Effect of CD8 T cells dedifferentiate into long-lived memory cells

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Memory CD8 T cells that circulate in the blood and are present in lymphoid organs are an essential component of long-lived T cell immunity. These memory CD8 T cells remain poised to rapidly elaborate effector functions upon re-exposure to pathogens, but also have many properties in common with naive cells, including pluripotency and the ability to migrate to the lymph nodes and spleen. Thus, memory cells embody features of both naive and effector cells, fuelling a long-standing debate centred on whether memory T cells develop from effector cells or directly from naive cells6–8. Here we show that long-lived memory CD8 T cells are derived from a subset of effector T cells through a process of dedifferentiation. To assess the developmental origin of memory CD8 T cells, we investigated changes in DNA methylation programming at naive and effector cell-associated genes in virus-specific CD8 T cells during acute lymphohytic choriomeningitis virus infection in mice. Methylation profiling of terminal effector versus memory-precursor CD8 T cell subsets showed that, rather than retaining a naive epigenetic state, the subset of cells that gives rise to memory cells acquired de novo DNA methylation programs at naive-associated genes and became demethylated at the loci of classically defined effector molecules. Conditional deletion of the de novo methyltransferase Dnmt3a at an early stage of effector differentiation resulted in reduced methylation and faster re-expression of naive-associated genes, thereby accelerating the development of memory cells. Longitudinal phenotypic and epigenetic characterization of the memory-precursor effector subset of virus-specific CD8 T cells transferred into antigen-free mice revealed that differentiation to memory cells was coupled to erasure of de novo methylation programs and re-expression of naive-associated genes. Thus, epigenetic repression of naive-associated genes in effector CD8 T cells can be reversed in cells that develop into long-lived memory CD8 T cells while key effector genes remain demethylated, demonstrating that memory T cells arise from a subset of fate-permissive effector T cells.

We used the mouse model of acute lymphohytic choriomeningitis virus (LCMV) infection to examine the transcriptional and epigenetic changes that occur as naive CD8 T cells differentiate into effector and memory cells. It is well known that many effector genes are turned on when naive CD8 T cells are stimulated by antigen, but it is less well appreciated that several genes expressed by naive T cells are also turned off upon T cell activation5–6. Notably, several of these naive-associated genes that are downregulated in effector CD8 T cells are expressed by central memory cells. This on–off–on pattern of gene expression is shown for LCMV-specific effector and memory CD8 T cells in Fig. 1a. Among the genes that show this pattern are those encoding L-selectin (Seli, also known as Cdlb2) (Fig. 1b) and CCR7, which are needed for homing to lymphoid organs, and BCL-2 and CD127, which are important for long-term survival of memory T cells6,7. To examine epigenetic changes associated with this on–off–on pattern, we analysed the DNA methylation profile of the Sell promoter. Previous studies have defined CpG sites in the Sell promoter region proximal to the binding sites for Klf2 and Ets1, two transcription factors known to regulate Sell expression5,9 (Extended Data Fig. 1a). To determine whether the methylation status of these CpG sites has a direct effect on gene expression, we used a reporter construct to show that these CpG sites do regulate the expression of L-selectin (Extended Data Fig. 1b, c). Having established that methylation of these CpG sites decreases L-selectin expression in vitro, we next examined the methylation status of these sites in LCMV-specific naive, effector, and memory P14 CD8 T cells (T cell receptor (TCR) transgenic CD8 T cells specific to the LCMV gp33 epitope) during acute LCMV infection in vivo (Fig. 1c). Consistent with the high level of Sell transcription in naive CD8 T cells, the CpG sites proximal to the Sell promoter were completely unmethylated in naive cells, whereas the Sell promoter was significantly methylated in LCMV-specific effector CD8 T cells taken at either day 4 or day 8 of infection, which did not express L-selectin (Extended Data Fig. 1d). Notably, memory P14 cells showed minimal methylation at this promoter site and, consistent with this permissive epigenetic state, there was expression of Sell mRNA (Fig. 1c, Extended Data Fig. 1d, e). However, as more than 95% of effector CD8 T cells undergo apoptosis, it is possible that these surviving L-selectin-positive memory P14 cells may never have been methylated during the effector phase of the T cell response. The pool of effector CD8 T cells consists of two subsets; the majority (95%) are terminal effectors (TEs) that are destined to die, and the minority (5%), termed memory precursors (MPs), survive to give rise to the pool of long-lived memory T cells7. These two subsets can be distinguished on the basis of their expression of cell surface markers KlrG1 and CD12710–12. We analysed the TE and MP effector subsets at day 8; notably, both subsets were equally methylated at the Sell promoter and they also expressed low levels of Sell mRNA (Fig. 1d, Extended Data Fig. 1f, g). We next analysed memory cells at day 37 of infection and found that the L-selectin13 promoter showed significant demethylation at the Sell promoter and expressed high levels of Sell mRNA (Fig. 1e, Extended Data Fig. 1f, h). Together, these results show that the MP effector CD8 T cell subset, which gives rise to memory cells, also becomes methylated at the Sell promoter during the acute phase of infection.

