Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory

Grégoire Jeannet[a,1], Caroline Boudousquié[a,1], Noémie Gardiol[a], Joonsoo Kang[b], Joerg Huelsken[c], and Werner Held[a,2]

[a]Ludwig Institute for Cancer Research Ltd., Lausanne Branch and University of Lausanne, 1066 Epalinges, Switzerland; [b]Department of Pathology, Graduate Program in Immunology and Virology, University of Massachusetts Medical School, Worcester, MA 01655; and [c]Swiss Cancer Research Institute, Federal University of Technology Lausanne, 1015 Lausanne, Switzerland

Edited* by Douglas T. Fearon, University of Cambridge School of Clinical Medicine, Cambridge, United Kingdom, and approved April 19, 2010 (received for review December 8, 2009)

Immune protection from intracellular pathogens depends on the generation of terminally differentiated effector and of multipotent memory precursor CD8 T cells, which rapidly regenerate effector and memory cells during recurrent infection. The identification of factors and pathways involved in CD8 T cell differentiation is of obvious importance to improve vaccination strategies. Here, we show that mice lacking T cell factor 1 (Tcf-1), a nuclear effector of the canonical Wingless/Integration 1 (Wnt) signaling pathway, mount normal effector and effector memory CD8 T cell responses to infection with lymphocytic choriomeningitis virus (LCMV). However, Tcf-1–deficient CD8 T cells are selectively impaired in their ability to expand upon secondary challenge and to protect from recurrent virus infection. Tcf-1–deficient mice essentially lack CD8 memory precursor T cells, which is evident already at the peak of the primary response, suggesting that Tcf-1 programs CD8 memory cell fate. The function of Tcf-1 to establish CD8 T cell memory is dependent on the catenin-binding domain in Tcf-1 and requires the Tcf-1 coactivators and Wnt signaling intermediates β-catenin and γ-catenin. These findings demonstrate that the canonical Wnt signaling pathway plays an essential role for CD8 central memory T cell differentiation under physiological conditions in vivo. They raise the possibility that modulation of Wnt signaling may be exploited to improve the generation of CD8 memory T cells during vaccination or for therapies designed to promote sustained cytotoxic CD8 T cell responses against tumors.

lymphocytic choriomeningitis virus infection | central memory | memory precursor | β-catenin | γ-catenin

Immune protection against intracellular pathogens and tumors is chiefly mediated by CD8 T cells. The first productive encounter with antigen initiates the clonal expansion and differentiation of very rare antigen-specific CD8 T cells, generating a large pool of cytokine producing and cytolytic effector T cells. Most effector CD8 T cells die once the pathogen is cleared, leaving behind a population of long-lived effector and memory CD8 T cells. Effector memory T cells respond to antigen reencounter with immediate effector function but limited proliferation. Long-lived central memory T cells have the unique ability to vigorously expand upon secondary antigen encounter, to give rise to effector cells, and to self-renew to maintain a functional memory CD8 T cell compartment. Memory CD8 T cells thus have attributes of tissue-specific stem cells (1, 2).

The progeny of a single naïve T cell are "programmed" during the initial encounter with antigen to differentiate into CD8 effector cells or to acquire a long-lived memory fate (3–5). Differentiation into CD8 effector cells is promoted when the duration or the intensity of exposure to antigen and inflammation is increased during CD8 T cell priming. This is mediated in part by cytokines such IL-12 or type I IFN (6). Furthermore, effector cell differentiation is favored by the transcription factors T-bet, Id-2, or BLIMP1 (7–10). Bcl6 reciprocally promotes memory formation and represses certain aspects of effector cell differentiation (11). Conversely, CD4 T cell help or IL-2 signals during the initial antigen encounter is selectively required to program the recall response of memory CD8 T cells to secondary infection (12–15) suggesting that activated CD4 T cells provide IL2 to promote CD8 T cell memory. Transcription factors selectively required for the development of functional CD8 T cell memory have not been identified. In addition, it is currently not clear whether the initial heterogeneity in cell fates arises due to quantitatively or qualitatively different signals and whether the commitment to the central memory lineage occurs before or after the adoption of the effector fate (16).

Based on the prominent role of the canonical Wingless/Integration-1 (Wnt) signaling pathway for stem cell maintenance and lineage specification in a variety of tissues (for a recent review see ref. 17), we addressed whether this pathway is required for the differentiation of CD8 T cells. Wnt ligands are lipid modified, secreted proteins, which bind to LRP5/6 and/or Frizzled cell surface receptors. A subset of Wnt proteins activates canonical Wnt signaling, which is characterized by the intracellular stabilization and accumulation of β-catenin. As a consequence, β-catenin translocates to the nucleus where it binds to transcription factors of the Tcf (T cell factor) family (Tcf-1, Lef-1, Tcf-3, and Tcf-4), which mediate transient transcription of Wnt responsive target genes. The β-catenin homolog γ-catenin (plakoglobin) is able to similarly activate certain Wnt target genes. In the absence of extracellular Wnt, β-catenin is recruited to a cytosolic destruction complex, phosphorylated, and degraded (17). In addition, Wnt target genes are actively repressed via the association of Tcf with Groucho corepressors (17). Much of the clinical interest in this pathway stems from the findings that deregulated Wnt signaling is implicated in a number of cancers (18).

Here we find that Tcf-1 is not essential for mounting a primary protective CD8 T cell response to viral infection and for the maintenance of a pool of virus-specific effector T cells. However, Tcf-1 is critical for the secondary expansion of virus-specific CD8 T cells upon reinfection and the generation of CD8 central memory precursor cells. A corresponding impairment of replicative CD8 T cell memory was also observed in mice deficient in β- and γ-catenin. These data indicate an essential role of the canonical Wnt signaling pathway for the generation of functional CD8 T cell memory.

