Transcriptional repressor ZEB2 promotes terminal differentiation of CD8\(^+\) effector and memory T cell populations during infection

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ZEB2 is a multi-zinc-finger transcription factor known to play a significant role in early neurogenesis and in epithelial–mesenchymal transition–dependent tumor metastasis. Although the function of ZEB2 in T lymphocytes is unknown, activity of the closely related family member ZEB1 has been implicated in lymphocyte development. Here, we find that ZEB2 expression is up-regulated by activated T cells, specifically in the KLRG1\(^+\) effector CD8\(^+\) T cell subset. Loss of ZEB2 expression results in a significant loss of antigen-specific CD8\(^+\) T cells after primary and secondary infection with a severe impairment in the generation of the KLRG1\(^+\) effector memory cell population. We show that ZEB2, which can bind DNA at tandem, consensus E-box sites, regulates gene expression of several E–protein targets and may directly repress Il7r and Il2 in CD8\(^+\) T cells responding to infection. Furthermore, we find that T-bet binds to highly conserved T-box sites in the Zeb2 gene and that T-bet and ZEB2 regulate similar gene expression programs in effector T cells, suggesting that T-bet acts upstream and through regulation of ZEB2. Collectively, we place ZEB2 in a larger transcriptional network that is responsible for the balance between terminal differentiation and formation of memory CD8\(^+\) T cells.

In response to intracellular pathogens, CD8\(^+\) T cells are activated to proliferate and differentiate into a heterogeneous population of effector T cells, which are armed to eliminate infected cells. After pathogen clearance, the majority of effector CD8\(^+\) T cells die; however, a subset survives and differentiates to long-lived memory T cells. Should reinfection occur, these memory cells undergo rapid expansion and redifferentiation into effector cells, providing superior protection compared with naive T cells and protecting the host for decades in many cases (Harty and Badovinac, 2008). The ability to selectively induce T cell memory would provide novel methods for provoking protective immunity and inform vaccine strategies.

Identification of effector and memory precursor CD8\(^+\) T cells within the effector population is facilitated by their distinct expression of several surface receptors. Both subsets express high levels of CD44, whereas IL-7 receptor-\(\alpha\) (CD127) is selectively up-regulated during the transition to long-lived memory cells (Kaech et al., 2003). Killer cell lectin-like receptor G1 (KLRG1) expression is inversely correlated with CD127 expression (Joshi et al., 2007) and identifies, in both mice and humans, a subset of terminally differentiated effector cells that possess limited proliferative potential and a shorter lifespan (Voehringer et al., 2002; Joshi et al., 2007).
Thus, differential expression of CD127 and KLRG1 identifies two populations of T cells during the peak of an infection: KLRG1hiCD127lo cells that consist of shorter-lived effector and effector memory cells and KLRG1hiCD127hi effector cells that include the long-lived memory precursors (Kaech and Wherry, 2007; Kallies, 2008). Notably, both populations undergo contraction as the infection is cleared; however, the KLRG1hi subset continues to contract over the months after antigen exposure, whereas the CD127hi subset provides stable, persistent memory (Sarkar et al., 2008).

The differentiation of CD8+ T cells into KLRG1hi shorter-lived effector cells in response to antigen is accompanied by dramatic changes in gene expression (Kaech et al., 2002; Goldrath et al., 2004). Although much is known about how antigen exposure and inflammatory signals impact this differentiation, the specific transcriptional pathways that control terminal differentiation versus memory formation have yet to be fully elucidated. It is now clear that multiple transcription factors work in concert during differentiation of CD8+ effector T cells to instruct terminal differentiation versus memory cell fates. These factors include, but are not limited to, T-bet, Blimp-1, Id2, and STAT4 promoting the formation of KLRG1hiCD8+ effector and effector memory T cells and Eomesodermin, Bcl-6, Id3, STAT3, FOXO1, and TCF1 favoring differentiation of CD127hi effector and memory precursor CD8+ T cells (Kaech and Cui, 2012). Many of these factors are expressed by both KLRG1hi and CD127hi effector T cells, albeit at higher levels in the subset that their expression supports. Thus, it is not yet clear how these factors assemble into a network that allows bifurcation into distinct fates.

Analysis of the transcriptional network responsible for CD8+ T cell activation and differentiation led to the identification of transcriptional regulators, including ZEB2 (also known as Zfhx1b and Sip1) not previously associated with T cell immunity (Joshi et al., 2007; Wirth et al., 2010; Best et al., 2013). ZEB2 is a two-handed zinc-finger transcription factor and one of two members of the ZEB family in vertebrates; ZEB1 and -2 bind DNA at tandem, separated (Remacle et al., 1999) consensus E-box sites (Sekido et al., 1994) and may be in direct competition for E-protein-binding sites. ZEB2 can also mediate transcriptional repression via cooperation with activated Smads or through recruitment of the corepressor CtBP as well as histone deacetylase complexes, particularly NuRD (Verschueren et al., 1999; Postigo and Dean, 1999b; van Grunsven et al., 2007; Verstappen et al., 2008).

