Title

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Journal

Journal of Experimental Medicine, 215(3)

ISSN

0022-1007

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Publication Date

2018-03-05

DOI

10.1084/jem.20171584

Peer reviewed

JEM

Brief Definitive Report

Sustained Id2 regulation of E proteins is required for terminal differentiation of effector CD8$^+$ T cells

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CD8$^+$ T cells responding to infection differentiate into a heterogeneous population composed of progeny that are short-lived and participate in the immediate, acute response and those that provide long-lasting host protection. Although it is appreciated that distinct functional and phenotypic CD8$^+$ T cell subsets persist, it is unclear whether there is plasticity among subsets and what mechanisms maintain subset-specific differences. Here, we show that continued Id2 regulation of E-protein activity is required to maintain the KLRG1^{hi} CD8$^+$ T cell population after lymphocytic choriomeningitis virus infection. Induced deletion of *Id2* phenotypically and transcriptionally transformed the KLRG1^{hi} "terminal" effector/effector-memory CD8$^+$ T cell population into a KLRG1^{lo} memory-like population, promoting a gene-expression program that resembled that of central memory T cells. Our results question the idea that KLRG1^{hi} CD8$^+$ T cells are necessarily terminally programmed and suggest that sustained regulation is required to maintain distinct CD8$^+$ T cell states.

Journal of Experimental Medicine

INTRODUCTION

In response to acute infection, naive antigen-specific CD8$^+$ T cells become activated, proliferate, and differentiate into a heterogeneous population of effector cells with the functional capacity to eliminate the pathogen. Many effector CD8$^+$ T cells within this population are thought to be terminally fated to undergo apoptosis upon resolution of the infection. Others appear to be programmed for long-term survival and uniquely suited to protect the host upon reinfection (Chang et al., 2014). Considerable work in the field has focused on relating effector CD8$^+$ T cell phenotype to cell fate. Two cell-surface receptors, killer cell lectin-like receptor G1 (KLRG1) and interleukin 7 receptor α (CD127), have been valuable in predicting the fates of CD8$^+$ T cell populations at the peak of the effector response. During the effector phase of infection, CD8$^+$ T cells expressing KLRG1 and low levels of CD127, called terminal effector (TE) cells, are often defined as terminally differentiated, have a shorter life span and exhibit minimal memory potential in adoptive transfer experiments. CD8$^+$ T cells with low KLRG1 and high CD127 surface expression in the effector phase have been defined as memory-precursor (MP) T cells and show a greater propensity to survive after infection and exhibit increased stem-like properties such as self-renewal (Kaech et al., 2003; Joshi et al., 2007; Sarkar et al., 2008).

At memory time points, the relationship of the canonical markers, KLRG1 and CD127, to cell fate becomes less clear. Memory CD8$^+$ T cells have been classified into subsets based on several criteria including location, effector function, capacity for self-renewal, and trafficking patterns. The best characterized distinction is that of effector memory (T_{EM}) and central memory (T_{CM}) T cells, based on CD62L and CCR7 expression (Sallusto et al., 1999). T_{EM} cells that lack CD62L and CCR7 expression circulate through nonlymphoid tissues and the blood and are poised to provide immediate effector function but have limited proliferation potential upon recall (Mueller et al., 2013). T_{CM} cells express CD62L and CCR7 and thus home to lymphoid tissues and provide a long-term, self-renewing pool of T cells (Mueller et al., 2013). Overlaying the KLRG1 and CD127 phenotypic characterization of T cells adds a level of complexity to defining memory T cell subsets. Although CD127 expression supports long-term survival of memory T cells, the classification of T_{EM} and T_{CM} has not explicitly included the expression of CD127 or exclusion of KLRG1. Within the T_{EM} population, KLRG1 expression can be detected on a portion of cells (Masopust et al., 2006; Hikono et al., 2007; Phan et al., 2016; Kakaradov et al., 2017). This observation is consistent with T_{EM} exhibiting more effector-like properties and being more terminally differentiated (Kaech and Cui, 2012); however, variable KLRG1 expression suggests the T_{EM} population itself is heterogeneous. Furthermore, a sizeable population of CD8$^+$ T cells defined as KLRG1^{hi}CD127^{lo} TE T cells at the effector stage survive after the infection has resolved and persist at memory time points, but the population continues to diminish relative to the KLRG1^{lo} population, further supporting the idea that these cells are terminally fated (Olson et al., 2013).

Unique transcriptional programs have been described that drive the differentiation of CD8$^+$ T cells during infec-

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The Rockefeller University Press
J. Exp. Med. 2018 Vol. 215 No. 3 773–783
https://doi.org/10.1084/jem.20171584

tion—with T-bet, Blimp-1, IRF4, Zeb2, and Id2 acting as critical regulators of the TE $CD8^+$ T cell population and Tcf1, Eomes, Bcl6, Foxo1, Id3, and E proteins regulating the MP $CD8^+$ T cell population (Kaech et al., 2003; Joshi et al., 2007; Zhou et al., 2010; Chang et al., 2014). Although it is clear that these transcriptional regulators are key for the generation of effector and memory $CD8^+$ T cell populations, little is known about their roles in maintaining subset-specific gene-expression programs. When considering the transition of $CD8^+$ effector T cells to memory populations, important questions arise: are effector $CD8^+$ T cell populations unconditionally committed to their specified fate after infection resolution, or does plasticity exist and is active transcriptional regulation necessary to continually enforce subset specificity?

E-protein transcription factors (TFs) and their repressors, Id (inhibitor of DNA binding) proteins, have emerged as key regulators of effector and memory $CD8^+$ T cell differentiation (Omilusik et al., 2013). E-protein activity increases upon T cell activation to induce a transcriptional network that promotes the formation of MP $CD8^+$ T cells; and loss of E2A and HEB, two E-protein family members, results in increased frequencies of $KLRG1^{hi}$ effector $CD8^+$ T cells after infection (D'Cruz et al., 2012). Id2 and Id3 are both thought to function by antagonizing E-protein activity, yet they differentially impact the formation of $CD8^+$ effector and memory T cell populations after acute infection (Cannarile et al., 2006; Ji et al., 2011; Yang et al., 2011; Knell et al., 2013; Masson et al., 2013). Loss of Id2 in naive $CD8^+$ T cells results in an effector $CD8^+$ T cell population that is highly susceptible to apoptosis with impaired survival after several bacterial or viral infections (Cannarile et al., 2006; Knell et al., 2016). Importantly, Id2-deficient $CD8^+$ T cells fail to form the $KLRG1^{hi}$ TE subset and instead adopt an MP phenotype (Cannarile et al., 2006; Knell et al., 2013; Masson et al., 2013). Conversely, $CD8^+$ T cells lacking Id3 have defects in formation and survival of long-lived memory (Ji et al., 2011; Yang et al., 2011). Here, we consider the transcriptional networks that actively maintain $CD8^+$ T cell commitment and the acquired phenotypic and functional subset-specific properties. In particular, we examine the role of Id2 and E-proteins in enforcing the TE $CD8^+$ T cell fate after resolution of infection. We find that memory T cell fates are more plastic than anticipated and that sustained regulation of E-protein activity is necessary to maintain the effector or TE $CD8^+$ T cell phenotype.