Results

Normal Primary CD8 T Cell Response to Lymphocytic Choriomeningitis Virus (LCMV) Infection in Mice Lacking Tcf-1 (*Tcf7*). The *Tcf7* gene, coding for Tcf-1, is expressed highly in naïve and central memory but at low levels in effector and effector memory CD8 T cells (19) (www.immgen.org). To identify the functional role of Tcf-1 and of the ca-

Author contributions: G.J., C.B., and W.H. designed research; G.J., C.B., and N.G. performed research; G.J., C.B., N.G., and W.H. analyzed data; J.K. and J.H. contributed new reagents/analytic tools; and J.K. and W.H. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]G.J. and C.B. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: werner.held@licr.unil.ch.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0914127107/-/DCSupplemental.

nonical Wnt signaling pathway for CD8 T cell differentiation, we infected *Tcf7*$^{-/-}$ mice with LCMV WE strain, which is controlled by CD8 T cells in normal mice. Even though T cell development is impaired in *Tcf7*$^{-/-}$ mice (20, 21), they have only a moderately reduced peripheral CD8 T cell compartment (Table S1) and mount an efficient CD8 T cell response to the LCMV epitopes D^b/gp33-41 (gp33) (Fig. 1 *A–C*), D^b/NP396-404 (NP396), and K^b/NP205-212 (NP205) (Fig. S1 and Table S2). At day 8 after infection *Tcf7*$^{-/-}$ splenocytes mediated efficient cytolysis of gp33 peptide pulsed target cells in vitro (Fig. 1*D*). Moreover, upon stimulation with gp33 peptide, in vitro mutant CD8 T cells readily produced IFNγ (Fig. 1*E*). Indeed, *Tcf7*$^{-/-}$ mice had efficiently controlled splenic LCMV titers at day 8 post-inoculation (Fig. 1*F*). Hence, during acute LCMV infection, Tcf-1 is dispensable for the expansion of antigen-specific CD8 T cells, the acquisition of effector function, and the control of viral infection.

Impaired Recall Response of Tcf-1–Deficient CD8 T Cells. Similar to wild-type mice, the frequency and absolute number of *Tcf7*$^{-/-}$ gp33-specific CD8 T cells started to decline after day 8 following primary infection (Fig. 1*B*). Despite the contraction, a sizeable population of gp33-specific CD8 T cells was maintained in Tcf-1–deficient mice for >40 and >100 days after LCMV infection (Fig. 1 *A*, *B*, and *G*). Similar results were obtained for NP396- and NP205-specific CD8 T cells (Table S2). In addition, gp33-specific CD8 T cells were readily detected in nonhematopoietic tissues, such as the lung (Fig. S2). Around day 50 after primary infection we assessed the functionality of memory CD8 T cells. LCMV-immune *Tcf7*$^{-/-}$ mice killed gp33 peptide pulsed spleen cells in vivo (Fig. 2*A*). Moreover, CD8 T cells from wild-type and *Tcf7*$^{-/-}$ mice readily produced IFN-γ upon stimulation with gp33 peptide in vitro (Fig. 2*B*). These data show that *Tcf7*$^{-/-}$ mice efficiently generate and maintain LCMV-specific CD8 effector memory T cells.

We next assessed the ability of LCMV-specific CD8 T cells to expand upon secondary stimulation, a hallmark of central memory CD8 T cells. First, LCMV-immune mice were challenged with