The role of ZEB proteins in mature T cell function has not previously been investigated. However, Goossens et al. (2015) recently found that ZEB2 drives immature T cell lymphoblastic leukemia in humans and mice, and in the mouse model this is caused by enhanced leukemia initiation potential and induction of CD127 expression. Additionally, ZEB1, likely through repression of E-box–containing genes such as CD4, IL-2, GATA–3, and δ4-integrin, plays a crucial role in early T cell development (Williams et al., 1991; Higashi et al., 1997; Brabletz et al., 1999; Grégoire and Roméo, 1999; Postigo and Dean, 1999a). Here we investigate a role for ZEB2 in the differentiation of CD8+ T cells in vivo. We show that ZEB2 is necessary for the formation of KLRG1hi effector cells and that ZEB2 is directly regulated by and acts downstream of the transcription factor T-bet to repress the memory gene expression program and support terminal differentiation of effector CD8+ T cells.

**RESULTS**

The transcriptional repressor ZEB2 is up-regulated by KLRG1hi effector CD8+ T cells

Through analysis of differential gene expression by CD8+ T cells over the course of an immune response, several potentially novel regulators of transcription in CD8+ T cells were identified (Best et al., 2013). One transcription factor of particular interest was ZEB2, whose homologous family member, ZEB1, was shown to be important for the formation of early thymic precursors (Higashi et al., 1997; Postigo and Dean, 1999a). We found that Zeb2 mRNA was coregulated with genes involved in differentiation of shorter-lived effector cells, including Tbx21 (encoding T-bet), Klrgl (Fig. 1A), and Id2 (not depicted). Notably, our previous immune system–wide analysis of gene expression patterns identified ZEB2 as a putative regulator of expression of key molecules (Best et al., 2013). Zeb2 mRNA was expressed at low levels in naive CD8+ T cells but was substantially increased in CD8+ T cells responding to lymphocytic choriomeningitis virus (LCMV) infection, correlating with the expansion of KLRG1hiCD8+ T cells (Fig. 1B). Indeed, Zeb2 mRNA was expressed at >30-fold greater levels in the KLRG1hi compared with KLRG1lo effector CD8+ T cell population. In contrast, ZEB1 was not dynamically regulated in T cells responding to infection (Best et al., 2013). We also found that Zeb2 mRNA expression was considerably decreased in Id2-deficient T cells, which fail to generate KLRG1hi terminally differentiated effector CD8+ T cells (Cannarile et al., 2006; Knell et al., 2013; Masson et al., 2013), compared with WT antigen–specific T cells (not depicted). Despite extensive efforts with available reagents, we were not able to successfully evaluate mouse ZEB2 protein levels. Both Id2 and ZEB2 can function by attenuating E-protein activity, by direct binding to E-protein transcription factors and via competitive binding to E-box sites, respectively. Thus, we considered the possibility that ZEB2 acts as a second transcriptional regulator, alongside Id2, that attenuates E-protein activity and is important for the fate decisions leading to the generation of KLRG1hi effector CD8+ T cells.

Impaired responses to infection by ZEB2-deficient CD8+ T cells

We next examined ZEB2-deficient CD8+ T cells over the course of infection. Mice bearing floxed alleles of Zeb2 were backcrossed to the C57BL/6 background and then to a CD4-driven cre recombinase transgenic line to induce deletion in T cells (Sawada et al., 1994; Higashi et al., 2002). ZEB2
deficiency did not affect the phenotype of the naive T cell compartment (not depicted). We infected WT mice and mice lacking T cell–specific expression of ZEB2 with the Arm- strong strain of LCMV and monitored CD8+ T cells specific for an epitope of LCMV using MHC class I–D b tetramers loaded with the gp33 peptide (Fig. 1 C). A significantly lower frequency of LCMV-specific CD8+ T cells was observed for ZEB2-deficient compared with WT mice at the peak and during contraction of the response. To rule out additional immune defects confounding the CD8+ T cell response, we generated ZEB2-deficient P14 TCR transgenic CD8+ T cells that recognized the same LCMV epitope, which were then adoptively transferred to naive recipients and distinguished from host cells by congenic CD45 expression. The frequency of ZEB2-deficient P14 cells transferred alone or mixed with WT P14 cells was monitored in the blood of recipients after infection with LCMV (Fig. 1, D and E) or Listeria monocytogenes (Lm) expressing the LCMV peptide gp33 (Lm-gp33; not depicted). In all experimental settings, we observed a significantly impaired accumulation of ZEB2-deficient antigen–specific cells in response to infection compared with WT CD8+ T cells, showing a cell-intrinsic role for ZEB2 in the expansion of CD8+ antigen–specific effector T cell population.