RESULTS AND DISCUSSION

Id2 is expressed in $CD8^+$ T cells throughout the effector and into the memory phase after infection (Fig. S1 A; Cannarile et al., 2006). Id2 promotes survival and terminal differentiation of $CD8^+$ T cells at the peak of several acute infections (Cannarile et al., 2006; Knell et al., 2013; Fig. S1, B–H). Effector and TE $CD8^+$ T cells deficient for Id2 fail to accumulate after infection, and the lack of $CD8^+$ T cells makes it difficult to attribute a functional role for Id2 in homeostasis of T cell populations at later time points. To circumvent this, we crossed mice with loxP-flanked *Id2* alleles ($Id2^{f/f}$; Niola et al., 2012) to *Rosa26*Cre-ERT2 mice (ERCre) to allow inducible deletion of *Id2* upon tamoxifen treatment. These mice were further mated with P14 transgenic mice that express an $H\text{-}2D^b$–restricted T cell antigen-receptor specific for lymphocytic choriomeningitis virus (LCMV) glycoprotein$_{33–41}$. Treatment of naive $Id2^{f/f}$-$ERCre^+$ P14 mice with tamoxifen induced a near complete deletion of *Id2* in $CD8^+$ T cells when compared with $Id2^{f/f}$-$ERCre^-$ P14 T cells that had been similarly treated (Fig. S1, B and C; mice called Id2WT or Id2KO after induced *Id2* deletion with tamoxifen). Upon mixed transfer of P14 T cells into congenically distinct hosts, $CD8^+$ T cells with induced deletion of *Id2* responding to LCMV infection had an accumulation and differentiation defect on day 8 of infection compared with Id2WT $CD8^+$ T cells consistent with our previously published results studying $CD8^+$ T cells with germline *Id2* deficiency after infection (Fig. S1, D and E; Cannarile et al., 2006; Knell et al., 2013). Further, when deletion was induced at the peak of infection (5–10 d), the *Id2*-deleted effector and TE $CD8^+$ T cells rapidly declined within days compared with the Id2WT cells (Fig. S1, F–H).

Having developed an efficient model in which to induce deletion of *Id2*, we sought to assess the role of Id2 in established memory $CD8^+$ T cells. Untreated $Id2^{f/f}$-$ERCre^+$ and $Id2^{f/f}$-$ERCre^-$ P14 $CD8^+$ T cells were co-transferred at a 1:1 ratio into congenically distinct recipient mice 1 d before LCMV infection. After day 30 of infection, recipient mice were treated with tamoxifen to induce deletion of *Id2* (Fig. 1 A; and Fig. S1, I and J). After tamoxifen treatment (15–16 d), Id2WT and Id2KO $CD8^+$ T cells persisted in the blood, spleen, and lymph nodes (LNs) of the host at equivalent frequencies and numbers (Fig. 1, B and C; and Fig. S1 K). Strikingly, in this short duration of Id2 deficiency, the phenotype of the Id2KO $CD8^+$ T cell population significantly changed compared with that of the Id2WT cells. Most prominently in the blood and spleen, the shorter-lived, more effector-like $KLRG1^{hi}CD127^{lo}$ and $KLRG1^{hi}CD127^{hi}$ Id2KO $CD8^+$ T cell subsets almost completely disappeared, whereas the frequency of the more long-lived $KLRG1^{lo}CD127^{hi}$ Id2KO $CD8^+$ T cells significantly increased (Fig. 1, B and D; Joshi et al., 2007; Sarkar et al., 2008). No differences in Ki67 were observed, indicating that the phenotypic changes occurred in the absence of proliferation (not depicted). Consistent with a conversion by the effector-like cells, the $CD27^{lo}CD43^{lo}$ $CD8^+$ T cell population, a subset of long-lived effector T cells (Olson et al., 2013), was also lost upon *Id2* deletion, whereas a proportional increase in the $CD27^{hi}CD43^{lo/hi}$ $CD8^+$ T cells was observed when compared with the Id2WT controls (Fig. 1, B and D). Furthermore, when analyzing the $CD8^+$ T cells from the blood and spleen based on CD62L, a marker classically used to define circulating memory $CD8^+$ T cells or T_{CM} cells (Sallusto et al., 1999), we found that the frequency of the $CD62L^{lo}CD127^{lo}$ long-lived effector Id2KO $CD8^+$ T cells declined compared with Id2WT $CD8^+$ T cells. (Fig. 1, E and F). The loss of cells with a $KLRG1^{hi}$ phenotype in the Id2KO

$CD8^+$ T cell population was accompanied by increased expression of memory-associated TFs, Tcf1 and Foxo1 (Zhou et al., 2010; Hess Michelini et al., 2013), and a decrease in the effector-associated protein, T-bet (Joshi et al., 2007; Fig. 1 G). There were not significant differences in CD122 or CD127 levels for the Id2-deficient cells that had converted to a $KLRG1^{lo}$ phenotype compared with WT cells, indicating a similar potential for cytokine responsiveness (not depicted). We did note that at >20 d after induced *Id2* deletion, the ratio of Id2KO to Id2WT donor cells began to fall, indicating that Id2 deficiency did eventually impair survival, in addition to profoundly affecting phenotype at earlier time points (Fig. S1 K). These results suggest that the effector differentiation state may be more actively controlled than previously appreciated and that continual Id2 transcriptional regulation is required to maintain the $KLRG1^{hi}$ effector/effector-memory $CD8^+$ T cell differentiation.

The maintenance of Id2WT and Id2KO $CD8^+$ T cells in a 1:1 ratio after *Id2* deletion at early time points indicates that, in the absence of regulation of E-protein activity, effector-like Id2KO $CD8^+$ T cells that have been presumed to be terminally fated dedifferentiate to resemble the long-lived $KLRG1^{lo}CD127^{hi}CD27^{hi}$ T cell population. To understand if the loss of day 30 $KLRG1^{hi}$ effector/effector-memory Id2KO $CD8^+$ T cells was due to altered cell fates, cell death, or increased $KLRG1^{lo}$ cell proliferation, we sorted, transferred, and induced *Id2* deletion in $CD8^+$ T cell subsets and followed them over time (Fig. 2 A). Untreated $Id2^{f/f}$-$ERCre^+$ and $Id2^{f/f}$-$ERCre^-$ P14 $CD8^+$ T cells were co-transferred into congenically distinct recipient mice that were infected the next day with LCMV. After day 30 of infection, $KLRG1^{hi}CD127^{lo}$ and $KLRG1^{lo}CD127^{hi}$ P14 $CD8^+$ T cells subsets that were $Id2^{f/f}$-$ERCre^+$ or $Id2^{f/f}$-$ERCre^-$ were sorted from the spleen and LN of recipient mice. $KLRG1^{hi}CD127^{lo}$ $Id2^{f/f}$-$ERCre^+$ and $Id2^{f/f}$-$ERCre^-$ ($KLRG1^{hi}$ transfer) or $KLRG1^{lo}CD127^{hi}$ $Id2^{f/f}$-$ERCre^+$ and $Id2^{f/f}$-$ERCre^-$ ($KLRG1^{lo}$ transfer) were mixed at a 1:1 ratio and transferred to new congenically distinct recipient mice, which were subsequently treated with tamoxifen to induce *Id2* deletion (Fig. S2, A and B). The frequency and number of Id2WT and Id2KO $CD8^+$ T cells in the spleens of recipient mice receiving the $KLRG1^{hi}$ $CD8^+$ T cell transfer was not affected on day 6 or 32 after tamoxifen treatment (Fig. 2, B and C). However, by day 32, the proportion and number of Id2KO $CD8^+$ T cells from the $KLRG1^{lo}$ transfer were significantly reduced compared with the Id2WT $CD8^+$ T cells. The phenotype of the transferred $KLRG1^{lo}$ $CD8^+$ T cells remained $KLRG1^{lo}$ $CD127^{hi}$ and $CD27^{hi}$ after *Id2* deletion, whereas the $KLRG1^{hi}$ Id2KO $CD8^+$ T cells significantly down-regulated KLRG1 and up-regulated CD27, resembling a more long-lived T cell population (Fig. 2, B and D). The Id2WT cells in the $KLRG1^{hi}$ transfer partially up-regulated CD127, albeit not to the degree as the Id2KO $CD8^+$ T cells, likely to support the long-term survival of the transferred cells. Notably, there is an increase in CD62L expression among $KLRG1^{hi}$ transferred cells after *Id2* deletion (Fig. 2, E and F). The Id2KO $KLRG1^{hi}$ $CD8^+$ T cells also significantly up-regulated Tcf1 expression and down-regulated T-bet expression consistent with reassignment from an effector-like phenotype to a memory-like phenotype (Fig. 2 G). The $KLRG1^{lo}$ transferred cells lost Eomes expression after *Id2* deletion (Figs. 1 G and 2 F), suggesting that Id2 differentially affects the $CD8^+$ T cell populations consistent with multiple roles—promoting survival in the $KLRG1^{lo}CD127^{hi}$ $CD8^+$ T cells and enforcing differentiation in the $KLRG1^{hi}CD127^{lo}$ $CD8^+$ T cells. These results imply that differentiation to a $KLRG1^{hi}$ effector $CD8^+$ T cell subset is a reversible process and that sustained Id2 regulation enforces the cell state of "terminal differentiation."