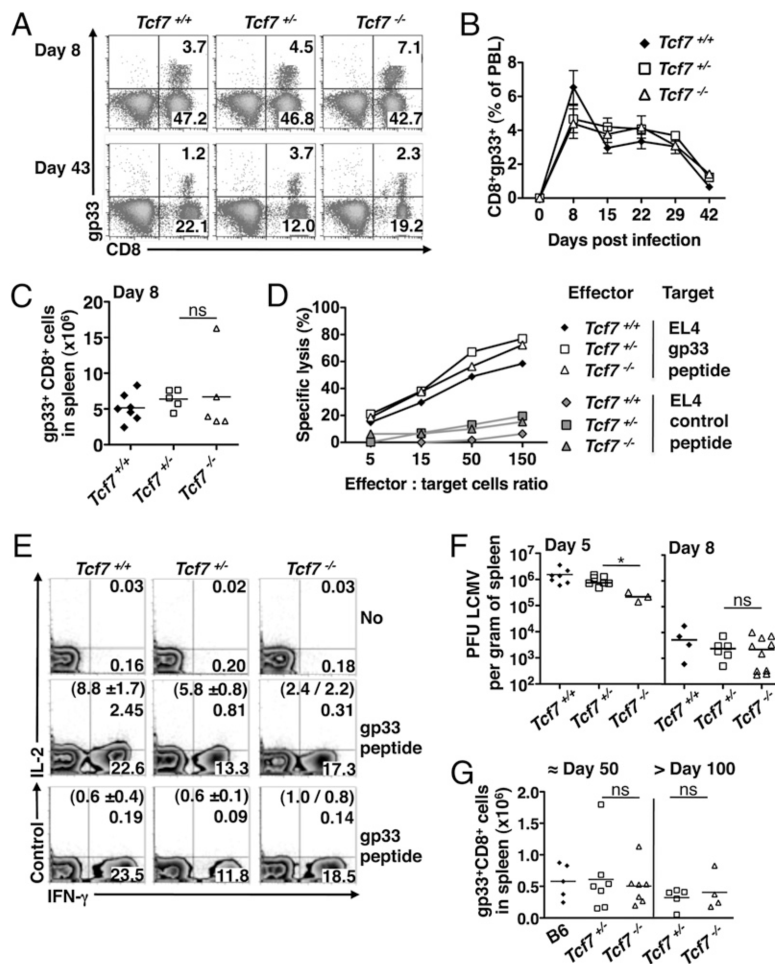

Fig. 1. Primary CD8 T cell response of *Tcf7*$^{-/-}$ mice to acute LCMV Infection. *Tcf7*$^{+/+}$ (B6), *Tcf7*$^{+/-}$ and *Tcf7*$^{-/-}$ mice were infected with LCMV. (*A*) The antigen-specific CD8 T cell response was determined at day 8 and day 43 post-LCMV infection. Numbers indicate the percentage of peripheral blood lymphocytes (PBL) in the respective quadrant. (*B*) The line graph depicts the mean percentage (±SD) of gp33+ CD8+ cells among PBL at the indicated time points post-LCMV infection. (*C*) The absolute number of gp33+ CD8+ cells per spleen was determined at day 8 after LCMV infection. Symbols depict individual mice. (*D*) EL4 cells (H-2^b) were pulsed with gp33 or Smcy (control) peptide and used as target cells in standard cytolysis assays using day 8 LCMV immune splenocytes as effectors. (*E*) Splenocytes from day-8 LCMV immune mice were stimulated in vitro with gp33 peptide before intracellular staining using IFN-γ and IL-2 or control mAbs. Density plots show gated CD8 T cells. Numbers indicate the percentage of cells in the respective quadrants. Numbers in brackets indicate the percentage (± SD or individual determinations) of IL-2+ cells among IFN-γ+ CD8+ cells from multiple determinations. (*F*). Recipient spleens were analyzed for infectious LCMV particles (pfus) at day 5 and day 8 postinfection. Symbols depict pfus in individual mice. (*G*) The number of gp33-specific CD8 cells per spleen was determined at the indicated time points post-LCMV infection. Symbols depict individual mice. (*) depict significant differences ($P < 0.05$); ns, not significantly different ($P > 0.05$) based on Student's *t* test.

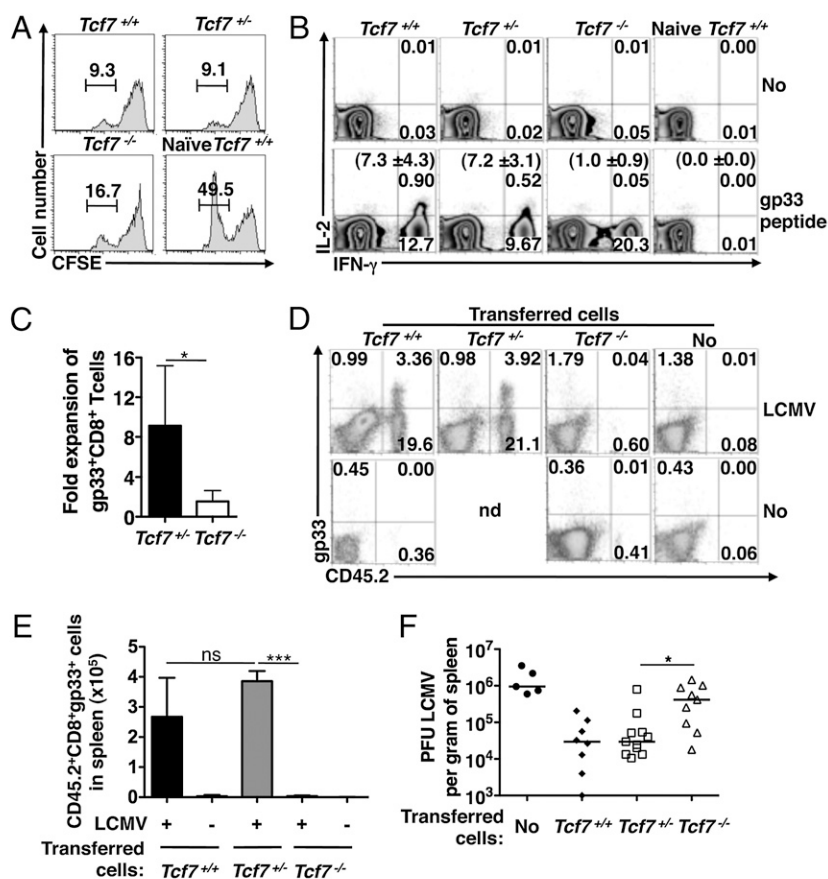

Fig. 2. Secondary CD8 T cell response of $Tcf7^{-/-}$ mice to LCMV infection. (*A*) B6 splenocytes were labeled with a high and with low concentration of CFSE and the latter were pulsed with gp33 peptide. A 1:1 mixture of these cells was injected into LCMV immune $Tcf7^{+/+}$ (B6), $Tcf7^{+/-}$, or $Tcf7^{-/-}$ mice around day 50 post-LCMV infection. Numbers indicate the percentage of residual $CFSE^{lo}$ (gp33 peptide pulsed) cells relative to $CFSE^{hi}$ control cells in recipient spleens 3 hr after transfer. (*B*) Splenocytes from LCMV immune mice (around day 80) were stimulated in vitro with gp33 peptide. Density plots show gated CD8 T cells stained for intracellular IFN-γ and IL-2. Numbers indicate the percentage of cells in the respective quadrants. Numbers in brackets depict the mean percentage (± SD) of IL-2 + cells among IFN-γ+ CD8+ cells of multiple determinations. (*C*) The frequency of gp33-specific CD8 T cells among PBL of LCMV immune mice (>day 60 to day 70) was estimated ($Tcf7^{+/+}$ 0.93 ± 0.44; $Tcf7^{-/-}$ 0.90 ± 0.36). These mice were challenged with gp33 peptide coupled to virus like particles and the frequency of antigen-specific CD8 T cells was determined 8 days later ($Tcf7^{+/+}$ 6.66 ± 2.42, n = 5; $Tcf7^{-/-}$ 1.18 ± 0.67, n = 7). The bar graph depicts the mean fold expansion (± SD) of gp33-specific CD8 T cells from individual mice. (*D*) Splenocytes from LCMV immune $Tcf7^{+/+}$, $Tcf7^{+/-}$, or $Tcf7^{-/-}$ mice (CD45.2) (around day 85) containing 3×10^4 gp33+ CD8+ cells were transferred into naïve recipients (CD45.1) followed or not (No) by LCMV infection 1 day later. Density plots show gated CD8 T cells stained with gp33 tetramer versus anti-CD45.2 at day 5 postinfection. Numbers indicate the percentage of cells in the respective quadrants. (*E*) The bar graph depicts the mean number (± SD) of donor-derived (CD45.2) gp33+ CD8+ T cells in recipient spleens at day 5 after infection (+) or not (–) with LCMV. (*F*) Naive wild-type mice received or not (No) LCMV immune cells followed by challenge infection as in *D*. Five days later, recipient spleens were analyzed for the abundance of infectious LCMV particles (pfu). Symbols depict individual mice. (*) and (***) depict significant differences ($P < 0.05$) and ($P < 0.001$), respectively; ns, not significantly different ($P > 0.05$) based on Student's *t* test.

