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Runx proteins and transcriptional mechanisms that govern memory CD8 T cell development

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Summary

Adaptive immunity to intracellular pathogens and tumors is mediated by antigen-experienced CD8 T cells. Individual naïve CD8 T cells have the potential to differentiate into a diverse array of antigen-experienced subsets that exhibit distinct effector functions, life-spans, anatomic positioning and potential for regenerating an entirely new immune response during iterative pathogenic exposures. The developmental process by which activated naïve cells undergo diversification involves regulation of chromatin structure and transcription but is not entirely understood. This review examines how alterations in chromatin structure, transcription factor binding, extracellular signals and single cell gene expression explain the differential development of distinct effector (T_{EFF}) and memory (T_{MEM}) CD8 T cell subsets that arise after infections. Special emphasis is placed on how Runx-proteins function with additional transcription factors to pioneer changes in chromatin accessibility and drive transcriptional programs that establish the core attributes of cytotoxic T lymphocytes, subdivide circulating and non-circulating T_{MEM} cell subsets, and govern terminal differentiation. The discussion integrates the roles of specific cytokine signals, transcriptional circuits and how regulation of individual nucleosomes and RNA Polymerase II activity can contribute to the process of differentiation. A model that integrates many of these features is discussed to conceptualize how activated CD8 T cells arrive at their fates.

Keywords

Runx transcription factors; nucleosome; chromatin remodeling; transcriptional elongation; CD8 T cell memory; viral infection

Introduction

During intracellular infections or malignancies, individual antigen specific naïve CD8 T cells that become activated have the potential to develop into a wide array of ‘effector’ (T_{EFF}) and ‘memory’ (T_{MEM}) CD8 T cell populations [1,2]. Protective T_{EFF} and T_{MEM} cells develop in response to various model intracellular pathogens in mice [3,4] and live viral vaccines in humans [5,6] that cause transient infections, which are efficiently cleared (‘acute infection’). These antigen-experienced CD8 T cell subsets manifest differences in their lifespans, effector functions, capacities for self-renewal and potential for proliferation

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upon secondary antigen encounter, and their ability to traffic between or localize within distinct lymphoid and non-lymphoid tissue and micro-anatomic locales [7,8]. Transcriptional regulation of gene expression governs the differential development of these cells but is incompletely understood and is of longstanding interest [9–12]. The central focus of this review is to consider how regulation of chromatin structure, transcription and gene expression during and after the activation of naïve CD8 T cells promotes their progenies to form specific subclasses of long-lived T_{MEM} cells, or to differentiate into T_{EFF} cells that are short-lived.

The CD8 T cell response to an acute infection derives from a small number of naïve antigen-specific CD8 T cells that undergo geometric expansion into a large and heterogeneous population of T_{EFF} cells. During this burst, a comparatively small number of T_{MEM} cells are generated that emerge as the infection resolves. This response follows a categorical pattern of T_{EFF} cell population accumulation, contraction, and T_{MEM} cell formation, but how cells select distinct T_{EFF} and T_{MEM} cell fates is still unclear. To gain insight into how transcriptional control contributes to the process, this review describes multiple established developmental relationships between phenotypically defined antigen-experienced CD8 T cell subsets, and examines how differential gene expression develops within nascent and definitive T_{EFF} and T_{MEM} cell populations. The roles of cytokines that differentially affect the formation of T_{EFF} and T_{MEM} subsets are redefined in the context of the transcriptional circuits and gene expression programs they promote. In addition, the regulation of chromatin structure that occurs as naïve CD8 T cells become activated, the similarities and differences in chromatin structure landscapes that manifest later in distinct T_{EFF} and T_{MEM} cell subsets, and several of the transcription factors (TFs) and chromatin regulatory factors (CRFs) that appear to be involved are discussed. Emphasis is placed on the role of individual nucleosomes and how Runx-family TFs cooperate with additional TF families to pioneer the reprogramming of chromatin accessibility during initial T cell receptor (TCR) stimulation of naïve cells, and how they establish transcriptional circuits that lead to T_{EFF} and T_{MEM} cell differentiation. The function of distal enhancers is discussed, and how regulation of RNA Polymerase II (Pol II) elongation activity might contribute to reinforcing the specific transcriptional programs that differentially drive formation of T_{EFF} and T_{MEM} cells. Finally, a model that integrates observations from all of these aspects is used to conceptualize how naïve CD8 T cells diversify into T_{EFF} and T_{MEM} populations.

The diversity of antigen-experienced CD8 T cells and their putative origins

Antigen-experienced CD8 T cell subsets—An extensive taxonomy of antigen-experienced CD8 T cells that arise during infections *in vivo* has been defined, and these cell subsets have been reviewed in detail [7,8,13]. Only salient points are emphasized here in order to provide the framework for conceptualizing how transcriptional regulation contributes to the diversification process.