The loss of Id2 in long-lived $KLRG1^{hi}$ effector $CD8^+$ T cells leaves them phenotypically resembling $CD8^+$ T cells with more memory potential, so we sought to test their ability to recall after reinfection. After day 30 of LCMV infection, untreated $KLRG1^{hi}CD127^{lo}$ and $KLRG1^{lo}CD127^{hi}$ $Id2^{f/f}$-$ERCre^+$ and $Id2^{f/f}$-$ERCre^-$ P14 $CD8^+$ T cells were sorted and co-transferred as above (Fig. 2 A), and 6 d after tamoxifen treatment to induce *Id2* deletion, the host mice were rechallenged with LCMV. Following the kinetics of the $CD8^+$ T cell response in the blood, we found that the Id2KO $CD8^+$ T cells of the $KLRG1^{hi}$ transferred cells, which had largely lost KLRG1 expression, peaked 1 d sooner than the equivalent Id2WT $CD8^+$ T cells after infection (Fig. 3 A). As previously observed in primary responses (Cannarile et al., 2006; Knell et al., 2013; Fig. S1), the $CD8^+$ T cells deficient for Id2 failed to accumulate in the late effector stage of the secondary response, and after day 7 of reinfection the proportion of Id2KO $CD8^+$ T cells of both the $KLRG1^{hi}$ and $KLRG1^{lo}$ transfer was largely diminished compared with the Id2WT $CD8^+$ T cells. We examined the phenotype of the responding Id2WT and Id2KO $CD8^+$ T cells in the spleen of recipients at day 9 after infection (Fig. 3, B–D). Although the Id2KO $CD8^+$ T cells of the $KLRG1^{lo}$ transfer were significantly impaired in their ability to differentiate into $KLRG1^{hi}CD127^{lo}$ effector cells much like in a primary response (Cannarile et al., 2006; Knell et al., 2013; Masson et al., 2013; Fig. S1), ∼60% of the Id2KO $CD8^+$ T cells of the $KLRG1^{hi}$ transfer, despite being largely $KLRG1^{lo}$ at the time of rechallenge (Fig. 2, B and D), were able to re-express KLRG1. Yet, the Id2KO $CD8^+$ T cells remained primarily $CD27^{hi}$ with increased expression of Tcf1 and Foxo1 and lower levels of T-bet compared with Id2WT $CD8^+$ T cells (Fig. 3, C and D; and Fig. S2 C). The Id2KO $CD8^+$ T cells likely persisted at a lower frequency at the late effector stage of rechallenge because of impaired survival demonstrated by reduced BCL2 (Fig. S2 C). Thus, $KLRG1^{hi}$ cells with induced *Id2* deletion responded more rapidly to reinfection than their WT counterparts, showing characteristics consistent with a memory phenotype.

To more thoroughly understand how Id2 affects the gene-expression profile of established $KLRG1^{hi}$ effector/effector-memory $CD8^+$ T cell populations, we studied global transcriptional changes in $CD8^+$ T cells that resulted from

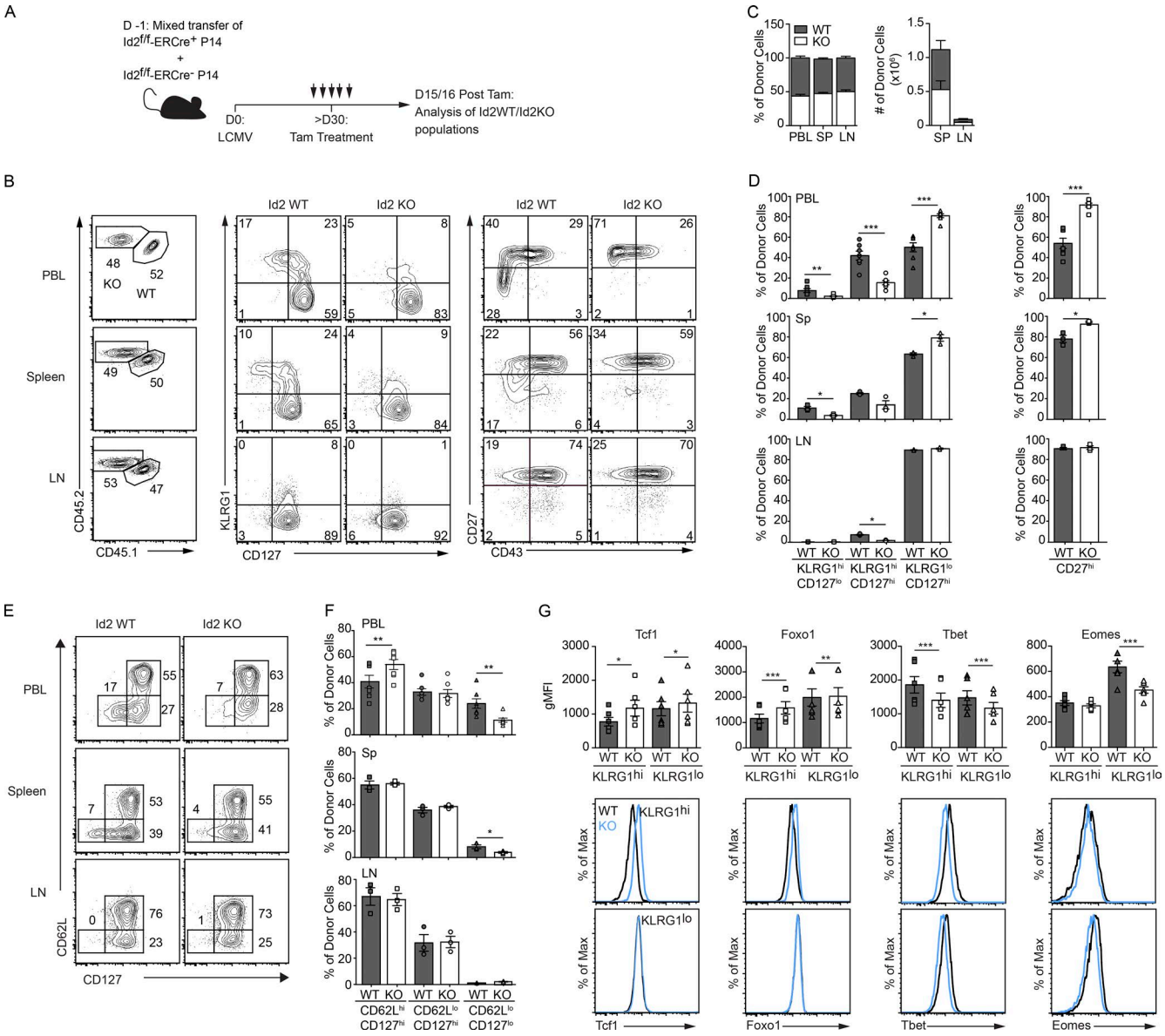
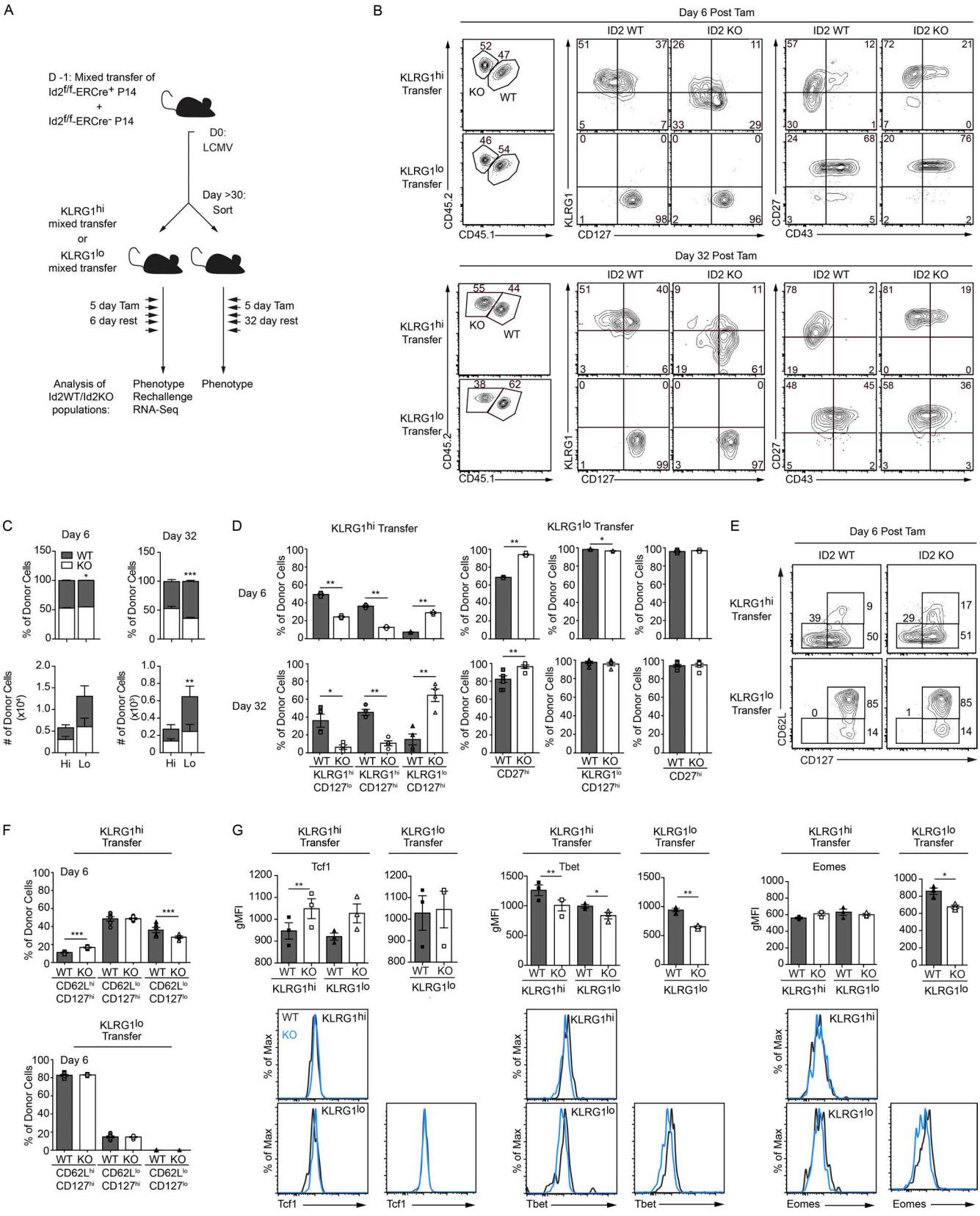


