and fig. S3M). Although remaining in brain and kidney, LCMV clone 13 is typically cleared from spleen and blood 3 months after infection (25). Consistent with their lack of antiviral T cell responses, high levels of virus were found in blood from cKO mice (Fig. 3K). Therefore, TCF1 is indispensible for sustained T cell responses during chronic viremia.

TCF1 is intrinsically required for persistence of virus-specific T cells during chronic viral infection

To address cell-intrinsic requirements for TCF1 in virus-specific T cells during chronic infection, we transplanted lethally irradiated WT CD45.1 mice with mixed bone marrow from WT CD45.1 donors and CD45.2 donors that were either WT or *Tcf7* cKO. In these chimeras, WT and cKO T cells were exposed to the same environment, allowing their direct comparison. Seven days after infection, there were no significant differences in the frequencies of either virus-specific CD4 (fig. S4A) or CD8 (Fig. 4A and fig. S4B) T cells between WT and cKO T cell compartments in chimeras. Thus, the initial expansion of virus-specific T cells after clone 13 infection did not require intrinsic TCF1 function. Nonetheless, TCF1 was indispensible for the generation of $Tim3^{low}$ virus-specific CD8 T cells in a cell-autonomous manner; frequencies of $Tim3^{low}$ cells were notably lower within virus-specific cKO CD8 T cells than within WT CD8 T cells in the same mice (Fig. 4B and fig. S4C).

However, by 4 weeks after infection, the frequencies of $tetramer^{+}$ CD4 T cells within cKO CD4 T cell compartments were considerably less than those within the WT compartments in chimeras (fig. S4D). The impact of TCF1 deficiency appeared even more profound in CD8 T cells: Frequencies of virus-specific CD8 T cells within cKO donor-derived CD8 T cells were sharply reduced relative to those within WT donor-derived CD8 T cells (Fig. 4, C and D, and fig. S4E). Frequencies of cKO CD8 T cells that produced IFN-γ in response to GP276 or GP33 peptide restimulation were also significantly lower than those of WT (Fig. 4E and fig. S4F). Nonetheless, viral titers did not differ between the two types of mixed chimeras, because WT cells were present in each case (fig. S4G). Thus, long-lasting antiviral T cell responses, especially those mediated by CD8 T cells, require TCF1 in a cell-autonomous manner.

Enforced expression of TCF1 enhances the generation of Tim3^{low} CD8 progenitor cells sustaining long-lasting antiviral CD8 T cell responses

To determine whether TCF1 is sufficient to drive the differentiation of Tim3^{low} virus-specific CD8 T cells, we transduced P14 T cell receptor (TCR) transgenic CD8 T cells, which recognize the GP33 epitope of LCMV (31), with MSCV (murine stem cell virus)–IRES (internal ribosomal entry site)–GFP (green fluorescent protein) (MIG) retroviral vectors that either did or did not encode TCF1. Transduced cells were transferred into naïve C57BL/6 recipients that were immediately infected with LCMV clone 13. On day 8 after infection, TCF1 overexpression strongly increased the percentages of Tim3^{low} P14 cells (Fig. 5A). Although only ~10% of control P14 cells were CXCR5^{high}Tim3^{low}, this subset constituted >30% of TCF1-overexpressing P14 cells (Fig. 5B). TCF1 overexpression also enhanced Bcl6 expression (Fig. 5C). Furthermore, whereas numbers of control and TCF1-overexpressing P14 cells were comparable on day 8 after infection (fig. S5A), ~10-fold more TCF1-overexpressing P14 cells were recovered relative to controls on day 14 after infection (Fig. 5D). Therefore, TCF1 overexpression both drives the differentiation of a T_{FH}-like CD8 progenitor population and enhances long-term persistence of virus-specific CD8 T cells during chronic viral infection.

TCF1 orchestrates a core network of genes regulating T cell differentiation

To determine whether TCF1 globally redirected virus-specific CD8 differentiation after chronic LCMV infection, we sorted tetramer^{+} WT and TCF1 cKO CD8 T cells on day 7 after infection and profiled their transcriptomes (fig. S6A). We first defined Tim3^{low}Blimp1^{low} and Tim3^{high}Blimp1^{high} signature gene sets on the basis of our earlier microarray data (table S1). Then, we performed GSEA to determine the enrichment of these gene sets in WT and TCF1 cKO CD8 T cells. cKO CD8 T cells showed greatly reduced Tim3^{low}Blimp1^{low} gene expression signatures and increased Tim3^{high}Blimp1^{high} signatures relative to WT cells (Fig. 6A). In addition, TCF1 cKO CD8 T cells had reduced mRNA for transcription factors such as *Aff3, Ikzf2*, and *Bcl6* and surface molecules such as *Il23r, Cxcr5, Tnfsf8* (encoding CD30L), *Sell* (encoding CD62L), and *Ccr7* (Fig. 6B and fig. S6B). *Aff3* is highly expressed by T_{FH} cells and memory precursors (21). CD30L is critical for the generation of long-lived memory CD8 T cells (32).

Next, we sorted control and TCF1-overexpressing P14 cells on day 8 after infection and profiled their transcriptomes (fig. S6C). TCF1 overexpression greatly enhanced the Tim3^{low}Blimp1^{low} gene expression signature and reduced the Tim3^{high}Blimp1^{high} signature (Fig. 6C). Thus, TCF1 drives the differentiation of virus-specific CD8 T cells toward the Tim3^{low}Blimp1^{low} lineage on a global scale. Microarray data further revealed that many genes down-regulated by TCF1 deficiency (Fig. 6B) were up-regulated by TCF1 overexpression (Fig. 6D). To examine whether TCF1 loss of function and gain of function perturbed expression of similar sets of genes, we generated four gene sets: genes down-regulated or up-regulated by TCF1 overexpression and genes down-regulated or up-regulated by TCF1 KO (tables S2 and S3). TCF1 cKO cells exhibited increased expression of genes down-regulated by TCF1 overexpression and decreased expression of genes up-regulated by TCF1 overexpression (fig. S6D). Similarly, TCF1-overexpressing P14 cells showed enhanced WT-associated and reduced cKO-associated gene signatures (fig. S6E).

Thus, TCF1 deficiency and TCF1 overexpression reciprocally affect the activity of the same core gene network. To explore pathways regulated by TCF1, we performed GSEA using the Reactome database and found that signatures related to branched-chain amino acid degradation and transfer RNA aminoacylation pathways were up-regulated by TCF1 overexpression and down-regulated by TCF1 deficiency (fig. S6, F and G). TCF1 deficiency also reduced branched-chain amino acid degradation genes in T_{FH} cells, suggesting that a common metabolic signature is affected by TCF1 (21).

