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Transcriptional and Epigenetic Regulation of Effector and Memory CD8 T Cell Differentiation

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Immune protection and lasting memory are accomplished through the generation of phenotypically and functionally distinct CD8 T cell subsets. Understanding how these effector and memory T cells are formed is the first step in eventually manipulating the immune system for therapeutic benefit. In this review, we will summarize the current understanding of CD8 T cell differentiation upon acute infection, with a focus on the transcriptional and epigenetic regulation of cell fate decision and memory formation. Moreover, we will highlight the importance of high throughput sequencing approaches and single cell technologies in providing insight into genome-wide investigations and the heterogeneity of individual CD8 T cells.

Keywords: CD8 T cell, memory differentiation, cell fate decision, transcriptional, epigenetic, single cell sequencing

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INTRODUCTION

During an acute viral or bacterial infection, pathogen-specific T cells robustly proliferate, acquire effector functions, and migrate to the site of infection to eliminate the pathogen. The majority (>90%) of antigen-specific CD8 T cells die via apoptosis upon pathogen clearance, leaving behind distinct memory subsets with unique phenotypic and functional properties. However, the molecular and genetic mechanisms that guide how these cell fate decisions are made remains incompletely understood. Additionally, although it is well-appreciated that antigen specific memory CD8 T cells can persist for extended periods of time in a functionally quiescent state, and that this is important for conferring long-term protective immunity against previously encountered pathogens, the underlying mechanisms that endow memory CD8 T cells with this longevity remain unclear. Moreover, the molecular pathways that help maintain the phenotypic and functional heterogeneity of memory subsets, and enable memory CD8 T cells to remain poised to quickly recall their effector function are still incompletely understood. Current evidence suggests that multiple signals, such as T-cell receptor (TCR), co-stimulation, inflammation, and metabolic signals can orchestrate CD8 T cell fate decisions, with some of these commitment choices occurring early in the immune response (1, 2). As the incorporation of multiple distinct signals received by individual T cells likely triggers diverse transcriptional programs, it is important to discuss the key transcription factors that have been known to orchestrate CD8 T cell fate decision. Moreover, we highlight the field's current understanding of CD8 T cell differentiation on the epigenetic and single-cell level, and provide a brief discussion on how modern technologies may help to refine the CD8 T cell differentiation paradigm.

MEMORY CD8 T CELL DIFFERENTIATION AND CELL FATE DECISION

The process of memory CD8 T cell selection is not entirely stochastic, as originally proposed (3), as effector cells can display inherently distinct memory cell potential, with some CD8 T cells being intrinsically better at persisting and populating the memory pool. It was previously identified that a small subset of effector T cells survive the contraction phase and serve as the precursors of the memory CD8 T cell compartment (4–8). This minor population of effector cells, termed memory precursor effector cells (MPECs), can be distinguished based on their high expression levels of CD127, the IL-7 receptor alpha (IL-7Rα), and their decreased expression of killer cell lectin-like receptor G1 (KLRG1) (5, 6). Other surface proteins that co-segregate with increased IL-7Rα expression on MPECs include CD27, CD28, CD62L, and CXCR3 (1). By contrast, a larger proportion of effector CD8 T cells display high expression of KLRG1 and low expression of IL-7Rα and are more terminally differentiated than their MPEC counterparts. This subset of KLRG1^{hi} effector CD8 T cells is collectively referred to as short-lived effector cells (SLECs). Of note, although MPECs and SLECs were observed in various infectious settings in different species including humans, these phenotypic distinctions are not exclusive criteria for forming memory T cells nor do they represent universal markers for memory precursor cells across all types of immune response (9–11). Furthermore, several studies previously demonstrated that MPECs can give rise to both T central memory (T_{CM}) and T effector memory (T_{EM}) populations (1, 5–7) and recent evidence further indicates that the precursors of tissue resident memory cells (T_{RM}) in the skin and small intestine are also derived from less differentiated KLRG1^{lo} memory T cell precursor cells (12, 13). It is important to note however that these phenotypic distinctions are not exclusive criteria for memory T cell formation, as cell death may also occur among IL-7Rα^{hi} effector T cells following infection, and many long-lived KLRG1^{hi}IL-7Rα^{hi} memory CD8 T cells have been observed following secondary infections (14–17). Moreover, the frequency of KLRG1^{hi} cells can vary widely depending on the type of infection or vaccination. Indeed, a recent study further highlighted the limitations of these markers and elegantly demonstrated that some KLRG1^{hi} cells can downregulate KLRG1 during the contraction phase and differentiate into all memory T cell lineages (18). Thus, a higher degree of developmental plasticity than previously appreciated may exist during the effector to memory CD8 T cell transition phase. Importantly, however, these cell surface markers do offer a useful framework of determining the relative memory cell potential of effector CD8 T cells in several circumstances, and they have become invaluable for identifying molecular pathways that regulate these effector-to-memory cell fate decisions.