Figure 1. **Late deletion of Id2 in antigen-specific CD8⁺ T cells results in conversion from a KLRG1^{hi} to KLRG1^{lo} population. (A)** Schematic of experimental set-up. CD45.1 hosts were given a cotransfer of Id2$^{f/f}$-ERCre^{-} (CD45.1.2) and Id2$^{f/f}$-ERCre^{+} (CD45.2) P14 CD8^{+} T cells then subsequently infected with LCMV. More than 30 d after infection, host mice were treated for 5 consecutive days with tamoxifen (Tam) to induce *Id2* deletion. Id2$^{f/f}$-ERCre^{-} and Id2$^{f/f}$-ERCre^{+} CD8^{+} T cells are called Id2WT or Id2KO, respectively, after Tam treatment. **(B)** Flow cytometry of transferred P14 CD8^{+} T cells from host peripheral blood lymphocytes (PBL) or spleen (Sp) and LN 15 or 16 d after the last Tam treatment, respectively. Frequency of Id2WT and Id2KO cells among P14 CD8^{+} T cells (left), KLRG1 and CD127 expression (middle), and CD27 and CD43 expression (right) are shown. Numbers in plots represent the percentage of cells. **(C)** Quantification of donor cell frequency and number or **(D)** frequency of populations from B. **(E)** Flow cytometry of cells from B for CD62L and CD127 expression. **(F)** Quantification of donor populations from E. **(G)** Expression of indicated proteins on KLRG1^{hi} or KLRG1^{lo} Id2WT or Id2KO donor populations. Data shown are representative of three (B and D–F) independent or cumulative of two or three (C and G) independent experiments; n = 3–5 mice per group. Data are expressed as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 (two-tailed paired Student's t test).

induced deletion of *Id2*. On day 26 of LCMV infection, KLRG1^{hi}CD127^{lo} and KLRG1^{lo}CD127^{hi} Id2$^{f/f}$-ERCre^{+} and Id2$^{f/f}$-ERCre^{-} P14 CD8^{+} T cells were sorted and co-transferred as in Fig. 2 A, and 6 d after tamoxifen treatment Id2WT and Id2KO T cell populations were sorted for RNA sequencing (RNA-Seq) analysis. Differential expression of genes associated with KLRG1^{hi}CD127^{lo} or KLRG1^{lo}CD127^{hi} CD8^{+} T cell populations from 30 or more days of infection was confirmed by comparison of Id2WT CD8^{+} T cells from the KLRG1^{hi} or KLRG1^{lo} transfers (Fig. 4 A,


A
D -1: Mixed transfer of
Id2f/f-ERCre+ P14
+
Id2f/f-ERCre- P14
D0:
LCMV
Day >30:
Sort
KLRG1hi
mixed transfer
or
KLRG1lo
mixed transfer
5 day Tam
6 day rest
5 day Tam
32 day rest
Analysis of
Id2WT/Id2KO
populations:
Phenotype
Rechallenge
RNA-Seq
Phenotype
B
Day 6 Post Tam
ID2 WT
ID2 KO
KLRG1hi
Transfer
KLRG1lo
Transfer
KO
WT
CD45.2
CD45.1
KLRG1
CD127
CD27
CD43
Day 32 Post Tam
C
Day 6
Day 32
WT
KO
% of Donor Cells
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(x10⁴)
(x10³)
Hi
Lo
D
KLRG1hi Transfer
KLRG1lo Transfer
Day 6
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WT KO
KLRG1hi
CD127lo
KLRG1hi
CD127hi
KLRG1lo
CD127hi
CD27hi
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CD127hi
CD27hi
E
Day 6 Post Tam
ID2 WT
ID2 KO
KLRG1hi
Transfer
KLRG1lo
Transfer
CD62L
CD127
F
KLRG1hi
Transfer
KLRG1lo
Transfer
Day 6
% of Donor Cells
WT KO
CD62Lhi
CD127hi
CD62Llo
CD127hi
CD62Llo
CD127lo
G
KLRG1hi
Transfer
KLRG1lo
Transfer
Tcf1
Tbet
Eomes
gMFI
WT KO
KLRG1hi
KLRG1lo
% of Max
WT
KO
KLRG1hi
KLRG1lo
Tcf1
Tbet
Eomes

left). We compared gene expression between the Id2WT and Id2KO CD8$^+$ T cells from the KLRG1^{hi} transfer and found that Id2KO KLRG1^{hi} CD8$^+$ T cells, in addition to down-regulating KLRG1 and T-bet protein expression, down-regulated mRNA of effector-associated genes (*Prdm1*, *Tbx21*, *Zeb2*, and *Gzmb*) and up-regulated mRNA of memory-associated genes (*Id3*, *Ccr7*, and *Tcf7*) upon *Id2* deletion (Fig. 4 A, right). Principal component analysis confirmed that the induced deletion of *Id2* resulted in the Id2KO KLRG1^{hi} CD8$^+$ T cells rapidly adopting a transcriptional profile that was distinct from WT KLRG1^{hi} CD8$^+$ T cells and more closely resembling MP or memory (T_{EM}, T_{CM}) cells (Fig. 4 B).