TCF1 directly regulates the Bcl6-Blimp1 axis and genes related to immune checkpoints

Our microarrays showed that TCF1 down-regulates genes in multiple pathways mediating T cell exhaustion, including *Havcr2* (Tim3), *Prdm1* (Blimp1), and *Cish* (Fig. 6D). In contrast, Bcl6, a master regulator of T_{FH} cell differentiation that counteracts Blimp1 activity (33, 34), was induced by TCF1 overexpression. To test whether Bcl6 also affected T_{FH}-like CD8 progenitor cells, we overexpressed Bcl6 in P14 cells and transferred transduced cells into recipients that were immediately infected. The majority of P14 cells overexpressing Bcl6 were $CXCR5^{high}Tim3^{low}$, indicating that Bcl6 enforced the progenitor fate (Fig. 6E) and suggesting that TCF1 promotes the differentiation of $CXCR5^{high}Tim3^{low}$ progenitor cells by inducing Bcl6.

To test whether TCF1 directly binds to these gene loci, we performed chromatin immunoprecipitation (ChIP) assays on sorted $Tim3^{low}PD1^{+}CD44^{high}$ and $Tim3^{high}PD1^{+}CD44^{high}$ CD8 T cells from mice 7 days after infection (Fig. 6F). TCF1 bound to a promoter region of *Bcl6* important for *Bcl6* expression in T_{FH} cells (23) and showed stronger binding in $Tim3^{low}$ than in $Tim3^{high}$ CD8 T cells. Similarly, there was a greater enrichment of TCF1 binding at a major regulatory site in the third intron of *Prdm1* (35) in $Tim3^{low}$ CD8 T cells. TCF1 also preferentially bound to a region 7 kb upstream of *Havcr2* transcription start site and to the promoter of *Cish* in $Tim3^{low}$ cells. Both are potential regulatory regions based on published ATAC-seq (assay for accessible chromatin using high-throughput sequencing) and histone modification data (36). Thus, TCF1 directly binds to regulatory regions of genes affecting CD8 T cell exhaustion.

T cell–intrinsic type I IFN signaling inhibits the formation of TCF1-dependent $Tim3^{low}$ CD8 T progenitors

In our analyses, we noted enrichment of genes related to type I IFN signaling in $Tim3^{low}Blimp1^{low}$ ($TCF1^{high}$) CD8 T cells (fig. S1I). Excessive type I IFN is induced early after chronic viral infection and drives T cell exhaustion (15, 16). Type I IFN also inhibits T_{FH} cell differentiation during acute viral infection (37). The similarities between the bifurcation of $TCF1^{high}$ and $TCF1^{low}$ CD8 T cell fates during chronic viral infection and the bifurcation of T_{FH} and T_H1 fates suggested that type I IFN signaling might also affect the differentiation of CD8 T cells after LCMV clone 13 infection. Treatment with a blocking anti-IFNAR1 (IFN-α/β receptor chain 1) antibody (15, 16) increased the frequency and the number of $TCF1^{high}Tim3^{low}$ virus-specific CD8 T cells on day 7 after infection (Fig. 7, A to C, and fig. S7, A to C) while reducing the number of $TCF1^{low}Tim3^{high}$ cells (Fig. 7D and fig. S7D). To determine whether the effects of type I IFN were CD8 T cell–autonomous, we transferred IFNAR1 KO and WT P14 cells mixed in 1:1 ratios into natural killer (NK)–

depleted hosts that were subsequently infected with LCMV clone 13, because IFNAR1-deficient activated CD8 T cells are prone to NK cell–mediated killing (38, 39). The percentage of TCF1^{high}Tim3^{low} cells within IFNAR1 KO P14 cells was greater than twofold higher than that within WT P14 cells on day 7 after infection (Fig. 7E). Similarly, knockdown of IFNAR1 increased the frequency of TCF1^{high}Tim3^{low} P14 cells (fig. S7E). Moreover, TCF1 deficiency almost completely abrogated the effects of type I IFN blockade on Tim3^{low} CD8 T cells (Fig. 7F and fig. S7F), suggesting that these effects depended on TCF1. Thus, early programming of TCF1-dependent CD8 progenitors is suppressed by type I IFN signaling in a cell-autonomous manner.

DISCUSSION

Here, we demonstrate a bifurcation of CD8 T cell fates into a TCF1^{high} progenitor-like and a TCF1^{low} terminally differentiated population after chronic LCMV infection and in human and mouse tumors. Virus-specific TCF1^{high}Tim3^{low} CD8 T cells are less exhausted, persist and recall better, and act as progenitors that can either maintain their cell fate or differentiate into terminally differentiated TCF1^{low} cells. Moreover, TCF1 is both necessary and sufficient to program these CD8 progenitors early after LCMV clone 13 infection. In addition to suppressing expression of pro-exhaustion factors, such as Tim3, Cish, and Blimp1, TCF1 also up-regulates Bcl6, which strongly enforces the progenitor fate. Last, we found that type I IFN suppresses the differentiation of this progenitor-like CD8 subset in a cell-autonomous manner. Thus, we have found evidence for a CD8 progenitor population that is required for long-lasting antiviral T cell responses and is governed by a type I IFN versus TCF1-Bcl6 axis early after chronic viral infection.

One of the most prominent features of TCF1^{high}Tim3^{low} CD8 T cells is their notable similarity with T_{FH} cells both at the transcriptomic level and in the expression of the canonical T_{FH} markers CXCR5 and Bcl6. We have shown that TCF1 acts upstream of Bcl6-Blimp1 axis in both T_{FH} (21) and TCF1^{high} CD8 T cells, suggesting a possible shared mechanistic program. Moreover, given the requirement for TCF1 for memory CD8 cells, it is possible that a TCF1-driven program may be common to multiple lineages. Like T_{FH} cells, TCF1^{high}Tim3^{low} CD8 T cells also express lower levels of canonical T_H1 markers, including Blimp1 (33) and Il2ra (40). IL-2 signaling can repress virus-specific T_{FH} differentiation via signal transducer and activator of transcription 5 (STAT5)– and Blimp1-mediated pathways (41, 42), raising the possibility that IL-2 might also affect the differentiation of TCF1^{high}Tim3^{low} CD8 T cells. Given that TCF1 enhances IL-6 signaling (22), it is also possible that IL-6 regulates differentiation of TCF1^{high}Tim3^{low} progenitors.

We find that TCF1^{high}Tim3^{low} CD8 T cells persisted and recalled better than TCF1^{low}Tim3^{high} cells. TCF1^{high}Tim3^{low} CD8 T cells also gave rise to both TCF1^{low} and TCF1^{high} populations, a characteristic of progenitor cells. Furthermore, cell-intrinsic TCF1 deficiency resulted in a loss of the Tim3^{low} CD8 population and undermined long-term persistence of virus-specific CD8 T cells, further supporting the role of this population as progenitors that sustain long-term CD8 response. Our results are corroborated by papers published during the preparation of this article, which identified a CXCR5^{+}, TCF1-dependent stem cell–like CD8 population responsible for therapeutic effects of PD1

checkpoint blockade during chronic LCMV infection (43–45). Our findings extend these studies by demonstrating a cell-intrinsic role for type I IFN signaling in suppressing these TCF1-dependent progenitor-like CD8 cells and by providing insight into TCF1-driven programs of gene expression. Furthermore, we now provide evidence for a parallel bifurcation of CD8 T cell populations in solid tumors, in both mice and humans.

