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ARTICLE

ZEB1, ZEB2, and the miR-200 family form a counterregulatory network to regulate CD8⁺ T cell fates

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Long-term immunity depends partly on the establishment of memory CD8⁺ T cells. We identified a counterregulatory network between the homologous transcription factors ZEB1 and ZEB2 and the *miR-200* microRNA family, which modulates effector CD8⁺ T cell fates. Unexpectedly, *Zeb1* and *Zeb2* had reciprocal expression patterns and were functionally uncoupled in CD8⁺ T cells. ZEB2 promoted terminal differentiation, whereas ZEB1 was critical for memory T cell survival and function. Interestingly, the transforming growth factor β (TGF-β) and miR-200 family members, which counterregulate the coordinated expression of *Zeb1* and *Zeb2* during the epithelial-to-mesenchymal transition, inversely regulated *Zeb1* and *Zeb2* expression in CD8⁺ T cells. TGF-β induced and sustained *Zeb1* expression in maturing memory CD8⁺ T cells. Meanwhile, both TGF-β and *miR-200* family members selectively inhibited *Zeb2.* Additionally, the miR-200 family was necessary for optimal memory CD8⁺ T cell formation. These data outline a previously unknown genetic pathway in CD8⁺ T cells that controls effector and memory cell fate decisions.

Introduction

Our immune system has two primary goals upon infection: (1) to rapidly fight off and eliminate the current invading pathogen, and (2) to generate long-term immunity, protecting us from future infection. This phenomenon, called immunological memory, is the basis for vaccination—one of the greatest achievements of modern medicine (Pulendran and Ahmed, 2011). However, not all vaccines (or infections) effectively induce protective and long-lasting memory, which accounts in part for the existing failure of effective prophylactic vaccines against many types of infections.

CD8⁺ T cells are a vital arm of adaptive immunity because they directly locate and kill virus-infected cells, limiting viral dissemination. Achieving present and future protection is accomplished within the CD8⁺ T cell population by the simultaneous generation of shorter-lived effector and longer-lived memory CD8⁺ T cells. Understanding how these cell fate decisions are regulated within CD8⁺ T cells is an important endeavor for developing better forms of vaccination and immunotherapy. Here, we describe a genetic network, previously not known to function in immune cells, that plays a critical role in establishing both the effector response and future immunity through the coordinated activities of two transcription factors (TFs), zinc-finger E-box–binding homeobox 1 (ZEB1) and ZEB2, and the *miR-200* microRNA family.

Our understanding of the biological processes and molecular mechanisms regulating effector and memory CD8⁺ T cell development has extensively advanced over the past two decades. During many acute viral infections, naive CD8⁺ T cells expand into a heterogeneous population of effector cells, the majority of which become highly differentiated CTLs that we refer to as terminal effector (TE) cells and distinguish by high killer cell lectin-like receptor G1 (KLRG1) and fractalkine receptor (CX3CR1) and low IL-7 receptor α (IL-7R) expression (Kaech et al., 2003; Huster et al., 2004; Joshi et al., 2007; Gerlach et al., 2016). Most of these TE cells undergo apoptosis after viral clearance, but some persist long-term, mainly circulating in the blood (Joshi et al., 2007; Jameson and Masopust, 2009; Olson et al., 2013; Gerlach et al., 2016). A smaller fraction of effector cells, referred to as memory precursor (MP) cells, up-regulate IL-7R and seed multiple

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memory cell compartments, including central memory (T_{CM}), effector memory (T_{EM}), peripheral memory, and resident memory (T_{RM}) T cells (Joshi et al., 2007; Kaech and Cui, 2012; Gerlach et al., 2016; Mackay and Kallies, 2017). Several TFs have been identified that regulate the ability of CD8$^+$ T cells to adopt effector or memory CD8$^+$ T cell fates, with many operating in a dynamic and graded manner, generating an intricate layered system of transcriptional states reflecting the integration of environmental signals individual cells experience over the course of an infection (Chang et al., 2007; Kaech and Cui, 2012; Kakaradov et al., 2017; Yu et al., 2017). For example, runt-related TF3 (RUNX3), IFN regulatory factor 4 (IRF4), T-box TF21 (*Tbx21*, also known as T-BET), eomesodermin (EOMES), inhibitor of DNA binding 2 (ID2), and B lymphocyte–induced maturation protein 1 (BLIMP-1) promote the early stages of cytotoxic effector CD8$^+$ T cell differentiation (Intlekofer et al., 2005, 2008; Cannarile et al., 2006; Joshi et al., 2007; Cruz-Guilloty et al., 2009; Kallies et al., 2009; Kwon et al., 2009; Rutishauser et al., 2009; Banerjee et al., 2010; Yang et al., 2011; Knell et al., 2013; Xin et al., 2016). However, as the expression and/or activities of some of these pro–effector TFs is intensified and prolonged, they can promote the terminal differentiation of effector cells, namely through the transcriptional and epigenetic repression of pro–memory TFs, such as T cell factor 1 (TCF1; *Tcf7*), BTB domain and CNC homologue 2 (BACH2), Forkhead box protein O1 (FOXO1), B cell lymphoma 6 (BCL6), EOMES, and ID3, that govern circulating memory cell differentiation (Ichii et al., 2004; Intlekofer et al., 2005; Banerjee et al., 2010; Jeannet et al., 2010; Zhou et al., 2010; Ji et al., 2011; Yang et al., 2011; Kim et al., 2013; Tejera et al., 2013; Roychoudhuri et al., 2016; Gray et al., 2017; Kakaradov et al., 2017; Yu et al., 2017). Less is understood about the transcriptional networks governing T_{RM} formation, but in contrast to circulating T_{EM} and T_{CM} cells, T_{RM} cell development relies on up-regulation of BLIMP-1 and homologue of Blimp-1 in T cells (HOBIT) and down-regulation of Krüppel-like factor 2 (KLF2), T-BET, EOMES, and TCF1 (Mackay et al., 2016; Mackay and Kallies, 2017). There are still many open questions regarding how these TFs interact with one another to instruct or repress effector and memory fates, which has important implications on the generation of a diverse array of memory T cells. Furthermore, as memory T cells traffic to many different tissue compartments, what are the environmental signals and underlying genetic modules regulated by such signals that influence their development and maintenance?

Prior work investigating the graded action of T-BET in terminal differentiation of effector CD8$^+$ T cells identified ZEB2 as a translator of high T-BET expression that switched on TE cell differentiation (Dominguez et al., 2015; Omilusik et al., 2015). Both ZEB2 and its highly conserved homologue, ZEB1, are well-known activators of the epithelial-to-mesenchymal transition (EMT), a process centrally involved in embryogenesis and tumorigenesis, particularly metastasis, in which they operate in a well-described negative-feedback pathway with the *miR-200* microRNA family (Bracken et al., 2008; Brabletz and Brabletz, 2010; Brabletz et al., 2011; Gregory et al., 2011). Given our discovery of ZEB2 in CD8$^+$ T cell differentiation, we sought to investigate whether ZEB1 and the *miR-200* family also played a role in this process. Surprisingly, rather than cooperating with ZEB2, we found that ZEB1 and ZEB2 were expressed in a reciprocal manner at temporally distinct phases of the immune response. Although ZEB2 promoted TE cell differentiation and survival, ZEB1 was critical for normal maintenance of memory CD8$^+$ T cells and protective immunity. Our data also demonstrated that well-described regulators of the EMT, miR-200 family members and TGF-β differentially regulated *Zeb1* and *Zeb2* expression in CD8$^+$ T cells. In contrast to the EMT, where *miR-200* family members repress both *Zeb1* and *Zeb2* to maintain epithelial states, in CD8$^+$ T cells, *miR-200* members selectively inhibited *Zeb2*, but not *Zeb1*, expression. Likewise, TGF-β induced *Zeb1* yet repressed *Zeb2* expression in CD8$^+$ T cells. Ectopic expression of *miR-200* family members in CD8$^+$ T cells impaired TE and promoted memory cell development, whereas *miR-200* deficiency resulted in the loss of memory CD8$^+$ T cells. Altogether, our study revealed a novel gene regulatory network involving ZEB1, ZEB2, and the *miR-200* family that governs effector and memory CD8$^+$ T cells fate decisions during viral infection.

Results

Zeb1 and *Zeb2* are reciprocally expressed in CD8$^+$ T cells during lymphocytic choriomeningitis virus (LCMV) infection

To investigate the role of ZEB1 in CD8$^+$ T cell differentiation and determine whether it cooperated with ZEB2 in this process, we first examined *Zeb1* and *Zeb2* expression in CD8$^+$ T cells during the course of viral infection. WT C57BL/6 (B6) mice were infected with LCMV-Arm, which led to an acute viral infection. Naive (CD44^{lo} CD62L^{hi}) CD8$^+$ T cells (day 0) were sorted from uninfected mice and MHC class I D^{b}GP$_{33-41}$ tetramer$^+$ LCMV-specific effector and memory CD8$^+$ T cells were isolated from 8 and 80 d postinfection (dpi), respectively. *Zeb1* and *Zeb2* mRNA expression was determined by quantitative RT-PCR, which revealed their reciprocal pattern of expression in naive, effector, and memory CTLs. *Zeb1* mRNA was expressed in naive CD8$^+$ T cells, reduced approximately fourfold in effector CD8$^+$ T cells, and then up-regulated again in mature memory CD8$^+$ T cells. Conversely, *Zeb2* mRNA displayed the opposite expression pattern, as previously reported (Dominguez et al., 2015; Omilusik et al., 2015), and was below detection in naive CD8$^+$ T cells, increased substantially in effector cells (8 dpi), and then decreased in memory CD8$^+$ T cells (Fig. 1 a). Further subdividing of the LCMV-specific CD8$^+$ T cells into more terminally differentiated TE (KLRG1^{hi} IL-7R^{lo}) and less differentiated MP (KLRG1^{lo} IL-7R^{hi}) cell subsets at 8 dpi showed an inverse expression pattern, with *Zeb1* being more highly expressed in MP cells and *Zeb2* being more dominant in TE cells (Fig. 1 b). ZEB1 expression in TE and MP subsets was examined using Western blot on protein lysates from LCMV-specific CD8$^+$ T cells, and this confirmed MP cells contain more ZEB1 compared with the TE cells (Fig. 1 c). These data demonstrate that during an immune response, *Zeb1* and *Zeb2* are expressed in a reciprocal manner in CD8$^+$ T cells, suggesting that rather than functioning cooperatively, as observed during the EMT, ZEB1 and ZEB2 may function in distinct processes in CD8$^+$ T cells.