virus-like particles (VLP) coupled with gp33 peptide (22). Although specific CD8 T cells from $Tcf7^{+/+}$ mice expanded efficiently (on average ninefold), those from $Tcf7^{-/-}$ mice expanded poorly (1.5-fold) (Fig. 2*C*). Next, we adoptively transferred splenocytes from LCMV immune mice (expressing the congenic marker CD45.2) containing equal numbers of gp33-specific CD8 T cells into naïve recipients (CD45.1). Upon LCMV challenge infection. gp33-specific $Tcf7^{-/-}$ CD8 T cells failed to expand, whereas those from $Tcf7^{+/-}$ mice expanded efficiently (Fig. 2 *D* and *E*). An impaired secondary expansion was confirmed for NP205-specific CD8 T cells and using competitive adoptive transfer experiments (Fig. S3) indicating a T cell-intrinsic defect. The absence of a secondary response was not based on a lack of cell transfer or homing because $Tcf7^{-/-}$ CD8 T cells were detectable in recipient spleens in the absence of virus infection (Fig. 2*D*). Hence, even though Tcf-1–deficient mice mount an efficient primary immune response to LCMV infection and maintain a sizeable pool of virus-specific CD8 T cells, these cells fail to expand upon secondary stimulation.

We next addressed whether the lack of secondary expansion of LCMV immune CD8 cells resulted in a reduced protection against repeated viral infection. To this end, we determined LCMV titers in the spleen of naive mice that had received LCMV immune CD8 cells. As compared to naive mice that received no such cells, the adoptive transfer of LCMV immune cells from $Tcf7^{+/+}$ or $Tcf7^{+/-}$ mice significantly reduced the viral load (>10-fold) at day 5 postchallenge infection (Fig. 2*F*). In contrast, the adoptive transfer of LCMV immune cells from $Tcf7^{-/-}$ mice was very inefficient at reducing the viral load (Fig. 2*F*). This was in contrast to the primary LCMV infection (day 8), which was efficiently controlled in $Tcf7^{-/-}$ mice (Fig. 1*F*). These data show that the reduced secondary expansion of LCMV immune CD8 T cells in the absence of Tcf-1 leads to an impaired viral clearance during recurrent infection.

Tcf-1–Deficient Mice Lack LCMV-Specific CD8 Memory Precursors. Because secondary expansion is mediated by central memory CD8 T cells, we analyzed a number of cell surface markers to

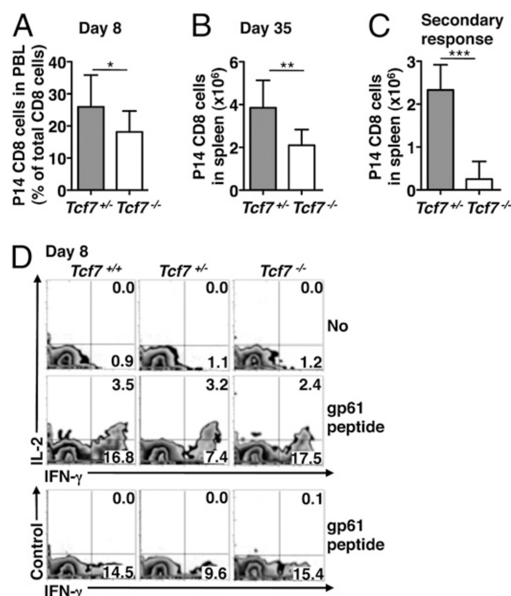

Fig. 3. Cell intrinsic role of Tcf-1 for the recall response of CD8 T cells. (*A*) Purified *Tcf7*$^{+/-}$ or *Tcf7*$^{-/-}$ P14 CD8 T cells (3×10^4) (CD45.2) were transferred into wild-type (CD45.1/2) recipients followed by LCMV infection 1 day later. The bar graph shows the mean percentage (± SD) of P14 CD8 T cells (CD45.2) among recipient PBL at day 8 postinfection. (B) The bar graph shows the mean number (± SD) of P14 CD8 T cells in recipient spleens at day 35 to day 60 postinfection. (*C*) Splenocytes from primary recipients containing 3×10^4 immune P14 CD8 T cells (day 42 to day 60) were transferred into secondary wild-type (CD45.1/2) recipients 1 day before LCMV challenge infection. The bar graph shows the mean number (± SD) of P14 CD8 T cells (CD45.2) in recipient spleens 5 days later. (*), (**) and (***) depict significant differences ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$), respectively based on Student's *t* test. (*D*) Splenocytes from *Tcf7*$^{+/+}$, *Tcf7*$^{+/-}$, and *Tcf7*$^{-/-}$ LCMV immune mice at day 8 postinfection were stimulated in vitro with gp61 peptide. Density plots show gated CD4 T cells stained intracellularly for IFN-γ and IL-2 or using a control Ab. Numbers indicate the percentage of cells in the respective quadrants.