Heterogeneity of Memory CD8 T Cell Subsets

As CD8 T cells transition from naïve to effector to memory cells, their overall gene expression profiles changed, resulting in phenotypic and functional variations among the different populations. As such, several fundamental studies have demonstrated that memory CD8 T cells can be compartmentalized into at least 3 distinct subsets on the basis of their effector function, proliferative potential, migration patterns and transcriptional program (19–23). For well over a decade, the population of circulating memory CD8 T cells has been broadly categorized into two distinct subsets, conventionally designated T_{CM} and T_{EM} (20, 24). These two subsets can be distinguished based on their differential expression of CCR7 and CD62L (L-selectin), with T_{CM} cells expressing both of these lymph node homing receptor molecules which facilitates their trafficking to and retention within secondary lymphoid tissues (19, 20). By contrast, T_{EM} cells lack expression of CCR7 and CD62L and are most commonly found in the blood and in non-lymphoid tissues (e.g., lung, liver, intestine) (20, 21, 25). Compared to T_{EM}, T_{CM} cells display an enhanced proliferative potential and an increased capacity to produce the cytokine IL-2, but are unable to immediately produce effector molecules until they undergo secondary proliferation and differentiate into effector cells (20, 26–28). Conversely, T_{EM} cells constitutively display effector functions such as cytolytic activity and IFN-γ production (1, 21, 29, 30). Notably, within the past 10 years, T_{RM} have emerged as the third major memory CD8 T cell subset and have been identified to permanently reside in peripheral tissues after pathogen clearance and provide site-specific protection upon re-infection (22, 23). The specific anatomical location of where T_{RM} cells develop and are maintained can depend on the nature or route of the infection and the inflammatory signals experienced during the effector phase of the T cell response (31). T_{RM} cells can generally be distinguished from T_{EM} cells infiltrating non-lymphoid tissues based on their high expression of CD69 and the integrin CD103 (12, 32–35), although not all T_{RM} cells constitutively express CD103 (31, 34). An important component of T_{RM} differentiation is the migration of T effector cells to target sites (such as the skin or intestines) and their subsequent downregulation of tissue egress receptors, such as S1PR1 (35) and upregulation of adhesion molecules, such as CD103 (12). Other distinguishable features of T_{RM} cells are their sustained expression of granzyme B (that may vary by location) and their maintained high levels of mRNAs encoding TNFα, IFNγ, and IL-2 (31), which allow them to eliminate any re-occurring microbial threat at portal entry sites. Whether T_{RM} undergo homeostatic proliferation to maintain a stable population has not been clearly demonstrated. T_{RM} cells from brain, skin and mucosal sites showed much lower homeostatic proliferation ability and turnover rate compare to their circulating counterparts (22, 31, 36, 37). Interestingly, T_{RM} cells in the lung airway may require constant replenishment from recirculating memory cells (38). As T_{RM} and T_{EM} subsets display constitutive effector functions and occupy the frontline sites of pathogen entry, they are uniquely positioned to be among the first responders of the adaptive recall response. Conversely, T_{CM} recall is critical for the rapid generation of a pool of secondary effector cells that may help contain pathogens that breach the initial containment. In humans and mice, there is a newly defined subset, called T memory stem (T_{SCM}) cells.

The characteristic of T_{SCM} is stemness. T_{SCM} cells represent increased proliferative, self-renewal and long-term persistence capacity (39, 40). Additionally, only naive T cells and T_{SCM} cells were able to reconstitute the entire heterogeneity of memory T cell subsets, indicating that T_{SCM} cells are multipotent (39). In patients undergoing haploidentical hematopoietic stem cell transplantation (HSCT), T_{SCM} cells are preferentially generated from naïve cells and the dominant long-term clonotypes appeared to preferentially originate from infused T_{SCM} rather than T_{CM} clones (41, 42). Gene expression data also showed that there is a progressive change moving from naïve to T_{SCM} to T_{CM} and T_{EM} cells (39). These evidences indicate that T_{SCM} cells are at the apex of the hierarchical tree of T cell differentiation and at a hierarchically superior level over the T_{CM} cells (40). Moreover, memory T cells can be further subdivided based on differential expression of additional phenotypic markers. As one example of such endeavor, CX_3CR1 has been recently used to identify a peripheral memory (T_{PM}) subset that possesses high cytotoxicity and provides global immune surveillance (43, 44). Collectively, the formation of these distinct memory CD8 T cell subsets and their division of labor likely ensures optimal protective immunity upon pathogen re-challenge. However, a key question that remains to be addressed is whether these distinct memory subsets are maintained by signals from the tissue microenvironment or preprogrammed by cell-intrinsic mechanisms, such as transcription profiles and the chromatin landscape.

The Impact of Signal Strength on CD8 T Cell Fate

During infection or vaccination, naïve CD8 T cells engage with antigen-presenting dendritic cells (DCs) and are presented cognate peptide in a major histocompatibility complex (MHC) class 1-restricted manner (45, 46). Upon TCR-mediated recognition of the MHC-peptide complex, antigen-specific CD8 T cells will start to rapidly proliferate and acquire effector functions and the ability to migrate to sites of infection. During this process of T cell priming, newly activated T cells will integrate multiple signals in the form of TCR signaling, co-stimulation, cytokine, chemokine, and metabolic signals, all of which can have a major impact on the accumulation, survival, and cell-fate decision of effector T cells (1, 2, 47) (**Figure 1**).

TCR signaling is one of the initiating signals that helps shape T cell memory. The strength and quality of TCR signaling, which is determined by the affinity of the TCR for peptide–MHC molecules (pMHC), the dose of antigen presented by APCs, the duration of the TCR–pMHC interaction, and the timing of TCR recognition (early or late during infection phase) have been shown to partially contribute to memory commitment, function and the diversity of the memory pool (48). The balance between co-stimulatory and co-inhibitory signaling is not only required for effector T cell activation and expansion, but also determines the size and quality of the memory T cell pool (49). Co-stimulatory molecules, such as CD28, 4-1BB, CD27 and OX40 have been shown to promote memory formation as well as contribute to secondary responses (50, 51). As for co-inhibitory signals, it has previously been reported that lower PD-1 expression may drive T cell differentiation away from a SLEC fate and skew toward T_{EM} memory generation (52). TIM-3 is another inhibitory receptor and blockade of TIM-3 increases transcription of genes involved in T cell effector function and differentiation but decreases expression of genes associated with memory T cell formation (52, 53). Further studies are required to determine how co-stimulatory and co-inhibitory signaling pathways coordinately regulate memory T cell development.

Several studies have identified that exposure to certain inflammatory signals can play a major role in regulating the differentiation of effector and memory CD8 T cell subsets