Given the reciprocal mRNA expression pattern of *Zeb1* and *Zeb2*, we next asked whether they could directly antagonize each other's expression in CD8$^+$ T cells. To explore this

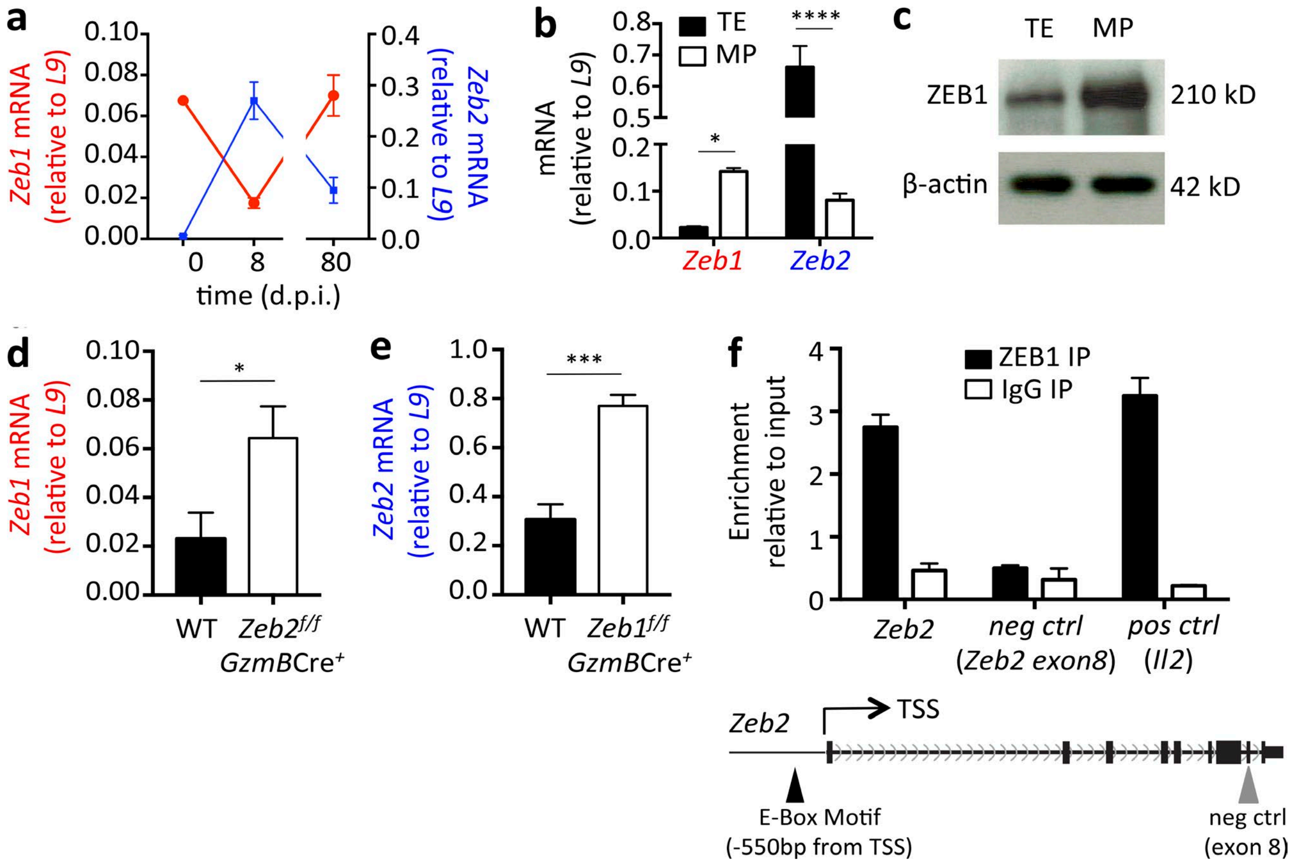


Figure 1. ***Zeb1* and *Zeb2* are reciprocally expressed in CD8⁺ T cells during LCMV infection. (a)** *Zeb1* and *Zeb2* mRNA was measured in purified $CD44^{lo}$ $CD62L^{hi}$ naive CD8⁺ T cells (day 0) or $D^bGP_{33-41}{}^+$ LCMV-specific CD8⁺ T cells from 8 and 80 dpi using quantitative RT-PCR. LCMV-specific CD8⁺ T cells were purified based on D^bGP_{33-41}-tetramer staining. **(b and c)** Effector P14⁺ CD8⁺ T cells were isolated 8 dpi based on the expression of $KLRG1^{hi}$ $IL\text{-}7R^{lo}$ (TE) and $KLRG1^{lo}$ $IL\text{-}7R^{hi}$ (MP) and *Zeb1* and *Zeb2* mRNA was measured using quantitative RT-PCR (b) or ZEB1 protein expression was measured using Western blotting (c; β-actin was used as a loading control). **(d)** *Zeb1* mRNA expression was compared between WT and *Zeb2*$^{f/f}$ *GzmB*Cre⁺ P14⁺ CD8⁺ T cells at 8 dpi. **(e)** *Zeb2* mRNA expression was compared between WT and *Zeb1*$^{f/f}$ *GzmB*Cre⁺ P14⁺ CD8⁺ T cells 30 dpi. **(f)** ZEB1 ChIP-quantitative PCR was performed on purified $CD44^{lo}$ $CD62L^{hi}$ naive CD8⁺ T cells using anti–ZEB1 (black bars) or IgG control (white bars) antibodies and primers to *Zeb2* promoter (−550 bp), exon 8 and *Il2* promoter. Data shown are representative of two (c) independent or cumulative of three (a, b, d, and e) independent experiments; *n* = 3–4 mice/group/experiment (c), *n* = 9–12 mice/group (a, b, d, and e). Data are expressed as mean ± SEM. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

possibility, *Zeb1* mRNA was quantified in *Zeb2*-deficient LCMV-specific effector CD8⁺ T cells 8 dpi (Fig. 1 d), and *Zeb2* mRNA was measured in *Zeb1*-deficient memory CD8⁺ T cells at 30 dpi (Fig. 1 e). Mice containing floxed alleles of *Zeb2* (*Zeb2*$^{flox/flox}$) have been previously described (Higashi et al., 2002; Dominguez et al., 2015; Omilusik et al., 2015). To generate *Zeb1* loss-of-function T cells, we created mice containing two loxP sites flanking *Zeb1* exon 6 using CRISPR/Cas9 technology, because exon 6 is the largest exon within *Zeb1* and encodes the central homeodomain and part of both N- and C-terminal zinc-finger clusters (see Fig. S1 a for more details on generation of *Zeb1*$^{flox/flox}$ mice). *Zeb1*$^{flox/flox}$ and *Zeb2*$^{flox/flox}$ mice were crossed to mice expressing the *Granzyme B-Cre* (*GzmB-Cre*) transgene to inducibly delete *Zeb1* or *Zeb2,* respectively, after CD8⁺ T cell activation during LCMV infection (we refer to these mice as *Zeb1*$^{f/f}$ *GzmB*Cre⁺ and *Zeb2*$^{f/f}$ *GzmB*Cre⁺ mice, and littermate controls lacking GzmB-Cre are referred to as WT). This analysis indicated that *Zeb1* and *Zeb2* mutually repress each other's expression, because *Zeb1* mRNA was significantly elevated in *Zeb2*$^{f/f}$ *GzmB*Cre⁺ effector CD8⁺ T cells and, likewise, *Zeb2* mRNA was increased in *Zeb1*$^{f/f}$ *GzmB*Cre⁺ memory CD8⁺ T cells (Fig. 1, d and e).

Given their reciprocal expression and the fact that ZEB1 and ZEB2 have both been described as transcriptional repressors that bind to paired E-boxes, we hypothesized that perhaps they might directly repress each other's expression. TF motif analysis indicated both *Zeb1* and *Zeb2* loci contain conserved E-box domains in their proximal promoter regions. Unfortunately, based on our experience, none of the commercial anti-mouse ZEB2 antibodies specifically recognize ZEB2, thereby preventing analysis of ZEB2 binding at the *Zeb1* locus. However, to determine whether the repression by ZEB1 on *Zeb2* transcription could be regulated via direct binding of ZEB1 to the *Zeb2* locus, chromatin immunoprecipitation (ChIP) using a ZEB1-specific antibody followed by quantitative RT-PCR of two regions of the *Zeb2* locus was performed. As a positive control, we also probed ZEB1 binding to a previously described target in the *Il2* locus (Williams et al., 1991; Wang et al., 2009). The results of this analysis showed clear enrichment of ZEB1 binding at the *Zeb2* promoter and the *Il2* locus, but not an irrelevant region in exon 8 of *Zeb2* (Fig. 1 f). This result demonstrated that ZEB1 can directly bind *Zeb2,* supporting the possibility ZEB1 may directly repress *Zeb2* to coordinate their inverse expression patterns.

ZEB1 promotes the homeostasis of memory CD8$^+$ T cells

To determine the role of ZEB1 in CD8$^+$ T cell differentiation during the course of viral infection and compare it with the role of ZEB2 previously described (Dominguez et al., 2015; Omilusik et al., 2015), *Zeb1$^{f/f}$ GzmB*Cre$^-$ (WT) and *Zeb1$^{f/f}$ GzmB*-Cre$^+$ mice were infected with LCMV-Arm, and splenic D^{b}GP$_{33-41}$ and D^{b}NP$_{396-404}$ tetramer$^+$ CD8$^+$ T cells were enumerated and characterized at 8, 15, and 60 dpi. *Zeb1*-deficient CD8$^+$ T cells underwent clonal expansion upon LCMV challenge and displayed frequencies of tetramer-specific CTLs at 8 dpi similar to controls, but there was an approximately twofold reduction in cell numbers (Fig. 2, a and b). The decline of *Zeb1*-deficient CD8$^+$ T cells became more apparent with time, demonstrating a profound defect in the establishment and/or maintenance of memory CD8$^+$ T cells (Fig. 2, a and b). In general, there were approximately six- to sevenfold fewer *Zeb1*-deficient memory CD8$^+$ T cells than WT cells, and this memory cell attrition was associated with a decreased ratio of BCL-2 to BIM within the *Zeb1$^{f/f}$ GzmB*Cre$^+$ memory CD8$^+$ T cells (Fig. 2 c). Accordingly, *Zeb1$^{f/f}$ GzmB*Cre$^+$ memory CD8$^+$ T cells contained a higher frequency of early- and late-stage apoptotic cells according to Annexin V and 7-AAD staining (Fig. 2 d). Homeostatic proliferation based on Ki67 staining revealed a modest but consistent decrease in the frequency of proliferating cells in *Zeb1*-deficient as compared with the WT memory CD8$^+$ T cells (Fig. 2 e). Altogether, these data demonstrate that ZEB1 plays an important role in maintaining survival and homeostasis of memory CD8$^+$ T cells, distinguishing it as a novel regulator of memory T cell development.

Surprisingly, despite the defect in long-term survival of *Zeb1$^{f/f}$ GzmB*Cre$^+$ memory cells and preferential expression of *Zeb1* mRNA in IL-7R^{hi} MP cells, there was no discernible defect in the development of IL-7R^{hi} effector and memory cells in *Zeb1$^{fl/fl}$ GzmBCre+* CD8$^+$ T cells 8 dpi (Figs. 1 b and 2 f). That is, similar frequencies of MP- or TE-like subsets formed in both groups of mice based on KLRG1 and IL-7R expression (Fig. 2 f). However, fewer numbers of these phenotypically distinct subsets formed in the absence of *Zeb1* (Fig. 2 a). These data suggest that unlike ZEB2, ZEB1 was dispensable for specialized aspects of CTL differentiation, but it played a more dominant role in the survival of all virus-specific CD8$^+$ T cells during the effector to memory transition.