Referring to antigen-experienced CD8 T cells as T_{EFF} and T_{MEM} cells is an imprecise but handy terminology that has its origins in the temporal pattern of a prototypical T cell response to acute infection of mice, which is categorized into effector, contraction and memory phases [11,14,15]. The effector phase generally encompasses the time from

primary infection to the peak in accumulation of the responding cell population and roughly coincides with the time of pathogen clearance. Cells during this period are termed T_{EFF} cells, although it turns out there is substantial differences in the nature of T_{EFF} cells within the population [11]. As the pathogen is cleared, the expanded T_{EFF} cell population 'contracts' because many T_{EFF} cells are terminally differentiated and short-lived [16]. Over the next few weeks, the declining numbers of T_{EFF} cells ultimately stabilize, which marks the beginning of the memory phase, generally about 30 days after a prototypical acute infection in mice. Cells that persist to this time are considered T_{MEM} cells [16]. Although T_{MEM} cells have their origins in the effector phase [17], they are not considered completely manifest *in vivo* until late times. The identity of T_{EFF} cells early in the response that serve as precursors to T_{MEM} cell subsets later, and the transcriptional regulation that controls whether T_{EFF} cells terminally differentiate or develop into long-lived T_{MEM} cells, are still incompletely understood. Exactly how the entire process occurs remains a matter of intense interest [9,10,12].

Protective T_{MEM} cell subsets that arise after an acute infection resolves are broadly subdivided into circulating (T_{CIRC}) memory cells and tissue-resident memory (T_{RM}) cells [18,19]. Within these broad classifications, cells can be further delineated based on phenotype, function, migratory properties and anatomic distribution [8,13]. Cells in these subclasses manifest distinct patterns of gene expression and chromatin structure, which suggests that they could be phylogenetically distinct subsets. T_{CIRC} cells consist of effector memory (T_{EM}) and long-lived effector (T_{LLE}) cells (also referred to as terminal-T_{EM}, or effector-like T_{MEM} cells) that patrol the vasculature [20–22], peripheral memory (T_{PM}) cells that recirculate through peripheral tissues and secondary lymphoid organs [23], and central memory (T_{CM}) cells that localize within T cell areas of secondary lymphoid organs [20,23]. In addition, memory cells with stem cell like qualities (T_{STEM}) also localize in lymphoid organs, exhibit overlapping features with T_{CM} cells and some exhibit features of T cells that interact with B cell follicles [24–29]. In contrast, T_{RM} cells enter, mature and establish residence in multiple non-lymphoid tissues, wherein they remain largely positioned without re-circulating [13,30]. There is additional heterogeneity among T_{RM} cells that is partly related to the tissue in which they reside, and their persistence at different times after infection within the same tissues, implying tissue and temporal dependent regulation [13,31].

Theories of initial T_{MEM} cell development—Despite this taxonomy, and although many population-level precursor-progeny relationships have now been defined, the developmental process by which naïve cells differentiate into distinct T_{MEM} cells is still unclear. Results from longitudinal analysis of cell phenotypes throughout the course of infection, studies using adoptive transfer of phenotypically defined populations, and mathematical modeling have led to linear models of differentiation in which naïve cells initially pass through a T_{EFF} cell stage, before some cells differentiate into T_{MEM} cells [4,11,15,32,33]. In contrast, results from lineage tracing using DNA barcodes and single cell transfers support an alternative linear differentiation model in which T_{CM} cells develop directly from activated naïve cells, some of which proliferate more than others and undergo differentiation into T_{EM} and ultimately terminally differentiated T_{EFF} cells [34–37]. Separate lineage models have also been described [11,38], and imply that cells early in the response

select distinct developmental pathways that lead to the alternative fates of terminally differentiated T_{EFF} cells or long-lived self-renewing T_{MEM} cells [38,39]. The separate lineage models provide a solution for the obvious heterogeneity among T_{EFF} cells, and also account for naïve and activated cells that undergo asymmetric cell division [39,40], but do not clarify when divergence into these pathways occurs. More recently, another model leaves behind the notion of defined 'lineages', and describes T_{MEM} cell formation as a continuum of cell states that are subdivided into T_{RM} and T_{CIRC} realms that are both arrayed in a tiered descent whereby cells that move into lower tiers lose potential to form or re-form other cells in the continuum [19]. Thus, a unifying framework is still outstanding, but will likely include attributes of all of these concepts. In any case, accurately defining this developmental framework will be instrumental for clarifying how gene regulation governs the process *in vivo*. Insight on both fronts is likely to serve as the basis for more effective vaccination and immunotherapeutic approaches that provide durable immunity against infections and cancers.

CD8 T_{EFF} cell heterogeneity near the peak response to acute infection— Following several model systemic acute infections, the T_{EFF} cell population generally peaks numerically around one week after infection. At this time, T_{EFF} cells with distinguishing phenotypes can be discerned that exhibit distinct proclivities for contributing to formation of T_{CIRC} cell subsets [10,11]. During primary TCR stimulation, all naïve CD8 T cells initially downregulate expression of IL-7Rα (CD127), a cytokine receptor that is essential for naïve and T_{MEM} cell survival [41–43]. Only some cells re-express CD127 as the infection clears, and these cells preferentially give rise to long-lived T_{CIRC} cells [41,44]. Differential expression of both KLRG1 and CD127 on cells has become one of the most extensively used experimental schemes for delineating cells late in the effector phase that possess different potentials to form T_{MEM} cells [45].