To obtain a more comprehensive assessment of methylation changes during naive-to-effector differentiation, we performed whole-genome bisulfite sequencing (WGBS) of antigen-specific MP and TE CD8 T cell subsets at days 4.5 and 8 (Extended Data Fig. 2a, b). Both effector subsets showed an increase in DNA methylation at about 1,000 regions.
relative to naive cells, with marked enrichment of these methylation events near or within genes (Fig. 2a, Extended Data Fig. 3a). Most of these gain-of-methylation events occurred within the first 4 days of the effector response, and more than half of these differentially methylated regions (DMRs) were similarly acquired in both TE and MP effector cells. These DMRs included the previously defined sites in the Sell promoter as well as several other naive-associated genes, such as Ccr7 and Tcf7 (Fig. 2b). Thus, MP effector CD8 T cells, which are the precursors of memory cells, acquire repressive DNA methylation marks at many genes expressed by naive cells. Notably, the MP subset not only showed methylation of naive-associated genes, but also showed demethylation of several effector-associated genes, such as Prf1, granzyme genes and Il6st (Fig. 2c, d, Extended Data Fig. 4a–c). These results are consistent with a model in which MP cells transition through an effector phase during their differentiation into memory cells. Although there is a high degree of overlap between TE and MP subsets in effector-associated programming, there were also some notable differences in the level of demethylation (Extended Data Fig. 4d)—for example, a DMR in the Prdm1 (also known as Blimp1) locus that remains methylated in the MP subset. This is consistent with prior reports showing that MP cells have lower Prdm1 expression than TE cells and that deletion of Prdm1 increases the number of effector cells with memory potential. We also detected differential methylation of Runx2 and Runx3, further suggesting that the fates of MP and TE cells are coupled to epigenetic programming of transcriptional regulators.

Having determined that epigenetic repression of naive-associated genes is a shared feature of both MP and TE CD8 T cells, we next investigated which enzyme(s) are involved in this DNA methylation. We hypothesized that the methylation would be de novo, because it was rapid and was retained even while the CD8 T cells were rapidly dividing (10–15 divisions) during the first week after infection. Expression of the de novo methyltransferase Dnmt3a increases upon activation of CD8 T cells, so we tested whether Dnmt3a was required for methylation of the Sell promoter. To do this we used Dnmt3a−/− mice to conditionally knock out (cKO) Dnmt3a in virus-specific CD8 T cells following T cell activation in vivo. To confirm successful deletion of the enzyme (Extended Data Fig. 5c). We then assessed the methylation status of wild-type and cKO gp33 tetramer-positive effector CD8 T cells and found that in the absence of Dnmt3a there was no de novo methylation of the Sell promoter (Extended Data Fig. 5d). However, loss of Dnmt3a did not have any effect on maintenance methylation of a set of CpG sites distal to the Sell promoter (Extended Data Fig. 5e, f).

To more extensively profile loci targeted for de novo DNA methylation in effector cells, we performed WGBS with DNA from wild-type and Dnmt3a cKO antigen-specific effector CD8 T cells (Fig. 2e–g). Comparison of data from naive and wild-type effector cells revealed that the total pool of effector cells acquired about 2,000 newly methylated regions (Fig. 2e). Of these newly methylated regions, about 1,000 were verified as targets of Dnmt3a, as they were not acquired in de novo methyltransferase Dnmt3a increases upon activation of CD8 T cells, so we tested whether Dnmt3a was required for methylation of the Sell promoter. To do this we used Dnmt3a−/− mice to conditionally knock out (cKO) Dnmt3a in virus-specific CD8 T cells following T cell activation in vivo. To confirm successful deletion of the enzyme (Extended Data Fig. 5c). We then assessed the methylation status of wild-type and cKO gp33 tetramer-positive effector CD8 T cells and found that in the absence of Dnmt3a there was no de novo methylation of the Sell promoter (Extended Data Fig. 5d). However, loss of Dnmt3a did not have any effect on maintenance methylation of a set of CpG sites distal to the Sell promoter (Extended Data Fig. 5e, f).

