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Differentiation and persistence of memory CD8+ T cells depend on T cell factor 1

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Abstract

T cell factor 1 (TCF-1) is a transcription factor known to act downstream of the canonical Wnt pathway and is essential for normal T cell development. However, its physiological roles in mature $CD8^+$ T cell responses are unknown. Here we showed that TCF-1 deficiency limited proliferation of $CD8^+$ effector T cells and impaired their differentiation towards a central memory phenotype. Moreover, TCF-1-deficient memory $CD8^+$ T cells were progressively lost over time, exhibiting reduced expression of the anti-apoptotic molecule Bcl-2, interleukin-2 receptor β chain and diminished IL-15-driven proliferation. TCF-1 was directly associated with the *Eomes* allele and the Wnt-TCF-1 pathway was necessary and sufficient for optimal Eomes expression in naïve and memory $CD8^+$ T cells. Importantly, forced expression of Eomes partly protected TCF-1-deficient memory $CD8^+$ T cells from time-dependent attrition. Our studies thus identify TCF-1 as a critical player in a transcriptional program that regulates memory CD8 differentiation and longevity.

$CD8^+$ T cells are critical in controlling infection by intracellular pathogens including viruses and intracellular bacteria. Upon encountering antigen, naïve $CD8^+$ T cells are activated and clonally expand to a large quantity of effector cells equipped with cytokines and cytolytic molecules. Most of the effectors succumb to apoptosis during the contraction phase, and

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SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 1 Supplemental Table, and 6 Supplemental Figures.

DATA ACCESSION NUMBERS

Microarray data analyzing differential gene expression in WT and *Tcf7*$^{-/-}$ memory T cells have been deposited at the NCBI Gene Expression Omnibus (GEO), accession number GSE20754.

only a small portion of them transition into memory $CD8^+$ T cells, capable of providing enhanced protection against the same pathogen. The transition of effector to memory $CD8^+$ T cells is affected by extracellular stimuli including the strength and timing of stimulatory signals derived from T cell receptor (TCR)-antigen interactions, costimulation, inflammatory cytokines including interferons and IL-12 (Harty and Badovinac, 2008; Kaech and Wherry, 2007; Williams and Bevan, 2007). Memory $CD8^+$ T cells are heterogeneous, consisting of at least two phenotypically and functionally distinct subsets, *i.e*., effector memory (Tem) and central memory (Tcm) (Sallusto et al., 1999). Whereas Tem cells patrol peripheral tissues, Tcm cells migrate through secondary lymphoid organs and are capable of more efficient homeostatic self-renewal and secondary proliferation than Tem (Lefrancois and Marzo, 2006; Wherry et al., 2003). Generation and differentiation of memory T cells are stipulated by intrinsic transcriptional programs. Inactivation of T-bet, Blimp-1, and Id2 and forced expression of Bcl-6 increased formation of Tcm cells (Cannarile et al., 2006; Ichii et al., 2004; Intlekofer et al., 2007; Kallies et al., 2009; Rutishauser et al., 2009). The longevity of memory T cells, in contrast, depends on IL-15 (Schluns and Lefrancois, 2003), and the IL-15 responsiveness is supported by eomesodermin-mediated positive regulation of IL-2 receptor β chain (IL-2Rβ) (Intlekofer et al., 2005).

T cell factor 1 (TCF-1) is a known effector transcription factor downstream of the canonical Wnt pathway, functioning as either transcriptional activator or repressor depending on its interacting partners (Staal et al., 2008). The most studied co-activator, β-catenin, is post-transcriptionally regulated by a multi-molecular “destruction complex” containing two scaffolding proteins, adenomatous polyposis coli and axis inhibition protein (Axin), and two protein kinases, casein kinase I and glycogen synthase kinase 3β (GSK3β). Phosphorylation of β-catenin by the kinases marks it for proteosome-mediated degradation. Wnt stimulation leads to inhibition of GSK3β and hence β-catenin stabilization. The accumulated β-catenin translocates into the nucleus where it displaces corepressor TLE-GRG (transducin-like enhancer-Groucho-related gene) proteins and complexes with TCF-1 to activate Wnt downstream genes (Staal et al., 2008). TCF-1 is required for normal T cell development as inactivation of TCF-1 partly blocked thymocyte maturation at several early stages (Verbeek et al., 1995), whereas TCF-1 deficiency did not affect proliferation and cytolytic activity of mature T cells when assayed *in vitro* (Schilham et al., 1998). However, several studies demonstrated that TCF-1-β-catenin pathway is operative in naïve or activated T cells (Jeannet et al., 2008; Wu et al., 2007) and can be modulated by TCR signaling (Xu et al., 2003). We and others have recently shown that during $CD8^+$ T cell responses, TCF-1 is dynamically regulated, being downregulated in effectors and partly restored in memory T cells (Willinger et al., 2006; Zhao et al., 2010). Simultaneous activation of TCR and the TCF-1-β-catenin Wnt pathways *in vitro*, however, prevented TCF-1 downregulation and promoted a $CD8^+$ memory stem cell phenotype (Gattinoni et al., 2009). In line with this, constitutive activation of the TCF-1-β-catenin pathway *in vivo* favored generation of memory $CD8^+$ T cells (Zhao et al., 2010). These observations suggest that TCF-1-β-catenin activity can be manipulated to positively regulate $CD8^+$ memory. In contrast to its well-elucidated roles in T cell development, it remains unknown what physiological roles TCF-1 may play in regulating mature $CD8^+$ T cells. This study revealed the critical requirements of TCF-1 for $CD8^+$ effector T cell expansion, Tcm differentiation, and persistence of $CD8^+$ memory T cells.

RESULTS

TCF-1 deficiency limited $CD8^+$ T cell response to *Listeria monocytogenes* infection

To circumvent potential alterations in TCR repertoire and precursor frequency caused by TCF-1 (encoded by *Tcf7*) deficiency (Verbeek et al., 1995), we crossed *Tcf7*$^{-/-}$ to OT-I TCR transgenic mice whose TCR recognizes the SIINFEKL epitope from chicken ovalbumin. To

mimic physiological precursor frequency (Badovinac et al., 2007), we transferred low numbers of WT or *Tcf7*$^{-/-}$ OT-I CD8^{+} T cells (expressing CD45.2) into CD45.1^{+} B6.SJL recipients, followed by infection with attenuated *Listeria monocytogenes* expressing Ova (*actA*$^{-}$LM-Ova). WT OT-I cells exhibited peak proliferation on day 7 after infection as tracked in peripheral blood leukocytes (PBLs), however, proliferation of *Tcf7*$^{-/-}$ OT-I cells reached peak on day 6 and was reduced in magnitude by approximately 50% (Figure 1A). *Tcf7*$^{-/-}$ OT-I effectors in the spleens were also decreased by about 50% (Figure 1B). Both WT and *Tcf7*$^{-/-}$ OT-I effectors were all positive for Ova-specific MHC-I tetramer and had similar capacity to produce interferon-γ (IFN-γ) when stimulated *in vitro* (data not shown). Compared with WT controls, *Tcf7*$^{-/-}$ OT-I effectors exhibited similar downregulation of IL-7Rα and CD62L, similar CD44 upregulation, and moderately increased upregulation of KLRG1 (Figure 1C). Further, *Tcf7*$^{-/-}$ effectors showed similar tumor necrosis factor-α (TNF-α) production, moderately increased granzyme B and decreased IL-2 production (Figure 1D). The perturbed effector proliferation due to TCF-1 deficiency was further substantiated in experiments transferring Thy1.2^{+} WT or *Tcf7*$^{-/-}$ OT-I (as test cells) together with Thy1.1^{+} OT-I cells (as reference cells) into CD45 disparate recipients where the test and reference cells were exposed to the same host environment (Figure S1A and S1B). Taken together, *Tcf7*$^{-/-}$ CD8^{+} T cells can be activated to acquire effector function but showed reduced magnitude of proliferation.