Most KLRG1^{hi} CD127^{lo} cells inefficiently persist into the memory phase and have been termed short-lived effector cells (SLEC) or terminal effector (TE) cells [3,41,45–47]. Some KLRG1^{hi} cells can give rise to T_{LLE} cells, although most KLRG1^{hi} cells do not survive contraction [20,22]. In contrast, KLRG1^{lo} CD127^{hi} cells are termed memory precursor (MP) effector cells, because they efficiently give rise to T_{EM} (CD62L^{lo} CD127^{hi}) and T_{CM} (CD62L^{hi} CD127^{hi}) subsets [3,41,45–47]. In addition, KLRG1^{hi} CD127^{hi} 'double positive' effector cells (DPECs) also contribute to several subsets in the memory compartment [3,21,48]. Finally, KLRG1^{lo} CD127^{lo} cells can give rise to all KLRG1/CD127 subsets and are referred to as early effector (EE) cells [3,45,46]. The differential expression of multiple additional markers including CD27, CD43, CD62L, CXCR3 and CX3CR1 on T_{EFF} cells near the peak response to infection has also been correlated with the development of T_{CM}, T_{EM} and T_{PM} subsets [21,23,34,36,47]. Classifying whether each of these phenotypically defined T_{EFF} and T_{MEM} cell subsets are variations along a continuum [7], or are separate cell lineages, is probably not as important as clarifying the regulation of gene expression that defines their development because they each contribute to primary and recall immunity depending on the context.

The point at which T_{EFF} cells become specified into distinct subsets with differential T_{MEM} cell potential prior to the peak response is still unresolved, but there is evidence that

lineage-bias develops well before maximum population expansion. The KLRG1/CD127 paradigm is less informative at early times because CD127 is not re-expressed until late in the effector phase, and KLRG1 expression is not an exclusive feature of terminal differentiation [41,48]. KRLG1^{hi} and KLRG1^{lo} cells isolated 4.5 days after infection with the Armstrong strain of lymphocytic choriomeningitis virus ($LCMV_{Arm}$) manifest both effector cell qualities and substantial T_{MEM} cell formation potential, although KLRG1^{hi} cells preferentially acquire a TE phenotype and have less T_{MEM} potential [45,49]. Differential expression of the interleukin-2 receptor alpha (IL-2Rα), as well as the transcriptional regulators Id2 and Id3, can demarcate cells at early time points that have differential potentials for developing into TE and T_{EM}, or T_{MEM} cells. IL-2Rα^{lo} cells isolated on day 5 after infection preferentially form T_{CM} cells, whereas IL-2Rα^{hi} cells preferentially become T_{EM} cells after adoptive transfer [50–52], which suggests that differential IL-2R signals might contribute to the early heterogeneity in T_{MEM} cell potential [42]. The source of this differential developmental potential was further defined using mice with gene-targeted *Id2-YFP* and *Id3-GFP* reporter alleles [53]. A subset of IL-2Rα^{lo}, KLRG1^{lo}, and CD62L^{lo} cells responding to either *Listeria monocytogenes* or Vesicular Stomatitis Virus on day 5 more highly express *Id3*-GFP and slightly less *Id2*-YFP than others. These Id3^{hi}/Id2^{lo} cells preferentially form MP cells several days later based on KLRG1/CD127 expression, and ultimately give rise to a T_{CIRC} cell population that develops into more secondary T_{EFF} cells upon rechallenge, compared to their *Id2*^{hi}/*Id3*^{lo} counterparts [53]. Likewise, CD8 T cells from mice with a targeted *Tcf7*-GFP allele have been used to trace cells expressing the transcription factor (TF) TCF1 (encoded by *Tcf7*) [40,54]. *Tcf7*-GFP^{hi} cells early in the response express less KLRG1 and other features of terminally differentiated cells, and preferentially give rise to self-renewing T_{CM} subsets after adoptive transfer [28,40,55]. The deficiency in either *Id3* or *Tcf7* compromises the formation of T_{CM} cells [53,55], whereas deficiency in *Il2ra* (IL-2Rα / CD25) impairs TE and T_{EM} cell formation [42,52,56,57]. Thus, cells that express differential amounts of these factors early in the response correlates with their capacity to form T_{EFF} and T_{CIRC} cells, which suggests that cells become lineage-biased prior to formation of canonical TE and MP cells defined later by KRLG1 and CD127 expression.

The origins of T_{RM} and T_{CIRC} subsets are likely to be different—T_{RM} cells and T_{CIRC} cells might develop from distinct T_{EFF} cell precursors. T_{RM} cells that develop in the skin following herpes simplex virus infection in mice derive from a KLRG1^{lo} T_{EFF} cell population [58], which could imply that canonical KLRG1^{lo} CD127^{hi} MP cells are the source of T_{RM} cells. However, a substantial fraction of T_{RM} cells in multiple non-lymphoid tissues develop from KLRG1^{hi} CD127^{hi} DPECs that downregulate KLRG1 [48]. In addition, MP cells isolated from the spleen, and T_{RM} precursors isolated from non-lymphoid tissues, one week after infection with $LCMV_{Arm}$ each exhibit distinct gene expression and chromatin accessibility profiles, which suggests that these subsets are distinct [12,31,59,60]. Consistent with this interpretation, T_{EFF} cells that seed T_{RM} cells in the intestinal epithelium are most frequent in the spleen ~4 days after systemic $LCMV_{Arm}$ infection [61], substantially earlier than when canonical MP cells become evident [45]. Finally, T_{EFF} cells that exhibit increased T_{RM}-associated gene expression while in the circulation preferentially form T_{RM} cells, and lineage tracing studies using DNA barcodes showed their formation is imprinted at the

clonal level before entering the skin [62]. These results indicate that the precursors of T_{RM} and T_{CIRC} cells might arise differentially at early times.