Although *Zeb1*-deficient memory CD8$^+$ T cells produced similar amounts of IFNγ and TNFα compared with WT cells when restimulated with D^{b}GP$_{33-41}$ peptide, they produced less IL-2, indicating they were less polyfunctional (Fig. 2 g). Coinciding with the reduction in IL-2, a property of central memory CD8$^+$ T cells (Sallusto et al., 1999; Wherry et al., 2003), we observed a modest reduction in other T$_{CM}$ properties in memory CD8$^+$ T cells lacking ZEB1, including decreased L-selectin (CD62L), IL-15 receptor β (IL-15Rβ, CD122), and EOMES expression (Fig. 2, h–j). EOMES sustains memory CD8$^+$ T cell homeostatic turnover by maintaining CD122 expression (Intlekofer et al., 2005; Banerjee et al., 2010), and this may explain the aforementioned decreased proliferation of *Zeb1*-deficient memory CD8$^+$ T cells.

To more rigorously validate that these defects in memory CD8$^+$ T cell development were caused by CD8$^+$ T cell–autonomous functions of ZEB1, we cotransferred equal numbers of WT (Thy1.1/1.1) and *Zeb1$^{f/f}$ GzmB*Cre$^+$ (Thy1.1/1.2) P14$^+$ CD8$^+$ T cells into congenically mismatched (Thy1.2/1.2) B6 mice and assessed their response to LCMV infection. The P14$^+$ TCR transgene is specific for the GP$_{33-41}$ epitope in LCMV. Relative to the WT donor cells, similar phenotypes were observed in the P14$^+$ *Zeb1*-deficient memory CD8$^+$ T cells as described above, including reduced survival and homeostatic proliferation and impaired IL-2 production and formation of T$_{CM}$ cells (Fig. S2).

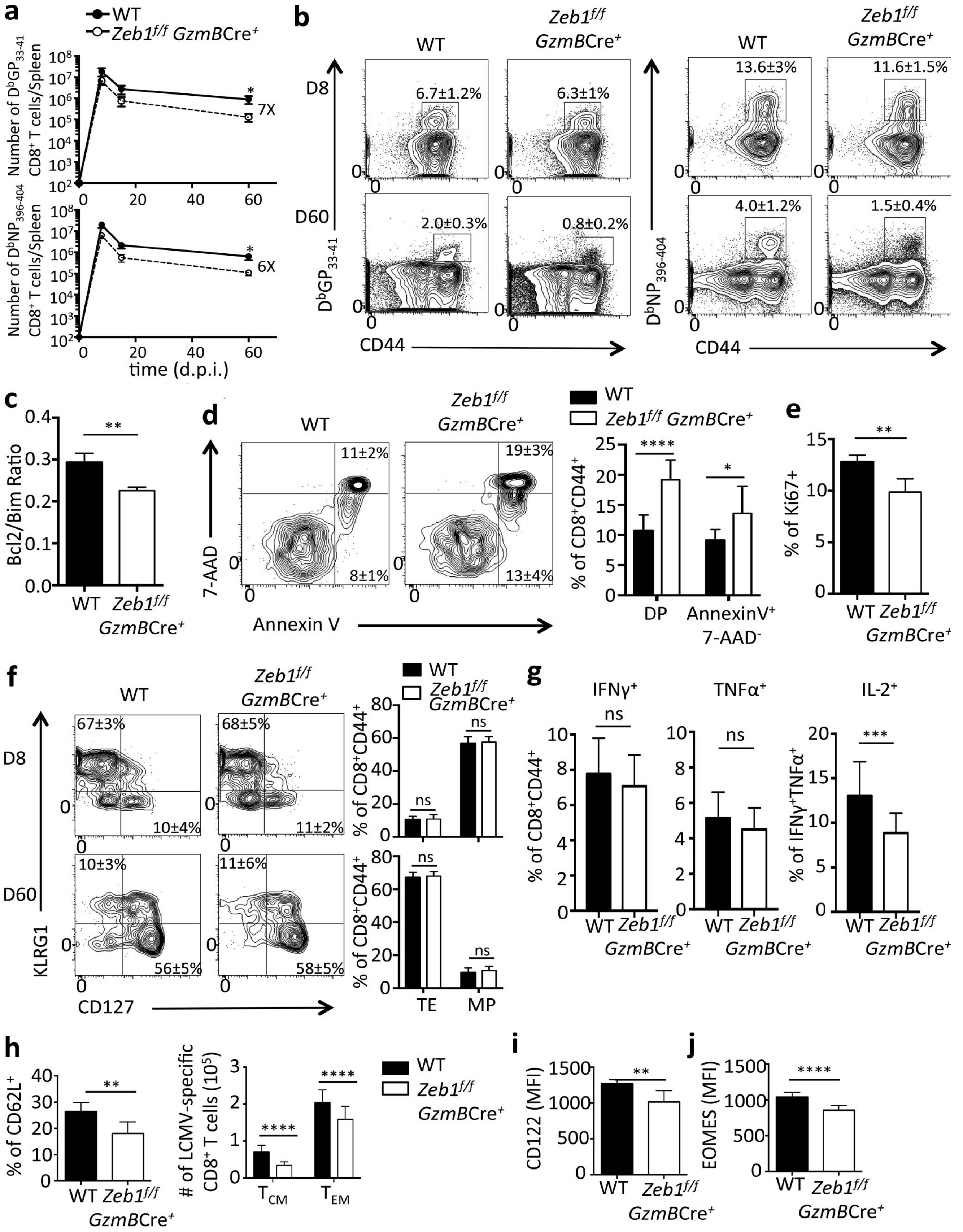
In summary, these data indicated that rather than functioning cooperatively, ZEB1 and ZEB2 have distinct roles in promoting memory and effector CD8$^+$ T cell fates, respectively. ZEB1 is not involved in TE CD8$^+$ T cell differentiation, unlike ZEB2, but rather is necessary for optimal memory cell survival, homeostasis, and T$_{CM}$ formation. These data indicate that ZEB1 and ZEB2 form a novel counterregulatory axis operating at distinct phases of the immune response to govern effector and memory CD8$^+$ T cell differentiation.

ZEB1 is necessary for memory CD8$^+$ T cell protective immunity

To assess whether the defect in *Zeb1$^{f/f}$ GzmB*Cre$^+$ memory CD8$^+$ T cells extended beyond survival to affect memory function, we compared the protective qualities of the WT and *Zeb1$^{f/f}$ GzmB*Cre$^+$ memory CD8$^+$ T cells during reinfection. WT and *Zeb1$^{f/f}$ GzmB*-Cre$^+$ mice were infected with LCMV-Arm, and at 30 dpi, equal numbers of D^{b}GP$_{33-41}$ tetramer$^+$ LCMV-specific CD8$^+$ T cells were isolated from each cohort and transferred separately into congenically mismatched mice that were subsequently challenged the next day with a recombinant strain of *Listeria monocytogenes* expressing the GP$_{33-41}$ epitope. Four days after the rechallenge, we compared the ability of transferred WT and *Zeb1$^{f/f}$ GzmB*Cre$^+$ cells to expand and mediate bacterial control upon secondary challenge. Although WT cells exhibited normal expansion and conducted robust bacterial clearance, *Zeb1$^{f/f}$ GzmB*Cre$^+$ memory CD8$^+$ T cells displayed substantial impairment in their proliferative capacity (~10-fold) and failed to reduce bacterial burden in the liver and spleen (Fig. 3, a–c). Additionally, secondary *Zeb1$^{f/f}$ GzmB*Cre$^+$ effector CD8$^+$ T cells produced less Granzyme B compared with WT cells (Fig. 3 d). These data demonstrate that ZEB1 is important not only for the survival of memory CD8$^+$ T cells but also for their protective recall responses.

Zeb1 and *Zeb2* are inversely regulated by TGF-β

Given the reciprocal expression pattern of *Zeb1* and *Zeb2* in naive, effector, and memory CD8$^+$ T cells, we hypothesized that they may be inversely regulated by environmental factors such as cytokines produced during or after viral infection. To examine this question, we tested the effects of several cytokines important for memory T cells, including TGF-β, IL-2, and IL-15, on *Zeb1* and *Zeb2* mRNA levels in in vitro activated P14$^+$ CD8$^+$ T cells. In addition to regulating the development of CD8$^+$ T$_{RM}$ cells, CD4$^+$ regulatory T cells, and Th17 cells, TGF-β is also a well-known inducer of ZEB1 and ZEB2 in mesenchymal cells during the EMT


a
WT
Zeb1f/f GzmBCre+
Number of DbGP33-41 CD8+ T cells/Spleen
108
107
106
105
104
103
102
0
20
40
60
*
7X
Number of DbNP396-404 CD8+ T cells/Spleen
*
6X
time (d.p.i.)
b
WT
Zeb1f/f GzmBCre+
D8
D60
6.7±1.2%
6.3±1%
2.0±0.3%
0.8±0.2%
13.6±3%
11.6±1.5%
4.0±1.2%
1.5±0.4%
DbGP33-41
DbNP396-404
CD44
c
Bcl2/Bim Ratio
0.4
0.3
0.2
0.1
0.0
**
WT
Zeb1f/f GzmBCre+
d
WT
Zeb1f/f GzmBCre+
7-AAD
Annexin V
11±2%
8±1%
19±3%
13±4%
% of CD8+CD44+
25
20
15
10
5
0
****
*
DP
AnnexinV+ 7-AAD-
e
% of Ki67+
15
10
5
0
**
f
WT
Zeb1f/f GzmBCre+
D8
D60
KLRG1
CD127
67±3%
10±4%
68±5%
11±2%
10±3%
56±5%
11±6%
58±5%
% of CD8+CD44+
80
60
40
20
0
ns
ns
ns
ns
TE
MP
g
IFNγ+
TNFα+
IL-2+
% of CD8+CD44+
10
8
6
4
2
0
ns
ns
% of IFNγ+TNFα+
20
15
10
5
0
***
h
% of CD62L+
40
30
20
10
0
**
# of LCMV-specific CD8+ T cells (105)
3
2
1
0
****
****
TCM
TEM
i
CD122 (MFI)
1500
1000
500
0
**
j
EOMES (MFI)
1500
1000
500
0
****
WT
Zeb1f/f GzmBCre+

(Comijn et al., 2001; Eger et al., 2005; Pearce et al., 2009; Lee et al., 2011; Casey et al., 2012; Mackay et al., 2013). IL-2 and IL-15 were examined, because they regulate CTL differentiation and memory cell fitness and long-term survival (Becker et al., 2002; Tan et al., 2002; Williams et al., 2006; Kalia et al., 2010; Pipkin et al., 2010). These experiments showed that *Zeb1* was down-regulated upon $D^bGP_{33–41}$ peptide stimulation and remained low with either IL-2 or IL-15 treatment. However, TGF-β treatment of the activated $CD8^+$ T cells led to significant induction of *Zeb1* mRNA (Fig. 4 a). In contrast, *Zeb2 mRNA* was lowly expressed in naive cells and not significantly affected by peptide stimulation alone or with IL-2 or IL-15 treatment. Furthermore, in contrast to the EMT, TGF-β further reduced *Zeb2* mRNA levels in activated $CD8^+$ T cells (Fig. 4 b). These opposing effects of TGF-β on *Zeb1* and *Zeb2* expression in activated $CD8^+$ T cells provide greater insight for how TGF-β may fine-tune memory $CD8^+$ T cell gene expression patterns and differentiation.