In mice, ZEB2 has been shown to be important in regulating directed migration (Van de Putte et al., 2003; Goossens et al., 2011; van den Berghe et al., 2013), raising the question of whether this defect in accumulation in the blood and spleen could be the result of differential trafficking of ZEB2-deficient cells. Alternatively, ZEB2 may be acting to promote cell survival (much like Id2 [Cannarile et al., 2006]) or in the control of proliferation. First, we examined the possibility that ZEB2-deficient cells were accumulating in other tissues confounding the CD8+ T cell response, we generated ZEB2-deficient P14 TCR transgenic CD8+ T cells that recognized the same LCMV epitope, which were then adoptively transferred to naive recipients and distinguished from host cells by congenic CD45 expression. The frequency of ZEB2-deficient P14 TCR transgenic cells transferred alone or mixed with WT P14 cells was monitored in the blood of recipients after infection with LCMV (Fig. 1 E, right), and antigen–specific cells in the spleen, lymph nodes, liver, and lung were assessed at the peak of infection. Defects in accumulation were observed for ZEB2-deficient compared with WT CD8+ T cells in all tissues examined, suggesting that ZEB2-deficient CD8+ T cells were not sequestered outside of the secondary lymphoid organs. Next, to discern potential defects in proliferation and survival by ZEB2-deficient cells during infection, we monitored incorporation of BrdU or Annexin V staining, respectively, in antigen–specific CD8+ T cells recovered from the spleen after LCMV infection (Fig. 1 F). We did not find notable differences between ZEB2-deficient and WT cells.
Loss of KLRG1hi effector CD8+ T cells with ZEB2 deficiency

We next examined the phenotype of ZEB2-deficient cells over the course of the immune response. Equal numbers of Zeb2-/- and Zeb2+/+ P14 CD8+ T cells were transferred into congenically distinct naive hosts that were then infected with LCMV. Throughout infection, the frequency and absolute number of KLRG1hiCD127loCD8+ T cells lacking ZEB2 were reduced compared with WT CD8+ T cells (Fig. 2, A and B). Conversely, the frequency of the KLRG1hiCD127hi longer-lived, memory precursor CD8+ T cell population was significantly increased. However, when the absolute numbers of the KLRG1hiCD127hi subset were calculated, consistently significant differences were not observed between Zeb2-/- and Zeb2+/+ populations, showing that the increase in frequency of this population was caused by a loss of the KLRG1hi subset and not by the generation of more memory cells (Fig. 2, B and C). Infection with a different pathogen, Lm-gp33, yielded comparable results (not depicted).

A more in-depth examination of the cell surface phenotype of ZEB2-deficient CD8+ T cells revealed that the majority of Zeb2-/- cells were KLRG1hiCD127hi; they also displayed expression of additional characteristic long-lived CD8+ T cell memory markers (Fig. 2 D). Although the CD44 activation marker appears unaffected, CD8+ T cells lacking ZEB2 were predominately CD43hi and CD27hi, which corresponds to T cell populations with increased memory potential and is consistent with the enhanced frequency of the CD127hi population (Hikono et al., 2006). Interestingly, CD27 has been shown to define a population of CD8+ T cells that contain central and effector memory T cell subsets (Olson et al., 2013). High levels of CXCR3, a marker suggested to predict recall ability of memory CD8+ T cells (Hikono et al., 2007), was also noted on ZEB2-deficient CD8+ T cells throughout the course of infection. Thus, ZEB2-deficient effector CD8+ T cells differentiate into phenotypically normal memory precursor populations but form ~60-fold fewer KLRG1hi terminally differentiated CD8+ T cells in the contraction phase of the immune response (Fig. 2 and not depicted).

Similarly, cytokine production by Zeb2-/- CD8+ T cells during the immune response was consistent with typical function of the memory precursor populations. Lymphocytes were harvested and restimulated with gp-33 peptide, and levels of effector cytokines IFN-γ and IL-2 were measured by flow cytometry. No difference in IFN-γ production was observed; however, a significant increase in the frequency of IL-2-producing cells was measured in the responding Zeb2-/- CD8+ T cells as compared with WT controls. This increase correlates with the loss of the KLRG1hi terminally differentiated effector CD8+ T cell population known to produce less IL-2 during infection (Fig. 2, E and F; Sarkar et al., 2008). These data provide phenotypic and functional evidence that ZEB2 plays a role in balancing the formation of KLRG1hi shorter-lived effector versus the CD127hi long-lived memory cells, during infection, with ZEB2 promoting the formation or accumulation of KLRG1hi effector cells.

Impaired secondary response by ZEB2-deficient memory CD8+ T cells

Given that ZEB2 deficiency leads to a higher percentage of KLRG1hiCD127hi memory precursor CD8+ T cells during a primary response to infection, it might be expected that a secondary encounter of antigen would induce a more robust memory recall response. Alternatively, ZEB2-deficient cells could generate a secondary effector population with similar defects observed in the primary response. To address this, Zeb2-/- and Zeb2+/+ mice were initially infected with vesicular stomatitis virus (VSV)-OVA and then reinfected with Lm-OVA 30 d later. MHC class I-Rb-OVAp tetramer staining was used to detect antigen-specific cells in the blood over the course of the secondary infection. Despite the overrepresentation of memory precursor cells in the ZEB2-deficient primary response, the defect in expansion was not rescued in the secondary response (Fig. 3 A, left). Similar kinetics were also observed when Zeb2-/- and Zeb2+/+ P14 cells were co-transferred to congenically distinct hosts that were first infected with Lm-gp33 and then subsequently infected with LCMV (Fig. 3 A, right). In both secondary infections, we observed impaired accumulation of KLRG1hiCD127hi terminally differentiated effector CD8+ T cells (Fig. 3, B and C); however, in contrast to the primary response, we find that antigen-specific CD8+ T cells form an intermediate KLRG1hiCD127mh population. We observed a significant reduction in the frequency of KLRG1hiCD127mh cells generated by ZEB2-deficient compared with WT effector cells with a corresponding significant increase in KLRG1hiCD127hi and KLRG1loCD127hi populations, suggesting that ZEB2 may be involved in the down-regulation of CD127 rather than the up-regulation of KLRG1 by effector cells. This observation fits with the published data showing that KLRG1 is not essential for the generation of terminally differentiated effector CD8+ T cells (Gründemann et al., 2010), whereas CD127 is necessary but not sufficient for memory precursor formation (Hand et al., 2007; Haring et al., 2008).