Additional evidence that supports this conclusion is the fact that the TFs Runx3 (and its obligatory partner, *Cbfb*) and Blimp1 (encoded by *Prdm1*), which are each essential for normal T_{RM} formation [59,63], are most highly expressed by T_{EFF} cells on day 5 compared to day 8 after $LCMV_{Arm}$ infection [64]. Depletion of either Runx3 or Cbfb results in a near complete loss in formation of KLRG1^{hi} CD127^{hi} DPECs, but only a partial reduction in formation of MP cells, while reciprocally, enforced Runx3 expression reduces TE cell frequencies and drives increased formation of both DPECs and T_{RM} cells [59,64]. These studies provide molecular evidence for distinct, early transcriptional programming of T_{CIRC} and T_{RM} precursors during systemic infection. Additional analyses using single cell genomics, lineage tracing, and the identification of additional differentially expressed markers on T_{EFF} cells that correlate with the development of T_{RM} cells could help to continue defining how and when responding CD8 T cells become subdivided into T_{RM} and T_{CIRC} subsets [31,60].

Flexibility in the differentiated states of some T_{EFF} and T_{MEM} cells—To what extent antigen-experienced CD8 T cells that have adopted specific phenotypic characteristics have actually ‘fixed’ their differentiation status remains an open question [19,65]. Several examples exist in which cells isolated on the basis of one cell phenotype undergo a transition into an alternative type. For example, some mature T_{RM} cells isolated from the small intestinal epithelium differentiate into T_{CM}, T_{EM} and new T_{RM} upon secondary challenge [66]. In addition, although virtually all T_{EFF} cells downregulate CD62L expression during the effector phase by the peak response, transfer studies have shown that CD62L^{lo} MP cells, but not CD62L^{lo} TE cells, re-induce CD62L expression prior to their initial homeostatic cell division as a consequence of DNA demethylation in the *Sell* (CD62L) locus [47]. Reciprocally, but in a similar vein, not all cells during the effector phase that initially upregulate expression of effector-associated genes sustain their expression and commit to terminal differentiation at later times. Analysis of mice in which Cre recombinase expression is driven by either the *Klrg1* (KLRG1) locus or a BAC-transgenic *Gzmb* (Granzyme B) locus, to permanently mark cells having expressed these genes by using Cre expression to activate constitutive reporter gene expression, demonstrated that many cells which initially expressed *Klrg1* or *Gzmb* downregulate their expression later, and ultimately become T_{MEM} cells [48,67]. Thus, gene expression in many T_{EFF} cells is not permanently stabilized, and appears to be reversible given the appropriate circumstances, which is consistent with the natural reversibility inherent to transcriptional control in response to fluctuating levels of transcriptional regulatory proteins [68]. For example, a population of cells with an apparently uniform phenotype might appear similar at the protein level (e.g., KLRG1^{hi}), but be composed of individual cells that manifest distinct underlying metastable transcriptional states that ultimately favor alternative developmental outcomes (e.g., TE or T_{MEM} cells) [69]. Defining how regulation of chromatin structure and transcription govern the stability and flexibility of these gene expression states is central to understanding how distinct CD8 T cell subsets are initially established and then maintained.

Regulation of gene expression during the formation of T_{EFF} and T_{MEM} CD8 T cell subsets

Heterogeneous gene expression in activated CD8 T cells leads to lineage-bias early during infection—CD8 T cells begin manifesting gene expression that is biased toward one or another T_{EFF} or T_{MEM} cell lineage early during their response to infection, but do not solidify these gene expression programs until later during differentiation. Recent single cell RNA-seq (scRNA-seq) analysis has shown that distinct groups of cells within the overall T_{EFF} population early after $LCMV_{Arm}$ infection are enriched with gene expression signatures specific to T_{EFF} or T_{MEM} cell subsets that develop later [31,60]. This suggests that early developmental decisions could be made that set some cells on distinct trajectories.

Consistent with this, bulk gene expression in FACS-purified subsets that are 'T_{EFF}-like' based on being $KLRG1^{hi}$ or $Id2^{hi}$ at early times after infection are more enriched with gene expression from mature TE cells, whereas those that are more 'T_{MEM}-like' based on being $KLRG1^{lo}$, or $Id3^{hi}$, are more enriched with gene expression characteristic of T_{MEM} or MP cells [49,53,64,70]. However, discerning the cells that are actually 'differentiated' at early times on the basis of only a handful of surface receptors or reporter genes is likely limited, because of transcriptomic variation between single cells [69], and gene expression patterns that do not stabilize until later. For example, gene expression on day 5 after infection is more similar between $KRLG1^{hi}$ and $KLRG1^{lo}$ cells than it is between either subset and naïve cells, or between either subset and mature EE, TE or MP phenotypic cells on day 8 post $LCMV_{Arm}$ infection [49,64]. In addition, unsupervised clustering of genes based on the kinetics of their expression throughout the CD8 T cell response has shown that the expression of groups of genes associated with mature T_{EFF} and T_{MEM} cell subsets is dynamic at early times, before their expression is consolidated at later times [71]. Thus, gene expression programs become more differentiated over time and gene expression of mature T_{EFF} and T_{MEM} cells develops progressively at the population level.