Nonetheless, although we have shown that $CD8^+$ TILs contain $TCF1^{high}$ and $TCF1^{low}$ subsets, we do not yet know the proportion of tumor-specific CD8 T cells within these subsets or whether $TCF1^{high}$ CD8 T cells exhibit functional differences, such as cytotoxicity, from their $TCF1^{low}$ counterparts. Another recent study showed that a $CXCR5^+$ TCF1-dependent cytotoxic CD8 population resides in B cell follicles after LCMV-Docile infection and eliminates infected T_{FH} cells (46). However, we note that both $TCF1^{high}$ and $TCF1^{low}$ CD8 populations infiltrating MCA205 tumors exhibit low CXCR5 expression. The bifurcation of CD8 T cells into a less exhausted $TCF1^{high}$ and a more exhausted $TCF1^{low}$ population during chronic viral infection and cancer is therefore unlikely to be strictly dependent on B cell follicle homing or CXCR5 expression and thus may have broader implications for T cell exhaustion.

In summary, we have identified a $TCF1^{high}Tim3^{low}$ progenitor population generated early after chronic viral infection that is critical for long-lasting antiviral T cell responses against chronic viremia. The fate of $TCF1^{high}Tim3^{low}$ progenitors is programmed by a type I inteferon versus TCF1-Bcl6 axis before the chronic phase of infection. Our results raise the possibility of therapeutic potential for such cells and thus shed new light on the development of improved immune therapies against chronic viral infection and cancer.

MATERIALS AND METHODS

Study design

The goals of this study were to characterize a progenitor-like CD8 population during chronic LCMV infection and mouse and human cancers and to dissect the molecular pathways governing the differentiation of this population. Pilot experiments were performed to determine the number of replicates to ensure enough statistical power while minimizing animal usage. Each experimental group contained three to nine biological replicates. Statistical significance was defined by $P < 0.05$. Results were confirmed by at least two independent experiments.

Mice and infection

Tcf7$^{loxP/loxP}$; CD4-Cre (cKO) (21), Blimp1-YFP (47), P14 TCR transgenic mice recognizing LCMV GP33 (31), and *Ifnar1* KO P14 (38) were previously described. Animal husbandry and experiments were approved by the National Human Genome Research Institute (NHGRI), the National Institute of Neurological Disorders and Stroke (NINDS), or the National Cancer Institute (NCI) Animal Use and Care Committees, or by the authorization of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen in accordance with German laws for animal protection. For viral infections, age- and sex-matched mice were intravenously injected with 2×10^6 plaque-forming units (PFU)

of LCMV clone 13. For type I IFN blockade, mice were injected intraperitoneally with 1 mg of anti-IFNAR1 (MAR1-5A3) 1 day before infection. For overexpression or knockdown studies, activated P14 CD8 T cells were spin-infected with retroviral constructs, sorted for transduced cells, and injected into C57BL/6 recipients, which were subsequently infected with LCMV. Viral plaque assay, antibody and tetramer (National Institute of Allergy and Infectious Diseases Tetramer Core Facility) staining, dyes, flow cytometry, and cell sorting are described in the supplementary materials and methods (48, 49).

Human melanoma TIL

Human samples were obtained as part of U.S. NCI Institutional Review Board–approved clinical trials (NCT01236573 and NCT01319565). Phenotyping is described in the supplementary materials and methods.

Microarray and ChIP assays

Microarrays were performed using GeneChip Mouse Gene 2.0 ST Arrays (Affymetrix), and data were analyzed with Partek Genomics Suite (Partek Inc.). ChIPs were performed on ~5 × 10^6 $Tim3^{low}PD1^{+}CD44^{high}$ or $Tim3^{high}PD1^{+}CD44^{high}$ CD8 T cells with a ChIP assay kit (Millipore; see the supplementary materials and methods).

Statistical analyses

Statistical analyses were conducted with GraphPad Prism 6 using two-tailed paired or unpaired Student's *t* tests. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Bar graphs are represented as means + SD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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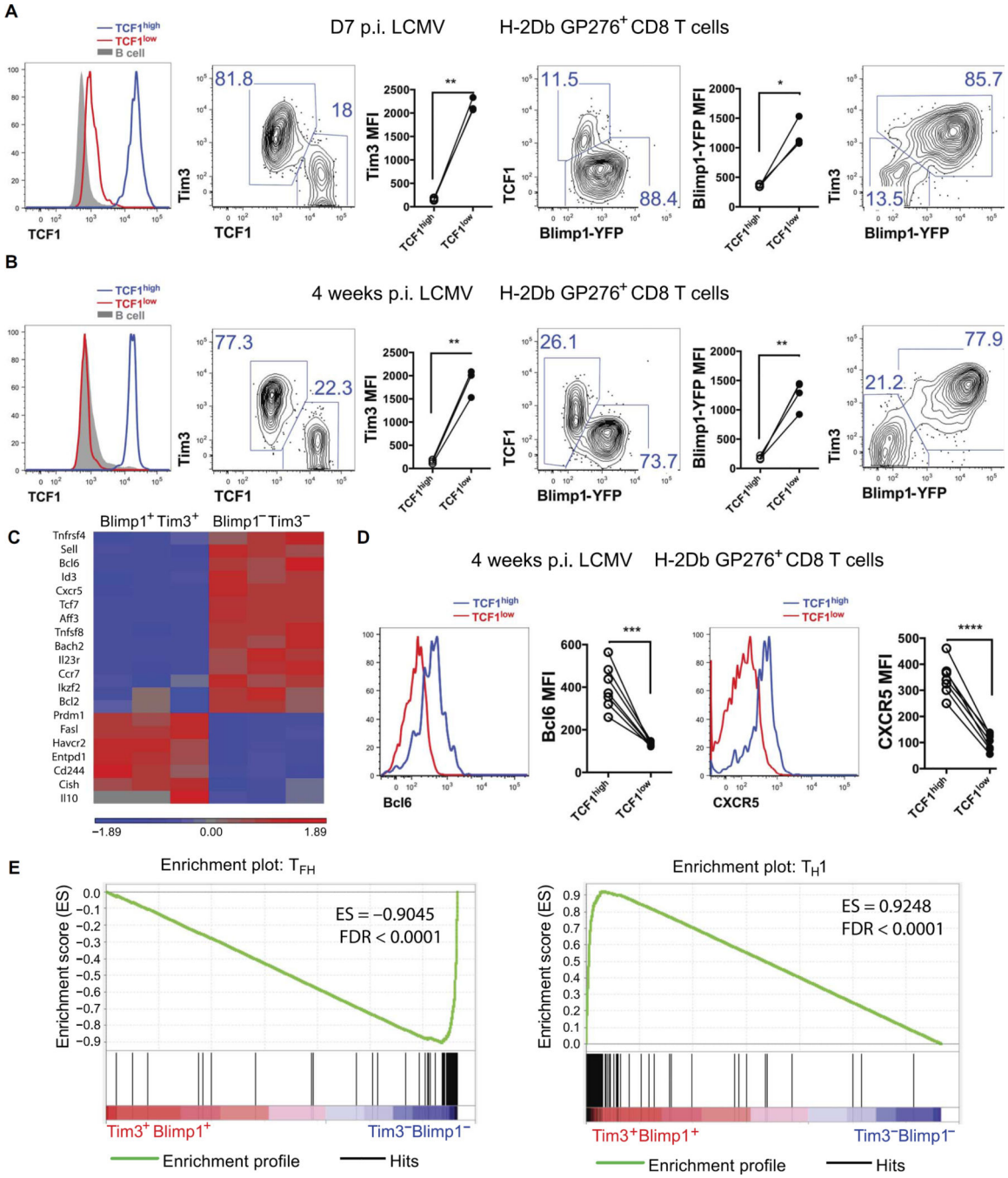