To more carefully characterize the effect of induced *Id2* deletion on genes that are effector or memory associated, we compared expression of genes differentially expressed between Id2WT and Id2KO CD8$^+$ T cells from the KLRG1^{hi} transfer to TE and MP gene signatures and T_{EM} and T_{CM} gene signatures previously established in our laboratory (Milner et al., 2017; Yu et al., 2017). It should be noted that T_{EM} and T_{CM} populations were isolated by sorting on the CD62L^{lo} or CD62L^{hi} P14 CD8$^+$ T cell populations and did not exclude KLRG1 to be consistent with other studies (Chang et al., 2014); therefore, it is likely that long-lived TE may be a component of our T_{EM} signature. Id2KO CD8$^+$ T cells lost expression of 79% of the TE signature genes and up-regulated expression of 69% of the MP-associated genes compared with the Id2WT CD8$^+$ T cells (Fig. 4 C, top; $P < 0.001$). Similarly, Id2KO CD8$^+$ T cells lost expression of 83% or up-regulated expression of 88% of T_{EM} or T_{CM} signature genes, respectively (Fig. 4 C, bottom; $P < 0.001$). These analyses indicate a substantial skewing away from an effector-like and toward a T_{CM} CD8$^+$ T cell transcriptional program by the so-called "terminally differentiated" KLRG1^{hi}CD127^{lo} Id2KO CD8$^+$ T cells, rapidly following induction of *Id2* deletion.

The dedifferentiation of long-lived KLRG1^{hi} CD8$^+$ T cells to memory-like KLRG1^{lo}CD127^{hi} CD8$^+$ T cells in the absence of continual Id2 regulation suggests that restrained E-protein activity is necessary to maintain a KLRG1^{hi} effector CD8$^+$ T cell phenotype. To confirm that E-protein activity, the presumed target of Id2, determines and maintains the fate of CD8$^+$ T cells after infection, we analyzed genes differentially expressed between Id2WT or Id2KO KLRG1^{hi} CD8$^+$ T cells that are identified E2A targets (Leong et al., 2016) and are defined in the TE and MP or T_{EM} and T_{CM} gene signatures (Fig. 4 D). We found a substantial number of E2A-bound genes that were differentially up-regulated (*Id3*, *Ccr7*, *Tcf7*, and *Cd27*) and down-regulated (*Prdm1*, *Ccl3*, and *Gzma*) upon induced deletion of *Id2*. This suggests that Id2 mediation of E-protein activity plays a key role in enforcing effector CD8$^+$ T cell terminal differentiation. It then follows that diminished levels of E2A would "rescue" the effector CD8$^+$ T cell dedifferentiation observed in Id2-deficient cells. Therefore, we transduced Id2$^{f/f}$-ERCre$^+$ and Id2$^{f/f}$-ERCre$^-$ P14 CD8$^+$ T cells with a retroviral shRNA vector targeting the gene encoding E2A (sh*Tcf3*) or control shRNA (sh*Cd19*; Fig. 4 E and Fig. S3 A) as previously described (Shaw et al., 2016). Id2$^{f/f}$-ERCre$^+$ and Id2$^{f/f}$-ERCre$^-$ P14 CD8$^+$ T cells transduced with sh*Tcf3* or sh*Cd19* were mixed in a 1:1 ratio and co-transferred to congenically distinct recipients that had been infected 1 d earlier with LCMV. On day 15 of infection, recipient mice were treated with tamoxifen to induce deletion of *Id2*, and the phenotype of the transferred CD8$^+$ T cells in recipient spleens was analyzed. As expected, the Id2KO CD8$^+$ T cells expressing control sh*Cd19* were unable to maintain a differentiated population of CD8$^+$ T cells defined as KLRG1^{hi}CD127^{lo} and CD27^{lo}CD43^{lo}. However, the Id2KO CD8$^+$ T cells with diminished expression of *Tcf3* were almost completely rescued with the frequency of KLRG1^{hi}CD127^{lo} and CD27^{lo}CD43^{lo} populations nearly equivalent to Id2WT CD8$^+$ T cell population (Fig. 4, F and G). Therefore, the loss of the effector-like CD8$^+$ T cell population we observed upon *Id2* deletion can be attributed largely to increased E-protein activity.

Loss of Id2 and enhanced E-protein activity disproportionally impact the KLRG1^{hi} effector-like CD8$^+$ T cell population at memory time points with a rapid and dramatic shift in phenotype and overall gene expression of KLRG1^{hi} CD8$^+$ T cells to a memory-like state, whereas the KLRG1^{lo} CD8$^+$ T cells showed moderately lower Eomes expression and impaired survival at later time points (by day 32). Al-

Figure 2. **Id2 is necessary to maintain the terminal differentiation of antigen-specific CD8$^+$ T cells. (A)** Schematic of experimental set-up. CD45.1 hosts were given a cotransfer of Id2$^{f/f}$-ERCre$^-$ (CD45.1.2) and Id2$^{f/f}$-ERCre$^+$ (CD45.2) P14 CD8$^+$ T cells and then subsequently infected with LCMV. At >30 d after infection, KLRG1^{hi}CD127^{lo} and KLRG1^{lo}CD127^{hi}, Id2$^{f/f}$-ERCre$^-$, and Id2$^{f/f}$-ERCre$^+$ P14 CD8$^+$ T cells were sort purified. Id2$^{f/f}$-ERCre$^-$ and Id2$^{f/f}$-ERCre$^+$ KLRG1^{hi}CD127^{lo} (KLRG1^{hi} transfer) or Id2$^{f/f}$-ERCre$^-$ and Id2$^{f/f}$-ERCre$^+$ KLRG1^{lo}CD127^{hi} (KLRG1^{lo} transfer) populations were co-transferred into naive CD45.1 hosts that were treated with tamoxifen (Tam). Id2$^{f/f}$-ERCre$^-$ and Id2$^{f/f}$-ERCre$^+$ CD8$^+$ T cells are called Id2WT or Id2KO, respectively, after Tam treatment. **(B)** Flow cytometry of transferred P14 CD8$^+$ T cells from host spleen 6 d (top) or 32 d (bottom) after last Tam treatment. Frequency of Id2WT and Id2KO cells among P14 CD8$^+$ T cells (left), KLRG1 and CD127 expression (middle), and CD27 and CD43 expression (right) for the KLRG1^{hi} and KLRG1^{lo} transfer are shown. Numbers in plots represent the frequency of cells in that quadrant. **(C)** Quantification of donor cell frequency and number from KLRG1^{hi} (Hi) or KLRG1^{lo} (Lo) transfers from B. **(D)** Quantification of donor populations from KLRG1^{hi} (left) and KLRG1^{lo} (right) transfer. **(E)** Flow cytometry of transferred P14 CD8$^+$ T cells from host spleen 6 d after last Tam treatment. CD62L and CD127 expression for the KLRG1^{hi} and KLRG1^{lo} transfers is shown. **(F)** Quantification of donor populations from E. **(G)** Expression of indicated transcription factors on KLRG1^{hi} or KLRG1^{lo} Id2WT or Id2KO donor populations from hosts receiving KLRG1^{hi} transferred (left) and KLRG1^{lo} transferred (right) cells 6 d after last Tam treatment. Data shown are representative (B, E, and G) or cumulative (C, D, and F) of two independent experiments; $n = 3$–4 mice per group. Data are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two-tailed paired Student's *t* test).