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Figure 2 | MP CD8 T cells acquire genome-wide effector-associated DNA methylation programs. a, Summary of the number of newly methylated DMRs in TE and MP subsets relative to naive cells identified from WGBS analyses. b, Normalized CpG methylation in the Ccr7, Tcf7, and Sell loci from WGBS datasets. Each vertical line indicates a CpG site and the ratio of red to blue indicate the per cent of methylated versus unmethylated CpGs at these sites. c, Summary of the number of demethylated DMRs between the effector subsets and naive cells. d, Normalized methylation at CpG sites in Gzmb, Ifng and Prf1 loci from TE and MP WGBS datasets. e, Heat-map representation of the top 3,000 newly methylated regions (relative to naive CD8 T cell methylation) from WGBS analysis of tetramer+ wild-type and Dnmt3a cKO effector CD8 T cells. f, Summary of de novo methylated regions in wild-type and Dnmt3a cKO effector CD8 T cells. g, Normalized Dnmt3a-mediated de novo methylation at CpG sites in the Sell, Ccr7 and Tcf7 loci. h, Top canonical pathways and upstream regulators from ingenuity pathway analysis of gene-associated Dnmt3a-mediated DMRs.

marked effect on de novo methylation, deletion of Dnmt3a did not have any effect on maintenance DNA methylation (Extended Data Fig. 6b). Given the selective nature of the Dnmt3a-targeted loci, we next assessed these genes for potential connections using ingenuity pathway analysis (IPA; Fig. 2h). Among the top canonical pathways identified by IPA were gene networks that were related to T cell receptor signalling and differentiation, further documenting the relationship between Dnmt3a-mediated programming and effector differentiation. Notably, our analysis revealed that loci targeted for de novo methylation were significantly associated with the transcription factors Id2 and Id3, two well-established regulators of effector and memory T cell differentiation8,19 (Extended Data Fig. 6c). Collectively, these results demonstrate that downregulation of naive-associated genes is coupled to acquisition of Dnmt3a-mediated de novo DNA methylation programs during development of both MP and TE cells.

To determine how Dnmt3a-mediated de novo methylation regulates effector and memory T cell formation in vivo, we performed longitudinal analysis of the numbers and phenotypes of virus-specific CD8 T cells following LCMV infection of wild-type and Dnmt3a cKO mice. The magnitude of the virus-specific CD8 T cell response was similar in wild-type and Dnmt3a cKO mice; there were equivalent numbers of LCMV-specific CD8 T cells in multiple tissues at both effector and memory time points, and both groups of mice controlled the viral infection (Fig. 3a, Extended Data Fig. 7a–c). We confirmed that the memory cells in the Dnmt3a cKO mice were indeed deficient in Dnmt3a and did not represent an outgrowth of wild-type cells (Extended Data Fig. 7d). Thus, the magnitude of the virus-specific CD8 T cell response was not affected by Dnmt3a-mediated de novo methylation. Longitudinal analysis of phenotypic markers showed that L-selectin and CD127 were downregulated in both wild-type and Dnmt3a cKO effector cells eight days after infection (Fig. 3a, b, Extended Data Fig. 7e). Although the Dnmt3a cKO effector cells indeed lack the repressive DNA methylation programs, the transient downregulation of CD62L probably reflects the role of TCR signalling in promoting eviction of transcription factors from this promoter. However, following viral clearance, re-expression of L-selectin occurred significantly earlier in Dnmt3a cKO virus-specific CD8 T cells than in wild-type cells (Fig. 3b). Dnmt3a cKO CD8 T cells also showed enhanced conversion to a central memory phenotype compared to wild-type CD8 T cells (Fig. 3c). Similar to Dnmt3a-deficient cells in the peripheral blood, CD8 T cells in the spleen and lymph nodes of Dnmt3a cKO mice had significantly higher levels of Sell expression and contained more central memory cells when compared to those of wild-type mice (Fig. 3d, Extended Data Fig. 7f). Not only did Dnmt3a cKO mice...
and MP (Klrg1lo L-selectinhi; CD8 T cells underwent effector differentiation. We first assessed our Dnmt3a to memory cells, we investigated the extent to which cKO L-selectin ted an increased rate of conversion to central memory cells (CD127 + type P14 cells (Fig. 3e). In addition, after infection, but they re-expressed Sell similar downregulated Sell acutely infected with LCMV (Fig. 3e). P14 cells, were co-transferred into wild-type mice that were then cKO naive P14 cells, along with congenically distinct wild-type naive T cells with a TCR specific to the LCMV gp33 epitope. These Dnmt3a crossed with P14 transgenic mice to generate Dnmt3a cKO CD8 cells was due to a cell-intrinsic mechanism. As we next investigated whether the faster re-expression of naive-associated genes in the Dnmt3a cKO cells compared to wild-type cells was due to a cell-intrinsic mechanism, Dnmt3a cKO mice were crossed with P14 transgenic mice to generate Dnmt3a cKO CD8 T cells with a TCR specific to the LCMV gp33 epitope. These Dnmt3a cKO naive P14 cells, along with congenically distinct wild-type naive P14 cells, were co-transferred into wild-type mice that were then acutely infected with LCMV (Fig. 3e). Dnmt3a cKO P14 cells showed similar downregulated Sell expression to wild-type P14 cells at 8 days after infection, but they re-expressed Sell much faster than did wild-type P14 cells (Fig. 3e). In addition, Dnmt3a cKO P14 cells exhibited an increased rate of conversion to central memory cells (CD127+ L-selectin+ CD27+ Klrg1hi) compared to wild-type P14 cells (Fig. 3f). Thus, Dnmt3a intrinsically regulates the execution of the memory CD8 T cell program.