To investigate whether TGF-β inversely regulates *Zeb1* and *Zeb2* expression in a $CD8^+$ T cell–intrinsic manner in vivo, we transferred small numbers of naive *Tgfbr2$^{f/f}$ Lck*Cre^- (WT) and *Tgfbr2$^{f/f}$ Lck*Cre^+ $P14^+$ $CD8^+$ T cells into B6 mice that were subsequently infected with LCMV-Arm. Memory $CD8^+$ T cells were isolated from the two groups of mice 45 dpi, and the amounts of *Zeb1* and *Zeb2* mRNA was measured by quantitative RT-PCR. In agreement with the in vitro data, LCMV-specific memory $CD8^+$ T cells lacking TGFβR2 failed to up-regulate *Zeb1* and sustained abnormally high expression of *Zeb2* (Fig. 4, c and d). Thus, in contrast to that observed in the EMT, where TGF-β induces both *Zeb1* and *Zeb2* expression, in developing memory CD8 T cells, TGF-β has differential effects and promotes *Zeb1* while repressing *Zeb2* mRNA expression.

miR-200 family directly represses *Zeb2* but not *Zeb1* in $CD8^+$ T cells

Our aforementioned findings identified a fundamental difference between the EMT and $CD8^+$ T cell immune responses. In the former, *Zeb1* and *Zeb2* are coordinately expressed to promote the EMT, whereas in the latter, they are expressed in opposition to promote TE and memory $CD8^+$ T cell development. This raises the question as to how *Zeb1* and *Zeb2* expression becomes uncoupled in $CD8^+$ T cells. To explore this question, we turned our attention toward the *miR-200* family members because they function in a negative-feedback pathway with *Zeb1* and *Zeb2* in the EMT. The *miR-200* family members maintain epithelial identity by repressing expression of EMT drivers, principally *Zeb1* and *Zeb2,* each of which contain several *miR-200*–binding targets in their 3′ untranslated regions (UTRs; Fig. 5 a; Mongroo and Rustgi, 2010). However, induction of the EMT by signals such as TGF-β induce ZEB1 and ZEB2, which in turn, directly bind to the highly conserved E-box–binding sequences in *miR-200* gene promoters and transcriptionally repress *miR-200* family expression (Brabletz et al., 2011; Fig. 5 a). The five members of *miR-200* family are separated into two clusters on chromosomes 2 and 4 in *Mus musculus* (Fig. 5 a) and are distinguished by their conserved binding sequences (seed sequences). *miR-200b*, *c*, and *429* share the same seed sequence (AAUACUG), which differs by only one nucleic acid from the seed sequenced shared by *miR-200a* and *141* (AACACUG).

First, we examined the RNA expression patterns of the *miR-200* family members in naive and LCMV-specific effector and memory $CD8^+$ T cells using TaqMan microRNA quantitative RT-PCR. This revealed that all five members of *miR-200* were expressed in naive $CD8^+$ T cells, down-regulated in effector CTLs 8 dpi, and up-regulated again in memory $CD8^+$ T cells (Fig. 5 b). Thus, the miR-200 members were expressed in a coordinated manner with *Zeb1,* but not *Zeb2* (Fig. 1 a), suggesting that *Zeb1* may escape *miR-200* repression in $CD8^+$ T cells, and vice versa. To better examine this point biochemically, we cross-linked LCMV-specific $CD8^+$ T cells from 12 dpi under UV light, immunoprecipitated the RNA-induced silencing complex using anti-Argonaut (anti-AGO) antibodies, and measured the amount of *Zeb1* or *Zeb2* mRNA captured in the complex using quantitative RT-PCR with primers specifically designed to the predicted *miR-200*–binding sites within *Zeb1* and *Zeb2* 3′ UTRs (Guo et al., 2014, 2015). We observed significantly greater enrichment of the *Zeb2* 3′ UTR in the RNA-induced silencing complex than *Zeb1* 3′ UTR (Fig. 5 c; note that *Zeb1* and *Zeb2* 3′ UTRs both contain seed sequences of *miR-200b/c/429* or *miR-200a/14*; Fig. 5 a). This finding fit nicely with the model proposed above in which *Zeb2* mRNA is targeted by *miR-200* repression in activated $CD8^+$ T cells, whereas *Zeb1* mRNA is not. This represents a novel mechanism uncoupling ZEB1 and ZEB2 expression that evolved in $CD8^+$ T cells.

In addition to the binding assay, we also assessed how overexpression of *miR-200* family members affects expression of *Zeb1* and *Zeb2* in $CD8^+$ T cells. Consistent with the aforementioned data, retroviral overexpression of *miR-200a* or *miR-200c* (one representative member of each *miR-200* seed sequence) selectively reduced *Zeb2* mRNA levels, leaving *Zeb1* mRNA unaffected

Figure 2. **ZEB1 promotes the survival of memory $CD8^+$ T cells. (a)** WT (filled circle) and *Zeb1$^{f/f}$ GzmB*Cre^+ (open circle) mice were infected with LCMV-Arm, and splenic D^bGP_{33-41} and $D^bNP_{396-404}$ tetramer$^+$ $CD8^+$ T cells were quantified at 8, 15, and 60 dpi. **(b)** Representative FACS plots of D^bGP_{33-41} and $D^bNP_{396-404}$ tetramer$^+$ $CD8^+$ T cells at 8 and 60 dpi in WT and *Zeb1$^{f/f}$ GzmB*Cre^+ mice (percentage of tetramer$^+$ $CD8^+$ T cells ± SEM is indicated). **(c–e)** Graphs are gated on D^bGP_{33-41}-tetramer$^+$ $CD8^+$ T cells from WT or *Zeb1$^{f/f}$ GzmB*Cre^+ mice at 30 dpi and show the ratio of BCL-2/BIM expression (c), percentage of Annexin V^+ and 7-AAD$^+$ cells (d), or percentage of Ki67$^+$ cells (e) based on flow cytometry. **(f)** Expression of KLRG1 and IL-7R in D^bGP_{33-41} tetramer$^+$ $CD8^+$ T cells at 8 and 60 dpi in WT and *Zeb1$^{f/f}$ GzmB*Cre^+ mice based on flow cytometry. **(g–j)** WT and *Zeb1$^{f/f}$ GzmB*Cre^+ $CD8^+$ T cells from 30 dpi were analyzed for production of IFNγ, TNFα, or IL-2 as indicated using intracellular cytokine staining (g) or expression of CD62L (together with absolute number of T_{CM} and T_{EM} cells; h), CD122 (i), and EOMES (j). Data shown are representative of five (b and f) or two (d) independent or cumulative of three (a) or five (c, e, and g–j) independent experiments; n = 3–5 mice per group per experiment (b, d, and f), n = 8–14 mice/group (a), amd n = 15–25 mice/group (c, e, and g–j). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

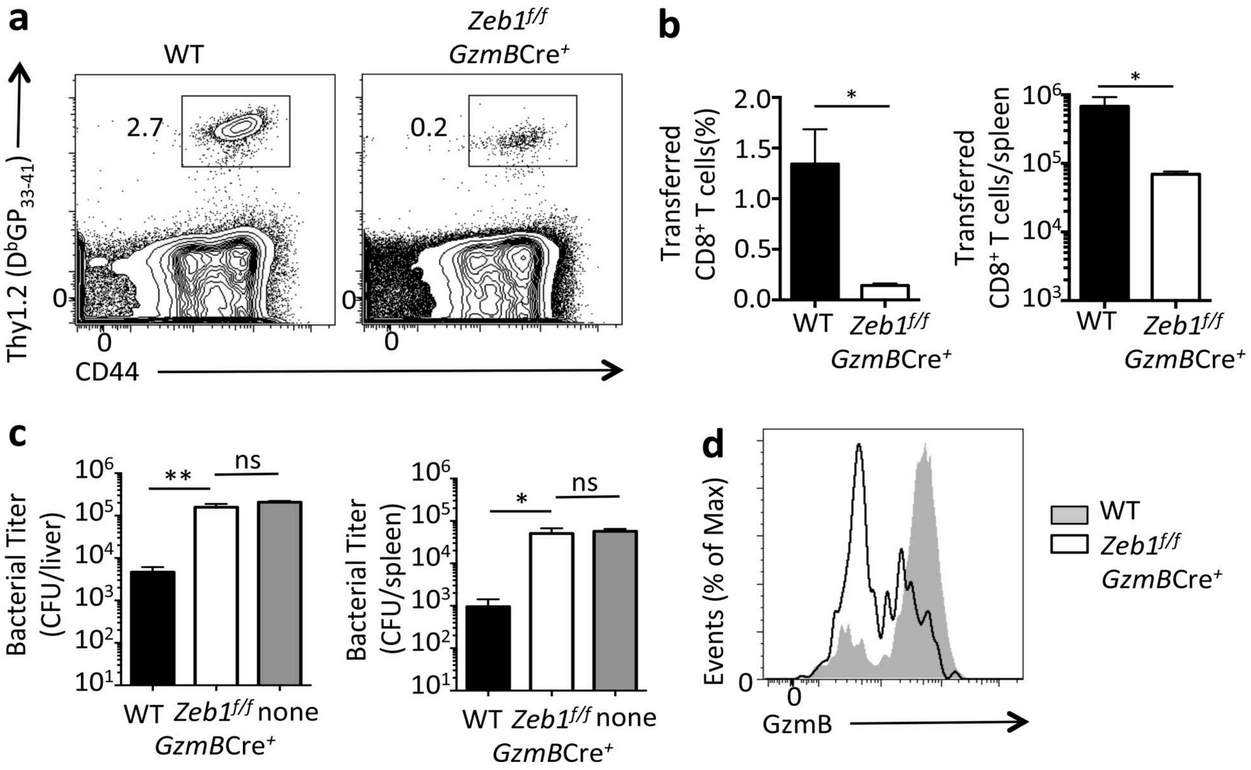


Figure 3. **ZEB1 is necessary for memory CD8⁺ T cell protective immunity.** 100,000 GP_{33-41}–specific WT or *Zeb1$^{f/f}$ GzmB*Cre⁺ memory CD8⁺ T cells (Thy1.2/1.2) were transferred into congenically mismatched (Thy1.1/1.1) naive B6 mice that were then infected with recombinant *L. monocytogenes* expressing the GP_{33-41} epitope (LM-33). **(a and b)** At day 4 after challenge, the frequency and numbers of recalled GP_{33-41}–specific WT or *Zeb1$^{f/f}$ GzmB*Cre⁺ CD8⁺ T cells were analyzed in the spleen. **(c)** LM-33 bacterial titers (CFUs) in the liver and spleen were determined at day 3 after challenge. **(d)** Histogram shows the expression of Granzyme B in GP_{33-41}–specific WT or *Zeb1$^{f/f}$ GzmB*Cre⁺ CD8⁺ T cells day 4 after LM-33 infection. Data shown are representative of two (a and d) or cumulative of two (b and c) independent experiments; *n* = 3–5 mice/group/experiment (a and d), *n* = 6–10 mice/group (b and c). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