By measuring 5'-bromo-2'-dexoxy-uridine (BrdU) uptake, we found that *Tcf7*$^{-/-}$ OT-I effectors proliferated similarly to WT during days 4-5 after infection but their proliferation was not sustained during days 6-7 (Figure S1C and S1D). On the other hand, WT and *Tcf7*$^{-/-}$ effectors at days 5 and 7 exhibited similar rates of apoptosis, as measured by caspase-3 and -7 activation (Figure S1E), in key contrast to increased caspase-3 and -7 activity in *Tcf7*$^{-/-}$ thymocytes (Figure S1F). Our results demonstrate that TCF-1 is essential for sustained proliferation of effector T cells, highlighting a distinct regulatory role of TCF-1 in activated mature T cells.

Tcf7$^{-/-}$ memory T cells were impaired in secondary expansion but not cytotoxity

Tcf7$^{-/-}$ effector OT-I T cells contracted similarly to WT effectors, giving rise to proportionally lower frequency of early memory CD8^{+} T cells in the PBLs (Figure 2A). Memory T cells are characteristic of enhanced proliferation upon reencounter of the same pathogen. To measure the secondary expansion on a per cell basis, we transferred the same number of sorted WT or *Tcf7*$^{-/-}$ OT-I memory T cells into naïve B6.SJL hosts. After infection with virulent LM-Ova, secondary proliferation of *Tcf7*$^{-/-}$ memory T cells was greatly diminished in the spleens (Figure 2B), indicating that secondary effectors are more dependent on TCF-1 for expansion compared with the primary response.

To assess the cytolytic activity of WT and *Tcf7*$^{-/-}$ memory CD8^{+} T cells, we used a short-term *in vivo* killing assay which does not involve extensive secondary expansion (Barber et al., 2003). To this end, we transferred 2,500 WT or *Tcf7*$^{-/-}$ OT-I cells prior to LM-Ova infection so that the endogenous antigen-specific T cells in the B6.SJL hosts were outcompeted (Badovinac et al., 2007), chose the recipients containing similar frequencies of WT and *Tcf7*$^{-/-}$ early memory OT-I T cells for the assay. Whereas the killing target cells that were Ova peptide-pulsed and labeled with low concentration of carboxyfluorescein succinimidyl diester (CFSE) were not affected in naïve mice, they were substantially eliminated in the immune recipients of either WT or *Tcf7*$^{-/-}$ OT-I cells (Figure 2C). Thus, lack of TCF-1 did not detectably alter the cytolytic activity of memory CD8^{+} T cells.

Tcf7$^{-/-}$ memory CD8^{+} T cells exhibited predominantly a Tem phenotype

Effector CD8^{+} T cells are generally IL-7Rα^{lo} and KLRG1^{hi}, but IL-7Rα upregulation and KLRG1 downregulation are associated with differentiation of effectors to long-lived protective memory CD8^{+} T cells (Joshi et al., 2007). Tcm and Tem memory T cells have distinct expression of homing receptors including CD62L and CCR7 (Sallusto et al., 1999). Analysis of *Tcf7*$^{-/-}$ memory CD8^{+} T cells at 75-85 days after infection revealed that substantially fewer cells expressed CD62L and CCR7 than WT controls and that most of them retained high expression of KLRG1 (Figure 2D). In contrast, IL-7Rα was upregulated similarly in both WT and *Tcf7*$^{-/-}$ memory T cells (Figure 2D). Upon peptide stimulation *in vitro*, WT and *Tcf7*$^{-/-}$ memory T cells were both capable of producing IFN-γ and TNF-α, and increased portion of *Tcf7*$^{-/-}$ CD8^{+} memory expressed granzyme B (Figure 2E). Importantly, IL-2 production, another characteristic feature of Tcm cells (Wherry et al., 2003), was impaired in *Tcf7*$^{-/-}$ memory T cells (Figure 2E). Thus, *Tcf7*$^{-/-}$ memory T cells predominantly manifested an effector memory phenotype even at >75 days after infection, indicating an essential role of TCF-1 in differentiation of Tcm cells. The intrinsic requirements of TCF-1 for memory CD8^{+} differentiation were further substantiated using a viral infection model, where *Tcf7*$^{-/-}$ memory OT-I T cells generated in response to infection with vaccinia virus expressing Ova (VacV-Ova) exhibited similarly predominant Tem phenotypes (Figure S2A and S2B).

Tcf7$^{-/-}$ mice are moderately lymphopenic, and *Tcf7*$^{-/-}$ T cells show increased portion of CD44^{hi} subset, probably resulting from increased homestatic proliferation (Schilham et al., 1998). *Tcf7*$^{-/-}$ OT-I T cells retained this phenotype and were positive for CD62L, IL-7Rα and CCR7 but negative for KLRG1 (Figure S2C). Whereas CD44^{hi} CD8^{+} T cells produced IFN-γ after brief TCR stimulation (Cho et al., 2000; Goldrath et al., 2000), the TCR-stimulated IFN-γproduction was negligible in both WT and *Tcf7*$^{-/-}$ CD44^{lo} CD8^{+} T cells (Figure S2D). Thus, *Tcf7*$^{-/-}$ CD44^{lo} T cells manifested phenotypic and functional features that resembled naïve T cells. To exclude the possibility that the increased CD44^{hi} subset in *Tcf7*$^{-/-}$ T cells may account for the defects observed above, we sorted CD62L^{+}CD44^{lo} naïve OT-I cells from WT and *Tcf7*$^{-/-}$ spleens and repeated the adoptive transfer and LM-Ova infection experiments. *Tcf7*$^{-/-}$ OT-I effectors in PBLs derived from the naïve precursors were also limited in proliferation (Figure S2E). Further, the *Tcf7*$^{-/-}$ memory CD8^{+} T cells derived from naïve precursors were also predominantly Tem cells, showing reduced CD62L expression and IL-2 production (Figures S2F and S2G). These findings further corroborated an intrinsic requirement of TCF-1 for Tcm cell differentiation, in spite of previous exposure to a lymphopenic environment.