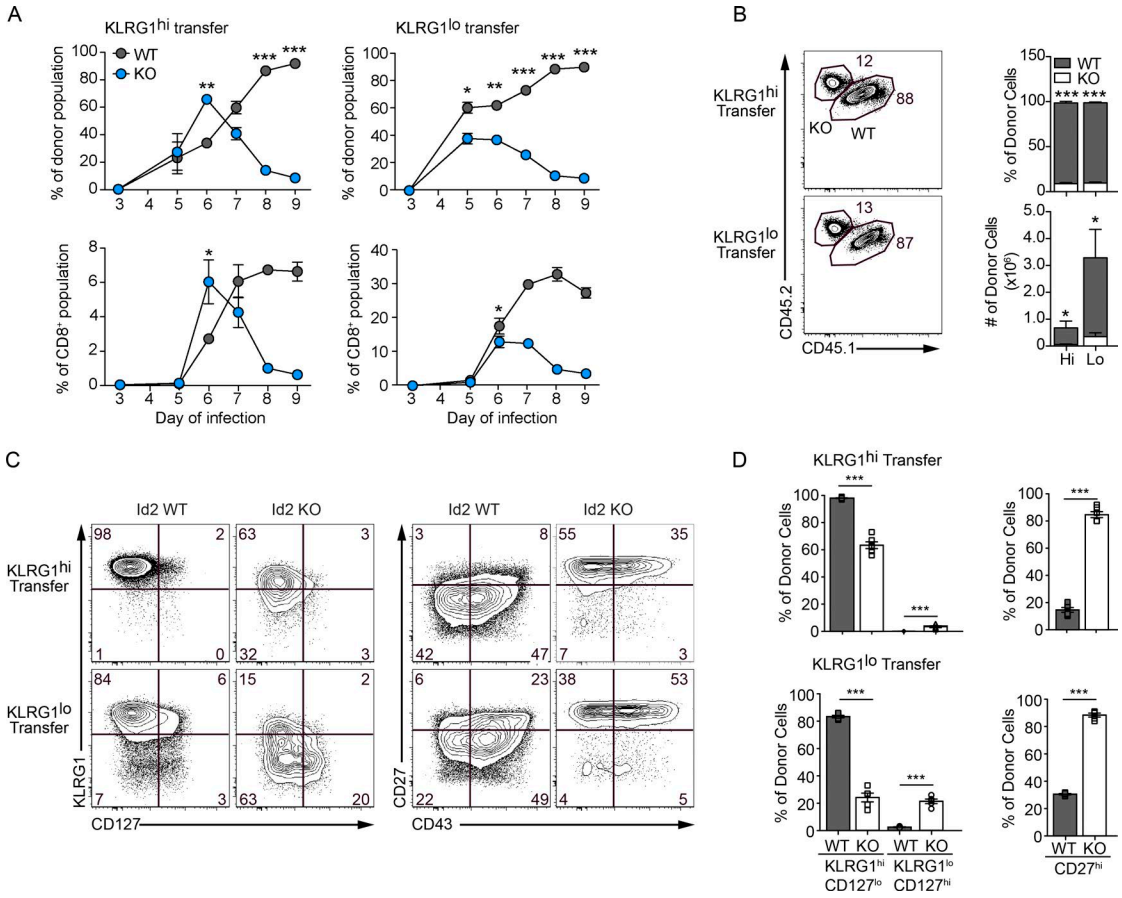

Figure 3. **Deletion of Id2 in KLRG1^{hi} memory CD8$^+$ T cells results in the faster initiation of a recall response.** Id2$^{f/f}$-ERCre$^-$ and Id2$^{f/f}$-ERCre$^+$ KLRG1^{hi} or KLRG1^{lo} P14 CD8$^+$ T cells were co-transferred into naive CD45.1 hosts, and *Id2* deletion was induced as described in Fig. 2 A. Host mice were infected with LCMV 7 d after the last tamoxifen treatment. Id2$^{f/f}$-ERCre$^-$ and Id2$^{f/f}$-ERCre$^+$ CD8$^+$ T cells are called Id2WT or Id2KO, respectively, following tamoxifen treatment. **(A)** The frequency of total Id2WT and Id2KO P14 donor cells (top) or Id2WT and Id2KO P14 cells of total CD8$^+$ T cells (bottom) in PBL on indicated days after infection is shown. **(B)** Flow cytometry (left) and quantification (right) of the frequency of Id2WT and Id2KO P14 CD8$^+$ T cells from host spleen 9 d after infection for KLRG1^{hi} and KLRG1^{lo} transfers. **(C)** Flow cytometry of KLRG1 and CD127 expression (left) and CD27 and CD43 expression (right) for KLRG1^{hi} and KLRG1^{lo} transfers are shown. **(D)** Quantification of donor populations from C after KLRG1^{hi} (top) and KLRG1^{lo} (bottom) transfer. Numbers in plots represent the percentage of cells. Data shown are cumulative (total donor frequency; A, top) or representative (total CD8$^+$ population frequency; A, bottom), representative (B), or cumulative (C) of two independent experiments; n = 2–4 mice per group. Data are expressed as mean ± SEM. *, P ≤ 0.05; **, P < 0.01; ***, P < 0.001 (one-tailed paired [A and B] or two-tailed paired [D] Student's t test).

though we noted a 1.9-fold increase in mRNA expression of *Id2* in the KLRG1^{hi}CD127^{lo} compared with the KLRG1^{lo} CD127^{hi} CD8$^+$ T cells (Fig. 4 A, left) and higher Id2 reporter expression has been observed in T_{EM} cells versus T_{CM} cells after influenza infection (Masson et al., 2013), we did not detect differential Id2 reporter levels between KLRG1^{hi} CD127^{lo} and KLRG1^{lo}CD127^{hi} CD8$^+$ T cells or Id2 protein levels between T_{EM} and T_{CM} in our experiments (Fig. S3 B). This suggests that the increased dependency on Id2 observed in the KLRG1^{hi} compared with KLRG1^{lo} CD8$^+$ T cells was not due to substantially differing expression levels. Using the assay for transposase-accessible chromatin–sequencing (ATAC-Seq) data for naive, TE, and MP or T_{CM} and T_{EM} CD8$^+$ T cells assessed 0, 7, 30, or 180 d after LCMV infection, we analyzed chromatin accessibility of MP or T_{CM} E-protein target genes that were differentially regulated upon *Id2* deletion (Fig. 4 D). E-protein binding sites in *Tcf7*, *Cd27*, *Cxcr5*, *Ccr7*, and *Id3*, identified by using E2A chromatin immunoprecipitation–sequencing (ChIP-Seq; Leong et al., 2016), were accessible in the naive, TE, MP, T_{CM}, and T_{EM} populations analyzed (Fig. S3 C and not depicted). This suggests that other factors contribute to CD8$^+$ T cell subset-specific E-protein regulation. For example, Zeb2, a transcription factor highly expressed in TE and T_{EM} CD8$^+$ T cells and found to be important for terminal differentiation (Dominguez et al., 2015; Omilusik et al., 2015; Kakaradov et al., 2017), can bind DNA at tandem, separated E-protein binding sites (Sekido et al., 1994; Remacle et al., 1999). We identified such a site within the *Tcf7* gene (Fig. S3 C), suggesting Zeb2 may function in KLRG1^{hi} effector-like CD8$^+$ T cell populations