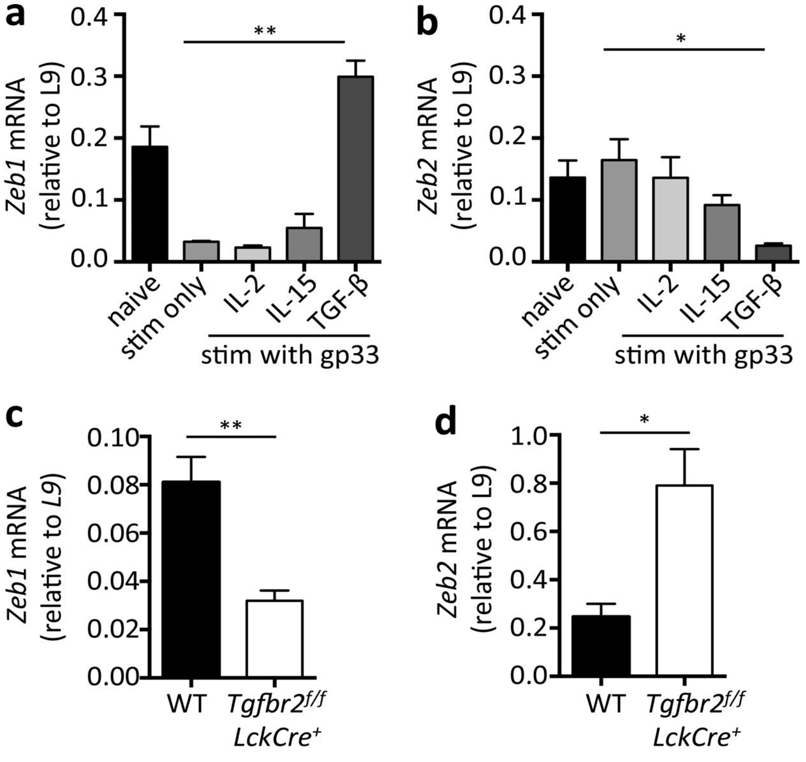


Figure 4. ***Zeb1* and *Zeb2* are inversely regulated by TGF-β. (a and b)** *Zeb1* and *Zeb2* mRNA expression in naive P14⁺ CD8⁺ T cells or those stimulated (stim) with GP_{33-41} peptide for 3 d followed by 2-d culture alone or in the presence of the indicated cytokines (IL-2, IL-15, and TGF-β). **(c and d)** 50,000 WT or *Tgfbr2$^{f/f}$ Lck*Cre⁺ P14⁺ CD8⁺ T cells were transferred to naive B6 mice followed by LCMV-Arm infection. 45 dpi, the donor cells were purified using FACS and the amount of *Zeb1* and *Zeb2* mRNA was measured by quantitative RT-PCR. Data shown are cumulative of two (a–d) independent experiments; *n* = 8 mice (a and b), *n* = 6–10 mice/group (c and d). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

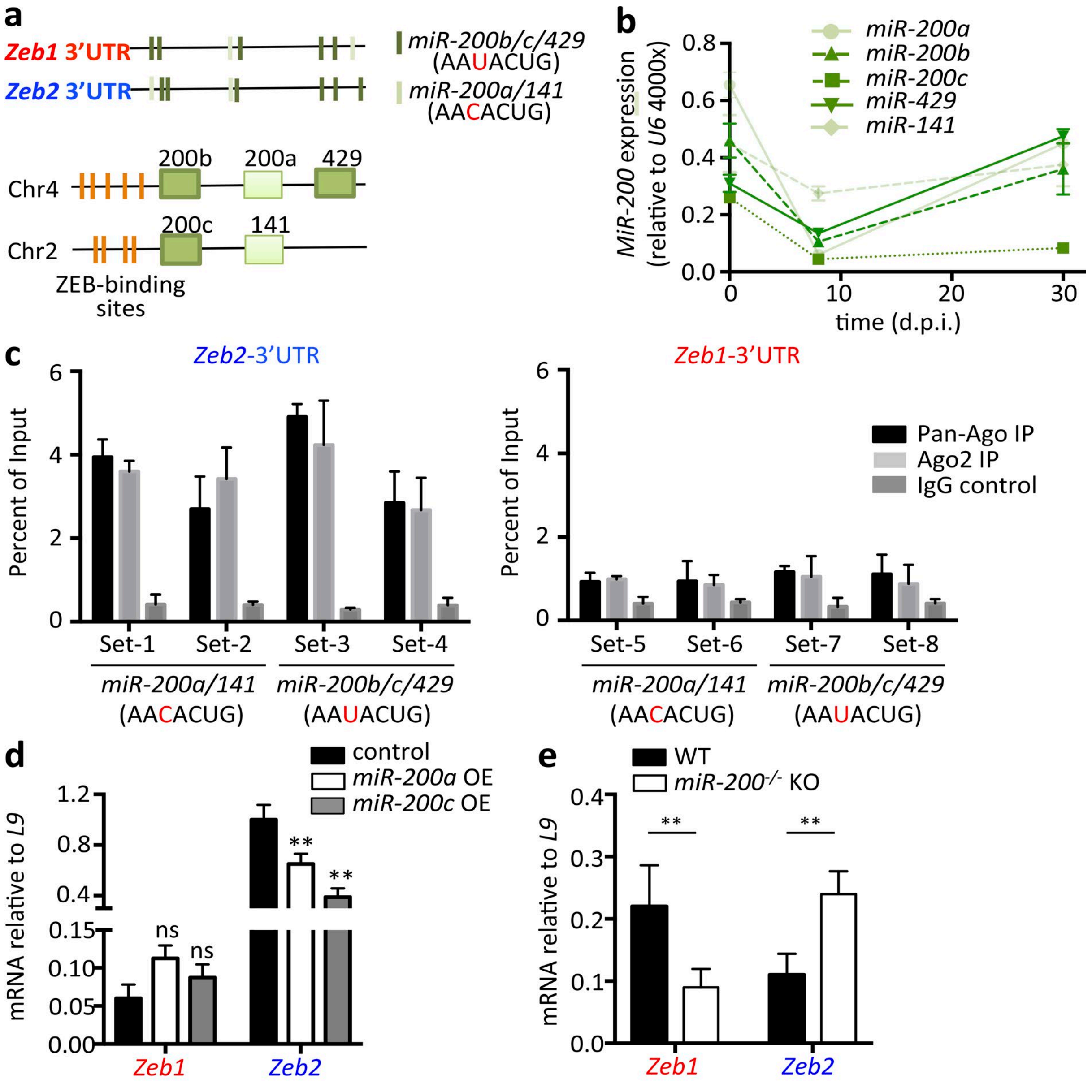


Figure 5. ***miR-200* family directly represses *Zeb2* but not *Zeb1* mRNA in CD8⁺ T cells. (a)** Schematic representation of *Zeb1 and Zeb2* 3′ UTRs (top) showing the *miR-200* family seed sequences (dark and light green) and both clusters of *miR-200* family genes (bottom) showing the corresponding conserved ZEB-binding sites (orange). **(b)** Naive (d0) and LCMV-specific effector (8 dpi) and memory (30 dpi) CD8⁺ T cells were sorted and measured for *miR-200* family microRNA expression using quantitative RT-PCR. **(c)** LCMV-specific CD8⁺ T cells were sorted 12 dpi followed by UV cross-linking and immunoprecipitating (IP) with pan-Ago (black), Ago2 (light gray), or IgG control (dark gray) antibodies. Enrichment of specific *miR-200a/141* or *miR-200b/c/429* binding regions within *Zeb2* (left) or *Zeb1* (right) 3′ UTR over input was measured by site-specific primers using quantitative RT-PCR. **(d)** P14⁺ CD8⁺ T cells transduced with RVs designed to overexpress (OE) *miR-200a* (white bar) or *miR-200c* (gray bar) were sorted at 8 dpi, and *Zeb1* and *Zeb2* mRNA was measured by quantitative RT-PCR. **(e)** LCMV-specific CD8⁺ T cells were purified from WT (black bar) and *miR-200*$^{-/-}$ KO (white bar) mice at 30 dpi, and *Zeb1* and *Zeb2* mRNA was measured by quantitative RT-PCR. Data shown are cumulative of two (b, c, and e) or three (d) independent experiments; *n* = 6–10 mice/group (b, c, and e), *n* = 9–15 mice/group (d). Data are expressed as mean ± SEM. **, P < 0.01.

or marginally increased in effector CD8⁺ T cells 8 dpi (Fig. 5 d). Conversely, memory CD8⁺ T cells lacking both miR-200 clusters (referred to as *miR-200*$^{-/-}$ and described in detail later) simultaneously displayed increased *Zeb2* and decreased *Zeb1* mRNA (Fig. 5 e). Altogether, these data illustrate the selective regulation of *Zeb2* expression by *miR-200* family members in CD8⁺ T cells, thus adding an additional layer of genetic control in the ZEB1 and ZEB2 counterregulatory network that guides effector and memory CD8⁺ T cell fate decisions.

miR-200 overexpression impairs TE and promotes MP cell development

To determine whether *miR-200* family members modulate *Zeb2* expression and differentiation of virus-specific effector and memory CD8⁺ T cells, we individually overexpressed (OE) each *miR-200* family member in P14⁺ CD8⁺ T cells using retroviruses (RVs) and transferred equal numbers of RV-transduced cells into naive B6 mice that were subsequently infected with LCMV-Arm. Control P14⁺ CD8⁺ T cells were transduced with empty RVs.