determine whether phenotypic central memory precursor cells were present in *Tcf7*$^{-/-}$ mice. Antigen-specific CD8 T cells in Tcf-1–deficient mice normally expressed CD27, CD44, and CD122 (IL2Rβ) both at the peak of the primary response and during the memory phase. However, LCMV immune *Tcf7*$^{-/-}$ mice contained very few central memory phenotype KLRG1^{low} cells at day 50 post-LCMV infection. The relative absence of gp33-specific KLRG1^{low} cells was already evident at the peak of the primary response (Fig. 4*D*) and this was confirmed for NP205-specific CD8 T cells. The absence of virus-specific KLRG1^{low} cells was due to a generalized KLRG1 overexpression, because naive CD8 T cells in *Tcf7*$^{-/-}$ mice were KLRG1^{low} (see Fig. 4*D* below). In addition, LCMV immune *Tcf7*$^{-/-}$ mice contained fewer (2- to 3-fold) central memory phenotype [CD127^{hi} (IL7Rα) CD62L^{hi}] CD8 T cells as compared to wild-type mice (Fig. S4*A*). The combination of the above markers revealed that phenotypic antigen-specific CD62L^{hi} CD127^{hi} KLRG1^{lo} CD8 memory precursor cells are reduced fivefold in LCMV immune *Tcf7*$^{-/-}$ ($5.0 \pm 1.3 \times 10^4$/spleen) as compared to *Tcf7*$^{+/-}$ mice ($1.0 \pm 0.6 \times 10^4$) (Fig. S4*B*). The remaining memory compartment of *Tcf7*$^{-/-}$ mice is CD127^{low} and KLRG1^{hi} CD127^{hi}.

Further, central memory precursor cells are functionally characterized by their ability to produce IL-2 (23). IL-2 producing CD8 cells were essentially absent in LCMV immune *Tcf7*$^{-/-}$ mice (Fig. 2*B*) and were significantly underrepresented at day 8 postinfection (Fig. 1*E*). These results suggest that Tcf-1–deficient mice essentially lack a pool of central memory precursor cells and that these cells fail to arise during primary LCMV infection.

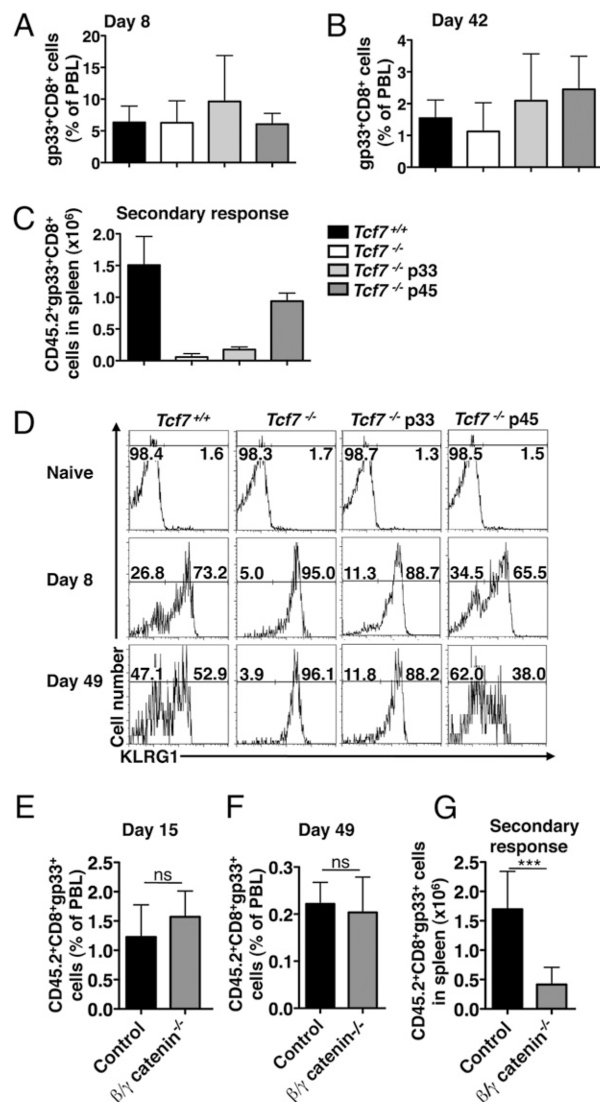

Fig. 4. The catenin-binding domain in Tcf-1 and β–/γ-catenin are essential for the formation of functional CD8 T cell memory. *Tcf7*$^{+/+}$ (B6), *Tcf7*$^{-/-}$, *Tcf7*$^{-/-}$ p33 and *Tcf7*$^{-/-}$ p45 Tg mice were infected with LCMV. Bar graphs depict the mean percentage (± SD) of gp33+ CD8+ T cells in peripheral blood at day 8 (*A*) and at day 42 (*B*) post-LCMV infection. (*C*) Splenocytes from day 50 LCMV immune mice (CD45.2), containing 3×10^4 gp33+ CD8+ cells, were transferred into naïve recipients (CD45.1) 1 day before LCMV infection. The bar graph depicts the mean number (± SD) of CD45.2+ gp33-specific CD8 T cells in recipient spleens 5 days later. (*D*) Histograms show KLRG1 expression among CD8 T cells of naïve mice and among gated gp33+ CD8+ T cells from day 8 and day 49 LCMV immune mice. Numbers indicate the percentage of cells in the respective gate. Chimeric mice with a wild-type (control) or β/γ-catenin-deficient (β/γ-catenin$^{-/-}$) hematopoietic system (>90% CD45.2+) were infected with LCMV. Bar graphs depict the mean percentage (± SD) of gp33+ CD8+ T cells among CD45.2+ PBL of the indicated chimera at day 15 (*E*) and day 49 (*F*) post-LCMV infection. (*G*) Splenocytes from the indicated LCMV immune chimera (day 50), containing 3×10^4 CD45.2+ gp33+ CD8+ T cells, were transferred into naïve recipients (CD45.1) 1 day before LCMV challenge infection. The bar graph depicts the mean number (± SD) of CD45.2+ gp33+ CD8+ T cells in recipient spleens 5 days later. (*) and (***) depict significant differences ($P < 0.05$) and ($P < 0.001$), respectively; ns, not significantly different ($P > 0.05$) based on Student's *t* test.