At the single cell level, gene expression in responding CD8 T cells is heterogeneous and also appears to be unstable at early times after infection. This attribute is a general characteristic of cells undergoing lineage choice and probably facilitates multilineage differentiation potential in the early T_{EFF} cell population [69]. Single cell analysis of gene expression in daughter cells resulting from the first naïve CD8 T cell division after infection found they clustered in two distinct groups [72]. One group is characterized by T_{CM}-like gene expression whereas the other TE-like, a result that is consistent with asymmetric cell division upon naïve cell activation [39]. However, this stark dichotomy in gene expression is not evident in T_{EFF} cells on day 4 after infection, and their gene expression is distinct from those after their first cell division [72], which suggests the initial gene expression patterns might not have been inherited. However, expression of multiple 'fate-classifier' genes that are associated with either T_{EFF} or T_{MEM} states could be used to categorize the day 4 T_{EFF} cells, suggesting that lineage-bias exists at this time [72]. An important unanswered question is whether T_{EFF} cells with a particular bias in gene expression on day 4 were the specific progeny of one category of daughter cells that were T_{CM} or TE cell biased after their first division, which would imply that the initially divergent gene expression patterns were maintained within a lineage of cells at later times. Given that lineage-tracing studies suggest individual naïve cells stochastically give rise to progenies comprising either terminal $T_{EFF,}$ or T_{MEM} cell fates [35],

it is likely that the initial divergence in gene expression among first generation daughter cells is not specifically retained in most of their progenies. Thus, naïve cells might adopt alternative transcriptional states after their first division, but it is unclear how frequently these states are preserved and account for distinct early differentiation trajectories.

Another possible interpretation that could explain cells exhibiting either T_{CM} or TE-biased gene expression after their first division *in vivo* is that they comprise daughter cells in different temporal stages of the same gene expression program, rather than qualitatively distinct programs. A separate scRNA-seq analysis using carefully controlled *in vitro* conditions demonstrated that all naïve cells activate a categorical gene expression program within the first 6 hours of stimulation, regardless of the initiating ligand's 'strength' [73]. The signal strength governs the rate at which cells in the population activate the program but does not change qualitatively the induced program's nature. Together, these studies demonstrate that early gene expression is dynamic and unstable and that differences observed at singular time points *in vivo* could relate to asynchrony. Future studies that link the kinships of single responding cells and their individual transcriptomes to trace how transcriptional states are propagated within lineages will likely provide insight as to when and how gene expression programs are initially established and stabilized *in vivo*.

Extracellular signals and transcriptional circuits that differentially regulate formation of TE and T_{MEM} cells—A single brief period of TCR and co-stimulatory receptor stimulation experienced by naïve CD8 T cells is sufficient to induce a complete program of memory cell differentiation [74,75]. Additional signals are necessary to drive terminal differentiation of T_{EFF} cells [3,42,45,51,76–79]. However, inferences from lineage tracing studies indicate that the differentiation fates of CD8 T cells are established before extensive population expansion [34,36], suggesting that signals delivered early in the response 'imprint' whether some activated cells will ultimately stabilize gene expression that preserves formation of T_{MEM} cells or drives terminal T_{EFF} cell differentiation. Described below are multiple signals and transcriptional networks that promote either terminal or T_{MEM} cell differentiation. The integration of these opposing signals early during infection is likely to foster the heterogeneity and instability of gene expression among responding cells in the effector phase at early times, before alternative transcriptional programs become dominant within distinct cell populations.

Signals and transcriptional circuits that promote terminal differentiation—Lineage tracing studies indicate that there is a positive correlation between protracted cell division history and terminal differentiation [34,36]. One part of the explanation for how activated cells might 'commit' at early times to terminal differentiation is that naïve cells integrate signals during activation that are delivered through the TCR, co-stimulatory receptors and receptors for inflammatory cytokines, such as IL-12 and IL-2, and translate their sum into the extent of cell division; greater sums predict more extended cell division [80]. Stimulation with type I IFNs, IL-12 and IL-2 determines the magnitude and duration of IL-2Rα expression, which is required for high affinity binding of IL-2 [42,45,51,77,78,81]. IL-2Rα expression is positively regulated by the continued presence of IL-2 [82], which promotes the proliferation and survival of stimulated cells by inactivating FoxO-family

TFs and other mechanisms [83–85]. Thus, early inflammatory cytokine signals establish a positive feedback loop that regulates expansion of the T_{EFF} cell population in response to the availability of IL-2, whose concentrations rise transiently and are sustained while it is produced by CD4 and CD8 T cells responding to the presence of antigen [51,52,86]. A second part of the explanation is that inflammatory cytokines (type I IFN, IL-12, and IL-2) enhance expression of TFs such as T-bet, Zeb2, Id2, and Blimp1, which each directly promote transcription of genes encoding factors that underlie terminal differentiation [42,45,51,53,79,87]. Thus, signals from multiple cytokines received early in the response that program extended proliferation also drive gene expression characteristic of TE cells. Coupling both of these features in activated cells is likely to increase the probability that cells stabilize gene expression underlying TE cells and become terminally differentiated.