Progressive loss of *Tcf7*$^{-/-}$ CD8^{+} memory T cells due to diminished responsiveness to IL-15

In addition to defective secondary expansion and Tcm differentiation, we noted that *Tcf7*$^{-/-}$ memory CD8^{+} T cells in the spleens underwent progressive loss over time, in contrast to relatively stably maintained WT CD8^{+} memory (Figure 3A). The decrease was not due to skewed tissue distribution, because *Tcf7*$^{-/-}$ memory CD8^{+} T cells were diminished similarly in bone marrow cells and did not accumulate in livers or lungs (Figure 3B). In fact, the decrease in *Tcf7*$^{-/-}$ CD8^{+} memory was most evident in lymph nodes (Figure 3B), consistent with its reduced CD62L and CCR7 expression and hence homing defects to secondary lymphoid tissues. IL-7 and IL-15 are known to be critical for maintaining memory CD8^{+} T cells (Schluns and Lefrancois, 2003). Although no apparent differences in IL-7Rα and IL-15Rα were observed, IL-2Rβ, a shared receptor subunit between IL-2 and IL-15, was decreased in expression in *Tcf7*$^{-/-}$ memory T cells (Figure 3C). Consistent with the reported roles of IL-15 in homeostatic proliferation and promoting survival of memory T cells (Berard et al., 2003; Prlic et al., 2002), *Tcf7*$^{-/-}$ memory T cells showed diminished BrdU uptake (Figure 3D) and Bcl-2 expression (Figure 3E), and exhibited substantially reduced

IL-15-driven proliferation compared with WT cells (Figure 3F). Importantly, reduced expression of IL-2Rβ and Bcl-2 was observed in *Tcf7*$^{-/-}$ memory OT-I cells that were generated in response to VacV-Ova infection (Figure S3A) and from naïve precursors (Figure S3C), supporting that TCF-1 is intrinsically required for sustaining IL-15 responsiveness in CD8^{+} memory.

Wnt-TCF-1 pathway was necessary and sufficient to induce eomesodermin expression

To gain mechanistic insights into the role of TCF-1 in maintaining memory CD8^{+} T cells, we performed transcriptomic analysis on sorted WT and *Tcf7*$^{-/-}$ memory OT-I cells. By the setting of $p < 0.05$ for significantly differential expression, 526 genes were upregulated and 776 genes downregulated for >1.5 fold due to TCF-1 deficiency. Functional annotation using DAVID (the Database for Annotation, Visualization, and Integrated Discovery) bioinformatics resources (Huang da et al., 2009) revealed that TCF-1 controls a wide spectrum of biological functions in memory T cells, including transcriptional regulation, cell cycle progression, and apoptosis, in addition to CD8^{+} effector molecules, cytokines, chemokines, and their receptors (Supplemental Table I). In line with our functional and phenotypic analyses, granzymes A and B, and KLRG1 were among the top 20 upregulated genes, and perforin transcript was increased by 1.45-fold in *Tcf7*$^{-/-}$ memory T cells ($p = 0.024$, Figure 4A). Additionally, *Tcf7*$^{-/-}$ memory T cells had decreased transcripts of CD62L (encoded by *Sell*), CCR7, and IL-2Rβ (Figure 4A, *Il2rb* transcript reduced by 30%, $p = 0.038$). Eomesodermin (Eomes) and c-Myc were among the most downregulated genes in *Tcf7*$^{-/-}$ memory CD8^{+} T cells (Figure 4A). Compared with another T-box factor T-bet, Eomes has been shown to directly bind to 5'-regulatory regions in the *Il2rb* gene, more effectively enhance IL-2Rβ expression, and confer IL-15 responsiveness to memory CD8^{+} T cells (Intlekofer et al., 2007). By intranuclear staining, we found that the protein expression of Eomes but not T-bet was substantially diminished in *Tcf7*$^{-/-}$ memory CD8^{+} T cells (Figure 4B). Similarly diminished Eomes expression was observed in *Tcf7*$^{-/-}$ memory OT-I T cells generated in response to VacV-Ova infection (Figure S3B) or from naïve precursors (Figure S3D).

To determine if Eomes expression is responsive to activation of Wnt signaling, we used 6-bromo-substituted indirubin-acetoxime (BIO-acetoxime), a specific inhibitor of GSK-3β, to block β-catenin phosphorylation and degradation (Meijer et al., 2004). BIO-acetoxime treatment stabilized β-catenin in naïve CD8^{+} T cells as expected and induced the expression of Eomes but not T-bet (encoded by *Tbx21*) (Figure S4A and S4B). c-Myc acts at least partly downstream of IL-15 in regulating homeostasis of memory CD8^{+} T cells (Bianchi et al., 2006). Although reported to be a Wnt target gene in human colorectal cancers (He et al., 1998), c-Myc did not respond to Wnt activation in mature T cells, suggesting that Wnt responsive genes could be highly cell context-dependent (Figure S4B). Treatment of sorted memory OT-I cells with BIO-acetoxime also induced Eomes expression but had little effects on T-bet and c-Myc (Figure 5A). In contrast, treatment with N-methylated BIO (MeBIO), an inactive analog of BIO-acetoxime, neither stabilized β-catenin nor apparently altered Eomes and other genes' expression (Figures S4 and 5A).

We next investigated if naïve and memory CD8^{+} T cells are responsive to bona fide Wnt ligands. Consistent with previous observations that both resting and activated human T cells expresses multiple Fzd receptors (Wu et al., 2007), our microarray analysis showed that WT memory CD8^{+} T cells expressed high amounts of Fzd5 and Fzd9, Lrp5 and Lrp6 co-receptors, and Dvl molecules connecting Fzd-Lrp receptor and the β-catenin destruction complexes (Figure S4C), most of which were not apparently affected by TCF-1 deficiency. Thus, memory CD8^{+} T cells are equipped with Wnt-responsive receptors. Axin2 has been suggested to be a universal Wnt-induced target gene as a negative regulator of the Wnt pathway (Jho et al., 2002; Lustig et al., 2002). We stimulated naïve CD8^{+} T cells with

Wnt3a, which has been shown to promote proliferation of hematopoietic stem cells via accumulation of β-catenin (Willert et al., 2003). We confirmed induction of Axin2 by Wnt3a and found that Wnt3a induced Eomes but not T-bet or c-Myc expression in naïve $CD8^+$ T cells (Figure 5B). Further, the induction of Axin2 and Eomes by Wnt3a was diminished in *Tcf7*$^{-/-}$ $CD8^+$ T cells, indicating its TCF-1 dependency (Figure 5B). The remaining induction of Eomes in *Tcf7*$^{-/-}$ $CD8^+$ cells may be ascribed to the presence of another Wnt effector transcription factor, lymphoid enhancer-binding factor 1 (LEF-1).

Eomes expression was reported to increase in effector T cells (Intlekofer et al., 2007). We found that TCR stimulation of naïve T cells did not detectably alter Eomes expression, at least during the observation period when the induction by Wnt3a was apparent (Figure 5C), suggesting that Eomes can be induced rapidly by Wnt ligand stimulation in the absence of TCR-derived signals. Interestingly, Wnt3a-induced Eomes upregulation was diminished when naïve T cells were stimulated by the combination of Wnt and TCR (Figure 5C). This is likely due to downregulation of TCF-1 in naïve T cells by TCR stimulation, regardless if Wnt3a is present (Figure 5C).