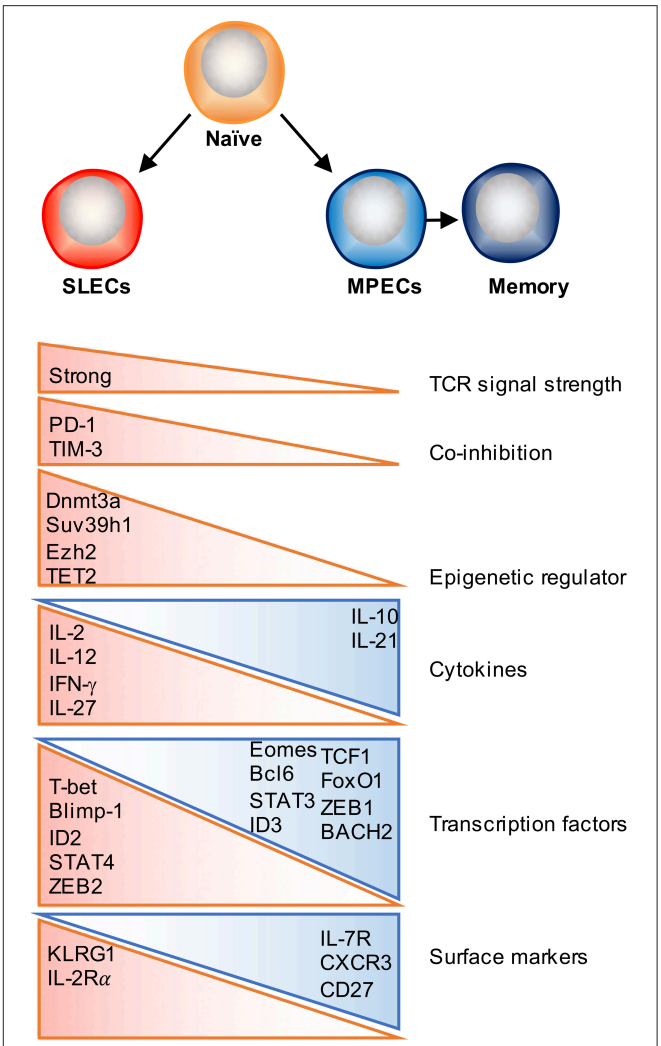


FIGURE 1 | Factors that regulate effector and memory cell fate decision. Following activation, antigen-specific naïve CD8 T cells proliferate and differentiate into a heterogeneous pool of effector T cells that consist of two major subsets: SLECs and MPECs. Majority of SLECs die by apoptosis during contraction phase, whereas MPECs survive and become long-lived memory cells. Numerous factors as depicted can contribute to this cell fate decision process, which include TCR signal strength, co-stimulatory/co-inhibitory molecules, cytokines, transcription factors, and epigenetic regulators.

in a context dependent manner. For example, IL-12 or IL-27 enhances SLEC formation during acute bacterial or viral infection, whereas type I and type II interferons can either promote memory or enhance SLEC differentiation under different settings (17, 54–59). Other studies have further identified that exposure to IL-15 helps skew CD8 T cells along a memory pathway (60–62), whereas IL-2 signaling is implicated in promoting the differentiation of short-lived effector T cells (63, 64). However, the effects of IL-2 signaling on CD8 T cell memory formation may be regulated in a temporal manner, as administration of recombinant IL-2 (rIL-2) during the expansion phase diminishes T cell survival, whereas treatment with rIL-2 during the contraction phase promotes T cell proliferation, survival, and memory formation (65). By contrast, IL-10 and IL-21 signaling through a STAT3-SOCS3 pathway was found to promote memory formation, potentially by insulating T cells from excessive inflammatory stimuli (66). Recent studies have also begun to shed light on potential cytokine signaling pathways that contribute to T_{RM} development, with recent findings elucidating an important role for the cytokine transforming growth factor β (TGF-β) and IL-15 in facilitating T_{RM} differentiation by inducing CD103 expression on T_{RM} precursor cells infiltrating the skin, lung, and small intestine (12, 13, 34, 67, 68).

TRANSCRIPTIONAL REGULATION OF EFFECTOR AND MEMORY CD8 T CELL DIFFERENTIATION

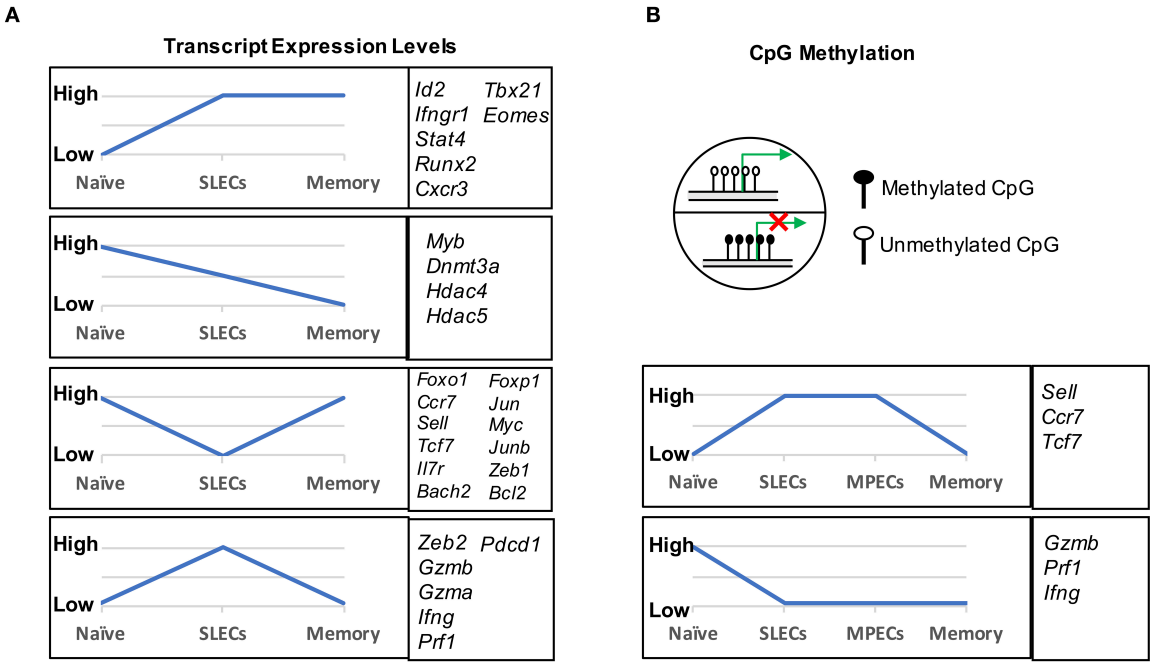
Pioneer Transcription Factors Initiate Effector Differentiation