ZEB2 deficiency favors expression of memory-associated genes

To gain insight into potential downstream targets of ZEB2, we looked for global gene expression differences between Zeb2-/- and Zeb2+/+ CD8+ effector T cells on day 6 after LCMV infection, before the dramatic differences in subset distribution were observed. Strikingly, relatively few genes were differentially expressed between the two populations on day 6 of infection (Fig. 4 A and Table S1). However, Tcf7 and Il7r both elevated in memory subsets were significantly higher in the Zeb2-/- cells, whereas effector-associated genes Zeb2, Gzma, and Klrg1 were all increased in WT cells.

In partnership with the Immunological Genome (ImmGen) Project, we previously examined the transcriptional programs initiated in CD8+ T cells during the response to in vivo infection. We grouped genes displaying differential gene expression into 10 unbiased clusters based on their kinetic patterns of
Figure 2. **Lack of ZEB2 leads to a selective loss of terminally differentiated shorter-lived effector CD8+ T cells.** (A) Co-transferred Zeb2+/+ and Zeb2−/− P14 T cells were analyzed on the indicated days after LCMV infection for KLRG1 and CD127 expression. Numbers indicate the percentage of the population in total P14 cells from a representative mouse. (B) Quantification of the frequency and total numbers of P14 populations represented in A on day 8 after LCMV infection. (C) Kinetics of the total KLRG1+CD127+CD8+ T cell population over the LCMV infection. (D) Expression of surface markers on Zeb2+/+ and Zeb2−/− P14 cells on the indicated days after LCMV infection. (E) Intracellular cytokine staining after gp33 peptide restimulation on day 10 of LCMV infection. Numbers indicate the frequency for each quadrant from a representative mouse. (F) Quantification of IFNγ+ and IFNγ+IL2+ P14 cells. Data are representative of two to three independent experiments with n = 2–3. Mean ± SEM is shown. Unpaired, two-tailed Student's t test was performed to determine significance. **, P < 0.01; ***, P < 0.001.
expression and predicted the biological function most likely associated with each cluster (Best et al., 2013). In Fig. 4B, we highlighted those clusters with the genes differentially expressed between Zeb2+/+ and Zeb2−/− P14 T cells in the PBL were assessed in mice primarily infected with Lm-gp33, followed 30 d later with LCMV (right). (A) Kinetic analysis of antigen-specific response to secondary infection. (B) KLRG1 and CD127 expression at the peak of infection after rechallenge. Numbers are the percentage of the population in total antigen-specific CD8+ T cells from a representative mouse. (C) Quantification of the frequency of antigen-specific CD8+ T cell populations represented in B. Data are representative of two independent experiments with n = 2–4. Mean ± SEM is shown. Unpaired, two-tailed Student’s t test was performed to determine significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

levels by Zeb2−/− compared with Zeb2+/+ CD8+ T cells, in spite of the fact that the differences in T cell subset composition were not significantly developed at this same time point. These data are consistent with the fact that ZEB2 deficiency ultimately leads to a smaller KLRG1hi effector population, favoring the generation of the CD127hi long-lived memory subset, and raises the possibility that ZEB2 represses the memory gene expression program.
tion (ChIP–seq data that examined the E-box occupancy of E2A (and the bHLH factor EBF1) in B cells (Lin et al., 2010) to cross reference against the genes we identified to be differentially expressed in Zeb2+/− and Zeb2−/− cells (not depicted). Although not all genes up- or down-regulated in Zeb2−/− CD8+ T cells were found to be potential E2A targets, many of the most differentially expressed did have putative E-box sites that could be bound by E2A. Of the 82 genes expressed 1.5-fold higher by Zeb2−/− T cells, 13 were found to be E2A targets, and of the 200 genes expressed 1.5-fold higher by WT cells, 21 were found to be E2A targets. These potential E-protein target genes that are differentially expressed by ZEB2−/− and WT cells include Cx3cr1, Zeb2 itself, Nsg2, Il7r, Tcf7, and Cxcr5 and suggest that at least a subset of ZEB2-regulated genes in CD8+ T cells are also E-protein targets.