We further validated that Eomes can be induced by Wnt3a in memory $CD8^+$ T cells (Figure 5D), albeit the induction was somehow smaller compared with that seen in naïve T cells. This is likely explained by the fact that memory $CD8^+$ T cells express higher amounts of Eomes (Intlekofer et al., 2007) but lower amounts of TCF-1 and LEF-1 than naïve T cells (Willinger et al., 2006; Zhao et al. 2010), or alternatively Wnt3a may not be the most potent Wnt ligand acting on memory T cells. In key contrast to naïve T cells, TCR stimulation of memory $CD8^+$ T cells did not apparently downregulate TCF-1 or diminish Wnt-mediated induction of Eomes (Figure 5D). These findings suggest distinct cross-talk between Wnt and TCR signaling pathways in naïve and memory T cells, which merits future investigations.

We recently demonstrated that constitutive activation of Wnt signaling pathway, via transgenic expression of p45 TCF-1 and stabilized β-catenin, favors generation of $CD8^+$ memory T cells (Zhao et al. 2010). In memory T cells generated in the double transgenic mice (dTg), protein expression of Eomes was elevated and T-bet expression was slightly decreased, if any (Figure 5E). These *in vivo* data further support the notion that in memory $CD8^+$ T cells, the Wnt-TCF-1 pathway is necessary and sufficient for inducing the optimal expression of Eomes transcription factor, which in turn positively regulates IL-2Rβ expression and IL-15 responsiveness.

TCF-1 bound to regulatory sequences in the *Eomes* gene *in vivo*

To investigate if TCF-1 is directly associated with the *Eomes* gene, we surveyed a 6 kb non-coding DNA sequence flanking the *Eomes* transcription initiation site (- 5 kb to + 1 kb) for the core consensus TCF-1 binding motif “CTTTG” (van de Wetering and Clevers, 1992). Among a total of 20 TCF-1 motifs found in the 5’-regulatory region, 6 were conserved among different species (Figure 6A and S5). Eight TCF-1 motifs (termed T1 to T8) within –1.5 kb to +2.6 kb of the *Aixn2* gene were previously defined to contribute to its Wnt responsiveness (Jho et al., 2002). Because Axin2 was induced by Wnt3a stimulation in mature $CD8^+$ T cells, we used 2 clusters of TCF-1 motifs (T2/3 and T7/8) in the *Axin2* gene (corresponding to its promoter and an intron region, respectively) as potential positive controls. Rag2 is silenced in mature T cells and T-bet does not respond to Wnt, we therefore used the *Rag2* promoter (-0.6 ~ +0.7 kb) and a *Tbx21* 5’-regulatory region (-2.6 ~ -1.2 kb) containing no conserved TCF-1 motifs as negative controls. Because Eomes is inducible by activated Wnt signaling in both naïve and memory T cells as shown above, we used naïve $CD8^+$ T cells to obtain sufficient cells for chromatin immunoprecipitation (ChIP). As shown in Figure 6B, enriched TCF-1 binding was found in the 2 *Axin2* regulatory clusters, 6 conserved 5’ consensus “CTTTG” motifs in *Eomes* (with first 3 in “cluster a” and the other

3 as "elements b-d"), but not in the *Tbx21* gene or -10 kb and +3 kb regions in the *Eomes* gene, although both locations in the *Eomes* allele harbored conserved TCF-1 motifs. Importantly, all the enriched TCF-1 bindings were abrogated when *Tcf7*$^{-/-}$ CD8^{+} T cells were used (Figure 6B). Additionally, ChIP with another irrelevant antibody against transcription factor GA binding protein did not enrich TCF-1-associated sequences, consistent with ChIP-seq data showing no binding of GA binding protein in *Eomes* or *Axin2* gene loci (data not shown). These results demonstrate direct and specific binding of TCF-1 with multiple cis-regulatory sequences in the *Eomes* and *Axin2* loci.

To further substantiate direct regulation of Eomes by the Wnt-TCF-1 pathway, we performed ChIP using an antibody recognizing all 4 isoforms of TLE-GRG, the corepressor proteins interacting with TCF-1 in the absence of active Wnt signaling. We also performed ChIP with a β-catenin antibody on transgenic CD8^{+} T cells expressing stabilized β-catenin. All the TCF-1-bound motifs were enriched by either TLE-GRG or β-catenin antibody (Figure 6C and 6D), indicating the co-occupancy of TLE-GRG or β-catenin with TCF-1 on regulatory sequences in the *Eomes* gene. Taken together, these findings identified *Eomes* as a direct Wnt target gene in mature CD8^{+} T cells.

Forced expression of Eomes protected time-dependent loss of *Tcf7*$^{-/-}$ memory T cells

To demonstrate that Eomes is functionally important in mediating regulation of memory T cell maintenance by TCF-1, we introduced the Eomes cDNA into primed WT or *Tcf7*$^{-/-}$ OT-I T cells using MigR1 bicistronic retroviral vector with GFP as an expression indicator. The retrovirally transduced OT-I T cells were then adoptively transferred into naïve mice, followed by LM-Ova infection (Figure S6A) (Joshi et al., 2007). We hypothesized that if Eomes is a key mediator of TCF-1-dependent persistence of memory CD8^{+} T cells, forced expression of Eomes should confer protection to *Tcf7*$^{-/-}$ memory OT-I T cells. Throughout effector to early and late memory phases, when infected with MigR1 control retrovirus, the percentages of GFP-positive WT or *Tcf7*$^{-/-}$ OT-I T cells remained relatively stable, indicating that retroviral infection itself or GFP expression did not detectably alter the behavior of effector and memory CD8^{+} T cells (Figure 7A and 7B). The GFP^{+} subset was also stably sustained in WT OT-I cells infected with MigR1-Eomes retrovirus. In key contrast, the portion of GFP^{+} *Tcf7*$^{-/-}$ OT-I cells that had forced expression of Eomes was elevated in early memory T cells compared with effector phase and continued to increase in late memory stage (Figure 7A and 7B), indicating that Eomes-expressing *Tcf7*$^{-/-}$ memory T cells gained a relative self-renewal and/or survival advantage whereas those without Eomes continues to disappear over time. We confirmed increased expression of Eomes in GFP^{+} WT or *Tcf7*$^{-/-}$ memory OT-I T cells infected with MigR1-Eomes (Figure S6B). We further determined that forced expression of Eomes substantially increased IL-2Rβ expression in *Tcf7*$^{-/-}$ memory CD8^{+} T cells, albeit it did not have an evident effect on IL-2Rβ expression in WT memory CD8^{+} T cells (Figure 7C). Additional phenotypic and functional analyses showed that increased Eomes expression in *Tcf7*$^{-/-}$ T cells did not improve generation of IL-2-producing CD62L^{+} Tcm cells (Figure S6C). Nonetheless, forced expression of Eomes can at least partly restore IL-2Rβ expression in TCF-1-deficient memory T cells and protect them from time-dependent attrition.