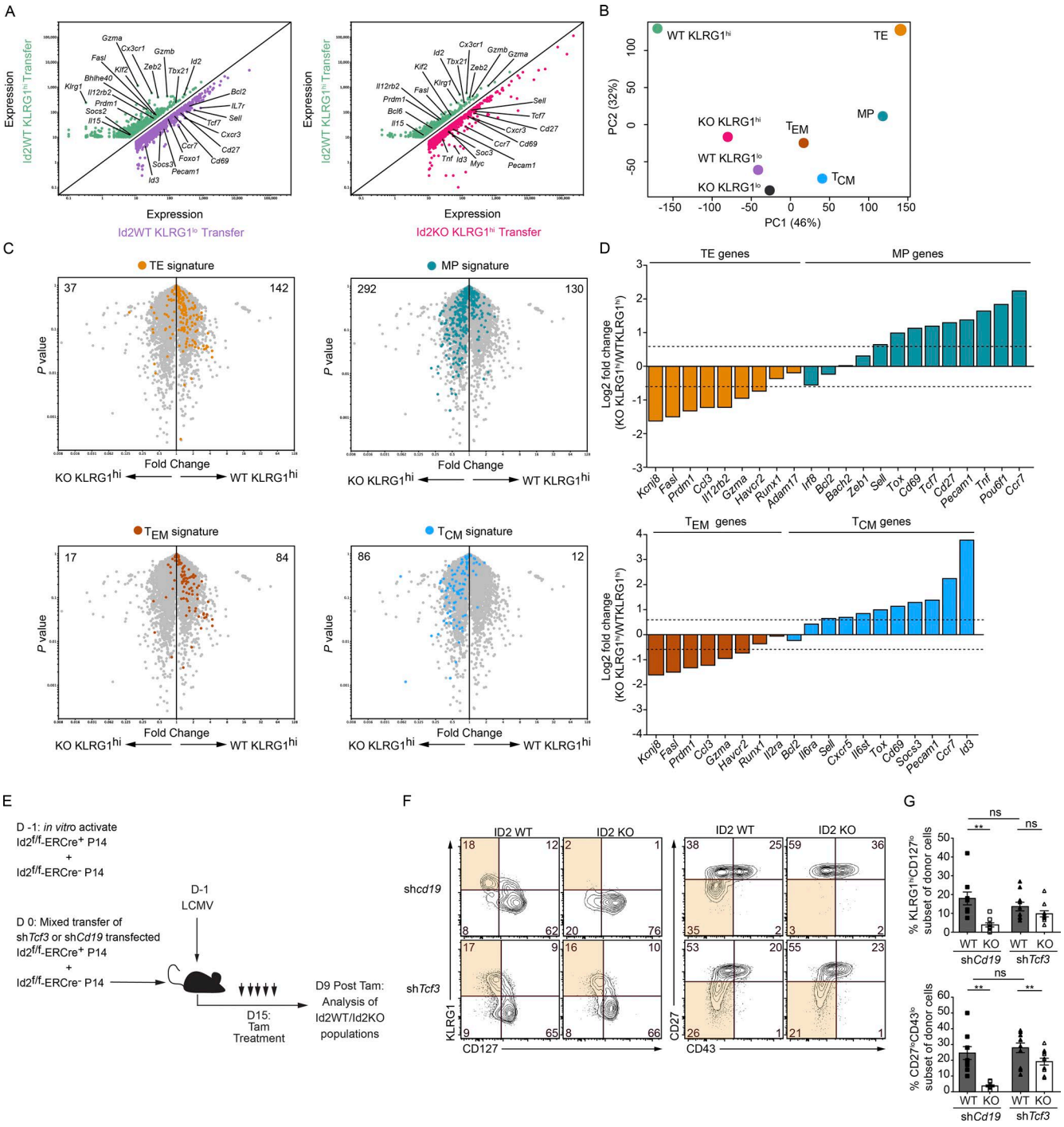

Figure 4. **Id2 regulation of E2A binding regulates expression of key effector CD8⁺ T cell genes. (A–D)** Id2$^{f/f}$-ERCre^{-} (CD45.1.2) and Id2$^{f/f}$-ERCre^{+} (CD45.2) KLRG1^{hi} or KLRG1^{lo} P14 CD8^{+} T cells were co-transferred into naive CD45.1 hosts, and *Id2* deletion was induced as described in Fig. 2 A. Id2$^{f/f}$-ERCre^{-} and Id2$^{f/f}$-ERCre^{+} CD8^{+} T cells are called Id2WT or Id2KO, respectively, after tamoxifen (Tam) treatment. On day 6 after the last Tam treatment, Id2WT and Id2KO cells were sorted for RNA-Seq. (A) Gene expression by cells from the Id2WT KLRG1^{hi} transfer versus Id2WT KLRG1^{lo} transfer (left) or from the Id2WT KLRG1^{hi} transfer versus Id2KO KLRG1^{hi} transfer (right) for genes differentially expressed by 1.5-fold or more, a coefficient of variation of ≤0.50, and an expression value of ≥10; colors indicate genes up-regulated 1.5-fold or more in the cells from the Id2WT KLRG1^{hi} transfer relative to their expression in cells from the Id2WT KLRG1^{lo} transfer (green) or vice versa (purple), or genes up-regulated in cells from the Id2WT KLRG1^{hi} transfer relative to cells from the Id2KO KLRG1^{hi} transfer (green) or vice versa (magenta). Labels in plots indicate genes of published relevance to CD8^{+} T cell differentiation and memory formation. (B) Principal component analysis of gene expression in Id2WT and Id2KO P14 CD8^{+} T cells from the KLRG1^{hi} and KLRG1^{lo} transfers, in TE and MP P14 CD8^{+} T cell populations at day 7 of LCMV infection, and in T_{CM}, and T_{EM} P14 CD8^{+} T cell populations at day 180 of LCMV infection. (C) Expression of TE- or

to repress E-protein activity and thus the memory-gene expression program. Perhaps enhanced E-protein activity upon the loss of Id2 expression may outcompete Zeb2 for E-box sites, resulting in greater expression of *Tcf7*, which then could promote the T_{CM}, long-lived memory phenotype.

Ultimately these results show that the concept of terminal differentiation may be flawed in the context of $CD8^+$ memory T cell differentiation. KLRG1 may not be a definitive marker of terminal differentiation, and bona fide, terminally differentiated $CD8^+$ T cells may die shortly after resolution of the infection, leaving a less-differentiated $KLRG1^{hi}$ population that persists at memory time points. Instead, we support the notion that $CD8^+$ T cell differentiation is flexible and sustained inhibition of E-protein activity by Id2 allows the survival of an effector-like population that can reacquire at least a portion of the T_{CM}, or long-lived memory gene-expression program, favoring the idea of plasticity of cell states over fixed cell fates among the heterogeneous, post-infection $CD8^+$ T cell population.

MATERIALS AND METHODS

Mice

All mouse strains 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. Id2-YFP mice (Yang et al., 2011), $Id2^{fl/fl}$ mice (Niola et al., 2012), *Rosa26*Cre-ERT2 (ERCre) mice (Hess Michelini et al., 2013), P14 mice (with transgenic expression of H-$2D^b$–restricted TCR specific for LCMV glycoprotein gp33), and CD45.1 and CD45.1.2 congenic mice were bred to obtain a fully C57BL/6J background. Both male and female mice were used throughout the study, with sex- and age-matched T cell donors and recipients.

Cell transfer, LCMV infection, and tamoxifen treatment

Congenically distinct $Id2^{f/f}$-$ERCre^-$ and $Id2^{f/f}$-$ERCre^+$ P14 $CD8^+$ T cells were mixed at a 1:1 ratio and adoptively transferred at 5×10^4 cells per CD45.1 recipient mouse. Mice were then infected with 2×10^5 PFU LCMV-Armstrong by intraperitoneal injection. At day 5 or >30 d after infection, *Rosa26*Cre-ERT2-mediated deletion of floxed alleles was induced by intraperitoneal injection for 5 consecutive days of 1 mg tamoxifen (Cayman Chemical Company) emulsified in 100 ml sunflower seed oil (Sigma-Aldrich). Alternatively, >30 d (or at 26 d for RNA-Seq analysis) after infection, $KLRG1^{hi}CD127^{lo}$ and $KLRG1^{lo}CD127^{hi}$ populations from total P14 $CD8^+$ T cells were sorted from recipient spleen and LN, mixed in a 1:1 ratio of $KLRG1^{hi}CD127^{lo}$ $Id2^{f/f}$- $ERCre^+$/$Id2^{f/f}$-$ERCre^-$ or $KLRG1^{lo}CD127^{hi}$ $Id2^{f/f}$- $ERCre^+$/$Id2^{f/f}$-$ERCre^-$ and then transferred at 1.5×10^5 total cells per CD45.1 recipient mouse. The next day, tamoxifen was administered as above for five consecutive days to induce *Id2* deletion. At 6 or 32 d after the last tamoxifen treatment, Id2WT and Id2KO populations were assessed by flow cytometry or sorted for RNA-Seq (as described in the RNA-Seq section), or recipients were rechallenged intraperitoneally with 2×10^5 PFU LCMV-Armstrong.