As Dnmt3a cKO cells showed faster conversion than wild-type cells to memory cells, we investigated the extent to which Dnmt3a cKO CD8 T cells underwent effector differentiation. We first assessed our whole-genome methylation analysis of day 8 cKO antigen-specific CD8 T cells for DNA demethylation events at effector-associated loci, including Ifng, Prf1, and Gzmk. Similar to the level of demethylation we observed in wild-type MP and TE cells, these loci were fully demethy- lated in the day 8 Dnmt3a cKO CD8 T cells, demonstrating that they had acquired effector-associated epigenetic programs (Extended Data Fig. 8a, b). The day 8 Dnmt3a cKO virus-specific CD8 T cells also expressed wild-type levels of Tbet (also known as Tbx21) and Ki67, providing further evidence that the Dnmt3a cKO cells had mounted an effector response (Extended Data Fig. 8c). Furthermore, the Dnmt3a cKO effector and memory cells were able to rapidly express effector cytokines upon ex vivo peptide stimulation (Extended Data Fig. 8d). These results explain the viral clearance data shown in Extended Data Fig. 7b, where we observed similar viral control in wild-type and Dnmt3a cKO mice.

Our results so far document that MP CD8 T cells not only demethyl- ate effector-associated genes, but also methylate naive-associated genes. So a key question is whether virus-specific MP CD8 T cells that had downregulated naive-associated genes such as Sell re-express these genes and undergo associated epigenetic changes. To address this question, we isolated purified populations of L-selectin-negative day 8 P14 TE and MP cells, labelled them with carboxyfluorescein succinimidyl ester (CFSE) and adoptively transferred these cells into congenically distinct naive B6 mice (Fig. 4a). We then longitudinally tracked cell division, L-selectin expression, and promoter methylation of the transferred TE and MP populations. We took special care to avoid

Figure 3 | Dnmt3a-mediated de novo DNA methylation regulates the kinetics of gene re-expression during the effector-to-memory CD8 T cell transition. a, Longitudinal measurement of numbers of wild-type and Dnmt3a cKO gp33-specific CD8 T cells in PBMCs during acute LCMV infection. b, c, Longitudinal analysis of central-memory (L-selectinhi; b) and MP (Klrg1lo L-selectinhi; c) phenotypes of gp33-specific wild-type and Dnmt3a cKO CD8 T cells in PBMCs (n ≥ 5). d, Percentages of L-selectin+ gp33+ T cells in PBMCs (× 106) and np396 epitopes of the virus (Extended Data Fig. 7g).

We next investigated whether the faster re-expression of naive-associated genes in the Dnmt3a cKO cells compared to wild-type cells was due to a cell-intrinsic mechanism. Dnmt3a cKO mice were crossed with P14 transgenic mice to generate Dnmt3a cKO CD8 T cells with a TCR specific to the LCMV gp33 epitope. These Dnmt3a cKO naive P14 cells, along with congenically distinct wild-type naive P14 cells, were co-transferred into wild-type mice that were then acutely infected with LCMV (Fig. 3e). Dnmt3a cKO P14 cells showed similar downregulated Sell expression to wild-type P14 cells at 8 days after infection, but they re-expressed Sell much faster than did wild-type P14 cells (Fig. 3e). In addition, Dnmt3a cKO P14 cells exhibited an increased rate of conversion to central memory cells (CD127+ L-selectin+ CD27+ Klrg1hi) compared to wild-type P14 cells (Fig. 3f). Thus, Dnmt3a intrinsically regulates the execution of the memory CD8 T cell program.

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have significantly more L-selectin-positive memory CD8 T cells in lymphoid tissues than wild-type mice, but they also had a greater number of L-selectin-positive memory CD8 T cells in nonlymphoid tissues (lung and liver; Fig. 3d). Enhanced expression of L-selectin was also observed in LCMV-specific CD8 T cells that recognized the gp276 and np396 epitopes of the virus (Extended Data Fig. 7g).