Fig. 1. TCF1^{high} virus-specific CD8 T cells in chronic LCMV infection resemble T$_{FH}$ cells

(**A** and **B**) C57BL/6 or Blimp1-YFP (yellow fluorescent protein) reporter mice were infected with LCMV clone 13. Expression of TCF1, Tim3, and Blimp1 in H-2Db GP276 tetramer^{+} CD8 T cells in the spleen analyzed 7 days (A) and 4weeks post-infection (p.i.) (B). MFI, mean fluorescence intensity. (**C**) Heat map of selected differentially expressed genes from microarrays comparing Tim3^{low}Blimp1^{low} and Tim3^{high}Blimp1^{high} virus-specific CD8 T cells from mice 7 days after infection. (**D**) Expression of Bcl6 and CXCR5 on TCF1^{high} (blue) and TCF1^{low} (red) H-2Db GP276 tetramer^{+} splenic CD8 T cells analyzed 4 weeks

after infection. (**E**) GSEA of the microarray data in (C) comparing the enrichment of T_{FH} (left) and T_H1 (right) gene signatures between $Tim3^{low}Blimp1^{low}$ and $Tim3^{high}Blimp1^{high}$ virus-specific CD8 T cells. Data in (A), (B), and (D) are representative of at least two independent experiments with $n \geq 3$. Statistical significance was determined by paired *t* tests. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Microarrays (C and E) were performed with three biological replicates per group. ES, enrichment score; FDR, false discovery rate.

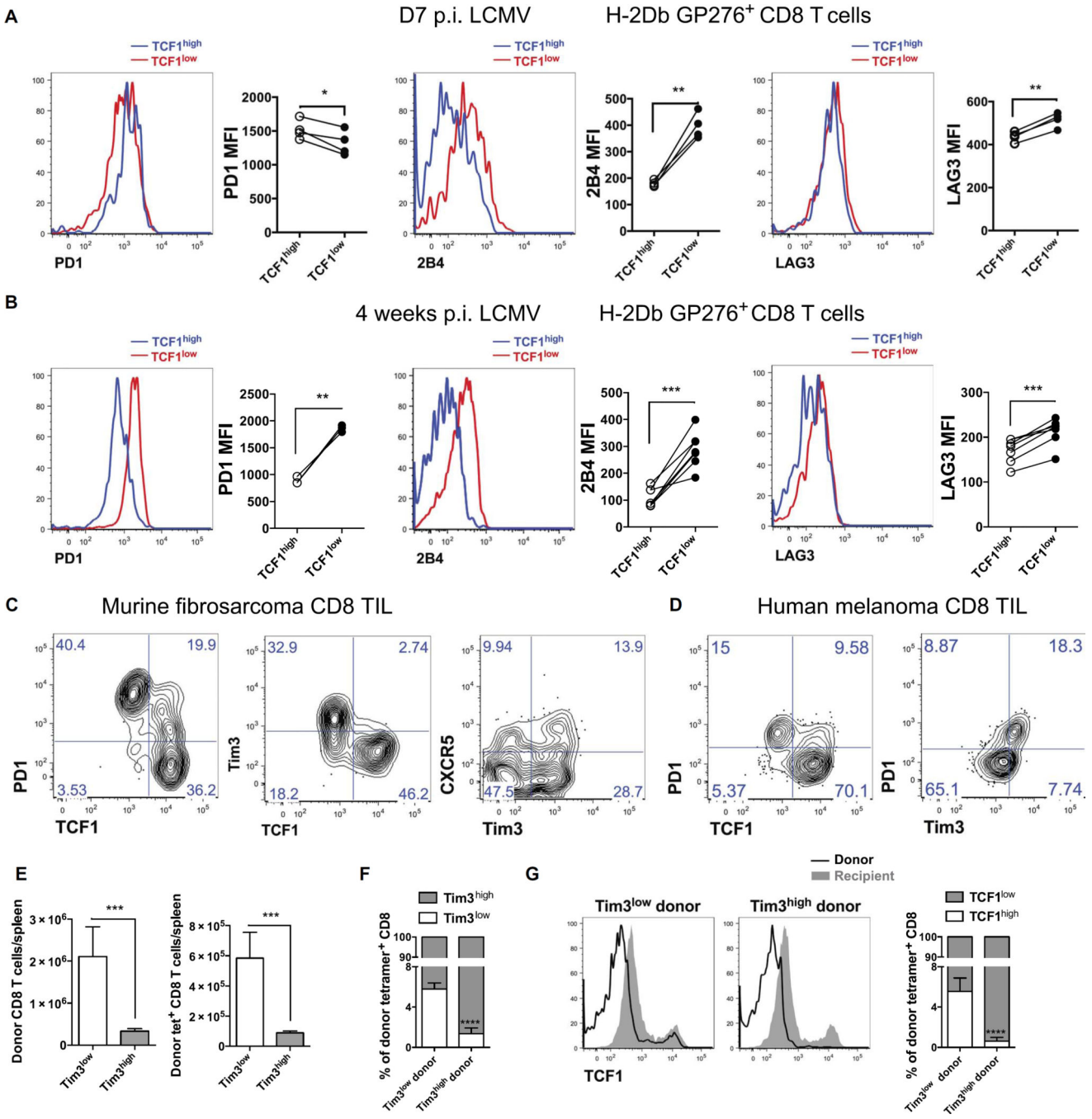

Fig. 2. TCF1 expression negatively correlates with T cell exhaustion in both chronic viral infection and solid tumors