Note, *miR-200a* and *miR-200c* were selected as representatives of each seed sequence and shown in Fig. 6, whereas the results of the remaining miR-200 family members are provided in Fig. S3. These experiments revealed that by 30 dpi, larger numbers (approximately three- to eightfold) of virus-specific CD8$^+$ T cells were present from the individual overexpression of the miR-200 family members, indicating that these *miR-200s* enhanced memory CD8$^+$ T cell formation (Fig. 6 a). At 8 and 30 dpi, the control and *miR-200* OE P14$^+$ CD8$^+$ T cells were analyzed and in line with their preferential binding to *Zeb2* mRNA, the overexpression of *miR-200* family members largely phenocopied CTLs lacking *Zeb2.* That is, at 8 dpi, the frequency and number of KLRG1^{hi} IL-7R^{lo} TE cells was greatly diminished, whereas the KLRG1^{lo} IL-7R^{hi} MP subset was markedly increased with *miR-200* overexpression compared with the control vector (Figs. 6 b and S3 a). Impressively, virtually the entire population of memory CD8$^+$ T cells overexpressing *miR-200* family members was IL-7R^{hi} and KLRG1^{lo} and contained a substantially larger percentage of CD62L$^+$ CD27$^+$ IL-2$^+$ T$_{CM}$ cells (Figs. 6c and S3 b). Moreover, pro-memory TFs such as EOMES and TCF1 were increased in *miR-200* overexpressing CD8$^+$ T cells, whereas the pro-TE TF T-BET was significantly down-regulated (Fig. 6 d and Fig. S3, c and d). Furthermore, at 8 dpi, we observed mildly reduced production of IFNγ and TNFα, but an increased proportion of IL-2–producing effector cells with *miR-200* overexpression upon D^{b}GP$_{33\text{-}41}$ peptide stimulation (Fig. 6 e). These data indicated that the *miR-200* family members are sufficient to inhibit the terminal differentiation of effector CD8$^+$ T cells and promote formation of memory cells and their progenitors, which is likely due in part to the direct repression of *Zeb2* by *miR-200* family. That we observed a more profound phenotype with *miR-200* OE compared with *Zeb2* deficiency with regard to the loss of TE cells and gain of central memory properties suggests that *miR-200* family members likely also target other pro-TE genes to achieve their biological function.

miR-200 family deficiency results in loss of memory CD8$^+$ T cells

To further investigate the requirement of *miR-200* for memory CD8$^+$ T cell development, we generated a *miR-200* double cluster KO mouse strain by crossing *miR-200b/a/429* cluster germline KO mice (Hasuwa et al., 2013) with mice that contain flanking loxP sites around the *miR-200c/141* cluster (*miR-200c/141*$^{flox/flox}$; Cao et al., 2013) and the *GzmB*-Cre transgene to conditionally delete both clusters in activated CD8$^+$ T cells. For simplicity, these mice will be referred to as *miR-200*$^{-/-}$. We then infected WT mice (*miR-200b/a/429*$^{+/+}$*; miR-200c/141*$^{flox/flox}$*; GzmB-Cre*$^-$) or those with heterozygous (*miR-200b/a/42*$^{+/-}$*; miR-200c/141*$^{+/flox}$*; GzmB-Cre*$^+$) or homozygous (*miR-200b/a/429*$^{-/-}$*; miR-200c/141*$^{flox/flox}$*; GzmB-Cre*$^+$) loss of both clusters with LCMV-Arm and analyzed effector and memory CD8$^+$ T cell formation. We refer to these two groups of mice as *miR-200*$^{+/-}$ HET or *miR-200*$^{-/-}$ KO, respectively. This experiment revealed a dose-dependent requirement of both *miR-200* clusters in the formation of virus-specific memory CD8$^+$ T cells, because there was marked decrease in numbers and frequency of memory CD8$^+$ T cell in *miR200*$^{+/-}$ HET mice and a further reduction in the *miR200*$^{-/-}$ KO mice (Fig. 7, a and b). Similar to the *Zeb1*-deficient memory CD8$^+$ T cells, there was no gross difference in the expression of KLRG1 or IL-7R among WT, *miR-200*$^{+/-}$ HET, and *miR-200*$^{-/-}$ KO mice, suggesting that *miR-200* is not required for phenotypic generation of these subsets of memory cells (Fig. 7 c); however, *miR-200*$^{-/-}$ mice exhibited a substantial decrease in the formation of CD62L$^+$ CD27$^+$ IL-2$^+$ T$_{CM}$ cells (Fig. 7, d and e). In summary, these findings demonstrate that *miR-200* members play a critical role in the development of long-lived memory CD8$^+$ T cells, particularly T$_{CM}$ cells, and the loss of *miR-200* family members in CD8$^+$ T cells strongly phenocopies the loss of *Zeb1.* Collectively, these data indicate that the *miR-200* family members temporally coordinate the reciprocal expression patterns of *Zeb1* and *Zeb2* during an immune response by selectively targeting *Zeb2* in CD8$^+$ T cells after effector cell development, allowing for *Zeb1* re-expression and memory cell formation and persistence.

Discussion

During viral infection, our immune system seeks to achieve two principle outcomes: to eliminate the present invading pathogen and generate immunological memory. A deeper understanding of the molecular mechanisms regulating the formation of different types of effector and memory CD8$^+$ T cells could propel the development of better vaccines and immunotherapies. This study identified a genetic circuit, previously unknown to function in the immune system, that regulates effector and memory CD8$^+$ T cell fate decisions involving the TFs ZEB1 and ZEB2 and the *miR-200* microRNA family, as well as the cytokine TGF-β—all critical regulators of the EMT. In particular, this is the first study of the functions of ZEB1 and *miR-200* family members in CD8$^+$ T cells and their important role in the establishment of a long-lived pool of memory CD8$^+$ T cells and T$_{CM}$ differentiation.

The ZEB1, ZEB2, TGF-β–, and *miR-200* network is best characterized in controlling epithelial versus mesenchymal cell fates as part of the EMT, wherein the *miR-200* family promotes epithelial states and TGF-β induces both ZEB1 and ZEB2, which cooperatively drive mesenchymal states. However, in CD8$^+$ T cells, we found that ZEB1 and ZEB2 work in opposition, possibly by inhibiting each other, in temporally distinct phases of an immune response to coordinate TE differentiation (ZEB2) and memory CD8$^+$ T cell survival and T$_{CM}$ maturation (ZEB1). Moreover, our data show that in CD8$^+$ T cells, *Zeb1* and *Zeb2* expression was inversely regulated by TGF-β and the *miR-200* family members, outlining a novel mechanism for "splitting" the functions of ZEB1 and ZEB2 to generate alternative cell fates. These data present a working model wherein *Zeb1* and *miR-200* expression is repressed upon CD8$^+$ T cell activation, allowing for the induction of *Zeb2* by T-BET in cells exposed to increasing inflammation and stimulation of TE cell development. Although there is no direct binding data in CD8$^+$ T cells, we postulate that ZEB2 operates to sustain repression of *Zeb1* and *miR-200* expression as TE cells form. Through TGF-β signaling, expression of *Zeb1* and *miR-200* is augmented in developing MP cells, which prevents *Zeb2* expression and promotes the development of long-lived circulating memory CD8$^+$ T cells, particularly T$_{CM}$ cells. Thus, this study identifies a new counterregulatory

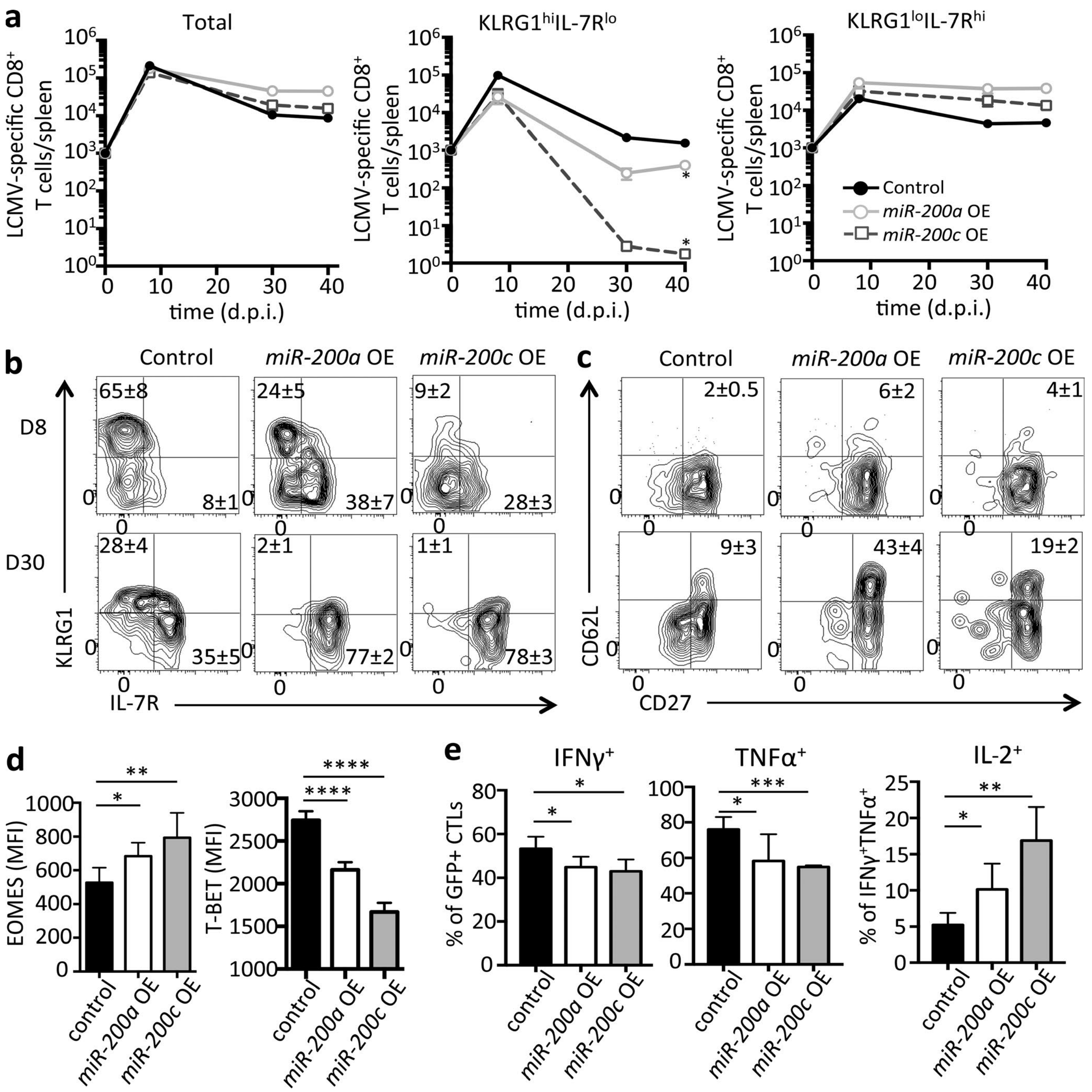


Figure 6. **Overexpression of *miR-200* family impairs TE and promotes memory cell formation.** As in Figure 5, small numbers of P14+ CD8+ T cells were transduced with *miR-200a* (light gray open circle) or *miR-200c* (dark gray open square) RVs or empty vector control (black filled circle) RVs and transferred into B6 mice that were subsequently infected with LCMV-Arm. **(a)** Line plots show total numbers of donor RV-transduced P14+ CD8+ T cells (left) or subsets of KLRG1^{hi} IL-7R^{lo} cells (middle) or KLRG1^{lo} IL-7R^{hi} cells (right) at 8, 30 and 45 dpi. **(b and c)** Flow plots show expression of KLRG1 and IL-7R (b) CD62L and CD27 (c) in control, *miR-200a* and *miR-200c* OE P14+ CD8+ T cells at 8 and 30 dpi. **(d and e)** Bar graphs show amounts of EOMES and T-BET in donor P14+ CD8+ T cells at 30 dpi (d) or production of IFNγ, TNFα or IL-2 as indicated using intracellular cytokine staining (e; note that IL-2–producing cells were gated on IFNγ+ TNFα+ CD8+ T cells). See Fig. S3 for additional data on effects of *miR-200b*, *miR-429*, and *miR-141* OE in LCMV-specific CD8+ T cells. Data shown are representative of five (b and c) or cumulative of two (a) or three (d and e) independent experiments; *n* = 3–5 mice/group/experiment (b and c), *n* = 6–8 mice/group (a), *n* = 8–10 (d and e). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

network in CD8+ T cells that temporally coordinates the formation of effector cells to fight present infection and memory cells to fight future infection.