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transferring any L-selectin-positive cells by sorting only L-selectin-negative TE and MP P14 CD8 T cells (Fig. 4a). The take of the adoptively transferred TE and MP populations was determined by killing a group of recipient mice one day after cell transfer and quantifying the number of P14 cells in several lymphoid and non-lymphoid tissues. The CFSE-labelled transferred cells were present in all tissues analysed, and all of these cells were L-selectin-negative. No L-selectin-positive P14 cells were detected in any of the tissues examined (Extended Data Fig. 9a, b). Thus, on day 1 we began with a pure population of L-selectin-negative P14 TE and MP cells. Longitudinal tracking showed that 28 days after transfer the TE cell population mostly remained L-selectin-negative and did not divide (Fig. 4b). By contrast, many of the transferred MP cells now expressed L-selectin and were undergoing an effector phase.

The most important finding from the above experiment was that even the undivided MP cells that were undivided and L-selectin-positive detected in the spleen on day 1 (input) and 28 days after transfer. d, Real-time PCR analysis of Sell, Bcl2, CD127 and Ccr7 from the indicated cell populations. e, Bisulfite sequencing DNA methylation analysis of the Sell promoter from the undivided L-selectinhi versus L-selectinlo memory populations.

We next sought to determine whether re-expression of L-selectin in the undivided CFSE+ memory CD8 T cells was coupled to demethylation of the promoter. Genomic DNA was isolated from sorted undivided L-selectin-positive and L-selectin-negative day 28 virus-specific MP cells (Extended Data Fig. 9d) and the Sell promoter methylation status was analysed. Notably, the undivided L-selectin-positive memory population acquired a demethylated Sell promoter, whereas the L-selectin-negative memory cells retained a level of promoter methylation equivalent to that of input effector cells (Fig. 4e). These data demonstrate that erasure of the previously acquired de novo DNA methylation program at the Sell promoter in CD8 T cells occurs concordantly with re-expression of L-selectin during the effector-to-memory transition. Together, these results support the idea that memory CD8 T cells are generated through a process of cellular
dedifferentiation that allows the re-expression of naive-associated genes.

As shown in Fig. 2 and Extended Data Fig. 4, MP CD8 T cells showed demethylation of many canonical effector genes, including Prf1 and Gzmb. To determine the methylation status of these effector genes in memory cells, virus-specific effector and memory CD8 T cells were isolated from mice 8 and 40 days after LCMV infection and the methylation status of the Gzmb and Prf1 DMRs was measured. Notably, both Gzmb and Prf1 loci remained demethylated in memory CD8 T cells (Extended Data Fig. 10a, b) even though these memory cells did not express high levels of perforin or granzyme B. Thus, these demethylation marks at effector genes are retained in memory cells.

The formation of memory CD8 T cells has been a topic of much interest, and debate over contrasting models of memory differentiation. One model describes effector and memory differentiation as distinct lineages arising from asymmetric cell division of the original naive T cell, allowing memory T cells to retain gene expression programs from the naive parental cell. A contrasting model describes memory T cell differentiation as a process whereby memory CD8 T cells arise from a subset of effector cells (MP cells) that have the ability to re-acquire key naive-like properties and still retain the ability to rapidly elaborate effector functions. Our results reveal that repression of the naive transcriptional program in MP effector cells is coupled to de novo DNA methylation of genes, which can then be erased as these cells re-acquire specific aspects of the naive-like gene expression program. Furthermore, we show that effector stage conditional deletion of the de novo DNA methytransferase Dnmt3a results in enhanced kinetics for development of memory cells. A recent study examining the role of Dmnt3a during T cell differentiation also reports that de novo DNA methylation programs regulate the development of memory CD8 T cells. However, the authors of this study conclude that TE cells acquire de novo programs at critical loci, including TcR, but the MP cells lack these de novo programs. By contrast, we show here that MP cells also acquire Dmnt3a-mediated methylation programs but have the capacity to erase their newly acquired methylation programs and re-express naive genes as they develop into memory CD8 T cells. Our findings do not argue against a model of memory T cell differentiation in which MP and TE cells undergo fate specification early in their development. However, our results provide evidence that the MP subset of effector CD8 T cells can dedifferentiate into memory T cells. In an accompanying manuscript, we show that human virus-specific CD8 T cells also follow a similar program of memory differentiation and that both mouse and human memory CD8 T cells retain an epigenetic signature of their past effector history.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions B.Y. and J.S.H. designed experiments, collected, analysed data and interpreted results. H.T.K. analysed data and interpreted results. All authors contributed to the preparation of the manuscript.

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METHODS
No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments and outcome assessment.