Tcf-1 Expression in CD8 T Cells Is Essential for Secondary Expansion. To address whether the impairment in secondary expansion is intrinsic to CD8 T cells and to rule out a role of the T cell receptor (TCR) as the basis for defective CD8 T cell memory formation in

$Tcf7^{-/-}$ mice, we introduced a transgenic (Tg) TCR specific for the LCMV gp33 peptide in the context of H-2D^b (P14) into Tcf-1–deficient mice. P14 $Tcf7^{+/-}$ and $Tcf7^{-/-}$ CD8 T cells were predominantly naive (CD44^{low}) (Table S1) and reacted equally efficiently with D^b gp33 tetramers. Purified P14 CD8 T cells (3×10^4) (CD45.2) were transferred into wild-type [CD45.1/CD45.2 heterozygous (CD45.1/2)] recipients followed by LCMV infection. Both types of transferred cells had efficiently expanded by day 8 postinfection (Fig. 3*A*) (although the accumulation of $Tcf7^{-/-}$ P14 cells was marginally lower) and LCMV immune CD8 cells were maintained accordingly over time (Fig. 3*B*).

To determine the recall response, day-35 splenocytes (containing equal numbers of P14 CD8 T cells) (CD45.2) were transferred to secondary recipients (CD45.1/2). Following challenge infection, $Tcf7^{+/-}$ P14 CD8 T cells expanded efficiently, whereas the recall expansion of $Tcf7^{-/-}$ P14 CD8 cells was very low (Fig. 3*C*). These data formally show that CD8 T cells from $Tcf7^{-/-}$ mice suffer from a cell-intrinsic defect in secondary replicative function, which is independent of the TCR.

Reciprocal adoptive transfers of wild-type P14 cells into $Tcf7^{-/-}$ recipients provided evidence that $Tcf7^{-/-}$ mice suffer from an additional, CD8 T cell-independent, defect, which impacts the CD8 recall response (Fig. S5). Because impaired functional memory is a hallmark of CD8 T cells that have been primed in the absence of CD4 T cell help or IL-2 signals (12–15), we verified whether $Tcf7^{-/-}$ mice mount a CD4 T cell response to LCMV infection. Stimulation of day-8 immune spleen cells with LCMV gp61-80 peptide, an immunodominant CD4 T cell epitope, induced IFN-γ production by a significant population of $Tcf7^{-/-}$ CD4 T cells (Fig. 3*D* and Table S3). In contrast to the impaired IL-2 production by CD8 T cells, a significant fraction of gp61-80-specific $Tcf7^{-/-}$ CD4 T cells produced IL-2 (Fig. 3*D*). These data suggest that $Tcf7^{-/-}$ mice do mount a CD4 helper T cell response and that the additional defect in $Tcf7^{-/-}$ mice may be independent of CD4 T cells. Irrespective of the precise basis for the additional, CD8 T cell-independent defect, the above transfer experiments establish that Tcf-1 expression in CD8 T cells is essential for the secondary replicative function of CD8 T cells.

Secondary CD8 T Cell Expansion Depends on the Catenin-Binding Domain in Tcf-1. Although Tcf-1 binds DNA, it has no intrinsic transcriptional activity. Target gene repression occurs through the association of Tcf-1 with Groucho corepressors. Activation of target transcription in response to Wnt signals depends on the coactivators β-catenin and γ-catenin binding to the NH_2 terminus of Tcf-1 (17, 24). Previously, we have used a genetic complementation approach to show the requirement of the catenin-binding domain of Tcf-1 for T cell development: A Tg Tcf-1 containing the catenin-binding plus the repressor domain (p45) rescued T cell development in $Tcf7^{-/-}$ mice, whereas a Tg containing only the repressor domain (p33) failed to do so (21). We used the same approach to determine the mechanism of Tcf-1 function in CD8 T cell memory formation.

We observed a significant population of gp33-specific CD8 T cells in $Tcf7^{+/+}$, $Tcf7^{-/-}$ and Tcf-1 Tg (both p45 and p33) $Tcf7^{-/-}$ mice at day 8 and >day 40 post-LCMV infection (Fig. 4 *A* and *B*). Spleen cells from LCMV immune mice (CD45.2) containing an equal number of gp33-specific CD8 T cells were then transferred into naïve recipients (CD45.1) followed by viral challenge. Whereas gp33-specific CD8 T cells from $Tcf7^{+/+}$ mice expanded efficiently, those from $Tcf7^{-/-}$ mice essentially failed to expand (Fig. 4*C*) in agreement with Fig. 2*E*. The expression of the p33 Tg in $Tcf7^{-/-}$ mice did not restore secondary CD8 T cell expansion. In contrast, the expression of the p45 Tg significantly restored the secondary expansion of CD8 T cells upon challenge infection (Fig. 4*C*). These data show that the catenin-binding domain of Tcf-1 is essential for the recall response of CD8 T cells.

The functional data were corroborated by the analysis of KLRG1 expression on gp33-specific CD8 T cells. Virus-specific CD8 T cells from $Tcf7^{-/-}$ and $Tcf7^{-/-}$ p33 Tg mice were essentially devoid of KLRG1^{low} memory precursor cells both at day 8 and day 50 after LCMV infection. In contrast, a KLRG1^{low} memory precursor population was evident in p45 Tg mice (Fig. 4*D*). These results show that Tcf-1-dependent transcriptional activation, putatively downstream of the canonical Wnt pathway, is necessary to generate CD8 memory precursor cells.

Secondary CD8 T Cell Expansion Depends on β-Catenin and γ-Catenin. To definitely demonstrate an involvement of the canonical Wnt signaling pathway in central memory CD8 T cell differentiation we employed bone marrow chimeric mice with a hematopoietic compartment (CD45.2) that is doubly-deficient in β-catenin and γ-catenin (24). In these mice, steady state hematopoiesis is entirely normal (24). Upon primary infection with LCMV, the gp33-specific CD8 T cell response of double-deficient chimeras was marginally lower than that of control chimeras at day 8, but was comparable at day 15 (Fig. 4*E*), although the magnitude of the primary response in the chimeras was overall lower than that of nonchimeric mice (Fig. 4*A*). Virus-specific CD8 T cells were maintained equally efficiently in both types of chimeric mice for >40 days (Fig. 4*F*). Around day 50 after primary infection, spleen cells from the chimeras (>90% CD45.2+), containing an equal number of gp33-specific CD45.2-derived CD8 T cells, were transferred into naïve recipients (CD45.1). Upon challenge with LCMV, virus-specific CD8 T cells from control chimeras expanded efficiently, whereas those from β–/γ-catenin-deficient chimeras expanded poorly (Fig. 4*G*). These data demonstrate an essential role of γ- and/or β-catenin for the recall response of CD8 T cells. Together, these results implicate canonical Wnt/catenin/Tcf signaling as a key pathway controlling the generation of central CD8 T cell memory and protection from recurrent viral infection.