RT-PCR and qPCR

To confirm *Id2* deletion or *Tcf3* knockdown, total RNA was extracted by using Trizol (Life Technologies) from sorted Id2WT and Id2KO P14 $CD8^+$ T cells. cDNA was synthesized by using Superscript II kit (Life Technologies) following the manufacturer's instructions. For qPCR, cDNA was quantitatively amplified by using Stratagene Brilliant II Syber Green master mix (Agilent Technologies). The abundance of transcripts was normalized to that of the housekeeping gene *Hprt*. The following primers were used: *Id2* forward, 5′-TCCCTTCTGAGCTTATGTCG-3′ and *Id2* reverse, 5′-GTCCATTCAACGTGTTCTCC-3′; *Tcf3* forward, 5′-CATCCATGTCCTGCGAAGCCA-3′ and *Tcf3* reverse, 5′-TTCTTGTCCTCTTCGGCGT-3′; *Hprt* forward, 5′-GGCCAGACTTTGTTGGATTT-3′, and *Hprt* reverse: 5′-CAACTTGCGCTCATCTTAGG-3′.

Flow cytometry and cell sorting

Single-cell suspensions were prepared from spleen, LN, or blood. The following antibodies were used for surface staining (all from eBioscience): CD8 (53-6.7), CD27 (LG-7F9), CD43 (1B11), CD45.1 (A20-1.7), CD45.2 (104), CD62L (MEL-14), CD122 (TM-b1), CD127 (A7R34), CXCR3 (CXCR3-173), and KLRG1 (2F1). Cells were incubated for 30 min at 4°C in PBS supplemented with 2% bovine growth serum and 0.1% sodium azide. Intracellular staining was performed by using the Foxp3 Transcription Factor Staining Buffer kit (eBioscience) and the following antibodies (all from eBioscience unless otherwise specified): BCL2 (3F11; BD PharMingen), Eomes (Dan11mag), Foxo1 (C29H4; Cell Signaling), Gzmb (GB12; Invitrogen), Id2 (ILCID2; Thermo Fisher Scientific), Tbet (eBio4B10), and Tcf1 (C63D9; Cell

MP-associated genes (top) or T_{EM}- or T_{CM}-associated genes (bottom) assessed in cells from the Id2KO $KLRG1^{hi}$ transfer versus cells from the Id2WT $KLRG1^{hi}$ transfer and plotted against p-value. Numbers in the corners indicate the total of those genes up-regulated in cells from the Id2KO $KLRG1^{hi}$ transfer (top left) or from the Id2WT $KLRG1^{hi}$ transfer (top right). $P < 0.001$ (χ^2 test). (D) Relative expression of TE and MP genes (top) and T_{EM} and T_{CM} genes (bottom) from C that are putative E2A-target genes identified by ChIP-Seq (bar colors match dot colors in C). **(E)** Schematic of experimental set-up. CD45.1 host mice infected 1 d before received a cotransfer of $Id2^{f/f}$-$ERCre^-$ and $Id2^{f/f}$-$ERCre^+$ P14 $CD8^+$ T cells transduced with control shRNA targeting *Cd19* or with sh*Tcf3*. At 15 d of infection, host mice were treated with tamoxifen (Tam) to induce Id2 deletion. **(F)** Flow cytometry of transferred cells 9 d after the last Tam treatment for KLRG1 and CD127 expression (left) and CD27 and CD43 expression (right). Numbers in the plots represent the percentage of cells. **(G)** Quantification of donor populations from highlighted populations in F. Data are representative of two (A–D) or three (F) and cumulative of three (G) independent experiments; $n = 3$–5 mice per group. Data are expressed as mean ± SEM. **, $P < 0.01$ (two-tailed paired Student's *t* test).

Signaling). Stained cells were analyzed by using LSRFortessa or LSRFortessa X-20 (BD) and FlowJo software (TreeStar). All sorting was performed on BD FACSAria or BD FACSAria Fusion instruments.

shRNA-mediated knockdown by retroviral transduction

DNA fragments encoding shRNA targeting mouse *Tcf3* or *Cd19* were subcloned into a custom retroviral vector containing ametrine as a reporter. Naive congenically distinct $Id2^{f/f}$-ERcre^{+} and $Id2^{f/f}$-ERcre^{-} P14 CD8^{+} T cells were stimulated for 18 h in 6-well plates precoated with anti-CD3 and anti-CD28. After stimulation, cells were transduced by adding retroviral supernatants supplemented with 100 U/ml human IL-2 and 8 µg/ml polybrene, followed by centrifugation for 90 min at 2000 *g* at 37°C. After transduction, cells were incubated for 3 h at 37°C. $Id2^{f/f}$- ERCre^{+}/$Id2^{f/f}$- ERCre^{-} P14 CD8^{+} T cells were mixed 1:1 and 10^{6} cells were transferred into day −1 LCMV-infected hosts, and remaining cells were cultured in vitro with 50 U/ml human IL-2 for 4 d to assess for knockdown efficiency by qPCR. On day 15 of infection, tamoxifen was administered as described in the tamoxifen treatment section for 5 consecutive days to induce *Id2* deletion. At 9 d after the last tamoxifen treatment, Id2WT and Id2KO populations were assessed by flow cytometry.

RNA-Seq, ChIP-Seq, and ATAC-Seq

6 d after the last tamoxifen treatment, 10^{3} Id2WT and Id2KO P14 cells from recipients receiving mixed transfers of KLRG1^{hi}CD127^{lo} or KLRG1^{lo}CD127^{hi} transfer were sorted into Buffer TCL (Qiagen) with 1% 2-mercaptoethanol. RNA was isolated, and libraries were prepared following Immgen protocols (https://www.immgen.org/Protocols/11Cells.pdf). Libraries were analyzed on an Illumina HiSeq2500 sequencer. Samples were generated from two biological replicates, and ∼10 million paired-reads were generated per sample. Reads were mapped by using Tophat, and aligned reads in transcripts were counted with HTseq. Analysis was performed by using the GenePattern Multiplot Studio module. TE and MP (Yu et al., 2017) and T_{EM} and T_{CM} gene signatures (Milner et al., 2017) represent genes that have a 1.5-fold increased expression in that CD8^{+} T cell population. Principal component analysis used RNA-Seq data for TE and MP or T_{EM} and T_{CM} P14 CD8^{+} T cells from day 7 or 180 after LCMV infection. E2A Bio-Chip analysis was deposited by Leong et al. (2016) (GEO accession code GSE84974), and ATAC-Seq data from Immgen (http://rstats.immgen.org/Chromatin/chromatin.html) was analyzed in the University of California, Santa Cruz, genome browser.

Statistics

The one- or two-tailed paired Student's *t* test was used for comparisons between groups as stated in the figure legends. The χ^2 test was used to determine significant enrichment of gene sets in the RNA-Seq data. Statistical analysis was performed by using GraphPad Prism software.

Online supplemental material

Fig. S1 shows analysis of CD8^{+} T cells responding to LCMV infection when *Id2* deletion is induced in naive cells or at the peak of infection, as well as knockout efficiency when *Id2* deletion is induced >30 d after infection. Fig. S2 shows the mix of Id2WT and Id2KO CD8^{+} T cells for KLRG1^{hi} and KLRG1^{lo} transfers and additional phenotyping after LCMV rechallenge. This relates to Figs. 2 and 3. Fig. S3 shows *Tcf3* knockdown efficiency in Id2WT and Id2KO P14 CD8^{+} T cells transduced with retroviral shRNA constructs. Also, ATAC-Seq and E2A ChIP-Seq analysis of the *Tcf7* loci for CD8^{+} T cell populations after LCMV infection is shown. This relates to Fig. 4.

ACKNOWLEDGMENTS

We thank Toan C. Nguyen and the Flow Cytometry Core at the La Jolla Institute for Allergy and Immunology for their assistance with cell sorting. We also thank Dr. John Chang for helpful discussion and critical review of this manuscript.

This study was supported by the National Institutes of Health (1F31AG043222-01A1 to L.A. Shaw; AI067545 and A1072117 to A.W. Goldrath) and the Leukemia and Lymphoma Society (to K.D. Omilusik and A.W. Goldrath).

The authors declare no competing financial interests.

Author contributions: K.D. Omilusik designed and performed experiments, analyzed the data, and wrote the paper. M.S. Nadjsombati, L.A. Shaw, B. Yu, and J.J. Milner performed experiments and assisted with data analysis. A.W. Goldrath supervised the project, designed the experiments, analyzed the data, and wrote the paper.

Submitted: 28 August 2017

Revised: 7 December 2017

Accepted: 18 January 2018

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