During naïve to effector transition, dynamic changes occur at both the transcriptional and epigenetic level (**Figures 2A**, **3**). Learning from CD4 differentiation (69), the fundamental identity of these heterogeneous effector CD8 T cells can generally be established by upstream "pioneer transcription factors" that regulate the entire transcriptional network to initiate early effector differentiation. Additionally, current evidence suggests that the majority gain-of-methylation and loss-of-methylation events, which represent a repressed and active transcription state respectively, happen within the first 4 days of activation, and more than half of these differentially methylated regions (DMRs) were similarly acquired in both SLECs and MPECs (70) (**Figure 2B**). Furthermore, effector and memory CD8 T cells have been found to share a more similar pattern of chromatin accessibility as compared to naïve CD8 T cells (71). Among these shared accessible regions, the binding motifs for bZIP, IRF, and T-box transcription factors are highly enriched (71–73) (**Figure 2D**). This then brings to a question, which transcription factors are initiating this early effector differentiation? Among naïve CD8 T cells, bivalency ($H3K4me3^{+}H3K27me3^{+}$) was observed at the promotors of transcription factors that are known to be crucial for initiating an effector program, such as T-bet, Eomes, Blimp1, and IRF4 (74) (**Figure 2C**). This finding indicates that these transcription factors may remain poised in naïve T cells but rapidly start transcription by acquiring a permissive histone methylation signature upon TCR stimulation within 24 h (74). Indeed, it has been demonstrated that IRF4 cooperates with BATF (belongs to AP-1 family) to serve as "pioneer transcription factors" that promote chromatin accessibility and gene expression associated with various aspects of effector CD8 T cell differentiation (75–78). In addition, Runx3 is another potential "pioneer transcription factor" that can initiate changes in chromatin accessibility after CD8 T cell activation, especially at the binding sites of IRF, bZIP transcription factors, and Blimp1 (73) (**Figure 2D**).

Transcriptional Regulation of CD8 T Cells Fate Decisions: Terminal Differentiation or Memory Formation?

After the initial expansion phase, effector T cells can be bifurcated into two distinct effector populations, SLECs and MPECs. Early on in the effector phase, the chromatin landscape has already been universally prepared by "pioneer transcription factors," and now lineage-specifying transcription factors start to take effect. Considering that TCR signal strength is negatively associated with memory formation (2, 48), it is possible that TCR-induced transcription factors can influence the type of progeny derived from a single T cell. One such transcription factor is IRF4, expression of which is highly dependent on the signal strength of TCR signaling (79). Indeed, IRF4 has been found to be crucial for initial expansion and promoting SLEC formation (79). In addition, the expression level of memory associated transcription factors, Eomes and TCF1 appear to be highly sensitive to graded expression levels of IRF4 both in acute and chronic viral infection, indicating potential mechanisms by which IRF4 may regulate CD8 T cell fate decision (79, 80). Furthermore, Nuclear receptor subfamily 4 group A member 1 (NR4A1) supports formation of MPECs and T_{CM} via inhibiting the expression of IRF4 by directly binding to its promoter region (81). Similarly, the transcription factor BACH2 represses genes associated with terminal differentiation by binding to their enhancer regions and attenuating the availability of AP-1 binding sites (82). In this manner, BACH2 suppresses the differentiation of SLECs and tips the balance in favor of generating memory cells (82). Collectively these findings indicate that TCR-responsive transcription factors, such as IRF4 and AP-1 family members establish effector differentiation while NR4A1 and BACH2 suppress effector-associated genes. Thus, these transcription factors may cooperatively or antagonistically regulate cell fate decisions in response to different TCR signal strength intensities.

Importantly, there is an ever-expanding list of transcription factors known to orchestrate various signals experienced during the effector phase to polarize terminal differentiation or memory formation. STATs are cytokine-induced lineage-specifying transcription factors. Inflammatory cytokines, such as IL-2, IL-12, IFN-γ, and type I IFNs, signal through STAT1, 2, 4, and 5 respectively, and direct effector CD8 T cell proliferation and differentiation by inducing T-bet and Blimp-1 expression, and downregulating Bcl6, TCF1, and IL-7Rα expression (6, 83, 84). Conversely, STAT3 activation, which is induced by IL-10



C

Histone modifications

Activating	
H3K4me1	Active enhancer regions. Enhancers associated with transcriptionally poised genes.
H3K4me3	Activating H3K4me3 and repressive H3K27me3 at gene promoters represents a bivalent chromatin signature.
H3K9ac	Lost in effector cells at memory associated genes
H3K27ac	Active enhancer regions
Repressive	
H3K27me3	Increased in SLECs at genes required for survival and memory
H3K9me2	
H3K9me3	

D

Transcription Factors Motifs Enrichment

	Naïve	SLECs	MPECs	Memory
TCF1	■			■
E2A	■			■
FoxO1	■			■
AICE (BATF/IRF4)		■	■	
AP-1 (bZIP)		■	■	
T-bet		■	■	
Blimp-1		■	■	
RUNX3		■	■	

FIGURE 2 | Transcriptional and epigenetic profiling during naïve to effector to memory transition. **(A)** Gene clusters defined by the mRNA expression levels in naïve, SLECs and memory cells. Four major expression patterns emerged: genes that were up- or downregulated during the effector stage and persisted into the memory phase, and genes that were up- or downregulated during the effector phase and then reverted to the naive state. **(B)** CpG methylation levels of different genes in naïve, SLECs, MPECs and memory cells. Naïve/memory genes are similarly acquired CpG methylation in both SLECs and MPECs. MPECs, not SLECs, have the capacity to erase their newly acquired methylation programs and re-express naïve/memory genes as they develop into memory CD8 T cells. SLECs and MPECs both show demethylation of several effector-associated genes which remain demethylated in memory cells for a long period of time. **(C)** Histones posttranslational modification (PTM) and their functions that are essential for CD8 T cell differentiation. For example, the epigenetic bivalency for H3K27me3 and H3K4me3 represent an epigenetic state from which a gene can be rapidly activated or repressed depending on the differentiation pathways. **(D)** Differentially enriched motifs of transcription factors in naïve, effector and memory cells. Motif analysis identified the cell-subsets specific transcription factors binding sites in enhancer or promoter regions. Gray depicts highly enriched motifs.

and IL-21, is necessary for memory formation by promoting the expression of memory related transcription factors, such as Bcl6, Eomes, the SOCS3 (66, 85, 86). Moreover, T-bet/Eomes, Id2/Id3 and Blimp-1/Bcl6 (1), and the newly defined ZEB1/ZEB2 axis (87, 88) have reciprocal expression patterns in SLECs and MPECs, and drive differentiation toward opposing cell fates (**Figure 1**). As cell identity determined by complicated gene regulatory networks, further studies should focus on how these networks cooperatively regulate downstream target genes and cell fate decisions.