(**A** and **B**) PD1, 2B4, and LAG3 expression on TCF1^{high} (blue) and TCF1^{low} (red) H-2Db GP276 tetramer^{+} (tet^{+}) splenic CD8 T cells analyzed 7 days (A) and 4 weeks after infection (B). (**C**) C57BL/6 mice were implanted with MCA205 fibrosarcoma cells. Expression of TCF1, PD1, Tim3, and CXCR5 on tumor-infiltrating CD8 T cells analyzed 20 days after injection. (**D**) Expression of TCF1, PD1, and Tim3 in CD8 T cells isolated from human melanoma samples. (**E** to **G**) Tim3^{low}PD1^{+}CD44^{high} and Tim3^{high}PD1^{+}CD44^{high} CD8 T

cells from C57BL/6 mice 7 days after infection were transferred separately into naïve CD45.1 mice that were subsequently infected with LCMV clone 13. (E) Numbers of total and tetramer^{+} donor CD8 T cells in the spleen 7 days after rechallenge. (F) Frequencies of Tim3^{low} (white) and Tim3^{high} (gray) cells within tetramer^{+} progeny from Tim3^{low} or Tim3^{high} donors. (G) Representative histograms of TCF1 expression in donors and recipient tetramer^{+} CD8 T cells. Frequencies of TCF1^{high} (white) and TCF1^{low} (gray) cells within tetramer^{+} progeny from Tim3^{low} or Tim3^{high} donors are shown. Data in (A) to (C) and (E) to (G) are representative of at least two independent experiments with $n \geq 3$. Data in (D) are representative of three melanoma patients. Statistical significance was determined by paired (A and B) and unpaired *t* tests (E to G). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

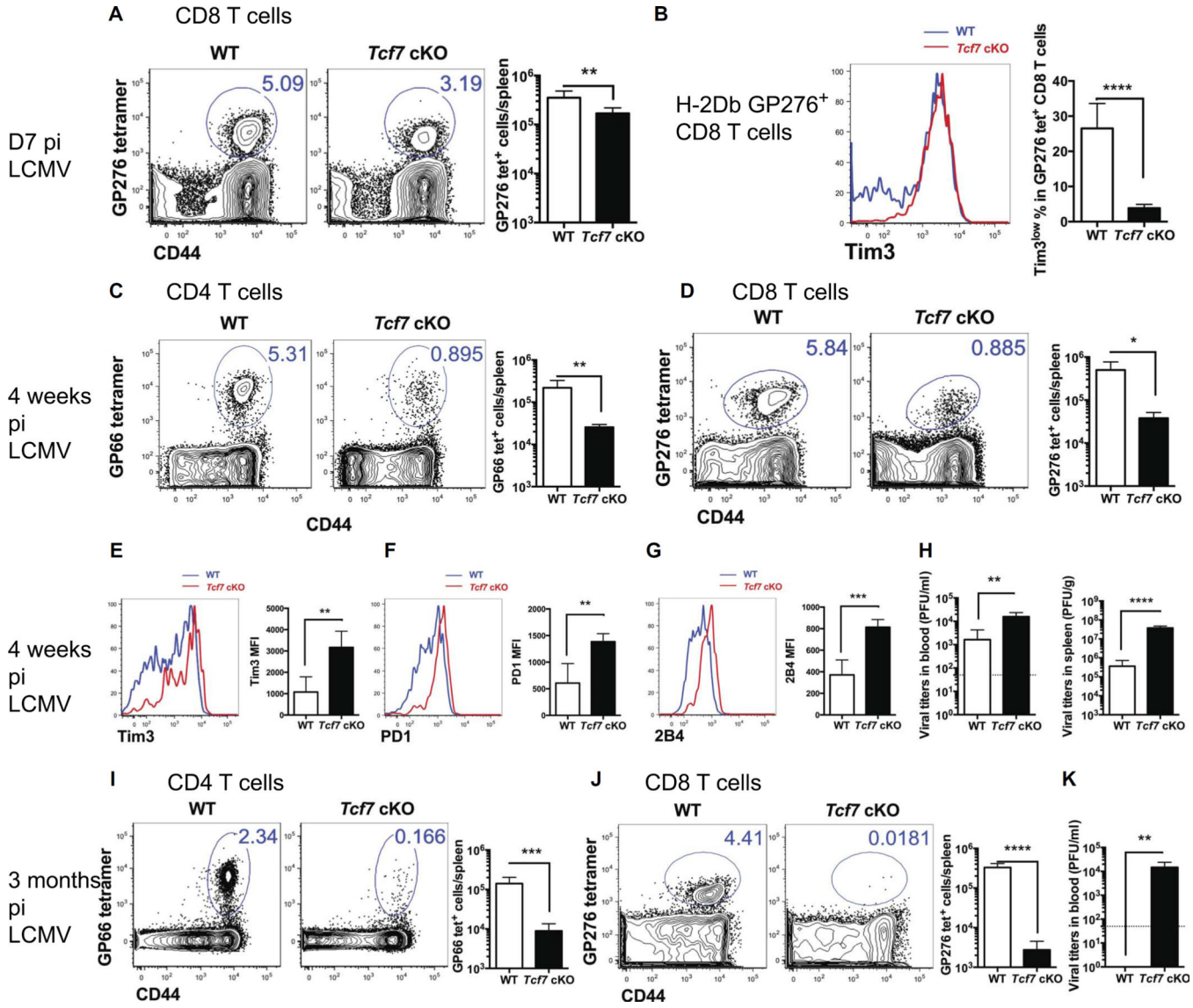

Fig. 3. TCF1 deficiency compromises Tim3^{low} virus-specific CD8 T cells and long-term persistence of T cell responses after chronic infection

Tcf7$^{loxP/loxP}$; CD4-Cre (*Tcf7* cKO) and littermate control (WT) mice were infected with LCMV clone 13. (**A**) Representative H-2Db GP276 tetramer staining and number of H-2Db GP276 tetramer$^+$ splenic CD8 T cells 7 days after infection. (**B**) Representative histograms of Tim3 expression and frequencies of Tim3^{low} cells within H-2Db GP276 tetramer$^+$ WT and cKO CD8 T cells 7 days after infection. (**C** and **D**) Frequencies and numbers of WT and cKO I-Ab GP66 tetramer$^+$ CD4 (C) and H-2Db GP276 tetramer$^+$ CD8 (D) splenic T cells 4 weeks after infection. (**E** to **G**) Expression of Tim3, PD1, and 2B4 on H-2Db GP276 tetramer$^+$ splenic CD8 T cells from WT (blue) and cKO (red) mice 4 weeks after infection. (**H**) Viral titers in blood and spleens 4 weeks after infection. (**I** and **J**) Frequencies and numbers of WT and cKO I-Ab GP66 tetramer$^+$ CD4 (I) and H-2Db GP276 tetramer$^+$ CD8 (J) splenic T cells 3 months after infection. (**K**) Blood viral titers 3 months after infection. Data are representative of at least two independent experiments with $n \geq 3$. Statistical

significance was determined by unpaired *t* tests. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