Generation of antigen-specific T cells. Wild-type male or female C57BL/6 mice (Jackson Laboratory), aged 6–8 weeks, were acutely infected with the Armstrong strain of LCMV (2 × 10^7 plaque-forming units (p.f.u.)), intraperitoneal injection (i.p.)). Efferector and memory antigen-specific CD8 T cells were identified and purified by FACs using H-2D^b tetramers bound to LCMV peptide gp33-41 conjugated to a fluorophore, along with CD8, CD44, CD127, Klrg1 and L-selectin–fluorophore conjugated antibodies as previously described11,14,16,27. To generate LCMV-specific CD8 T cell chimaeras, transgenic P14 CD8 T cells with an engineered TCR that recognizes the epitope gp33-41 of LCMV were collected from naive P14 TCR transgenic mice and adoptively transferred intravenously into C57BL/6 mice (cell numbers per mouse are specified in the figure legends)11,28–30. Congenically marked LCMV-specific CD8 T cells were sorted using fluorescently labelled CD90.1 (Thy1.1) and CD8 antibodies as previously described 31. Naive antigen-specific cells obtained from transgenic P14 mice31 were used as an antigen-specific naive control. Dnm3a cKO mice were generated by breeding previously characterized floxed Dnm3a mice with mice that contain a granzyme B-driven recombination transgene17,32. Genotyping and recombination of the Dnm3a locus were performed using primers listed in Supplementary Table 1. All mouse experiments were approved by Emory University Institutional Animal Care and Use Committee (IACUC).

Bisulfite sequencing methylation analysis. DNA was isolated from FACs-purified antigen-specific CD8 T cells using the Qiagen DNeasy kit. Genomic DNA was bisulfite-treated using the Zymo Research EZ DNA methylation kit. Bisulfite-induced deamination of cytosine was used to determine the allelic frequency of cytosine methylation of the target genomic region33. The bisulfite-modified DNA was PCR amplified with specific locus primers (Supplementary Table 1). The PCR amplicon was cloned into the pGEM-T TA cloning vector (Promega) then transformed into XL10-Gold ultracompotent bacteria (Stratagene). Individual bacterial colonies were grown and the cloning vector was isolated and sequenced.

Genomic-wide methylation analysis. Genomic DNA from FACs-purified wild-type naive P14, day 4.5 TE and MP effector P14, day 8 TE and MP effector P14, day 8 total tetracer wild-type and Dnm3a cKO effector, and day 35 tetracer wild-type memory CD8 T cells was isolated using the QIAgen DNeasy kit. Genomic DNA was bisulfite-treated using the Zymo Research EZ DNA methylation kit. The bisulfite-modified DNA sequencing library was generated using the Epicentre kit per the manufacturer’s instructions. Bisulfite-modified DNA libraries were sequenced using an Illumina HiSeq Sequencing. Sequencing was performed to yield more than 5× average coverage across the genome. Sequencing data were aligned to the mm10 mouse genome using BSMAP74,15. Differentially methylated regions (DMRs) were identified using Bioconductor package DSS35. We first performed statistical tests of differentially methylated loci (DML) using DMLtest function (smoothing = TRUE) in DSS; the results were then used to detect differentially methylated regions using CaliDMR function in DSS, with a P threshold for calling DMR set at 0.01. The minimum length for a DMR was set to 50 bp with a minimum number of three CpG sites. Genomic location classification was defined on the basis of RefSeq annotation as follows: promoter from within −2 kb to 1 kb of transcriptional start site (TSS), TSS proximal enhancer from −10 kb to −2 kb of TSS, distal from transcriptional termination site (TTS) from 1 kb to 2 kb of TTS, introns and exons defined in RefSeq annotation, and any other genomic regions were defined as intergenic. The distance was defined according to the strand of the gene. Each DMR was assigned in the order listed above and each DMR was assigned to one category only. The ShuffleBed function in bedtools25 was used to generate a set of random regions of the same number and size distribution as the original DMRs to assess enrichment of DMR distribution among relative genomic locations. Specifically, each DMR was repositioned on a random chromosome at a random position. The number of DMRs and the size of DMRs were preserved. The random set of regions was then annotated in the same way as the original DMRs. Differential methylation analysis of CpG methylation among the datasets was further assessed using a Bayesian hierarchical model to detect differences among methylation at three CpG sites37.

Real-time PCR analysis of mRNA. Splenocytes were collected from mice at the described time points after infection with LCMV. Antigen-specific CD8 T cells were purified by FACs. RNA was extracted from cells using the Qiagen RNeasy kit per the manufacturer’s instructions. Quantitative real-time PCR of Sell was performed using primers that have been previously described34. In vitro cellular proliferation. Virus-specific CD8 T cells were FACs-purified and resuspended in PBS at a concentration of 2 × 10^5 per ml. One volume of 5μM CFSE was mixed with one volume of cells for 7 min at room temperature. 100% fetal calf serum was added to the cell suspension at final volume of 20% to quench the labelling. Cells were washed with PBS once and then adoptively transferred into naive B6 mice.