ZEB2 could act directly or indirectly to turn off genes necessary for the formation of terminally differentiated effector cells and shorter-lived KLRG1hi effector memory cells during infection. This places ZEB2 at a potential branch point of two effector lineages early in a response: the shorter-lived effector cells, which express low levels of CD127 and IL-2 and high levels of KLRG1 and make up the bulk (up to 90%) of the CD8+ T cell response, versus the memory precursor effector cells, which have effector function and contain cells that will seed the long-lived memory T cell population. To establish whether differentially expressed genes were directly regulated by ZEB2, we examined individual genes that contain potential ZEB2-binding sites (bipartite E-box sites [Remacle et al., 1999]) by ChIP. As analysis of the ZEB2-deficient CD8+ T cell populations revealed increased levels of CD127 expression (see also Fig. 3 A), we hypothesized that ZEB2 may be directly repressing the Il7r gene. Consistent with this, we find that anti-ZEB2–precipitated chromatin was indeed enriched for sequences in the Il7r promoter and introns, which contain bipartite E-box sites (Fig. 4 C). It has been previously published that there are regulatory binding sites for ZEB1, which contain remarkable DNA-binding domain similarity to ZEB2, in the Il2 gene that can mediate repression of Il2 expression (Williams et al., 1991; Yasui et al., 1998). Our analysis indicates that ZEB2 occupies these sites in activated CD8+ T cells (Fig. 4 C). Thus, it appears that ZEB2 may share targets with its homologue ZEB1 and could be responsible for the diminished production of IL-2 by KLRG1hi effector memory cells (Joshi et al., 2007; Sarkar et al., 2008).

Zeb2 is a target of T-bet

Our data show that ZEB2 is key in CD8+ effector T cell differentiation and is likely part of a larger regulatory network. Interestingly, the T-box–binding transcription factor, T-bet, has also been shown to promote KLRG1hi effector cell differentiation (Joshi et al., 2007). T-bet protein levels were not notably affected by the absence or overexpression of ZEB2 in this system (Fig. 5 A and not depicted), suggesting that, if they are in the same transcriptional pathway, T-bet is likely upstream of ZEB2. In line with this, antigen-specific CD8+ T cells heterozygous for Tbx21 (Tbx21+−) isolated on day 7 of Lm-OVA infection showed lower Zeb2 mRNA levels compared with those expressing WT levels of T-bet. Upon examination of the regulatory regions of the Zeb2 gene, we found numerous, highly conserved predicted T-box sites upstream of the transcriptional start sight and in the 3′ untranslated region (UTR)–coding region of Zeb2, suggesting that T-bet could directly regulate Zeb2 expression. To address this, we performed ChIP analysis on WT bulk or KLRG1hiCD8+ T cells isolated on day 8 of infection with LCMV. We found that T-bet–precipitated chromatin was enriched for sequences in sites 5′ to the Zeb2 coding sequence and in the 3′ UTR and that this binding was lost in Tbx21−/− cells (Fig. 5 C). Thus, our data place ZEB2 downstream of T-bet in the regulation of gene expression, promoting the differentiation of KLRG1hi effector CD8+ T cells.

To further investigate the relationship between T-bet and ZEB2, we used microarray analysis of Tbx21−/− and Tbx21+/− CD8+ T cells at day 6 of infection and compared global expression differences to genes differentially expressed between Zeb2−/− and Zeb2+/− effector CD8+ T cells identified above (Fig. 4 A). Many genes were similarly regulated in the two comparisons (Fig. 5 D; R = 0.308). Consistent with the notion that T-bet is upstream of ZEB2 activity, T-bet expression was not elevated in ZEB2-deficient versus WT CD8+ T cells. Furthermore, when we focused on genes up-regulated in Tbx21−/− CD8+ T cells compared with their WT counterparts, 75% of those genes were also up-regulated by Zeb2−/− CD8+ T cells compared with their WT counterparts (Fig. 5 E, top). Similarly, of genes with lower expression in Tbx21−/− cells, 75% were also down-regulated in Zeb2−/− cells compared with WT cells (Fig. 5 E). These data suggest that a large subset of T-bet–regulated genes are also regulated by ZEB2, whereas 25% of the T-bet–regulated gene expression program is independent of ZEB2 activity. Conversely, we see that 100% of genes up-regulated with loss of ZEB2 were correspondingly up-regulated in T-bet−/− T cells and 85% of the genes down-regulated with loss of ZEB2 were also down-regulated by Tbx21−/− T cells (Fig. 5 E, bottom), again indicating that T-bet may regulate ZEB2-dependent and -independent gene expression and that ZEB2-dependent genes are largely T-bet dependent.