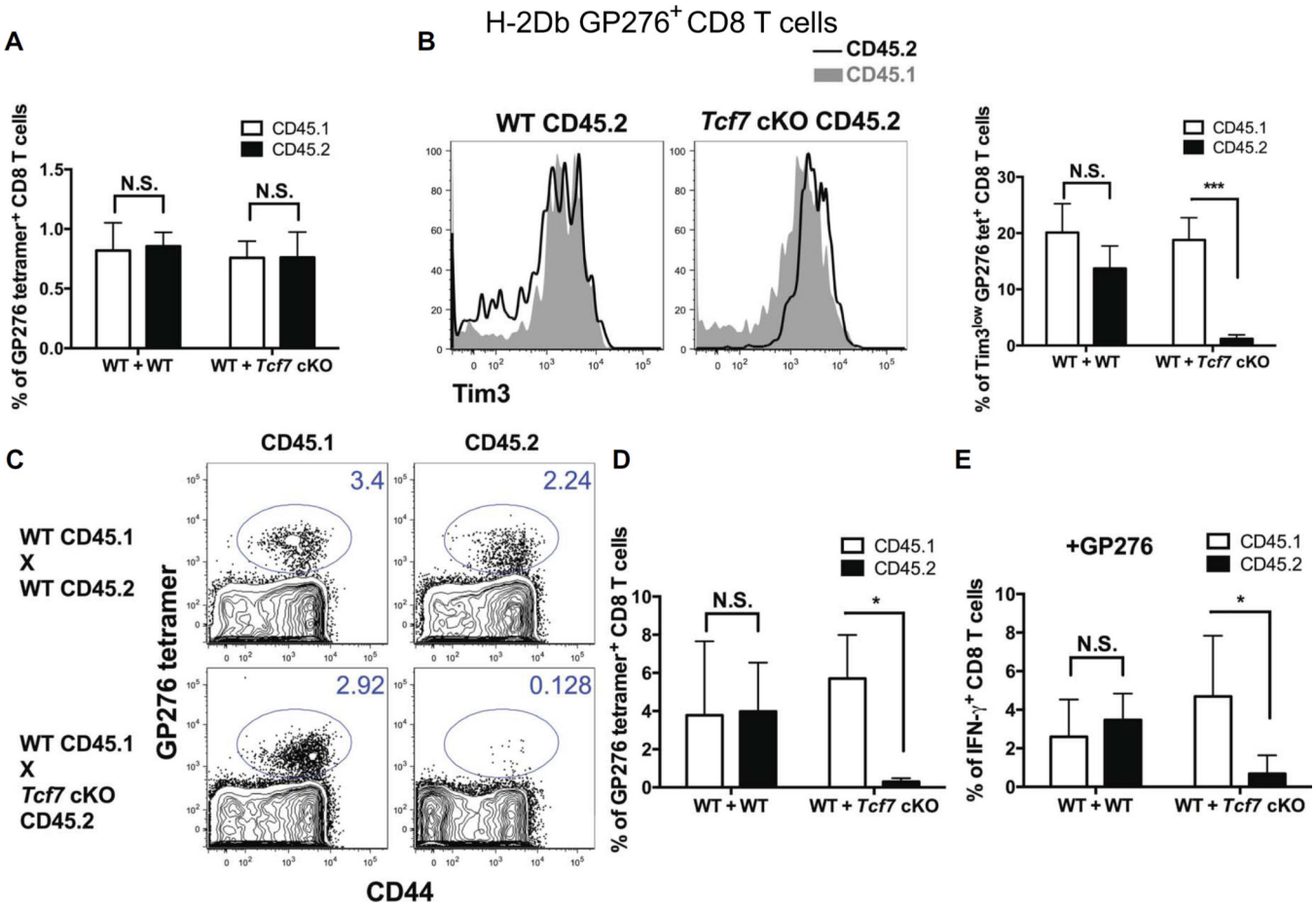

Fig. 4. Cell-intrinsic requirement of TCF1 for Tim3^{low} virus-specific CD8 T cells and persistence of T cell responses

Mixed bone marrow chimeras that received WT CD45.1 and WT CD45.2 (WT + WT) or WT CD45.1 and *Tcf7* cKO CD45.2 bone marrow (WT + *Tcf7* cKO) were infected with LCMV clone 13. (**A**) Frequencies of H-2Db GP276 tetramer$^+$ cells within CD45.1 (white) and CD45.2 (filled) CD8 splenic T cells 7 days after infection. (**B**) Tim3 expression on H-2Db GP276 tetramer$^+$ WT CD45.1 (shaded) and WT or cKO CD45.2 (solid line) CD8 T cells and frequencies of Tim3^{low} cells within H-2Db GP276 tetramer$^+$ CD45.1 WT (white) and CD45.2 WT or cKO (filled) splenic CD8 T cells from chimeras 7 days after infection. (**C**) Representative fluorescence-activated cell sorting plots of H-2Db GP276 tetramer staining on CD45.1 WT and CD45.2 WT or cKO CD8 compartments in spleens from chimeras 4 weeks after infection. (**D**) Frequencies of H-2Db GP276 tetramer$^+$ cells within CD45.1 WT (white) and CD45.2 WT or cKO (filled) CD8 T cells in spleens from chimeras 4 weeks after infection. (**E**) Frequencies of IFN-γ^+ cells within CD45.1 WT (white) and CD45.2 WT or cKO (filled) splenic CD8 T cells from chimeras 4 weeks after infection, restimulated with GP276 peptide. Data are representative of at least two independent experiments with $n \geq 4$. Statistical significance was determined by paired *t* tests. N.S., not significant. $*P < 0.05$, $***P < 0.001$.