DISCUSSION

TCF-1 is known to be important for normal thymocyte development, and loss of TCF-1 partly blocked T cell development at several early stages (Verbeek et al., 1995). Whereas deficiency in LEF-1, another Wnt effector transcription factor, did not cause detectable T cell defects, a combination of hypomorphic TCF-1 alleles and LEF-1 null alleles completely arrested T cell development at the immature single positive stage due to failure of TCRα rearrangements (Okamura et al., 1998). These observations indicated a partially overlapping

function of TCF-1 and LEF-1 in developing T cells. Our current studies revealed that TCF-1 is a key player in regulating mature T cell responses at several different phases and that TCF-1 may have both overlapping and non-redundant roles with LEF-1 during these processes. TCF-1 deficiency limited primary expansion of effector T cells, but this effect was relatively moderate. In addition, TCF-1 appeared to be dispensable for cytolytic activity of memory $CD8^+$ T cells. During revision of the current manuscript, Jeannet et al (2010) reported that the proliferation of *Tcf7*$^{-/-}$ $CD8^+$ effectors and cytolytic activities of *Tcf7*$^{-/-}$ memory $CD8^+$ T cells were relatively unaffected using a lymphocytic choriomeningitis (LCMV) infection model. These are likely explained by compensatory effect by LEF-1. In contrast, the requirements of TCF-1 in Tcm cell differentiation and longevity are non-redundant with LEF-1, although TCF-1 and LEF-1 are both expressed in memory T cells (Zhao et al. 2010). Additionally, the secondary expansion of *Tcf7*$^{-/-}$ memory $CD8^+$ T cells was more severely impaired in both LM and LCMV infection models (Jeannet et al. 2010), indicating a unique and strong dependence on TCF-1 in memory $CD8^+$ T cells. In line with this, a distinct function of TCF-1 has also been recently described in differentiation of $CD4^+$ T cells to a Th2 cell fate (Yu et al., 2009).

A transcriptional regulatory network has been elucidated that determines the fate of activated B cells to antibody-secreting plasma cells and long-lived memory B cells. In the network, B lymphocyte-induced maturation protein-1 (Blimp-1) and Bcl-6 are two key transcription factors that mutually repress each other (Martins and Calame, 2008). A similar transcription network regulating $CD8^+$ effectors to central memory fate is beginning to emerge from several recent studies. Inactivation of T-bet, Blimp-1, and Id2 and forced expression of Bcl-6 increased formation of Tcm cells (Cannarile et al., 2006; Ichii et al., 2004; Intlekofer et al., 2007; Rutishauser et al., 2009). Gene expression analysis showed that T-bet difficiency is associated with increased expression of Eomes and that loss of Blimp-1 resulted in downregulation of T-bet and Id-2 and upregulation of Eomes and Bcl-6 (Intlekofer et al., 2007; Kallies et al., 2009; Rutishauser et al., 2009). Our current study revealed that ablation of TCF-1 impaired Tcm formation. Coupled with our previous finding that forced expression of TCF-1 and stabilized β-catenin gave rise to increased numbers of IL-2-producing memory $CD8^+$ T cells (Zhao et al., 2010), TCF-1 has a positive regulatory role in Tcm cell differentiation. In line with this notion, TCF-1 expression was upregulated approximately 10-fold in Blimp-1-deficient $CD8^+$ effector cells (Rutishauser et al., 2009). Collectively, along with other transcription factors, TCF-1 contributes to an intricate yet interactive gene regulatory network to collaboratively program Tcm cell formation.

Wnt responsive genes were previously reported in double negative thymocytes, including transcription factors c-Jun and c-Fos (Staal et al., 2004), and direct binding of TCF-1 to regulatory elements of these genes *in vivo* has not been demonstrated in thymocytes. In mature T cells, information on Wnt targets is scarce. Recently, it has been shown using ChIP that TCF-1 can bind to the proximal promoter of Gata3 in CD4 T cells to promote a Th2 cell fate (Yu et al., 2009), and to the promoters of matrix metalloproteinases 2 and 9 genes in human T cells to facilitate T cell transmigration through epithelial cells (Wu et al., 2007). All the aforementioned Wnt-responsive or TCF-1-β-catenin-bound genes were not markedly altered in expression between WT and TCF-1-deficient memory $CD8^+$ T cells. In addition, c-Myc, another known Wnt target in human colorectal cancers (He et al., 1998), was decreased in *Tcf7*$^{-/-}$ memory T cells but its expression did not respond to stimulation by Wnt3a or β-catenin stabilization. These findings highlight that TCF-1-regulated genes are highly dependent on cell types and their developmental stages for T cells in particular. Axin2 has been characterized to be a Wnt target gene in many other tissue and cell types (Jho et al., 2002; Lustig et al., 2002). In the current study of mature $CD8^+$ T cells, we found that Axin2 was induced by Wnt3a stimulation in a TCF-1 dependent manner and further showed direct association of TCF1 with the *Axin2* allele *in vivo*. Using the *Axin2* gene as a

positive control, we demonstrated direct and specific binding of TCF-1 to 6 conserved consensus sequences in the *Eomes* 5'-regulatory region. The relevance of these TCF-1 binding sites in Wnt signaling was supported by the co-occupancy by TLE-GRG corepressor proteins in the absence of Wnt stimulation and by β-catenin upon stabilization. Along with TCF-1-dependent induction of Eomes by Wnt, these findings collectively identified Eomes as a direct target of the Wnt-TCF-1 pathway in mature $CD8^+$ T cells.

It is well known that TCF-1 activity is regulated by Wnt ligands which stabilize the coactivator β-catenin and lead to displacement of TLE-GRG corepressors (Staal et al., 2008). Recent studies suggest that the expression of TCF-1 in T cells can be modulated by signals derived from TCR and cytokines. *In vivo* activation of T cells downregulated TCF-1 in both mice and humans (Willinger et al., 2006; Zhao et al., 2010). This is also true for *in vitro* TCR-stimulated T cells, however, when combined with pharmacologically stabilized β-catenin, TCF-1 downregulation was blocked (Gattinoni et al., 2009). In addition, TCF-1 expression can be influenced by cytokines when primed *in vitro*, being induced by IL-21 but sustained at a low level by IL-2 and IL-15 (Hinrichs et al., 2008). Furthermore, β-catenin stabilization by TCR stimulation was described in thymocytes and CD4 T cells (Xu et al., 2003). Collectively, it is reasonable to assume that TCF-1-β-catenin pathway can integrate signals from Wnt ligands, TCR engagement, and various cytokines during T cell responses, especially during effector expansion and transition to memory T cells. Detailed dissection of the contribution of individual pathways to regulating TCF-1 activity and expression awaits future investigations. Nevertheless, data from the current study suggest a direct Wnt involvement in the persistence of memory $CD8^+$ T cells. We demonstrated that activation of the Wnt pathway by either GSK3β inhibition or Wnt3a stimulation, in the absence of signals from TCR or cytokines, induced the expression of Eomes in both naïve and memory $CD8^+$ T cells. *In vivo*, forced expression of TCF-1 and stabilized β-catenin expanded the $CD8^+$ memory T cell pool (Zhao et al., 2010). By the time when memory T cells are generated in response to acute viral or bacterial infection, inflammatory cytokines produced during early immune responses have subsided, and it has been demonstrated that persistence of memory $CD8^+$ T cells does not depend on signaling from MHC class I ligands but rather on IL-15 (Jabbari and Harty, 2005; Murali-Krishna et al., 1999; Schluns and Lefrancois, 2003; Williams and Bevan, 2007). In the absence of TCR stimulation, the endogenous IL-15 signal alone may not affect TCF-1 expression in memory $CD8^+$ T cells *in vivo*, and this notion is supported by our recent observation that memory T cells express higher amounts of TCF-1 than effectors (Zhao et al., 2010). Thus, TCF-1-β-catenin-mediated regulation of Eomes expression in memory $CD8^+$ T cells is more likely ascribed to Wnt, rather than TCR or cytokine-derived signals. Whereas the identity and source of Wnt ligands that contribute to the longevity of $CD8^+$ memory remain to be elucidated, it has been shown that macrophages and *in vitro* differentiated dendritic cells can produce Wnt7b and Wnt5a, respectively (Lehtonen et al., 2007; Lobov et al., 2005) and that multiple Wnt transcripts including Wnt 1, 2B, 4, 5A, and 8B were detected in vascular endothelial cells (Wu et al., 2007). Our findings, along with other studies, raised an intriguing possibility that Wnt morphogenic proteins may act upstream of the known Eomes-IL-2Rβ axis to confer cytokine responsiveness to memory $CD8^+$ T cells. The potential roles of Wnt agonists in regulating immunological memory may be explored to improve vaccination regimens and enhance tumor immunotherapy.