Transcription Factors Promote Memory Maintenance

Transcription factors that are critical for naïve T cell homeostasis have also been identified to promote memory CD8 T cell self-renewal and maintenance. For example, FoxO1, TCF1, and LEF1 are all highly expressed in naïve CD8 T cells, downregulated in effector cells, and re-acquired in memory cells (84, 89–93) (**Figures 2A,D**). Their expression is continuously required for the long-term survival and homeostatic proliferation but not the initial activation and clonal expansion, or effector function (91, 94–96). FoxO1 promotes the expression of pro-memory and pro-survival genes, such as *Il7r, Bcl2, Sell, Ccr7, Eomes, Tcf7, Bach2, Zeb1, and Socs3,* potentially by shielding these genes from deposition of repression associated histone 3 lysine 27 trimethyl (H3K27me3) chromatin modifications (91, 93). TCF1 and LEF1 are downstream factors of the Wnt-signaling pathway and their downregulation in effector cells is due to cell cycle and IL-12-dependent CpG methylation at the TCF1 promoter (84). Intriguingly, TCF1 and LEF1 can induce deacetylation at effector genes regions, such as *Prdm1*, to favor memory formation (97).

Transcriptional Regulation of Tissue-Resident Memory CD8 T Cells

In parallel with circulating memory cell subset differentiation, T_{RM} acquire a unique transcriptional program during differentiation and adaptation to a particular microenvironment (98–101). As early as 7 days after acute infection, a unique transcriptional signature and chromatin landscape is already established in intestinal intraepithelial lymphocytes (IELs) (102). The transcription factor Runx3 has been identified as a central regulator for T_{RM} specification by controlling a core tissue-residency gene-expression program in barrier tissues (such as lung, skin, and small intestine) and non-barrier tissues (such as salivary glands and kidney), as well as in tumors (102). Blimp-1 and its homolog protein, Hobit, establish a universal transcriptional program of tissue-residency in lymphocytes, and they have been shown to be required for T_{RM} retention in the gut, skin, liver, kidneys and lung by promoting CD103 expression while repressing *Klf2, S1pr1,* and *Ccr7* expression (99). In addition, Notch controls T_{RM} maintenance by promoting CD103 expression and regulating metabolic programs (98). Recently, NR4A1 was shown to be critical in regulating the tissue residence and function of human T_{RM} (103), and AhR was also shown to be required for skin T_{RM} (104). By contrast, the transcription factors ZEB2, T-bet (87), and KLF2 (100) have been demonstrated to inhibit T_{RM} formation by promoting tissue egress. Although T-bet and Eomes can inhibit T_{RM} formation, certain levels of T-bet expression are required for CD122 expression and IL-15 mediated T_{RM} survival (105).

THE ROLE OF EPIGENETICS IN THE CELL FATE DECISION OF CD8 T CELLS

A critical feature of memory CD8 T cells is their ability to rapidly re-acquire effector functions upon secondary challenge with the same pathogen. We are now learning that changes in the epigenetic landscape of memory CD8 T cells, including DNA methylation, histone modifications, and chromatin accessibility, play a substantial role in this phenomenon. In this section, we will discuss how these epigenetic changes shape the effector and memory fate decision as well as memory T cell formation and function (**Figure 3**).

Differences in the Epigenetic Landscapes of SLECs and MPECs Underlie Their Divergent Cell Fate Decisions

DNA methylation occurs primarily at CpG dinucleotides with the cytosine being methylated. Genomic regions with high frequencies of these CpG dinucleotide sequences are known as CpG islands and are often found in promoters. DNA methylation is commonly thought of as a repressive epigenetic mark, exerting its downstream effects by influencing transcription factor binding and acting as a docking site for various histone modifying enzymes (**Figure 2B**). In CD8 T cells, the DNA methyltransferase Dnmt3a has been shown to reduce MPECs formation by catalyzing DNA methylation at sites such as the promoter of *Tcf7*, a critical transcription factor for memory CD8 T cells (106). TET2 is methylcytosine dioxygenase and mediates active DNA demethylation. TET2 gene expression is rapidly and transiently induced by TCR signaling. TET2-deficient CD8 T cells rapidly acquired memory associated surface markers such as CD62L, CD27, and CXCR3 to promote memory formation (107). Interestingly, while naïve genes become methylated and effector genes become demethylated in both MPECs and SLECs, MPECs erase these DNA methylation marks at naïve genes as they develop into long-lived memory CD8 T cells, indicating that epigenetic repression in the form of DNA methylation can be reversed (70) (**Figure 2B**).

Genomic DNA is packaged in nucleosomes, comprised of DNA wrapped around histone octamers made up of two copies each of the histones H2A, H2B, H3, and H4. Each histone has a flexible N-terminal tail that is subject to post-translational modifications that subsequently influence transcription of nearby genes. These modifications can affect gene expression by recruiting other transcriptional regulators or, in the case of acetylation, by neutralizing the positively charged histone N-terminal tail and decreasing its interaction with negatively charged phosphates on DNA. Large-scale genomic studies have found patterns of histone modifications that can identify cis-regulatory elements such as promoters and