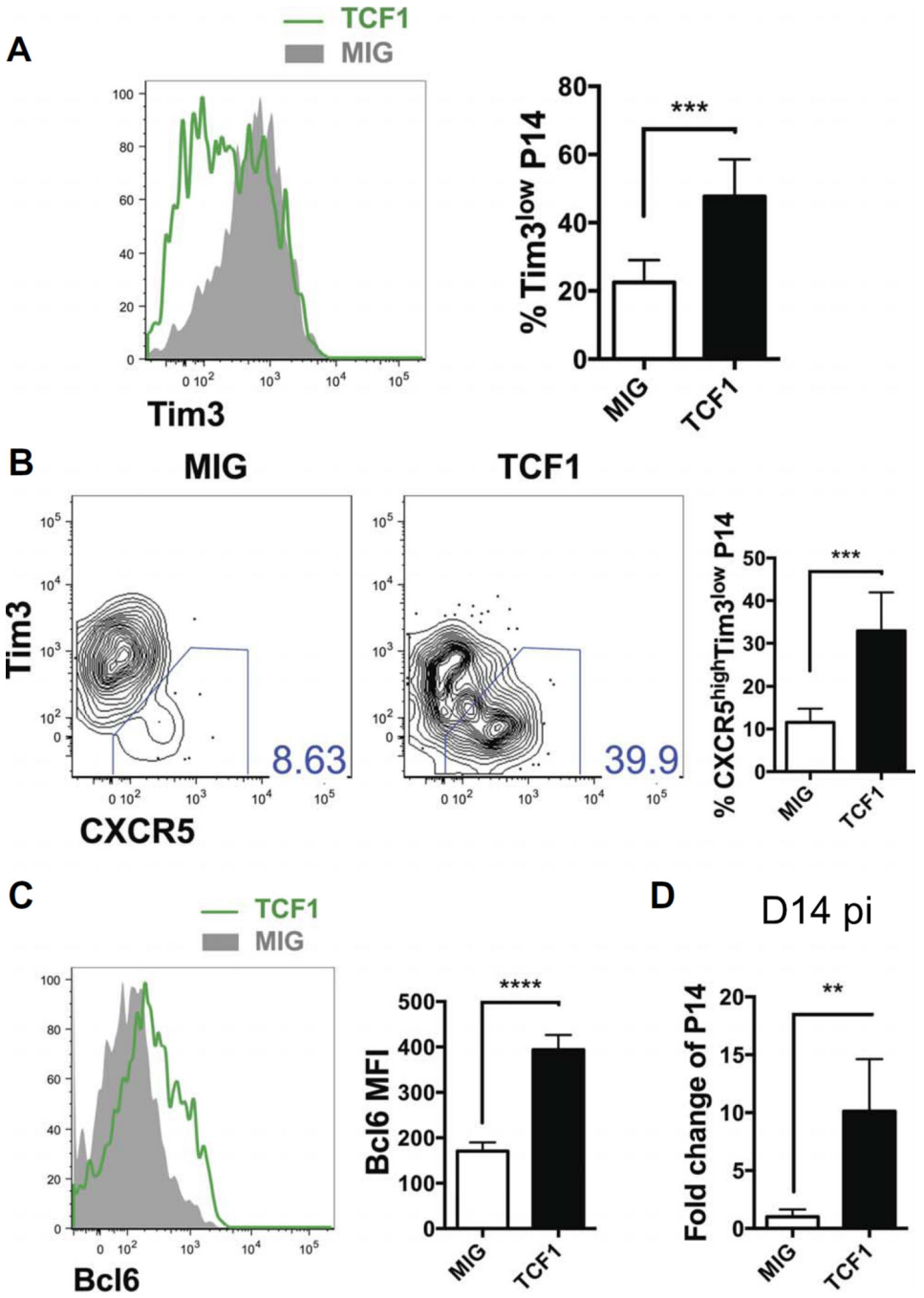

Fig. 5. TCF1 overexpression enhanced the differentiation of Tim3^{low}CXCR5^{high} CD8 T cells and persistence of virus-specific CD8 T cells

P14 cells transduced with either control (MIG) or TCF1 overexpression (TCF1) retroviral vectors were transferred to C57BL/6 recipients that were subsequently infected with LCMV clone 13. (**A** to **C**) Mice were evaluated on day 8 after infection. (A) Tim3 expression and frequencies of Tim3^{low} cells within transduced P14 cells. (B) Flow plot and frequencies of CXCR5^{high}Tim3^{low} cells in transduced P14 cells. (C) Bcl6 expression in control (MIG) or TCF1-overexpressing (TCF1) P14 cells. (**D**) Numbers of control (MIG) and TCF1-

overexpressing (TCF1) P14 cells in spleens of mice 14 days after infection. Data are representative of at least two independent experiments with $n \geq 3$. Statistical significance was determined by unpaired t tests. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

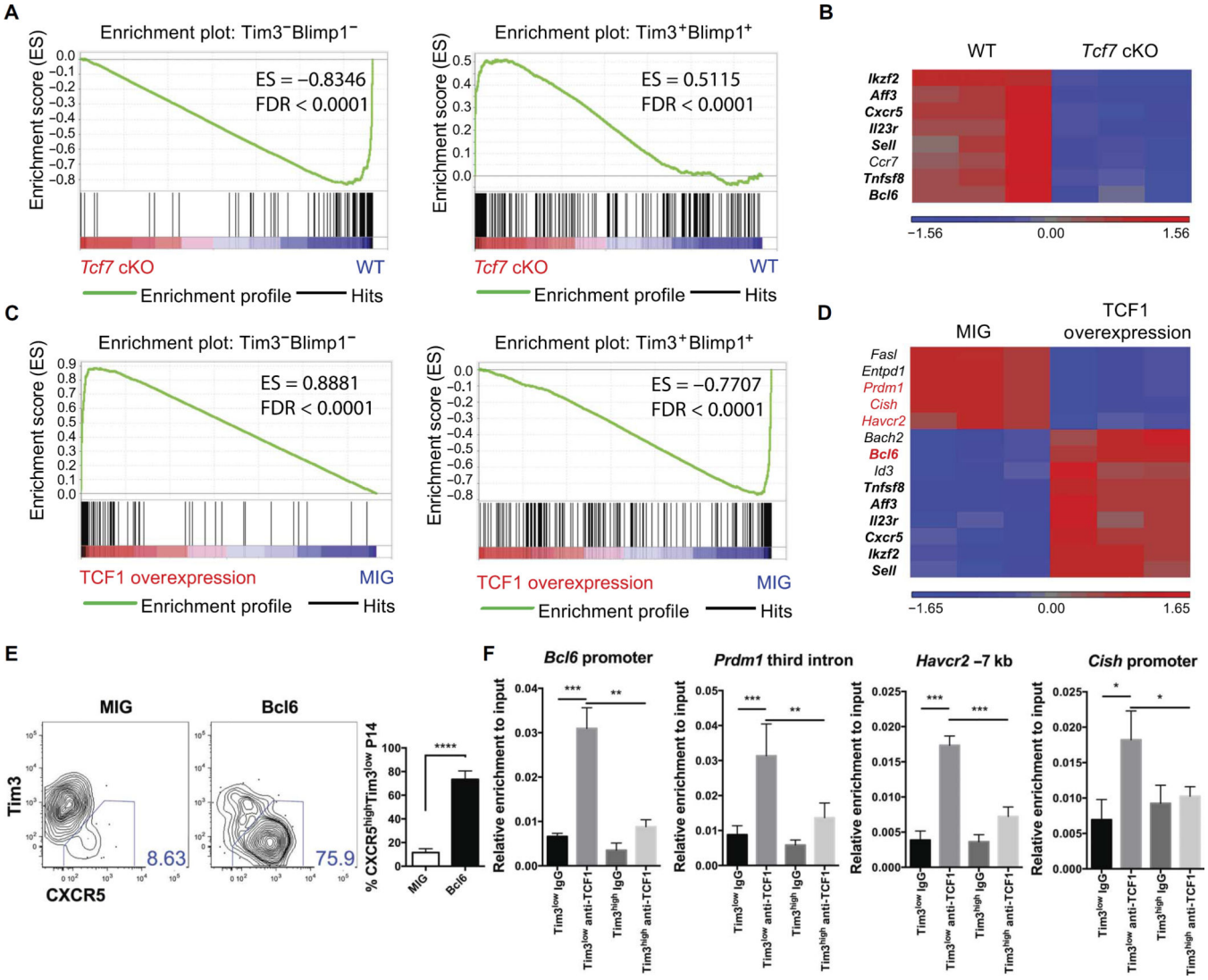

Fig. 6. TCF1 regulates the differentiation of Tim3^{low}Blimp1^{low} virus-specific CD8 T cells and the expression of Bcl6 and pro-exhaustion genes