There are other studies wherein ZEB1 and ZEB2 control alternative cell fate decisions in *Xenopus laevis* embryogenesis with ZEB1 guiding mesoderm fates and ZEB2 driving neural fates (Postigo, 2003; Postigo et al., 2003). In this context, it was found that ZEB1 and ZEB2 regulate TGF-β/BMP signaling in opposite ways wherein ZEB1 recruits transcriptional coactivators (p300 and P/CAF) and synergizes with Smad-mediated transcriptional

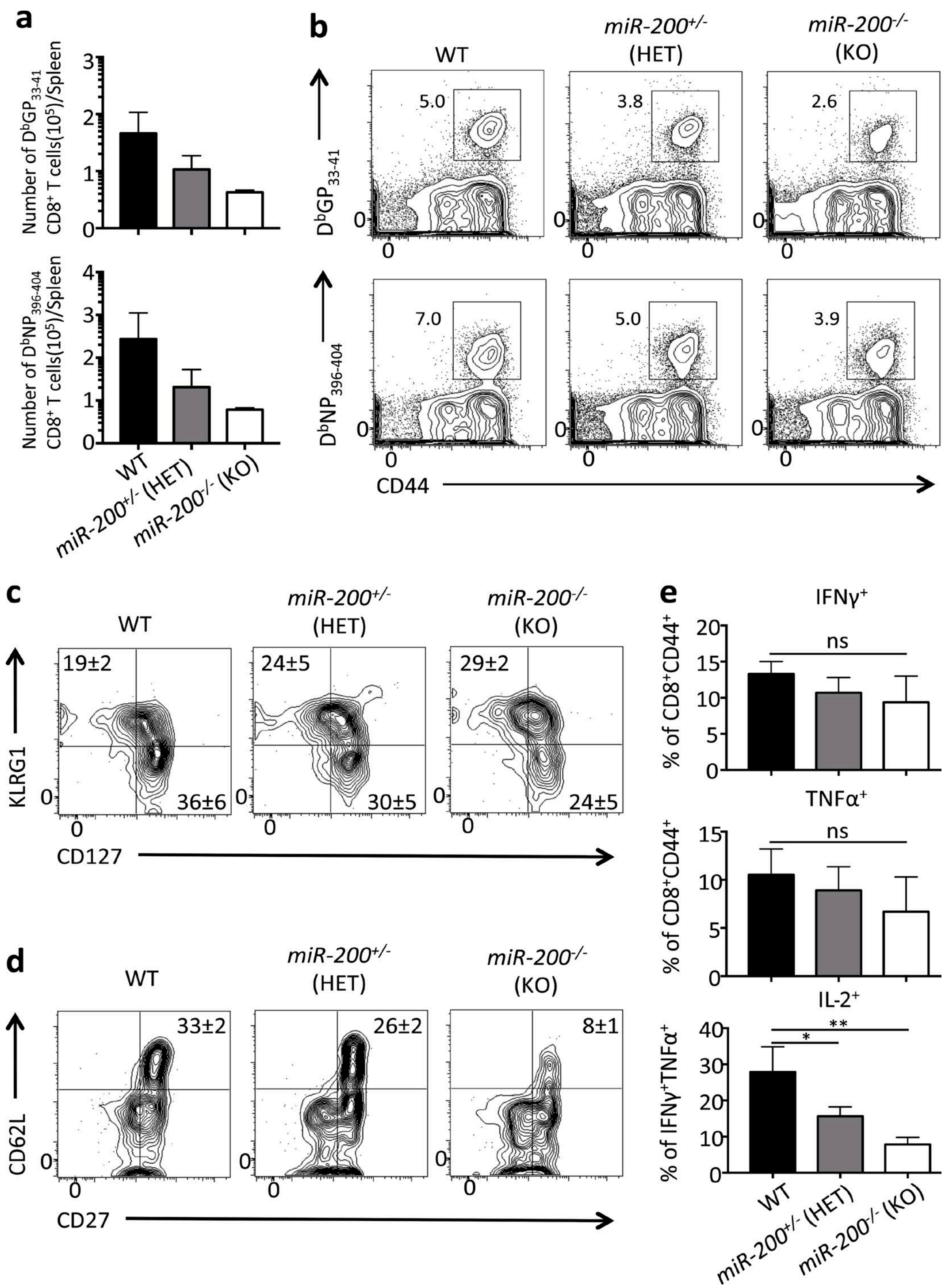


Figure 7. ***miR-200* family deficiency results in the loss of memory $CD8^+$ T cells. (a)** WT (black bar), *miR-200*$^{+/-}$ (HET, gray bar), and *miR-200*$^{-/-}$ (KO, white bar) mice were infected with LCMV Arm and splenic D^bGP_{33-41} and $D^bNP_{396-404}$ tetramer$^+$ $CD8^+$ T cells were quantitated at 45 dpi. **(b)** Representative contour plots of D^bGP_{33-41} and $D^bNP_{396-404}$ tetramer$^+$ $CD8^+$ T cells at 45 dpi in WT, *miR-200*$^{+/-}$ HET, and *miR-200*$^{-/-}$ KO mice. **(c and d)** Representative flow cytometry data measuring KLRG1 and IL-7R expression (c) and CD62L and CD27 expression (d) in WT, *miR-200*$^{+/-}$ and *miR-200*$^{-/-}$ LCMV-specific $CD8^+$ T cells 45 dpi. **(e)** WT (black bar), *miR-200*$^{+/-}$ (HET, gray bar), and *miR-200*$^{-/-}$ (KO, white bar) $CD8^+$ T cells from 45 dpi were analyzed for IFNγ and TNFα (top two bar graphs) or IL-2 (bottom bar graph) expression using intracellular cytokine staining after a 5-h GP_{33-41} peptide stimulation. Note that IL-2 producing cells were gated on IFNγ$^+$ TNFα$^+$ $CD8^+$ T cells. Data shown are representative of two (b–d) or cumulative of two (a and e) independent experiments; *n* = 2–3 mice/group/experiment (b–d), *n* = 5–6 mice/group (a and e). Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$.

activation, whereas ZEB2 binds to corepressors (CtBP) and suppresses downstream TGF-β signaling (Postigo, 2003; Postigo et al., 2003). Perhaps a similar scenario operates in $CD8^+$ T cells where the presence of ZEB1 or ZEB2 alters the quality of TGF-β signaling in T cells. Our data showed that TGF-β enhanced *Zeb1* while repressing *Zeb2* expression in $CD8^+$ T cells, but whether they modify downstream TGF-β signaling or T_{RM} formation requires more exploration. Our data provide greater mechanistic insight into a recent study that demonstrated constitutive TGF-β signaling was required to maintain mature IL-7R^{hi} memory CD8^{+} T cells (Ma and Zhang, 2015); likely, this is due in part, to TGF-β–mediated maintenance of *Zeb1* and *miR-200* expression. Interestingly, despite increased *Zeb2* mRNA in *Zeb1*-deficient CD8^{+} T cells, no overt changes in MP and TE cell differentiation was observed, indicating increased ZEB2 alone was not sufficient to repress MP cell fates. This result suggested that ZEB1 and ZEB2 do not simply compete for binding to the same gene loci with opposing effects on transcription, albeit there may be some loci for which this occurs (e.g., *Il2*). Rather, the data indicate that ZEB1 and ZEB2 largely drive different gene expression programs, with ZEB2 supporting TE differentiation during the naive→effector phase and ZEB1 supporting cell survival during the effector→memory transition. On this note, the expression patterns and loss-of-function phenotypes of *Zeb1* and *Zeb2* are highly overlapping with that of *Id3* and *Id2* in CD8^{+} T cells, respectively (Yang et al., 2011), and thus, it is tempting to speculate that functional cooperativity exists between ZEB1-ID3 and ZEB2-ID2 to control temporally and functionally distinct gene expression programs.

This study also identified a new manner by which ZEB1 and ZEB2 functions could be uncoupled through the selective actions of microRNAs. We observed that all five members of *miR-200* family are expressed in naive and memory CD8^{+} T cells but down-regulated in effector CTLs. Ectopic expression of *miR-200* family members markedly reduced TE subset formation and boosted memory CD8^{+} T cell maturation, phenotypes resembling a deficiency in *Zeb2*. Indeed, *Zeb2* mRNA was diminished in CD8^{+} T cells overexpressing *miR-200* family members, whereas *Zeb1* was not. Although *miR-200* family members can bind to both *Zeb1* and *Zeb2* 3′ UTRs and repress their expression based on luciferase reporter assays in HEK 293T cells (Wang et al., 2013), in virus-specific CD8^{+} T cells, we detected a strong preference for *miR-200* binding at the *Zeb2* 3′ UTR, but not the *Zeb1* 3′ UTR, using cross-linking immunoprecipitation assays. How *Zeb1* mRNA escapes binding to *miR-200* in CD8^{+} T cells is not clear, but several possibilities exist, the simplest being that CD8^{+} T cells express a *Zeb1* isoform lacking the miR-200–binding sites in 3′ UTR; however, analysis of the *Zeb1* transcripts in CD8^{+} T cells by RNA sequencing disproved this simple explanation (unpublished data). It is also possible that *Zeb1* mRNA forms secondary structures in CD8^{+} T cells that masks the *miR-200*–binding sites or that other RNA-binding proteins such as HuR or microRNAs that promote mRNA stability and translation (Mukherjee et al., 2011) bind to the *Zeb1* 3′ UTR and prevent *miR-200* recognition. Distinguishing between these mechanisms will be important to better understand the separation of ZEB1 and ZEB2 expression and function in T cells and possibly other immune cells in which they are expressed, such as CD4^{+} T cells, B cells, natural killer cells, dendritic cells (DCs), monocytes, and plasmacytoid DCs (Heng et al., 2008; van Helden et al., 2015). Interestingly, these data reveal that *Zeb1* is predominantly expressed in CD103^{+} DCs from skin draining LNs, whereas *Zeb2* is more highly expressed in spleen plasmacytoid DC populations (Heng et al., 2008). Thus, we postulate ZEB1 and ZEB2 may exhibit counterregulatory roles and modulate alternative cell fate decisions more broadly in the immune system.

Even though our data suggested that ZEB2 is a primary target of *miR-200*, clearly, *miR-200* overexpression displayed a more profound phenotype compared with the *Zeb2*-deficient CD8^{+} T cells (Dominguez et al., 2015). In particular, *Zeb2*-deficient CD8^{+} T cells did not display enhanced survival of memory CD8^{+} T cells or diminished effector functions, whereas *miR-200* overexpression affected both of these processes (Dominguez et al., 2015; Omilusik et al., 2015). These results suggest that, in addition to *Zeb2*, *miR-200* family members regulate other genes involved in CD8^{+} T cell differentiation. Indeed, using microRNA-binding target prediction software, we found *miR-200*–binding sequences within the 3′ UTR of *Prdm1* (*Blimp-1*), another critical pro–effector TF that suppresses memory CD8^{+} T cell formation and survival (Kallies et al., 2009; Rutishauser et al., 2009). Given the enhanced memory formation that resulted in the *miR-200* OE, further characterization of their targets may have therapeutic implications.