Haploinsufficiency of ZEB2 does not affect peripheral T cell populations

Upon examination of the Zeb2−/− mice, we found they exhibit a modest accumulation defect compared with the Zeb2+/− mice after infection with VSV-OVA (Fig. 6 A), with a trend toward a loss of KLRG1hi effector CD8+ T cells at the peak of infection (Fig. 6 B). To see whether this translates to a human phenotype, we examined Mowat–Wilson syndrome (MWS) patients, who display a rare disorder resulting in facial dysmorphism, congenital heart disease, and lack of innervation of the lower bowel caused by haploinsufficiency of the Zeb2 gene (Mowat et al., 1998). To our knowledge, the peripheral im-
The immune status of these patients has not been examined. Here, we analyzed peripheral blood lymphocytes of five MWS patients with confirmed Zeb2 mutations. Compared with controls, the frequency of the CD4+ and CD8+ T cells appeared unaffected, whereas a trend toward a reduced percentage of CD19+ B cells was observed (Fig. 6, C and D). Furthermore, we noted minimal differences in the KLRG1 and CD127 CD8+ T cell populations between the MWS and control peripheral blood samples (Fig. 6, E).

To further examine whether the CD8+ T cell differentiation defect extends from our Zeb2+/- mouse model to haploinsufficient human patients, we characterized the naive (CCR7+CD45RA+), central memory (CCR7-CD45RA+), effector (CCR7-CD45RA-), and effector memory (CCR7-CD45RA-) CD8+ T cells in the blood. Again, we observed similar representation of all populations between MWS patients and controls (Fig. 6, F). These results suggest that haploinsufficiency of Zeb2 in humans is not sufficient to manifest CD8+ T cell defects despite adversely affecting other systems (Garavelli and Mainardi, 2007).

**DISCUSSION**

Recent studies have revealed numerous transcriptional regulators that govern formation of terminal effector versus memory CD8+ T cell populations (Kaech and Cui, 2012). Many of these factors are expressed in a gradient within the differentiating CD8+ T cell effector population, but when their expression is lost they prove to be key in supporting either effector or memory fates, raising the question as to how small differences in expression of transcription factors can enforce distinct differentiation programs. Notably, ZEB2 is expressed almost exclusively in the KLRG1hi terminally differentiated effector and effector memory populations. This correlation of ZEB2 expression with degree of differentiation has also been reported in human memory T cell subsets (Gattinoni et al., 2011) and observed in a profile of repetitively stimulated mouse memory CD8+ T cells (Wirth et al., 2010). Here, we describe a novel role for the transcriptional repressor ZEB2 in regulating the differentiation of CD8+ T cells responding to infection, placing its activity in the transcriptional network that represses the
memory program and promotes terminal differentiation. In the absence of ZEB2, we see a dramatic reduction in the number of CD8⁺ T cells responding to primary and secondary infection, which can be accounted for by a loss of the KLRG1⁺CD127lo subset, consistent with high ZEB2 expression. Additionally, we find that expression of genes associated with naive, memory, and memory precursor cells is enhanced when ZEB2 is absent, suggesting ZEB2 functions to repress the memory program in effector CD8⁺ T cells. Our data highlight a previously undefined role for ZEB2 in the cell fate decision of effector CD8⁺ T cells.

We place ZEB2 as a downstream target of T-bet in the regulatory network that promotes the formation of the KLRG1hi terminally differentiated subset of activated CD8⁺ T cells. Our microarray analysis shows ZEB2-mediated gene expression patterns are largely overlapping with those regulated by T-bet. Our ChIP analysis confirms that T-bet binds sites both 5’ to the coding sequence and in the 3’ UTR–coding region of Zeb2 and thus may directly impact expression of Zeb2 in the context of infection. T-bet protein is expressed in a gradient across effector CD8⁺ T cell populations with the highest amounts observed in KLRG1hi shorter-lived effector cells and twofold lower levels in memory precursor effector cells (Takemoto et al., 2006; Joshi et al., 2007). Elevated levels in KLRG1hi shorter-lived effector cells may allow for increased T-bet–mediated gene expression, including induction of ZEB2 expression itself. In the absence of T-bet, a similar but more dramatic phenotype to that of ZEB2 deficiency is observed with a reduced accumulation of effector CD8⁺ T cells after infection and an almost complete lack of terminally differentiated effector CD8⁺ T cells (Joshi et al., 2007). This fits with the notion that T-bet mediates a gene expression program that largely involves cooperation of ZEB2 but that also includes ZEB2-independent targets.

We propose that ZEB2 may also act in concert or parallel to Id2 to regulate the transcriptional program of KLRG1hiCD8⁺ T cells. Id2 is expressed in both the KLRG1hi
and KLRG1<sup>lo</sup> effector subsets. Similar to ZEB2, Id2 is necessary for the survival and differentiation of mature CD8<sup>+</sup> T cell as CD8<sup>+</sup> T cells lacking Id2 do not generate a terminally differentiated effector population (Cannarile et al., 2006; Yang et al., 2011; Knell et al., 2013). The mechanisms of ZEB2 and Id2 likely allow for overlapping but not necessarily identical regulation. Id proteins have no DNA-binding domain but rather bind E-proteins and prevent them from binding their gene targets (Massari and Murre, 2000; Engel and Murre, 2001), whereas ZEB2 is able to compete with E-proteins to bind DNA (Sekido et al., 1994; Remacle et al., 1999). Additional selectivity may be garnered by slight variations in sequences bound by ZEB2 and E-proteins. E-proteins, in general, bind a canonical CANNNTG (Murre et al., 1989; Engel and Murre, 2001), whereas ZEB2 more specifically binds a bipartite repeat of the CACCT(G), often referred to as an E2-box, suggesting regulation of a smaller, more specific subset of E-protein target genes. KLRG1<sup>lo</sup>CD8<sup>+</sup> T cells may be more sensitive to E-protein activity than their memory precursor counterparts and require the dual inhibitory action of both Id2 and ZEB2 for development.