IL-2 and IL-12 mediate distinct and overlapping effects that promote terminal differentiation [42,79]. Deficiency in IL-2Rα results in impaired formation of TE cells [42,56,79]. IL-2 stimulation positively regulates expression *Irf4* and *Batf* after TCR stimulation (M.E. Pipkin unpublished observations), which encode TFs that co-bind composite bZIP-IRF motifs in genes whose expression promotes overall T_{EFF} cell accumulation and terminal differentiation [88]. Deficiency in either of these TFs impairs TE cell differentiation [88–91]. In addition, T-bet expression is impaired in activated CD8 T cells lacking IL-2Rα, however, increased IL-2 stimulation of wildtype cells does not increase *Tbx21* (T-bet) mRNA expression [42]. Thus, IL-2R stimulation is required for terminal differentiation, but sustained IL-2R signals most likely promote TE cell differentiation by mechanisms other than increasing *Tbx21* gene expression. In contrast, IL-12 potently induces *Tbx21* gene expression, which drives terminal differentiation [14,45]. T-bet cooperatively induces TE cell development by functioning with additional TFs, including Zeb2 and Blimp1 [9,10,79,92]. T-bet directly binds the *Zeb2* gene locus and enhances its expression. Zeb2 is required for optimal T-bet binding to *cis*-regulatory regions in other downstream genes that both TFs control [92]. Thus, T-bet activates its own facilitator (Zeb2), which creates a feed-forward circuit that crystalizes gene expression comprising the terminally differentiated state [92,93]. In addition, both IL-2 and IL-12 positively regulate *Prdm1* mRNA expression, and Blimp1 is essential for terminal differentiation [87,94,95]. Thus, both cytokines cooperatively promote terminal differentiation. However, Blimp1 represses *Il2ra* expression late in the effector phase, suggesting it is part of a negative feedback circuit that regulates the magnitude of the T_{EFF} cell population by desensitizing $KLRG1^{hi}$ cells to IL-2 signals during the contraction phase [56,96].

Although IL-2R signals are necessary for terminal differentiation, they also ensure the normal programming of T_{CM} cells, which indicates that IL-2 functions as a formatting factor that facilitates multiple differentiation outcomes, rather than only instructing one fate over the other. Although CD8 T cells deficient in IL-2Rα preferentially develop a phenotype resembling T_{CM} cells, these cells are not normal, and do not undergo robust recall proliferation during secondary infections [52,57,86]. The rapid kinetics of IL-2Rα expression early during infection suggest that the intensity or duration of transient IL-2 stimulation programs T_{CM} cell formation and regulates terminal differentiation early in the response [51]. Notably, gene expression has not been analyzed in IL-2Rα-deficient cells at early times during infection while IL-2 stimulation is normally underway. Future studies

of IL-2R-dependent nascent mRNA expression (in addition to mature mRNA expression), is likely to be important for determining how transcription-dependent regulation by IL-2 initially establishes both T_{MEM} and T_{EFF} cell populations. In addition, the transient and dynamic nature of IL-2Rα expression during T cell responses suggests IL-2 stimulation could function at multiple stages, and future studies using methods to conditionally control IL-2Rα expression could be important for delineating the physiological roles of IL-2 throughout T_{MEM} cell formation and homeostasis.

Signals and transcriptional circuits that establish and preserve T_{MEM} cell differentiation—The cytokines IL-10 and IL-21 each activate the TF STAT3 and are important for formation of MP cells and establishing the T_{CM} compartment, in part by counteracting IL-12 signals. Cells lacking STAT3 aberrantly develop a terminally differentiated phenotype, form fewer MP cells and fail to form or sustain T_{CM} cells during viral infection [97]. Blocking IL-10 in the context of IL-21 deficiency also reduces formation of T_{CM} cells, suggesting these cytokines are responsible for activating the STAT3 that promotes T_{CM} development [97]. STAT3-deficient cells express less of the inhibitory molecule suppressor of cytokine signalling 3 (SOCS3), and exhibit enhanced IL-12 responsiveness, resulting in reduced expression of the TFs Bcl6, Blimp1 and Eomes [97]. Disruption of *Bcl6* also results in reduced frequencies of MP cells and fewer T_{CM} cells in the memory phase [97]. In addition, *Eomes* deficient cells contribute poorly to the T_{CM} compartment, and its expression in wildtype cells is repressed by IL-12 stimulation [98,99]. Thus, these TFs appear to function downstream of STAT3 during infection, probably in response to both IL-10 or IL-21, and perhaps other cytokines. In addition, the stability of the IL-10 pathway appears to be sustained by a positive feedback loop involving Eomes [100]. In the absence of Eomes, T_{EFF} cells express less IL-10, Bcl6 and CD62L. Conversely, enforced Eomes expression is able to induce expression of Bcl6, IL-10 and CD62L, both in wildtype and IL-10-deficient T_{EFF} cells [100]. These results indicate that the ability of activated CD8 T cells to sense cytokines that activate STAT3 (e.g., IL-10 and IL-21, and potentially others), and retard signals from IL-12, is important for establishing or maintaining T_{CM} cell developmental potential during the effector phase.