Discussion

Here we demonstrate an essential and selective requirement for Tcf-1 and the canonical Wnt signaling pathway in the secondary expansion of virus-specific CD8 T cells and consequently for the protection from recurrent viral infection. Tcf-1 is not required for the expansion of virus-specific CD8 T cells in primary infection, the initial acquisition of effector functions, virus control, and the long-term persistence of effector cells, suggesting that the emergence of effector and effector memory cells is independent of Tcf-1. Tcf-1 is selectively implicated in the generation of long-lived CD8 T cell memory. Our data suggest a role of Tcf-1 for the specification of CD8 central memory fate during the primary response to LCMV infection.

Given that Tcf-1 has multiple functions, it was important to determine whether the generation of CD8 T cell memory relied on the canonical Wnt signaling pathway. Using two genetic approaches, we showed that the catenin-binding domain in Tcf-1 and β- and/or γ-catenin coactivators are essential in generating functional CD8 T cell memory in vivo. Thus, key components of the canonical Wnt signaling pathway are necessary for CD8 T cell differentiation. Consistent with these results based on loss-of-function approaches, enforced activation of the Wnt signaling pathway in CD8 T cells, using a pharmacological inhibitor of glycogen synthase kinase 3 β (GSK3β, a serine kinase implicated in numerous signaling pathways, including the degradation of β-catenin) or recombinant Wnt3a, promoted the differentiation of CD8 T cells into memory precursor cells at the expense of effector cell fate in vitro (25). In addition, enforced Wnt signaling in vivo favored the generation of CD8 T cell memory (26). Together with the data reported herein, these results suggest that the formation of CD8 T cell memory may depend on extracellular Wnt proteins. However, due to the significant number of Wnt proteins, Wnt receptors and the complexity of Wnt signaling (www.stanford.edu/~rnusse/wntwindow.html), it will be difficult to establish

IMMUNOLOGY

a functional link between specific extracellular Wnt proteins and the formation of CD8 T cell memory under physiological conditions in vivo. In addition, there is evidence that signaling through the TCR can increase β-catenin stability in CD4 T cells (27), raising the possibility that factors other than extracellular Wnt ligands that use the canonical Wnt signaling pathway may contribute to the generation of CD8 T cell memory.

Irrespective of the precise nature of extrinsic cues that modulate catenin-Tcf-1 transcriptional activation in CD8 T cells, our study shows that the canonical Wnt signaling pathway is implicated in the generation of functional CD8 T cell memory under physiological conditions in vivo. A major clinical implication of these findings is that Wnt signaling agonists might serve as adjuvants to improve the generation of CD8 memory T cells during vaccination or therapies designed to marshal sustainable cytotoxic T cell responses against tumors.

Materials and Methods

Mice. C57BL/6 (B6) mice were purchased from Harlan (Horst). CD45.1 and Thy1.1 congenic B6 mice were purchased from Jackson Lab. *Tcf7*$^{-/-}$ (20), Tcf-1 p33, and p45 Tg mice (B6 backcross > 10) have been described (21). As wild-type *Tcf7*$^{+/+}$ controls we used both B6 [referred to as *Tcf7*$^{+/+}$ (B6) in the respective figure legends] and *Tcf7*$^{+/+}$ littermate mice. There was no difference between the two. P14 TCR Tg (B6) mice (line 327) (expressing a TCR specific for amino acids 33–41 of the LCMV gp33 presented by H-2D^b). P14 Tg *Tcf7*$^{-/-}$ mice were obtained by breeding. Chimeric mice with a hematopoietic system lacking β- and γ-catenin were generated as described before (24). For adoptive transfer experiments, we used purified P14 CD8 T cells (3×10^4) or total splenocytes containing 3×10^4 gp33-specific CD8 T cells, unless indicated otherwise. As recipients for some transfer experiments we used (CD45.1 B6 x CD45.2 *Tcf7*$^{+/+}$)F_1 mice. Female or male mice were used at 6 to 20 weeks of age.

LCMV Infection, Virus Titration, and Virus-Like Particles. From primary and secondary infection mice were injected i.v. with 200 and 2,000 pfu of LCMV strain WE, respectively. LCMV titers per gram of spleen weight were determined in centrifuged spleen homogenates using a plaque forming assay as described (28). Secondary CD8 T cell responses were also induced by subcutanous injection of 100 μg of virus like particles conjugated with gp33 peptide (KAVYNFATM) (provided by M. Bachmann, Cytos, Zürich).

Flow Cytometry. Splenocytes were incubated with anti-CD16/32 (2.4G2) hybridoma supernatant before staining for multicolor flow cytometry with fluorescent mAbs (all from BD Biosciences) to CD4 (GK1.5), CD8α (53-6.7), KLRG1 (2F1), CD44 (IM7), CD62L (MEL-14), CD122 (TM-β1), and CD127 (A7R34). Phycoerythrin (PE)-labeled D^b gp33 (KAVYNFATA) tetramers were produced using standard procedures.

For intracellular cytokine detection, splenocytes were incubated for 5 hr in round-bottom 96-well plates in complete DMEM medium containing Brefeldin A (1 μM, Sigma), Monensin (1 μM) and recombinant IL-2 (50 ng/mL) in the presence of gp33 (KAVYNFATA) or gp61 (GLNGPDIYKGVYQFKSVEFD) peptide (0.1 μg/mL). The cells were harvested and fixed with 2%. parafomaldhyde for 30 min at 4 °C. After washing, cells were stained with mAbs to IFN-γ (XMG1.2; e-Bioscience), IL-2 (JES6-5H4), or a rat IgG control Ab (both from BD PharMingen) in PBS plus 0.5% saponin. Samples were run on FACS Calibur or FACScanto flow cytometers (Becton Dickinson) and analyzed with FlowJo (Tree Star).