One important question is what are the direct targets of ZEB2? ZEB2 likely mediates some of its regulation by binding to E-box sites in target genes. We find that a portion of genes showing differential expression between Zeb<sup>2</sup><sup>−/−</sup> and Zeb<sup>2</sup>+/− CD8<sup>+</sup> T cells are putative E2A targets. Specifically, we show an enrichment of E-boxes in both the Il2 and Il7r genes when we isolated DNA bound by ZEB2. IL-2 is expressed at lower levels in terminally differentiated effector CD8<sup>+</sup> T cells than their memory precursor counterparts (Sarkar et al., 2008). ZEB2 may directly target Il2, providing an explanation for the inability of KLRG1<sup>lo</sup>CD8<sup>+</sup> T cells to produce this cytokine as well as the increased responsiveness of this population to IL-2 (Rubinstein et al., 2008). Similarly, we find ZEB2 bound at proximal enhancers in the Il7r locus. Though not sufficient for memory precursor formation, CD127 surface expression correlates with memory potential during infection. In the secondary response, ZEB2-deficient T cells are capable of up-regulating KLRG1 but do not down-regulate CD127. Thus, ZEB2 may function to inhibit the expression of Il7r in terminally differentiated effector CD8<sup>+</sup> T cells. Notably, a recent study also provides evidence for ZEB2-mediated regulation of Il7r expression (Goossens et al., 2015). In this instance, ZEB2 expression in early T cell development initiated T cell leukemia via activation of the JAK/STAT signaling pathway downstream of elevated Il7r mRNA expression, and blockade of CD127 impaired tumor survival and growth, indicating an essential role for IL-7-mediated signaling in this model (Goossens et al., 2015). The role of ZEB2 as a driver of, as opposed to a repressor of, Il7r expression may be the result of additional factors that ZEB2 associates with (and additional posttranslational modification of components in the ZEB2 complex) in different cell types or differentiation stages or as a result of a transformation event. In addition to binding E-boxes, ZEB2 is able to bind activated Smads and mediate suppression of Smad targets (Verschueren et al., 1999). The role of Smad-mediated ZEB2 repression in CD8<sup>+</sup> T cell differentiation will be the focus of future studies.

Here we provide evidence that ZEB2 is essential in the generation of the CD8<sup>+</sup> T cell response to infection and is necessary for accumulation of terminally differentiated effector CD8<sup>+</sup> T cells. Our data support the finding that ZEB2 expression is downstream of T-bet and is most highly induced during infection and after repetitive antigen stimulation (Wirth et al., 2010). This places ZEB2 as one of the key regulators of effector CD8<sup>+</sup> T cell terminal differentiation and highlights a key fate decision point in the formation of CD8<sup>+</sup> T cell memory. The function of ZEB2 in regulating terminal differentiation of CD8<sup>+</sup> T cells, similar to other systems (see Conidi et al. [2011]), and the fact that it is a zinc-finger–containing transcription factor make it an interesting and specific therapeutic target as has been shown for other zinc-finger transcription factors, including the Snail family (Rice et al., 1997; Harney et al., 2009, 2012; Larabee et al., 2009). Furthermore, these data help foster a basal understanding of the mechanisms of memory formation, providing insight that may inform future vaccine design.

**MATERIALS AND METHODS**

**Mice.** Mice with a loxP-flanked ZEB2 allele were a gift of D. Huylenbroeck (Higashi et al., 2002). These mice were originally derived on the outbred Swiss CD-1 background and were backcrossed >10 times to C57BL/6j mice to achieve 99.9% similarity, as verified by GenCheck Speed Congenics services (Harlan Laboratories, Inc.). Mice were crossed to the CD4-cre recombinase strain (Sawada et al., 1994) and subsequently to either OT-I or P14 transgenic lines. WT littermates were used for controls.

For microarray experiments, male C57BL/6j mice were obtained from the Jackson Laboratory and housed in specific pathogen–free conditions for 7–10 d before experimentation. All mice were bred and housed in specific pathogen–free conditions in accordance with the Institutional Animal Care and Use Guidelines of the University of California, San Diego (UCSD).

**Human experiments.** After obtaining informed consent under a Human Research Protections Program–UCSD–approved protocol and from the ethics committee of the Medical Faculty at the University of Erlangen–Nuremberg, whole blood samples were obtained from five patients suffering from MWS and six healthy controls. Two patients have been previously published (Zweier et al., 2002).