Given that STAT5 and STAT3 can compete for occupying overlapping binding sites to produce distinct differentiation outcomes during CD4 T cell differentiation [101], it is possible that IL-2R stimulation, which activates STAT5, directly influences STAT3-dependent programs during the differentiation of activated CD8 T cells. In line with this hypothesis there is strong negative regulation of Bcl6 expression in response to IL-2R stimulation. *Bcl6* is expressed in naïve CD8 T cells, and its expression is maintained throughout TCR stimulation [42,51]. Upon cessation of TCR stimulation and provision of exogenous IL-2, *Bcl6* is quickly repressed, and reciprocally *Prdm1* is induced [42]. *Bcl6* is re-expressed in conjunction with *Il7ra* after several days, in a manner that is inversely related to the concentration of IL-2 in culture [42]. *Bcl6* is not repressed normally in IL-2Rα deficient CD8 T cells during LCMV infection, confirming that IL-2R signals are essential for repressing *Bcl6 in vivo* [42,51,56]. These results suggest that IL-2R signals might promote the antagonistic regulation of both Bcl6 and Blimp1 in CD8 T cells, analogous to what occurs in CD4 T cells, which requires STAT5 [102–107]. Thus, titration of Bcl6 and Blimp1 expression in

response to IL-2R signals and STAT5, and other cytokines that activate STAT3, could be important for establishing divergent gene expression programs that bias differentiation of distinct T_{EFF} and T_{MEM} cell subsets. Additional studies are needed to determine the order of operations in how this occurs downstream of TCR and IL-2R signals in CD8 T cells. One intriguing possibility is that variation in Bcl6 and Blimp1 expression influences the type of T_{MEM} cells that develop, because both factors are implicated in distinct T_{MEM} subsets. Although disruption of *Prdm1* clearly impairs terminal differentiation, and results in an increase in T_{CM}-like cells [87,94–96], its deficiency also reduces T_{RM} cell formation [12,63,108], suggesting it has roles in multiple T_{MEM} subsets. Reciprocally, whereas Bcl6 deficiency impairs formation of T_{CM} cells [97,109], it is also expressed in some T_{RM} cells at late memory time points [31]. In addition, its high expression in T_{CM} cells and cells that exhibit features of follicular CD4 T cells and stem cells suggests it might contribute those that are memory stem (T_{STEM}) cell-like [28,29,110]. Thus, the roles of Bcl6 and Blimp1 in the regulation of T_{EFF} and T_{MEM} cells is likely more complex than currently appreciated. Clarifying their regulation by IL-2R signals is likely to lead to a better understanding of how distinct T_{MEM} cell subsets develop.

In addition to cytokines that promote STAT3-mediated signals, TGF-β signals also counteract terminal differentiation, induce aspects of both T_{CIRC} and T_{RM} cells, and have tissue-specific effects. In the spleen, TGF-β promotes development of T_{CIRC} cells by inducing and perpetuating the expression of the TF Zeb1 during T_{EFF} and T_{MEM} cell maturation. Disruption of Zeb1 in activated T cells *in vivo* using Gzmb-Cre (which becomes expressed after T cell activation) reduces the number of T_{CM} cells that form after $LCMV_{Arm}$ infection [111]. Zeb1-deficient cells that persist into the memory phase express reduced amounts of genes encoding factors that normally ensure T_{CM} homeostasis, suggesting Zeb1 promotes T_{CM} gene expression. TGF-β interferes with terminal differentiation because Zeb1 and the *mir-200* family microRNAs both repress Zeb2 expression, which uncouples the T-bet/Zeb2 driven pathway that otherwise drives the terminal differentiation program [111]. Accordingly, post-thymic disruption of TGF-β receptor II (*Tgfbr2*$^{fl/fl}$ dLck-Cre^{+}) causes increased fractions of KLRG1^{hi} cells in the spleen during acute LCMV infection, and culture of human CD8 T cells with TGF-β represses KLRG1 expression [112].

In non-lymphoid tissues, TGF-β signals are essential for maturation of T_{RM} cells. The T_{RM} phenotype becomes fully manifest after establishing residence in particular non-lymphoid sites [58], and a key step in T_{RM} formation is their retention within particular non-lymphoid tissues, where TGF-β expression is plentiful. Expression of the E-cadherin-binding integrin CD103 (*Itgae*) on T cells is essential for T_{RM} cell retention in certain epithelial tissues, and is upregulated on T_{RM} cells that establish residency at these sites [113]. In the skin, salivary gland and in the intestinal epithelia, CD8 T cells lacking TGF-β receptor II do not upregulate CD103, and overexpress KLRG1 following LCMV infection [112]. Likewise, expression of a dominant negative form of TGF-βRII in LCMV-specific CD8 T cells also prevents normal upregulation of CD103 expression [113]. T_{EFF} cells from day 4 after $LCMV_{Arm}$ infection, which are enriched with T_{RM} precursors [61], induce CD103 expression when cultured with TGF-β, whereas splenic T_{CIRC} cells from later times do not [113]. Collectively, these results suggest a model in which TGF-β signals in the spleen retard terminal differentiation, and perhaps maintain competence of some T_{EFF} cells that

will emigrate to certain distal non-lymphoid tissues where they receive additional TGF-β stimulation *in situ* and complete T_{RM} maturation.