Lysis Assays. Splenocytes from naïve B6 mice were pulsed with 1 μM of gp33 peptide for 1 hr at 37 °C before labeling with 0.5 mM of 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes). Nonpulsed splenocytes were labeled with 5 mM of CFSE. Labeled splenocytes were mixed (2.5×10^6 cells of each type) and injected i.v. into LCMV immune mice. Three hours later recipient spleens were analyzed for the presence of CFSE-labeled cells. Specific lysis was determined based on the ratio between CFSE^{lo} versus CFSE^{hi} cells. For in vitro cytotolysis assays, ^{51}Cr labeled EL-4 (H-2^b) tumor cells were incubated for 1 hr at 37 °C with 1 μM of gp33 (KAVYNFATA) or control Smcy (KCSRNRQYL) peptide. After washing, target cells were incubated with splenocytes from day-8 LCMV-immune mice. After 4 hr of incubation at 37 °C, target cell lysis was estimated based on the release of ^{51}Cr into the supernatant.

Statistical Analysis. Data sets are considered significantly different when $P < 0.05$ is in a two-tailed Student's *t* test.

ACKNOWLEDGMENTS. We thank P. Guillaume (LICR Lausanne) for the preparation of tetramers, C. Mueller (University of Bern) for LCMV WE, M. Bachmann (Cytos, Zurich) for VLPs, H. Clevers (Utrecht University) for *Tcf7*$^{-/-}$ mice, H. P. Pircher (University of Freiburg) for P14 mice and for advice, and A. Ochsenbein (University of Bern) for advice. This research was supported in part by the Leenaards Foundation (to J.H. and W.H.), the Fondation Emma Muschamp (to W.H.), and a grant from the Swiss National Science Foundation (to W.H.).

1. Stemberger C, et al. (2009) Stem cell-like plasticity of naïve and distinct memory CD8+ T cell subsets. *Semin Immunol* 21:62–68.
2. Parish IA, Kaech SM (2009) Diversity in CD8(+) T cell differentiation. *Curr Opin Immunol* 21:291–297.
3. Mercado R, et al. (2000) Early programming of T cell populations responding to bacterial infection. *J Immunol* 165:6833–6839.
4. Kaech SM, Ahmed R (2001) Memory CD8+ T cell differentiation: Initial antigen encounter triggers a developmental program in naïve cells. *Nat Immunol* 2:415–422.
5. Stemberger C, et al. (2007) A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity* 27:985–997.
6. Williams MA, Bevan MJ (2007) Effector and memory CTL differentiation. *Annu Rev Immunol* 25:171–192.
7. Joshi NS, et al. (2007) Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. *Immunity* 27:281–295.
8. Intlekofer AM, et al. (2007) Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells. *J Exp Med* 204:2015–2021.
9. Cannarile MA, et al. (2006) Transcriptional regulator Id2 mediates CD8+ T cell immunity. *Nat Immunol* 7:1317–1325.
10. Rutishauser RL, et al. (2009) Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties. *Immunity* 31:296–308.
11. Yoshida K, et al. (2006) Bcl6 controls granzyme B expression in effector CD8+ T cells. *Eur J Immunol* 36:3146–3156.
12. Janssen EM, et al. (2003) CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421:852–856.
13. Sun JC, Bevan MJ (2003) Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* 300:339–342.
14. Williams MA, Tyznik AJ, Bevan MJ (2006) Interleukin-2 signals during priming are required for secondary expansion of CD8+ memory T cells. *Nature* 441:890–893.
15. Bachmann MF, Wolint P, Walton S, Schwarz K, Oxenius A (2007) Differential role of IL-2R signaling for CD8+ T cell responses in acute and chronic viral infections. *Eur J Immunol* 37:1502–1512.
16. Ahmed R, Bevan MJ, Reiner SL, Fearon DT (2009) The precursors of memory: Models and controversies. *Nat Rev Immunol* 9:662–668.
17. Grigoryan T, Wend P, Klaus A, Birchmeier W (2008) Deciphering the function of canonical Wnt signals in development and disease: Conditional loss- and gain-of-function mutations of beta-catenin in mice. *Genes Dev* 22:2308–2341.
18. Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. *Nature* 434:843–850.
19. Willinger T, et al. (2006) Human naive CD8 T cells down-regulate expression of the WNT pathway transcription factors lymphoid enhancer binding factor 1 and transcription factor 7 (T cell factor-1) following antigen encounter in vitro and in vivo. *J Immunol* 176:1439–1446.
20. Verbeek S, et al. (1995) An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature* 374:70–74.
21. Ioannidis V, Beermann F, Clevers H, Held W (2001) The β-catenin—TCF-1 pathway ensures CD4(+)CD8(+) thymocyte survival. *Nat Immunol* 2:691–697.
22. Storni T, et al. (2004) Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J Immunol* 172:1777–1785.
23. Sarkar S, et al. (2008) Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates. *J Exp Med* 205:625–640.
24. Jeannet G, et al. (2008) Long-term, multilineage hematopoiesis occurs in the combined absence of beta-catenin and gamma-catenin. *Blood* 111:142–149.
25. Gattinoni L, et al. (2009) Wnt signaling arrests effector T cell differentiation and generates CD8+ memory stem cells. *Nat Med* 15:808–813.
26. Zhao DM, et al. (2010) Constitutive activation of Wnt signaling favors generation of memory CD8 T cells. *J Immunol* 184:1191–1199.
27. Xu Y, Banerjee D, Huelsken J, Birchmeier W, Sen JM (2003) Deletion of β-catenin impairs T cell development. *Nat Immunol* 4:1177–1182.
28. Battegay M, et al. (1991) Quantification of lymphocytic choriomeningitis virus with an immunological focus assay in 24- or 96-well plates. *J Virol Methods* 33:191–198.