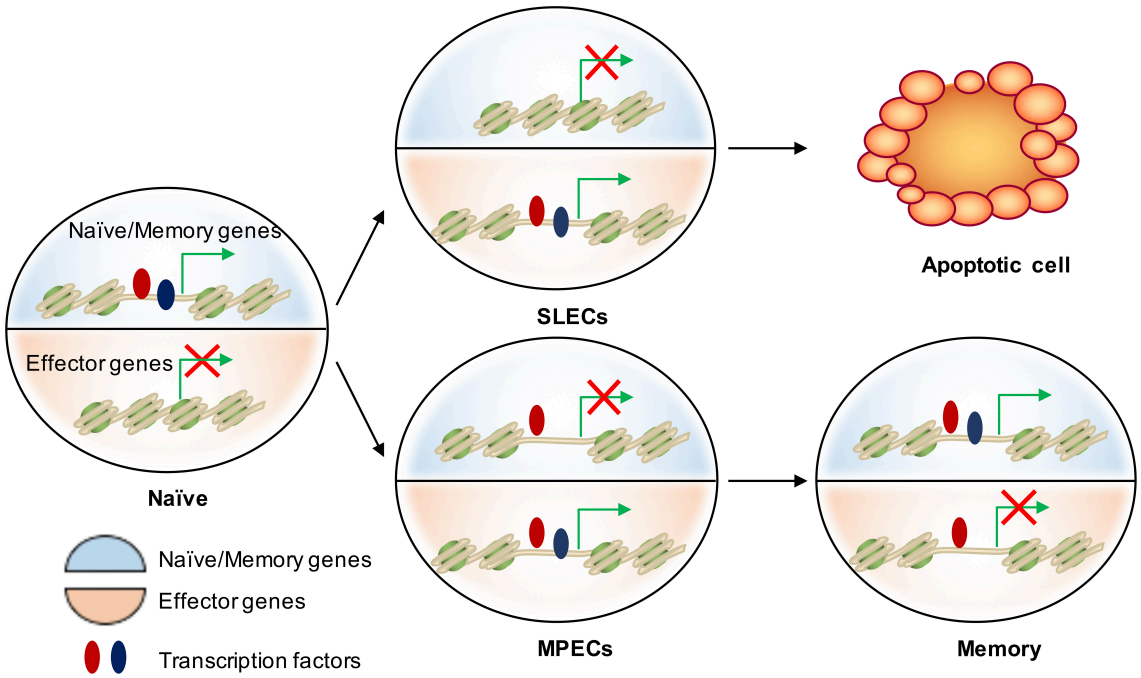


FIGURE 3 | Epigenetic regulation of cell fate decision during acute infection. There are two major transcriptional circuits in regulating CD8 T cell differentiation: one of them associated with effector function and another one is essential in naïve/memory cells controlling T cell quiescence and homeostasis. In naïve CD8 T cells, naïve/memory genes are open (epigenetically by TFs in red) and on (transcriptionally by TFs in blue), while the effector genes are closed and off. When naïve cells are activated, effector genes are turned on mainly by "pioneer TFs" (in red) in both SLECs and MPECs. These genetic regions remain open but poised as MPECs develop into memory cells. Transcriptional repression of naïve/memory genes in MPECs cells can be reversed in memory CD8 T cells through recruiting additional TFs (in blue) to restart gene expression. In contrast, SLECs lose the accessibility at these TF-bound cis-regulatory elements and therefore permanently turn off the naïve/memory gene expression. This leads to their loss of memory potential and long-term survival.

enhancers, as well as provide information regarding their activity (108–111) (**Figure 2C**). Additionally, active promoters and enhancers tend to have a central region that is depleted of nucleosomes, where transcription factors can more easily access their binding sites. It is therefore reasonable to suspect that a combination of histone modifications and accessible regions also contribute to the enhanced function of memory CD8 T cells. From studies investigating chromatin accessibility using assay for transposase-accessible chromatin (ATAC)-seq (112) and the deposition of histone modifications (H3K4Me1, H3K27Ac, H3K27Me3) by chromatin immunoprecipitation (ChIP)-seq in CD8 T cells during acute infections with *Listeria monocytogenes* and lymphocytic choriomeningitis virus (LCMV), we now have a genome-wide overview of the epigenetic changes accompanying memory CD8 T cell differentiation (71, 72, 113). These studies provide important insights into the epigenetic differences between MPECs and SLECs and through which their differentiation is regulated. Regulatory regions that are more open in MPECs than SLECs are genetic loci regulate feature genes related to naïve and memory T cell properties. However, these regulatory regions are less open or permanently silenced in terminally differentiated SLECs or exhausted CD8 T cells, suggesting that MPECs keep their memory potential through maintaining accessibility at critical memory-related cis-regulatory elements (71). Terminally differentiated SLECs have increased levels of the repressive histone modification H3K27Me3 at genes required for survival and memory cell formation, and deposition of this mark is catalyzed by the polycomb repressive complex 2 (PRC2) (93). The histone methyltransferase Suv39h1 also promotes terminal differentiation by trimethylating histone H3 lysine 9 at memory-related genes, repressing their expression (114). These differences in the epigenetic landscape between the two subsets of effector CD8 T cells provides a potential mechanism for their divergent gene expression profiles and cell fate decisions.

Epigenetic Changes in Memory CD8 T Cells Allow for Rapid Activation

The chromatin accessible regions of memory CD8 T cell are quite similar to effector cells, especially around effector gene regions (115). Moreover, their promoter regions remain demethylated from effector to memory transition (70, 115). Much work has been done investigating DNA methylation at the *Ifng* locus in CD8 T cells, which encodes the important cytokine IFNγ that is rapidly expressed by memory cells (116–120). Naïve CD8 T cells possess substantial DNA methylation at the *Ifng* promoter, at least in part due to the activity of the DNA methyltransferase Dnmt1 (117). After activation, effector CD8 T cells have this site demethylated and turn on the expression of *Ifng*. Despite no longer expressing *Ifng*, memory CD8 T cells maintain a

demethylated state at the *Ifng* promoter, thereby decreasing the number of steps required before gene expression. Help from CD4 T cells during initial activation appears to play a role in this process (119). Similar patterns seem to exist at the sites of other critical CD8 T cell effector molecules, including *Gzmb* and *Prf1*, which were found to maintain their demethylated state for at least 12 years in humans who received the yellow fever virus (YFV) vaccine (115). Therefore, regulation of DNA methylation provides a mechanism for the ability of memory CD8 T cells to quickly respond to infection.

Levels of histone H3 acetylation (119, 121) and, more specifically, H3 lysine 9 acetylation (H3K9Ac) contributes to the rapid reactivation in memory CD8 T cells (122–124). Furthermore, several studies have characterized a number of different histone modifications and chromatin accessibility at a genome-wide level over the course of a CD8 T cell response to infection or vaccination (71, 74, 93, 113, 115, 125–128). In the same study mentioned earlier, YFV-specific CD8 T cells in vaccinated humans maintain open, accessible chromatin at the promoters of the effector molecules *Ifng* and *Gzmb* (115). Overall, the establishment of specific patterns of DNA methylation, histone modifications, and chromatin accessibility prime memory CD8 T cells to more rapidly produce effector molecules and clear the pathogen.