EXPERIMENTAL PROCEDURES

Animals and Infectious Agents

TCF-1-null mice, p45 TCF-1 isoform transgenic mice, and stabilized β-catenin transgenic mice were as described (Ioannidis et al., 2001; Verbeek et al., 1995; Xie et al., 2005). $Thy1.1^+$ or $Thy1.2^+$ OT-I TCR transgenic mice, and B6.SJL mice were from Jackson

Laboratories. Attenuated or virulent LM-Ova, VacV-Ova, and their usage were as described (Zhao et al., 2010). All the animals were housed and handled following protocols approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Cell Isolations and Flow cytometry

Isolation and adoptive transfer of WT or *Tcf7*$^{-/-}$ OT-I cells, infection of B6.SJL recipients were described previously (Badovinac and Harty, 2007). Isolation of lymphocytes from the livers and lungs was carried out using an established protocol as described (Masopust et al., 2001). Identification and characterization of antigen-specific T cells were performed using cell surface, intracellular, or intranuclear staining following standard protocols (Intlekofer et al., 2007; Zhao et al., 2010). Detection of BrdU uptake and activated Caspsase-3/7 was as described (Zhao et al., 2010). Detailed methods are in Supplemental Experimental Procedures.

Chromatin immunoprecipitation

Splenic CD8^{+} T cells were isolated from WT, *Tcf7*$^{-/-}$, or β-catenin transgenic mice, cross-linked, and sonicated to generate chromatin fragments as previous described (Yu et al., 2010). The chromatin was immunoprecipitated with antibodies against TCF-1 (C63D9) and TLE1/2/3/4 (both from Cell Signaling Technology), β-catenin (clone 14, BD Biosciences), or respective normal rabbit or mouse IgG. Each DNA segment of interest was quantitatively determined by real-time PCR (Yu et al., 2010), and abundance of the segment by an antibody was first normalized to that by control IgG, followed by normalization to the *Rag2* promoter region to calculate the relative enrichment for the segment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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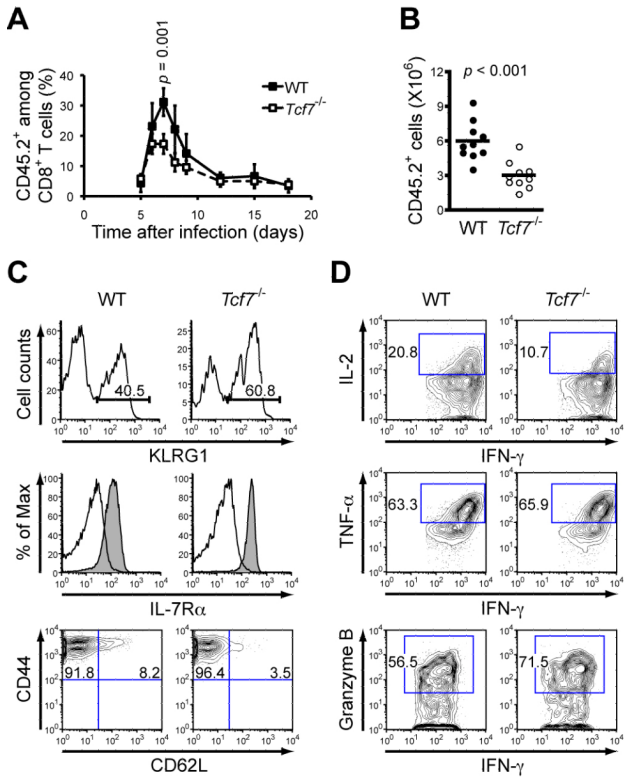

Figure 1. TCF-1 deficiency limits expansion of antigen-specific effector CD8$^+$ T cells

(A) Five hundreds of WT or *Tcf7*$^{-/-}$ OT-I T cells (CD45.2$^+$) were injected into CD45.1$^+$ B6.SJL recipients, followed by *i.v.* infection with 5 × 10^6 CFU *actA*$^-$LM-Ova. Kinetics of early responses of WT or *Tcf7*$^{-/-}$ OT-I cells were tracked in the PBLs, and their percentages in CD8$^+$ T cells are shown. Data are representative of 3 independent experiments with similar results (n ≥ 3 for each time point). All p-values, including those in following figures, were determined using Student's *t*-test.

(B) Numbers of effector OT-I T cells in the spleen on day 7 after infection. Data are pooled from 4 independent experiments.

(C) Cell surface phenotypes of effector OT-I T cells. Day 7 effector T cells were analyzed for KLRG1, IL-7Rα, CD62L, and CD44 expression. Percentages for KLRG1$^+$, CD44$^+$CD62L^{lo}, and CD44$^+$CD62L^{hi} populations are shown. Shaded histogram in IL-7Rα denotes its expression in naïve CD8$^+$ T cells for a direct comparison with the effector T cells.

(D) Production of effector molecules by effector OT-I T cells. Splenocytes were isolated on day 7 after infection and incubated with Ova peptide for 6 hrs *in vitro*. The cells were sequentially surface-stained, fixed and permeabilized, and intracellularly stained for IL-2, TNF-α, and granzyme B. Gating of positive populations was based on respective isotype controls. For (C) and (D), data are representative from at least 3 independent experiments with similar results.