In vitro promoter methylation and expression assay. The pCpGfree-basis reporter construct was purchased from InvivoGen and the Sell promoter was cloned into the MCS of the reporter construct. Proper orientation of the promoter was confirmed by sequencing. In vitro methylation of the plasmid was performed using MSII purchased from New England Biolabs. The plasmid was incubated with the methyltransferase and cofactor for 37°C and then additional cofactor was spiked into the reaction before incubation for another 2h. Methylation was confirmed using the established Sell bisulfite sequencing assay. Unmethylated and in vitro methylated plasmids (0.5μg) were then transfected into EL4 cells (this cell line was purchased from ATCC but was not independently confirmed or tested for mycoplasma contamination) using Lipofectamine 2000 according to the manufacturer’s instructions. Supernatant was isolated from the cell culture at various times and the lucia activity was measured using Quantiluc reagent on a Veritas luminometer. Statistical significance for all in vivo and in vitro studies, excluding the WGBS analyses, was determined on three or more biological replicate samples using Prism software. P values were determined using a two-tailed Student’s t-test. *P < 0.05; **P < 0.01; ***P < 0.001.

Data availability. The data that support the findings of this study are available from the corresponding author upon request. Whole-genome bisulfite sequencing datasets can be obtained from the NCBI Gene Expression Omnibus (accession no. GSE107150).
Extended Data Figure 1 | Sell gene expression changes during effector and memory CD8 T cell differentiation are coupled to epigenetic reprogramming of the Sell promoter. a, Cartoon of CpG positions within the Sell promoter region cloned into the CpG-free Lucia Promoter reporter construct. Putative transcription factor binding sites are indicated by coloured boxes. b, Representative methylation profiling of *in vitro* methylation efficiency of the reporter construct. c, Longitudinal measurement of relative light units from EL4 cells transfected with unmethylated and *in vitro* methylated reporter constructs. d, Real-time PCR analysis of Sell mRNA in virus-specific naive, effector, and memory P14 CD8 T cells. e, Summary of Sell proximal promoter methylation in naive, day 4 effector, day 8 effector and day 60+ memory P14 CD8 T cells. Each horizontal line represents an individual sequenced clone. Filled circles, methylated cytosine; open circles, non-methylated cytosine. f, Real-time PCR analysis of Sell mRNA expression from day 8 TE and MP P14 CD8 T cells, and day 37 L-selectinhi and L-selectinlo P14 CD8 T cells. Transcript data correspond to cell sorts used for DNA methylation measurements in Fig. 1f, g, h. Summary of Sell proximal promoter DNA methylation in TE and MP effector CD8 T cells and L-selectinlo and L-selectinhi memory CD8 T cells. Statistics were generated from three or more biological replicates.
Extended Data Figure 2 | Isolation of MP and TE CD8 T cells for whole-genome methylation profiling.  