Transcription Factors Regulating the Epigenetic Landscape of CD8 T Cells

Individual transcription factors can affect the epigenetic landscape through the recruitment of chromatin modifying enzymes or their own intrinsic activity. Blimp-1, for example, directly binds to the genes *Il2ra* and *Cd27*, recruits the histone methyltransferase G9a and the histone deacetylase HDAC2, and leads to increased deposition of the repressive marks H3K9Me2, H3K9Me3, and H3K27Me3 and decreased levels of permissive marks H3Ac and H3K4Me3 (129). The AP-1 factor BATF has been proposed to act as a pioneer transcription factor, in cooperation with its binding partner IRF4, by directly binding to tightly packed chromatin and promoting its accessibility to other transcription factors (130). Runx3 was recently shown to drive memory CD8 T cell formation by regulating chromatin accessibility of memory cell *cis*-regulatory elements (73). While TCF7 has not yet been shown to affect the epigenetic landscape during the differentiation of activated mature CD8 T cells, it establishes critical regions of open chromatin during T cell development in the thymus (131). Additionally, studies performed in thymocytes have shown that TCF7 has intrinsic histone deacetylase activity (97). Given its importance in memory CD8 T cell formation (94, 95, 132), it is likely that TCF7 uses a combination of these two methods to regulate the memory differentiation process. Other transcription factors will likely continue to be identified that either directly or indirectly lead to epigenetic changes in activated CD8 T cells, and untangling this complex network of transcription factors and the epigenetic changes they induce will help decode the differentiation of memory CD8 T cells.

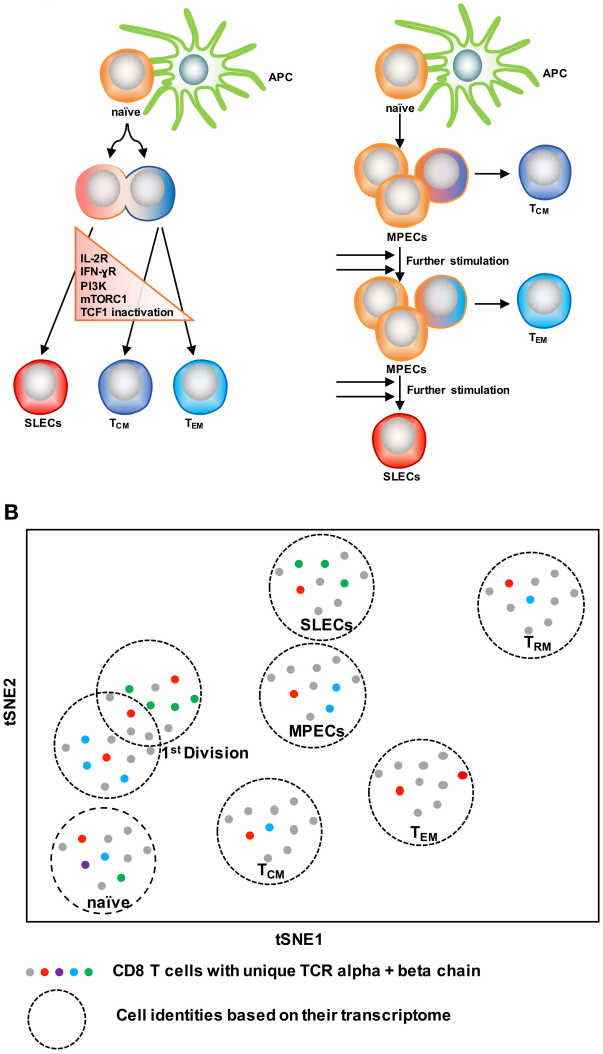


FIGURE 4 | Two CD8 T cell differentiation models. **(A)** The asymmetric division model emphasizes the significance of asymmetric segregation of cytokine receptors and signals pathways as early as the first division in dictating the memory or effector potential of T cells. The proximal daughter cell (red) inherits molecules that make it more likely to become an effector cell, while the distal daughter (blue) inherits more memory-related molecules. The progressive differentiation model is a linear model in which the cumulative history of encounters with antigen and inflammation dictate the cell fate from a memory-like stage to terminally differentiated cells. **(B)** A method to depict "one cell, one fate" and "one cell, multiple fates" models. Single cell identity of a T cell can be profiled based on their transcriptome using scRNA-seq. TCRs are nature molecular tags to track T cells. Integration of TCR clonotypes to a gene expression profile on a single-cell level can monitor the dynamics of effect and memory CD8 T cell fate decision during infection.

CELL FATE DETERMINATION OF CD8 T CELLS AT SINGLE CELL LEVEL

Previous studies show that there can be anywhere from ~80 to 1,200 naïve CD8 T cells or from ~20 to 200 CD4 T cells specific for a particular epitope in one mouse (133, 134). Following

infection, each antigen specific CD8 T cell can interpret and integrate signals in a distinct way to create differential responses in the generation of terminally differentiated effector cells and self-renewing memory T cells (6, 20). However, when and how this fate decision is made following infection has been a topic of research for many years (127, 135–139).

Different Experimental Approaches to Study the Cell Fate of Single CD8 T Cells

In terms of fate specification from a single T cell, two obvious possibilities can happen: (1) one T cell can give rise to two daughters cells with each being capable of choosing multiple fates or (2) one T cell can give rise to daughter cells with only one fate (140). Different experimental approaches have been applied to understand the *in vivo* fate of single CD8 T cells following acute viral or bacterial infections. Using an OT-I TCR transgenic adoptive cell transfer model, it has been demonstrated that diverse cellular progeny, including both effector and memory T cells, could develop out of a single naïve T cell following infection with *L. monocytogenes* (135). Similar results have been found using tetramer enrichment to isolate antigen specific naïve CD4 T cells followed by a single cell adoptive transfer approach for the *in vivo* fate mapping for CD4 T cells (141). Surprisingly, in both cases single naïve T cells displayed diverse patterns of differentiation, yet when combined together, they resembled the endogenous T cell response in the same individual mouse. Although these studies were instrumental in developing our understanding of T cell fate decision at the single cell level, a limitation of these approaches is that they only allow for deciphering the fate of one T cell at a time per mouse. To overcome this hurdle and to facilitate the analysis of multiple T cell families at the same time, one elegant study performed adoptive transfer experiments using barcoded TCR transgenic CD8 T cells. Upon bulk transfer of single barcoded naïve CD8 T cells the authors demonstrated that individual naïve T cells have multiple fates and can differentiate into both effector and memory subsets during acute infection (136). This approach offers the opportunity to analyze large numbers of barcoded TCR transgenic single naïve CD8 T cells and their fates at the same time. However, this experimental strategy is limited by its dependency on using indirect approaches (microarray, sequencing) for barcode identification and by its inability to conduct a functional assessment of T cells at the protein level. Notably, other powerful tools have emerged that help alleviate some of these pitfalls. To serve the purpose of analyzing multiple T cell families simultaneously, adoptive transfer experiments have been accompanied with the use of a matrix co-expressing congenic markers, followed by their breeding to TCR transgenic mice (137). This innovative approach allowed for the transfer and assessment of eight naïve TCR transgenic CD8 T cells at a time and revealed differential subset diversification by each single cell resulting in broad and vigorous CD8 T cell immunity. To rule out any TCR-based influence, a limiting dilution strategy has been developed with the aim of transferring a single naïve antigen specific CD8 T cell into recipient mice, which is plausible mathematically but *in vivo* difficult to prove (138). With this approach, single naïve CD8 T cells have been found to exhibit differential cell fates as well as display some extreme bias toward a particular cell fate. Importantly, using the latest powerful technology- single-cell RNA sequencing (scRNA-seq), it has recently been demonstrated that virus-specific CD8 T cells display vast transcriptional heterogeneity and can give rise to multiple cell fates, which unexpectedly was found to occur as early as the first cell division (127). This study highlights the power of using scRNA-seq and computational analyses to elucidate cell-fate decisions at the singe-cell level.