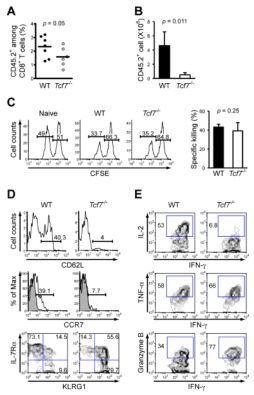

Figure 2. *Tcf7*$^{-/-}$ memory CD8^{+} T cells are impaired in secondary expansion and in differentiation to a Tcm phenotype

(A) Frequency of early memory OT-I cells in PBLs. B6.SJL recipients of either WT or *Tcf7*$^{-/-}$ OT-I cells were infected with *actA*$^{-}$LM-Ova, and the percentage of CD45.2^{+} OT-I in CD8^{+} T cells was determined on days 34-44 after infection. Data are pooled results from 2 independent experiments.

(B) Secondary expansion of memory CD8^{+} T cells in the spleens. Sorted WT or *Tcf7*$^{-/-}$ memory OT-I T cells (6×10^{3}) were transferred into naïve B6.SJL hosts, followed by infection with 2×10^{5} CFU of virulent LM-Ova. The numbers of CD45.2^{+} OT-I cells in the spleens were determined 6 days later. Data are means ± s.d. (n = 3). Similar results were obtained when total splenocytes containing equivalent numbers of WT or *Tcf7*$^{-/-}$ memory OT-I cells were transferred without sorting separation (not shown).

(C) *In vivo* killing capacity of memory CD8^{+} T cells. CD45.2^{+} splenocytes were differentially labeled with CFSE. Ova peptide-pulsed CFSE^{lo} and non-peptide pulsed CFSE^{hi} cells were injected at 1:1 ratio into naïve or immune chimeras (35 days after infection). The spleens were harvested 4 hrs later, and percentages of CFSE^{lo} and CFSE^{hi} cells in CD45.2^{+} splenocytes were determined. Data are representative of 2 independent experiments with similar results.

(D) Surface staining for CD62L, CCR7, IL-7Rβ, and KLRG1 on antigen-specific memory CD8^{+} T cells. During days 75-85 after infection with *actA*$^{-}$LM-Ova, splenocytes from WT or *Tcf7*$^{-/-}$ OT-I recipients were stained. Percentages of CD62L^{hi} and CCR7^{+} subsets were shown in histograms, with shaded histogram denoting isotype control. Data are representative of 2-3 independent experiments (n ≥ 4).

(E) Intracellular detection of IFN-γ, IL-2, TNF-α, and granzyme B in memory CD8^{+} T cells. Splenocytes were stimulated with Ova peptide for 6 hrs, followed by surface and intracellular staining. Gating of positive populations was based on respective isotype controls. All data are representative of 3 independent experiments (n ≥ 5).

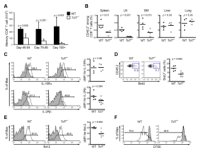

Figure 3. Long-term maintenance and IL-15 responsiveness of memory CD8$^+$ T cells depend on TCF-1

(A) Progressive loss of *Tcf7*$^{-/-}$ memory CD8$^+$ T cells. B6.SJL recipients of WT or *Tcf7*$^{-/-}$ OT-I cells were sacrificed on indicated days post-infection and memory OT-I T cells in the spleens were enumerated. Data are pooled from at least 3 independent experiments.

(B) Tissue distribution of memory CD8$^+$ T cells. During 75-85 days after infection, frequency of memory OT-I in CD8$^+$ T cells was determined in indicated tissues. Data are pooled from 2 independent experiments.

(C) Expression of IL-15Rα and IL-2Rβ on memory CD8$^+$ T cells. Memory OT-I cells were surface-stained, and percentages of positive cells were shown in representative histograms based on isotype staining (shaded). Cumulative results from 2-4 experiments were shown on the right panel as ΔMFI, the difference of MFI (mean fluorescent intensity) values of antibody- and isotype-stained entire cell populations without positivity gating. The same approach was used to present data in Figures 3E, 4B, 5E, and 7C.

(D) BrdU uptake in memory CD8$^+$ T cells. The B6.SJL recipients were *i.p.* injected with BrdU on day 70 after infection and fed with BrdU in drinking water for 1 week. The percentage of BrdU$^+$ population was shown in representative contour plots (on the left) or cumulative data from 3 independent experiments (on the right).

(E) Expression of Bcl-2 in memory CD8$^+$ T cells. Memory OT-I cells were intracellularly stained for Bcl-2. Data are either representative of or pooled from 3 independent experiments.

(F) Proliferation of memory CD8$^+$ T cells in response to IL-15. Memory OT-I cells were labeled with CFSE and cultured in the presence of IL-15 (50 ng/ml). The shaded histogram denotes the CFSE level in cells cultured without IL-15, and the percentage denotes actively dividing cells. Representative data after 4-day culture are shown for 2 independent experiments with similar results (n = 3).

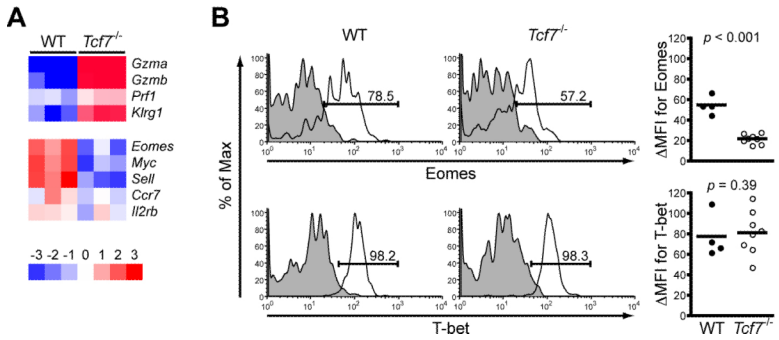

Figure 4. TCF-1 is necessary for optimal Eomes expression

(A) Transcriptomic analysis revealed expression changes in genes that are critical for regulating $CD8^+$ T cell activities. Day 70-80 memory $CD8^+$ T cells were sorted from 3 WT or 3 *Tcf7*$^{-/-}$ OT-I recipients and subjected to microarray analysis using Mouse GENE 1.0 ST arrays. Heatmaps of select genes and colour-coded scales are shown.

(B) Eomes but not T-bet was expressed at lower levels in *Tcf7*$^{-/-}$ memory $CD8^+$ T cells. Memory $CD8^+$ T cells (>70 days after infection) were intranuclearly stained for Eomes and T-bet. Shown on left are representative histograms with percentages of positive subsets, and on right are cumulative ΔMFI data from 3 independent experiments.

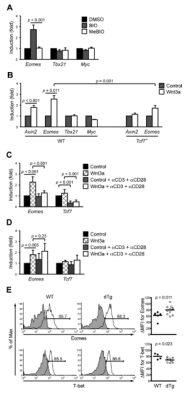

Figure 5. Activation of the canonical Wnt/TCF-1/β-catenin pathway is sufficient to induce Eomes expression

(A) Inhibition of GSK3β induces Eomes expression in memory CD8$^+$ T cells. Day 60 memory OT-I T cells were sorted from LM-Ova-infected B6.SJL recipients and treated with DMSO, BIO-acetoxime (BIO), or N-methylated BIO (MeBIO) for 12 hrs. The expression of select genes was quantitatively determined, and all normalized to the samples treated with DMSO.