Lastly, it is important to elucidate the connection between the EMT and T cell differentiation and why the ZEB1, ZEB2, TGF-β, and *miR-200* network is used in both. That is, how do the cellular processes involved in the EMT relate to T cell function? One connection may be cellular motility and trafficking and induction of EMT-like processes are needed for T cell trafficking. However, we did not notice severe defects in LN egress or gross tissue infiltration of effector T cells during acute LCMV infection in either ZEB1- or ZEB2-deficient CD8^{+} T cells relative to their WT counterparts (Dominguez et al., 2015; Omilusik et al., 2015; unpublished data). Nonetheless, this does not rule out the possibility that this genetic network may fine-tune CD8^{+} T cell trafficking within tissues, for example between interstitial spaces versus epithelial linings versus the vasculature or in the skin between the dermis versus epidermis (i.e., tissue-resident vs. circulating memory T cells). In addition to T_{RM} cells, it will be important to investigate how this network regulates the tissue surveillance of CX3CR1^{int} peripheral memory T cells (Gerlach et al., 2016), particularly because ZEB2 is needed for CX3CR1 expression (Dominguez et al., 2015; Omilusik et al., 2015). In summary, this work identified a novel genetic circuit involving both transcriptional and posttranscriptional programs to guide T cell fate decision and provided new insights into the molecular regulation of T cell plasticity and heterogeneity, which could allow considerable improvement of vaccine and therapeutic development against infection and cancer.

Materials and methods

Mice

C57BL/6 (B6) mice were obtained from the National Cancer Institute. *Zeb2*$^{flox/flox}$ mice were originally generated by D. Hoylebroeck (University of Leuven, Leuven, Belgium; Higashi et al., 2002) and obtained from R. Aslopp (John A. Burns School of Medicine, University of Hawaii, Honolulu, HI). Granzyme B-Cre (GzB-Cre^{+}) mice were provided by J. Jacobs (Emory University, Atlanta, GA) via R. Flavell's laboratory (Yale University School of

Medicine, New Haven, CT) and were crossed to *Zeb2*$^{flox/flox}$ mice for generation of GzB-cre$^+$; *Zeb2*$^{flox/flox}$ (*Zeb2*-deficient) mice and GzB-cre$^+$; *Zeb2*$^{+/+}$ or GzB-cre$^-$; *Zeb2*$^{flox/flox}$ (*Zeb2*-WT) mice. *Zeb2*$^{f/f}$ *GzmB*Cre$^+$ and *Zeb2*$^{f/f}$ *GzmB*Cre$^-$ mice were further crossed to P14$^+$ TCR transgenic mice so that P14$^+$ *Zeb2*$^{f/f}$ *GzmB*-Cre$^+$ and *Zeb2*$^{f/f}$ *GzmB*Cre$^-$ mice could be obtained.

Zeb1$^{flox/flox}$ mice were generated using the CRIPSR/Cas9 technique in collaboration with R. Flavell's laboratory under C57BL/6 (B6) mice background and were crossed to *GzmB*-Cre$^+$ mice to generate *Zeb1* conditional KO. P14$^+$ *Zeb1*$^{f/f}$ *GzmB*-Cre$^+$ mice were generated in the same way described above. To generate P14 chimeric mice, 10–50,000 P14$^+$ CD8$^+$ T cells were transferred into B6 mice by i.v. injection. *Tgfbr2*$^{f/f}$ *LckCre* mice were a gift from M. Bevan (University of Washington, Seattle, WA) and crossed to P14$^+$ mice to generate P14$^+$ *Tgfbr2*$^{f/f}$ *LckCre* mice. *miR-200c/141*$^{f/f}$ mice were purchased from The Jackson Laboratory and crossed to GzB-Cre$^+$ mice to generate *miR-200c/141*$^{-/-}$ mice. *miR-200b/a/429* KO mice were a gift from H. Hasuwa (Keio University, Keio, Japan). m*iR-200* family total KO mice were generated by crossing *miR-200c/141*$^{-/-}$ mice to *miR-200b/a/429* KO mice. All animal experiments were done with approved Yale institutional animal care and use committee protocols.

Infections and treatments

For infections of mice, 2 × 10^5 PFU of the LCMV Armstrong strain were administered i.p. For recall experiments, mice were administered 2 × 10^4 CFU recombinant *L. monocytogenes* expressing the LCMV GP$_{33–41}$ epitope.

Antibodies for surface and intracellular staining

Lymphocyte isolation, along with surface and intracellular staining, was performed as described previously (Joshi et al., 2007). For in vitro stimulation, splenocytes were stimulated with 100 ng/ml GP$_{33–41}$ and NP$_{396–404}$ peptides for 5 h in the presence of brefeldin A. Antibodies were purchased from eBioscience, BD Biosciences, or BioLegend and Cell Signal. Class I MHC tetramers were generated as described previously (Kaech et al., 2003). Flow cytometry data were acquired on BD LSRII with Diva software and analyzed with FlowJo software (Tree Star). Sorting was performed on a FACSAria (BD).

ChIP

ChIP experiments were performed with 10 million naive CD8$^+$ T cells. The cells were cross-linked with 1% formaldehyde for 10 min and sonication to obtain an ~200–500-bp DNA fragment. ChIP was performed with anti–ZEB1 antibody (Cell Signaling), and anti–mouse IgG was used as a negative control. Two independent experiments were performed. Immunoprecipitated DNA was analyzed by quantitative PCR.

Sybr-based quantitative PCR was performed with the following primers: Zeb1_*Zeb2* forward, 5′-CCACATCTGGAAGTCAGCAA-3′; Zeb1_*Zeb2* reverse, 5′-ACAAAACAGCAGAGCATTGG-3′; Zeb1_*il2* forward, 5′-GGAGCTCCTGTAGGTCCATC-3′; Zeb1_*il2* reverse, 5′-AAGCTCTACAGCGGAAGCAC-3′; *Zeb2* exon 8 forward, 5′-CACCTAAGTGCTGCATTGGA-3′; and *Zeb2* exon 8 reverse, 5′-TTAGTGGCAGCAGTCCCTTT-3′.

Cross-linking immunoprecipitation and quantitative RT-PCR

Cross-linking was performed using 5 million sorted LCMV-Specific CD8$^+$ T cells 12 dpi according to a published protocol (Guo et al., 2014). RNA immunoprecipitation was performed with 10 µg pan-AGO antibody (MABE56; Millipore) and Ago2 antibody (015–22031; Wako Chemical), and quantitative RT-PCR was performed following a published protocol (Guo et al., 2015). RT reaction was performed using the Invitrogen Superscript III cDNA synthesis kit according to the manufacturer's protocol. Sybr-based quantitative PCR was performed with the following primers: set-1 forward, 5′-GCAGTTCAGCCAAGACAGAG-3′: set-1 reverse, 5′-TGTAGTGATACATACGTAGAGTGCAA-3′; set-2 forward, 5′-CTGCAAGTGCCATCCTTGTA-3′; set-2 reverse, 5′-TGACCTAAAATTAAATGAATGCAAA-3′; set-3 forward, 5′-TTTAAAAGGTGCCCGCACTA-3′; set-3 reverse, 5′-TGCATCACTTCAAGTTCCTTCA-3′; set-4 forward, 5′-GGCAGCAGTTCCTTAGTTTACA-3′; set-4 reverse, 5′-GCCCAAATGATCAACGTCAT-3′; set-5 forward, 5′-GGCAGAATCAGTGTTCGTGA-3′; set-5 reverse, 5′-CAACAAACGAATCAACAACTGC-3′; set-6 forward, 5′-CAGTAGAGATGCAGTTGGTTCC-3′; set-6 reverse, 5′-AAAACTGGGGAAAGGGAGAA-3′; set-7 forward, 5′-AGGTTACAGGAGGCTGGATG-3′; set-7 reverse, 5′-TGCTCTGTGAAGGGAATTCTG-3′; set-8 forward, 5′-TTTGGTTCACAGCCGTTTTC-3′; and set-8 reverse, 5′-AAAAGTACGTGTCAGTAAGAAGGGTA-3′.

Retroviral transduction

Preparation of retroviral supernatants and transduction was performed as previously described (Dominguez et al., 2015). All five *miR-200* family overexpression constructs were cloned by PCR-amplification of genomic DNA into the pMIR WAY-GFP vectors.

Immunoblot analysis

Protein lysates from 10^6 day 15 post–LCMV-infected mice were sorted on CD8$^+$CD44$^+$ KLRG1^{hi} IL-7R^{lo} or KLRG1^{lo} IL-7R^{hi} sorted effector CTLs were lysed and resolved by SDS-PAGE. ZEB1 (Cell Signaling Technology) and β-actin (Santa Cruz Biotechnology) were detected by immunoblotting.

In vitro cultures with cytokines

Naive P14$^+$ CD8$^+$ T cells were cultured for 72 h in the presence of 10 ng/ml GP$_{33–41}$ peptide and 10 ng/ml IL-2 for 3 d followed by another 48 h culture in 10 ng/ml of IL-2, 20 ng/ml of IL-15, or 10ng/ml TGF-β (eBioscience or Peprotech). TGF-β was activated with citric acid as outlined in the product manual.

Gene expression by quantitative RT-PCR

For quantitative RT-PCR, RNA was isolated from 200,000–1,000,000 sorted cells using Qiagen RNeasy Mini kit. cDNA was synthesized using SSRTII (Life Technologies) and quantitative RT-PCR was performed on a Stratagene Mx3000P with iTaq Universal SYBR green super mix (Bio-Rad). Relative fold changes were calculated using *Rpl9* (*L9*) expression.

The following primers were used in these studies: *Zeb2* forward, 5′-GAGCAGGTAACCGCAAGTTC-3′; *Zeb2* reverse, 5′-TGTTTCTCATTCGG-3′; *Rpl9* forward, 5′-TGAAGAAATCTGTGGGTCG-3′; *Rpl9* reverse, 5′-GCACTACGGACATAGGAACTC-3′; *Zeb1* forward, 5′-CCGCCAACAAGCAGACTATT-3′; and *Zeb1* reverse, 5′-GGCGTGGAGTCAGAGTCATT-3′.

For microRNA expression, sorted cells were lysed with TRIzol (Invitrogen) and RNA extraction was performed according to the manufacturer's protocol. Taqman microRNA expression assays (for miR-200a, b, c, 429, and 141) were performed using Taqman-provided primers and following the manufacturer's protocol.

Statistical analysis

Prism 6 (GraphPad Software) was used to calculate statistics for all bar graphs shown. For comparisons of two groups, a two-tailed *t* test was performed. For multiple-group comparisons, a one-way ANOVA with Tukey's multiple comparisons test was used. For grouped multiple comparisons, a two-way ANOVA with Sidak's multiple comparison test was used (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$).

Online supplemental material

Fig. S1 shows the generation of *Zeb1* conditional KO mice and validation of deletion. Fig. S2 shows that ZEB1 plays an intrinsic role in promoting memory $CD8^+$ T cell survival. Fig. S3 shows that overexpression of *miR-200* family promotes memory $CD8^+$ T cell differentiation.

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