(**A** and **B**) Microarray experiments with tetramer^{+} WT and *Tcf7* cKO CD8 T cells on day 7 after infection. (A) GSEA comparing the enrichment of Tim3^{low}Blimp1^{low} (left) and Tim3^{high}Blimp1^{high} (right) gene signatures between WT and cKO virus-specific CD8 T cells. (B) Heat map of selected up-regulated or down-regulated genes in cKO cells relative to WT cells. (**C** and **D**) Microarrays of TCF1-overexpressing and control (MIG) P14 T cells on day 8 after infection. (C) GSEA comparing the enrichment of Tim3^{low}Blimp1^{low} (left) and Tim3^{high}Blimp1^{high} (right) gene signatures between TCF1-overexpressing and control P14 cells. (D) Heat map of selected up-regulated or down-regulated genes in TCF1-overexpressing relative to control P14 cells. Genes marked in bold were reciprocally expressed in *Tcf7* cKO cells. Genes marked in red were evaluated by ChIP. (**E**) Tim3 and CXCR5 expression, as well as frequencies of CXCR5^{high}Tim3^{low} cells in control (MIG), or Bcl6-transduced P14 cells on day 8 after infection. (**F**) ChIP assays performed on day 7 after infection. Tim3^{low}PD1^{+}CD44^{high} and Tim3^{high}PD1^{+}CD44^{high} CD8 T cells. Binding of

TCF1 to indicated loci determined by ChIP and quantitative reverse transcription polymerase chain reaction. Data in (E) and (F) are representative of two independent experiments with $n \geq 3$. Statistical significance in (E) and (F) was determined by unpaired *t* tests. IgG, immunoglobulin G. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

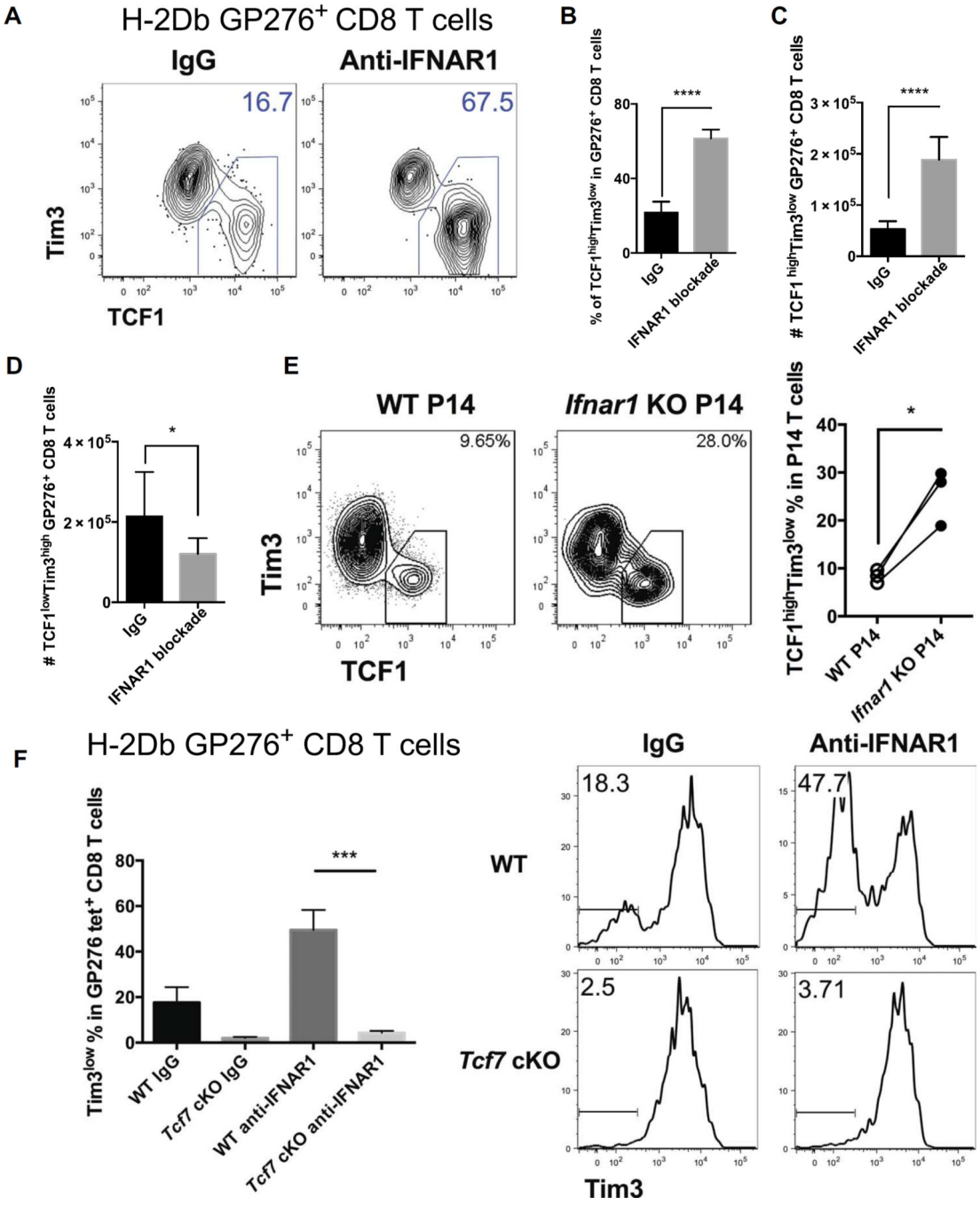

Fig. 7. Type I IFN signaling suppresses the generation of TCF1^{high}Tim3^{low} virus-specific CD8 T cells, upstream of TCF1

(**A** to **D**) C57BL/6 mice were treated with anti-IFNAR1 or IgG before LCMV clone 13 infection. Splenocytes analyzed 7 days after infection. (A) Representative TCF1 and Tim3 expression inH-2DbGP276 tetramer$^+$ CD8 T cells. Frequencies (B) and numbers (C) of TCF1^{high}Tim3^{low} GP276 tetramer$^+$ CD8 T cells. (D) Numbers of TCF1^{low}Tim3^{high} GP276 tetramer$^+$ CD8 T cells. (**E**) Ten thousand *Ifnar1* KO and WT P14 cells were mixed in a 1:1 ratio and transferred into NK-depleted WT hosts that were subsequently infected with

LCMV clone 13. TCF1^{high}Tim3^{low} CD8 T cells 7 days after infection. (**F**) WT and *Tcf7* cKO mice were treated with 1 mg of anti-IFNAR1 or isotype control IgG before LCMV clone 13 infection. Splenocytes analyzed 7 days after infection. Representative Tim3 expression and frequencies of Tim3^{low} cells within GP276 tetramer^{+} CD8 T cells in each group. Data are representative of two independent experiments with $n \geq 3$. Statistical significance in (B) to (D) and (F) was determined by unpaired *t* tests. Statistical significance in (E) was determined by paired *t* tests. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.