(B) Induction of Axin2 and Eomes by Wnt3a in naïve CD8$^+$ T cells. Freshly isolated naïve CD8$^+$ T cells from WT or *Tcf7*$^{-/-}$ mice were exposed to Wnt3a conditioned or control medium for 3 hrs. Gene expression was quantitatively determined and then normalized to the control medium-treated samples.

(C) Effect of Wnt and TCR stimulation in naïve CD8$^+$ T cells. WT naïve CD8$^+$ T cells were stimulated with Wnt3a and/or plate-bound anti-CD3 (5 μg/ml) + soluble anti-CD28 (1 μg/ml) for 3 hrs, and gene expression was quantitatively determined and normalized as in (B).

(D) Effect of Wnt and TCR stimulation in memory CD8$^+$ T cells. Memory CD8$^+$ T cells were isolated as in (A) and stimulated as in (C), and gene expression was quantitatively determined. For (A)-(D), data are representative of at least 2 independent experiments with similar results (n ≥ 4).

(E) Increased Eomes expression in memory CD8$^+$ T cells in the presence of constitutively active Wnt signalling. Double transgenic (dTg) mice with forced expression of p45 TCF-1 and stabilized β-catenin and their WT littermates were infected with *actA*$^-$LM-Ova. During days 45-55 after infection, memory CD8$^+$ T cells were identified by intracellular detection of IFN-γ after 6-hr Ova peptide stimulation, and Eomes or T-bet expression was determined in IFN-γ$^+$ CD8$^+$ T cells. Data shown are representative histograms (on the left) and cumulative ΔMFI from 2-3 independent experiments (on the right).

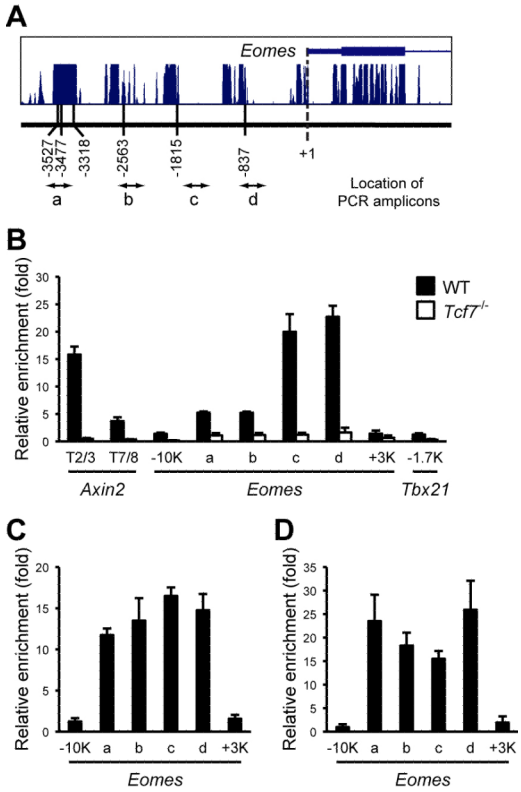

Figure 6. TCF-1 is directly associated with regulatory sequences in the *Eomes* gene

(A) Schematic showing locations of conserved consensus TCF-1 binding motifs in the 5'-regulatory region of the *Eomes* gene. Multiple species conservation from the UCSC genome browser is shown on the top, and locations of each conserved TCF-1 motif and corresponding PCR amplicon are marked below. The 3 TCF-1 motifs found in -3.5 kb were collectively defined as "cluster a", and the other 3 relatively scattered motifs were referred to as "element b to d". See Figure S5 for the sequence alignments among different species.

(B) TCF-1 binds to the Eomes regulatory sequences *in vivo*. Chromatin fragments from WT or *Tcf7*$^{-/-}$ splenic CD8^{+} T cells were immunoprecipitated with an anti-TCF-1 antibody. Enrichment of each segment was determined with quantitative PCR and normalized to the *Rag2* promoter region. Data are representative of 3 independent ChIP experiments with each sample measured in duplicates or triplicates.

(C) TLE-GRG and (D) β-catenin co-occupy Eomes regulatory sequences with TCF-1. Chromatin fragments from splenic CD8^{+} T cells of WT or β-catenin transgenic mice were immunoprecipitated with TLE-GRG or β-catenin antibody, respectively. Enrichment of each segment was quantitatively measured, and data are representative of 2 independent experiments with similar results.

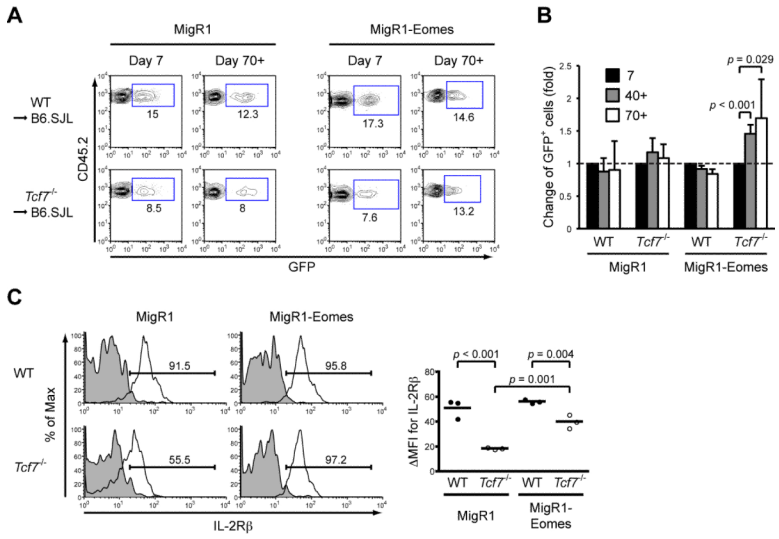

Figure 7. Forced expression of Eomes protects *Tcf7*$^{-/-}$ memory CD8^{+} T cells from attrition

(A) Representative flow profiles of retrovirally transduced effector (day 7) and memory (day 70+) CD8^{+} T cells. Percentages of GFP^{+} subsets, infected with either MigR1 control retrovirus or Eomes-expressing retrovirus, in CD8^{+}CD45.2^{+} cells are shown from 2 independent experiments (n ≥ 4).

(B) Fold changes of GFP^{+} cells during OT-I response. GFP^{+} percentage in each recipient mouse on day 7 after infection was arbitrarily set to 1, and that in each mouse during the memory phase was normalized to day 7 to calculate the fold changes. Data are means ± s.d. of at least 4 individual recipients for each group (pooled from 2 independent experiments).

(C) IL-2Rβ expression in retrovirally infected WT and *Tcf7*$^{-/-}$ memory CD8^{+} T cells. Splenocytes were isolated from recipients of retrovirally infected WT or *Tcf7*$^{-/-}$ OT-I T on day 40 after LM-Ova infection, and IL-2Rβ expression was determined on GFP^{+}CD45.2^{+}CD8^{+} T cells with representative histograms and cumulative ΔMFIs shown from 2 independent experiments.