**a**. Experimental setup for isolating MP and TE LCMV-specific CD8 T cells on days 4.5 and 8. 

**b**. Representative post-sort purity and phenotypic analysis of day 4.5 and day 8 MP and TE P14 CD8 T cells isolated from acutely infected mice used for WGBS methylation profiling.
Extended Data Figure 3 | Effector-associated changes in DNA methylation occur predominantly at or near genes and are highly similar between MP and TE CD8 T cells. Pie chart representation of newly methylated DMR genomic distribution relative to the TSS of the nearest gene.
Extended Data Figure 4 | Both MP and TE CD8 T cells acquire demethylated effector loci. a, Pie charts represent demethylated DMR genomic distribution relative to the TSS of the nearest gene. b, Venn diagrams of regions that undergo demethylation during differentiation of naive CD8 T cells into TE and MP subsets. c, Normalized methylation at CpG sites in the Gzmk locus from TE and MP WGBS datasets. d, Normalized differentially methylated CpG sites in the Klrg1, Prdm1 (also known as Blimp1), Runx2, and Runx3 loci from TE and MP WGBS datasets.
Extended Data Figure 5 | Conditional deletion of Dnmt3a in activated CD8 T cells inhibits effector-associated de novo DNA methylation but does not impair maintenance methylation. a, Cre recombinase expression is driven by the Gzmb promoter to initiate recombination of Dnmt3a exon 19 following T cell activation. b, Representative FACS analysis of virus-specific CD8 T cells sorted 8 days after acute viral infection of wild-type and Dnmt3a cKO mice. c, Recombination of genomic DNA from FACS-purified Dnmt3a cKO virus-specific CD8 T cells was assessed by PCR using primers that anneal to DNA outside the floxed target region. The larger PCR amplicon corresponds to the intact locus and the smaller PCR product is the amplicon of the recombined locus. d, Representative and graphical summary of Sell promoter methylation in wild-type and Dnmt3a cKO cells. Mean and s.d. were calculated from bisulfite sequencing analysis of six individually sorted populations. e, Diagram of Sell promoter CpG location proximal and distal to the TSS. f, Representative DNA methylation analysis of CpG sites distal to the Sell promoter regions in day 8 wild-type and Dnmt3a cKO antigen-specific effector CD8 T cells. Graphical summary of the average Sell distal CpG methylation in wild-type and Dnmt3a cKO cells calculated from bisulfite sequencing analysis of four individually sorted populations.
Extended Data Figure 6 | Effector-stage de novo DNA methylation is enriched at genes that regulate effector and memory T cell differentiation. a, Normalized Dnmt3a-mediated de novo methylation at CpG sites in the Lef1 and Il6st loci from WGBS datasets. b, Summary of maintenance methylated regions in wild-type and Dnmt3a cKO effector WGBS datasets. c, Connectivity plot showing IPA-predicted interactions of ID2 and ID3 with Dnmt3a-targeted loci.
Extended Data Figure 7   Dnmt3a-deficient CD8 T cells undergo effector differentiation.  a, Summary of gp33-specific CD8 T cell quantities at effector and memory time points in lymphoid and nonlymphoid tissues. b, c, Summaries of viral titres in spleen (b) and day 5 lung and liver (c) of acutely infected wild-type and Dnmt3a cKO mice. d, Quantitative PCR analysis of Dnmt3a exon 19 recombination using a primer set that binds to DNA internal to the floxed target region. The mean and s.d. of intact (non-recombined) floxed Dnmt3a alleles were determined by quantitative PCR from four individually sorted gp33-specific effector and memory CD8 T cell populations. e, Real-time PCR analysis of Sell mRNA expression of naïve and tetramer+ wild-type and Dnmt3a cKO effector CD8 T cells. f, Representative FACS analysis of Klrk1, CD127, CD27, and L-selectin expression on wild-type and Dnmt3a cKO effector and memory gp33-specific CD8 T cell splenocytes. g, Summary graph for the percentage of wild-type and Dnmt3a cKO L-selectin-positive gp276 and np396-specific CD8 T cells.
Extended Data Figure 8 | Effector molecule loci are demethylated during differentiation of virus-specific Dnmt3a cKO CD8 T cells.

a, Heat-map representation of top 3,000 demethylated regions in wild-type and Dnmt3a cKO effector CD8 T cell WGBS datasets relative to the naive WGBS dataset.
b, Normalized effector loci methylation at CpG sites in the Ifng, Prf1, and Gzmk loci from wild-type and Dnmt3a cKO WGBS datasets.
c, Representative FACS analysis of Tbet, Eomes, and Ki67 expression of gp33-specific effector CD8 T cells.
d, Representative FACS analysis of cytokine production from virus-specific memory CD8 T cells following 5 h of ex vivo gp33 peptide stimulation.
**Extended Data Figure 9 | L-selectin<sup>lo</sup> MP effector CD8 T cells develop into Tcm CD8 T cells.**

**a**, Representative FACS analysis of L-selectin expression on Thy1.1<sup>+</sup> CFSE<sup>+</sup> MP and TE CD8 T cells 1 day after transfer into naive recipient mice. The limit of our detection was approximately 10–20 CD62L<sup>+</sup> cells in each of the lymphoid and nonlymphoid tissues at 1 day post-transfer.

**b**, Summary of number of transferred TE and MP CD8 T cells in the spleen, blood, lymph node, IEL (intraepithelial lymphocytes), lung, and liver of the recipient mice 1 day post-transfer.

**c**, Summary of per cent undivided (undiluted CFSE) L-selectin-positive virus-specific memory CD8 T cells arising from adoptively transferred MP versus TE cells. Data are from three independent experiments.

**d**, Representative post-sort purity FACS analysis of undivided L-selectin<sup>hi</sup> and L-selectin<sup>lo</sup> MP P14 cells 28 days after adoptive transfer.
Extended Data Figure 10 | Memory CD8 T cells retain demethylated effector loci. Representative analysis and summary graphs of locus-specific methylation profiling of Gzmb (a) and Prf1 (b) DMRs in naive, effector (day 8 gp33 tetramer+) and memory (day 40+ gp33 tetramer+) CD8 T cells. s.d. calculated from three independently sorted samples.