Two Models of CD8 T Cell Differentiation

With the knowledge of possible cellular fates (single fate vs. multiple fates) at the single cell level, the next question to ask is: how does this subset diversification occur following infection? There are two possibilities to support this: the model of asymmetric division driven differentiation vs. progressive differentiation model (140, 142) (**Figure 4A**). According to the asymmetric division model, the generation of long and short-lived progenies from a single precursor T cell occurs at the immediate onset of response, i.e., as early as the first cell division (143). Asymmetric segregation of cytokine receptors like IL-2Rα and IFN-γR and intracellular signaling pathways like PI3K and mTORC1 during mitosis (92, 139, 143–146) can cause proximal and distal daughter cells to have differential cytokine signaling that may lead them toward an effector or memory cell differentiation process, respectively. Supporting this, three groups (127, 138, 139) have found that at the single cell level, cellular bifurcation is possible during early rounds of cellular division in response to acute infection. On the other hand, the progressive differentiation model supports the subset diversification process from a single cell via a gradual differentiation process, from a memory-like stage to terminally differentiated cells, which is affected by the signaling strength of the signals that are received in the priming phase (115, 142). This model has been supported by a study (147) using unbiased mathematical model and probabilistic framework. It has been shown that a linear developmental pathway is responsible for cell fate diversification, that progresses from slowly proliferating memory precursors to the rapidly expanding effector population. However, none of these models alone can explain why during differentiation some cells take multiple fates while some show extreme bias toward a singular fate. On this note, it is important to consider that cellular differentiation is a dynamic process and can be accompanied by encountering stochastic initial priming events, which can make a difference in the fate of every single cell, depending on their reception and interpretation of various signals. In this respect, both T cell intrinsic factors like: signaling strength, co-stimulation, amount of cell intrinsic signaling molecules, the epigenetic landscape, and cellular metabolism and also cell extrinsic factors like: anatomical location to interact with APC, and inflammation can affect the fate of a single T cell undergoing differentiation (1, 148–150). Intriguingly, a recent finding that showed, depending on the developmental origin of naïve CD8 T cells: either fetal derived CD8 T cells or adult bone marrow derived CD8 T cells, can give rise to either memory-like

CD8 T cells in adulthood or in the generation of naïve-like CD8 T cells, respectively (151). The diverse *in vivo* response generated at the single cell level during acute infections may potentially be a result of the recruitment of heterogeneous naïve CD8 T cells, a topic, which demands further research. In terms of technological advancement, it is now possible to do *in vivo* fate mapping of single naïve CD8 T cells in a way which was limited previously with the usage of cell number, while simultaneously accounting for the influence of TCR and more importantly to recapitulate an *in vivo* natural infection scenario without the reliance on adoptive transfer strategies.

ScRNA-seq has been emerged as an innovative platform to understand the cellular development and differentiation process (127, 152, 153). With the power of computational analysis, it also offers an assessment of the subset diversification and developmental trajectory in an unbiased manner without reliance on the preexisting knowledge of cellular types (153, 154). To understand single T cell fate and its kinship with subsequent progenies, it is ideal to trace the cell fate decision by using a natural T cell lineage barcode, the TCR sequence (155) (**Figure 4B**). Using TCR sequencing to uncover the identity of single T cells was limited with the determination of both TCR alpha and beta chain information in a single cell (156–158). With the use of more powerful algorithms, it is now possible to reconstruct TCR alpha-beta gene information from single cell RNA sequencing data and to couple the cellular identity of a T cell with its transcriptomic profile at the single cell level (159, 160). This approach can overcome the usage of TCR transgenic T cells and can allow for *in vivo* single cell fate mapping by observing and tracing thousands of single T cells simultaneously in a natural infection setting (152–154).

CONCLUDING REMARKS

Current studies on genome-wide transcriptional and epigenetic changes during infection have revealed that DNA methylation, histone modifications and transcriptional signatures define CD8 T cell subsets and regulate CD8 T differentiation. Eventually, an identification of a core set of transcription factors or epigenetic regulatory molecules that can regulate memory formation could potentially be sufficient to help reprogram terminally differentiated CD8 T cells. Such findings will undoubtedly have an impact on T cell-based therapies and vaccine designs. Although the epigenetic patterns associated with distinct T cell subsets are starting to be unraveled, additional functional analyses are needed to further reveal the role of epigenetic modifying proteins and their relationship to key transcription factors that coordinately work together to determine cell-fate decisions. Moreover, as naïve CD8 T cells go through tremendous changes in their cell cycle, metabolism, cell signaling, and genetic landscape, it is starting to become well-appreciated that individual effector cells may acquire distinct cell fates, that as a whole results in the generation of a heterogeneous pool of memory T cells. While our current understanding of CD8 memory formation is derived from investigations using pooled cell populations to study cell fate decisions, recent technological advances in scRNA-seq and computational approaches hold great promise for deciphering the true transcriptional heterogeneity of individual CD8 T cells.

AUTHOR CONTRIBUTIONS

YC, RZ, DS, and AK wrote the manuscript. YC, RZ, and WC edited the manuscript.

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