**Quantitative PCR (qPCR).** RNA was extracted using TRIzol reagent (Life Technologies), and cDNA was reverse transcribed with a Superscript III First-Strand Synthesis kit (Life Technologies). qPCR was performed using Stratagene Brilliant II Sybr Green master mix (Agilent Technologies). Quantities of transcripts were normalized to GAPDH levels. The following primers were used: ZEB2, 5′-CATGAACCATTTAGTGCCA-3′.
and 5′-AGCAAGTCTCCGTGAAATC-3′; T-bet, 5′-AGC AAGGAGGCGGAATTTT-3′ and 5′-GTGGACATATAAG CGGTTCCTCC-3′; and GAPDH, 5′-CGGATGATGACTCC ACTCAGC-3′ and 5′-GACTCCACGATACTACTGAC-3′.

**Infection.** Mice were infected intravenously with 5 × 10⁵ CFU recombinant Lm-OVA or Lm-gp33 or infected intraperitoneally with 5 × 10⁶ PFU recombinant VSV-OVA or 2 × 10⁶ PFU LCMV-Armstrong. For adoptive transfer experiments, 5 × 10⁵ CD45.1+ OT-I or P14 ZEB2−/− and CD45.1.2+ OT-I or P14 ZEB2 WT cells were singly or co-transferred (1:1 ratio) into CD45.2+ C57BL/6J recipients 1 d before infection. For secondary infections, mice were rechallenged intravenously with 5 × 10⁴ CFU (for VSV-OVA primary infections) or intraperitoneally with 5 × 10⁴ Lm-gp33 (for LCMV primary infections).

**Flow cytometry.** Single-cell suspensions were prepared from spleen, lymph node, liver, lung, or blood. The following antibodies were used (all from eBioscience unless specified otherwise): CD8 (53-6.7), CD27 (LG-7F9), CD44 (IM7), CD45.1 (A20-1.7), CD45.2 (104), CCR7-M21), and CCR7 (3D12). Samples were collected on a FACSCalibur, FACS LSRFortessa, or FACSAria (BD) and analyzed with FlowJo software (Tree Star).

**ChIP.** 4.0 × 10⁷ CD8+ T cells were FACs sorted from splenocytes prepared from mice infected with Lm-OVA, fixed in 1% formaldehyde for 10 min, and subsequently quenched by adding glycine to a final concentration of 0.125 M. Cells were lysed in buffer (1% SDS, 10 mM EDTA, pH 8, and 50 mM Tris-HCl, pH 8) and then sonicated to generate ~250–750-bp fragments. Immunoprecipitation was performed with 5 µg antibody (Seumtjens et al., 2009) on 10 µg DNA and allowed to rotate overnight at 4°C. 30 µl protein F agarose beads (Cell Signaling Technology) were then added and allowed to rotate for 2 h at 4°C. Beads were washed, spun, and eluted according to the Cell Signaling Technology protocol. DNA was reverse cross-linked overnight at 65°C and then treated for 30 min at 37°C with R.Nase, followed by 2 h at 55°C with Proteinase K. qPCR was performed using Stratagene Brilliant II Sybr Green kit (Agilent Technologies). Primer sets used were Il7ri, 5′-TGC TTAGATGTTCTCATTGAA-3′ and 5′-TTTGCAAG AGGTTCCTATTTAC-3′; Iflrp, 5′-TCCCGCAAATCTAT TTAGATTTCTC-3′ and 5′-TCTATTTGAGGGACATC ATTT-3′; Il22kh, 5′-CATGCGAGAGTTTTTGTGTGGT GTTTCTAG-3′ and 5′-GCCCTAATTGTCCTCACA AAGACAGA-3′; Il2prox, 5′-CAAGCTGACTCTTTAG AATATGTGTA-3′ and 5′-CATGGAGAGCAATTTTA CTGTGAATG-3′; Tbixa, 5′-ACCAACTGACACCGG AGA-3′ and 5′-ACTCTGCTTTGCTGCTGACACAG-3′; and Tbix3, 5′-TTGAGCCACCGCTGTGACGA-3′ and 5′-TGA CCTAAATATAAATGACAA-3′.

**Microarray analysis.** CD8+CD45.1.2+ (Zeb2−/−) or CD8+CD45.2+ (Zeb2+/+) P14 transgenic T cells were sorted into TRIzol reagent from three pooled mice 6 d after LCMV infection. CD8+CD45.1.2+ (T-bet−/−) and CD8+CD45.2+ (T-bet+/+) OT-I transgenic T cells were similarly sorted from three pooled mice 6 d after Lm-OVA infection. RNA was amplified and hybridized to the Mouse Gene 1.0 ST Array (Affymetrix). Microarray analysis was performed using the GenePattern suite and R. Using the ImmGen Project profiling and quality control (QC) pipelines, gene expression profiles were generated on Mouse Gene 1.0 ST arrays. All data analyzed passed ImmGen QC, with good replicate quality. The general ImmGen postnormalization threshold of 120 was taken to indicate positive expression (at 95% confidence), and probes were included in comparisons only if they were expressed by at least one cell type and with low variability within populations (CV < 0.5). The microarray data are deposited in the GEO database under accession no. GSE72162.

**Online supplemental material.** A list of genes with differential gene expression between ZEB2-deficient and -sufficient P14 CD8+ T cells on day 6 after LCMV infection can be found in Table S1. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20150194/DC1.

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