Runx-family proteins establish core transcriptional programs of CD8+ cytotoxic T lymphocytes

Transcriptional control of the Prf1 locus by Runx3 initially implicated Runx-TFs in the programming of T_{EFF} cell differentiation—A unique function of antigen-experienced CD8 T cells is their capacity to lyse infected or malignant cells using cytotoxic granules that contain the pore-forming protein perforin, and a family of serine esterases (the granzymes) that activate multiple cell death pathways following perforin-mediated delivery into target cells [114,115]. *Prf1* (encodes perforin) is specifically expressed in antigen-experienced CD8 T cells and regulation of the *Prf1* locus of humans and mice has served as a model to study in order to define molecules that could be critical for the differentiation of naïve CD8 T cells into T_{EFF} and T_{MEM} cytotoxic T lymphocytes (CTLs) [116,117]. Early transgenic analyses indicated *cis*-regulatory regions in the *Prf1* locus might become active in developing T cells as early as the double negative stage in the thymus (reviewed in [116]). However, the earliest point in which endogenous *Prf1* transcripts become detectable occurs as double positive thymocytes undergo positive selection into the CD8 lineage, and is conspicuously coordinated with upregulation of the TF Runx3 [118]. Runx-family TFs are essential for T cell development in the thymus, and Runx3 is essential for specification of the CD8 T cell lineage [119–121]. These studies formed the premise of a hypothesis that Runx3 would be critical for programming transcriptional control of the *Prf1* locus, and perhaps the differentiation of T_{EFF} and T_{MEM} cells.

Runx3 drives the transcriptional program of CTLs following naïve CD8 T cell activation—The Runx-family of TFs is encoded by three genes (*Runx1*, *Runx2* and *Runx3*) in mammals [122]. High affinity DNA recognition by each of the Runx proteins results from an allosteric change in their Runt domain that is induced by binding of their common partner Cbfb (encoded by *Cbfb*) [123,124]. Analysis of CD8 T cells from Runx3 deficient mice showed that Runx3 is essential for inducing *Prf1* and multiple additional key effector functions characteristic of CTLs (Fig. 1). Initially, the requirement for Runx3 during the development of antigen-experienced CD8 T cells was examined using purified CD8 T cells from an outbred strain of mice (ICR) that survive germline inactivation of Runx3 [122,125]. Thymic CD8 T cell development is substantially impaired in the absence of Runx3, but residual CD8 T cells with a mature phenotype exist, although most fail to repress *Cd4* expression [120,121]. Runx1 is strongly upregulated in Runx3 deficient CD8 T cells [125], and partial redundancy between Runx1 and Runx3 accounts for the incomplete block in CD8 T cell development in the absence of Runx3 [119,121]. In wildtype CD8 T cells, *Prf1* transcripts are strongly upregulated after naïve cells stimulated with TCR and co-stimulatory signals are cultured with IL-2 at concentrations that sustain IL-2R signals in the context of intermediate affinity receptors (i.e., IL-2Rβ and the common gamma chain (γc))[42,125,126]. In contrast, Runx3 deficient cells do not induce expression of either *Prf1* or *Gzmb* (encodes Granzyme B) under these conditions, and also inefficiently produce IFNγ, TNF and IL-2 upon restimulation, despite initially becoming activated and

accumulating (albeit less strongly) [125]. Thus, Runx3 is essential for establishing hallmark effector functions of cytotoxic lymphocytes in antigen-receptor stimulated CD8 T cells.

Runx3 functions in conjunction with proteins from the T-box family of TFs. The phenotype of Runx3 deficient cells is somewhat analogous to CD8 T cells deficient in the T-box TFs T-bet and Eomes, in that they are also unable to differentiate into *bona fide* CTLs [127,128], which suggested potential genetic interactions between Runx3 and these T-box TFs could promote CTL differentiation. Analysis of Runx3 deficient cells showed they upregulate T-bet after differentiation *in vitro*, but do not induce Eomes expression [125]. Complementation of Runx3 deficient CD8 T cells by retroviral transduction with a hyperactive form of Eomes (Eomes-VP16) that transactivates *Prf1* in wildtype cells is unable to do so in Runx3 deficient cells, but is able restore their capacity to produce IFNγ. In contrast, Runx3 complementation restores both Eomes expression and the CTL gene expression program [125]. In addition, Runx3 binds directly to *cis*-regulatory regions of both *Prf1* and *Eomes*, suggesting it functions at these genes directly [125,129]. These results suggest that both Runx3 and Eomes are important for inducing the CTL program, with Runx3 being located upstream of Eomes. It also indicates that T-bet is insufficient to activate *Prf1* and *Gzmb* expression in the absence of Runx3. Consistent with this, CD8 T cells lacking only *Tbx21* (encodes T-bet) do not exhibit a defect in *Prf1* mRNA upregulation during differentiation in cell culture, although they inefficiently induce IFNγ, prior to upregulation of Eomes [42,125]. These studies collectively establish Runx3 as a cornerstone TF that in conjunction with T-box proteins drives the gene expression program of cytotoxic lymphocytes in activated CD8 T cells.

Multiple Runx-TFs regulate terminal differentiation and formation of T_{CIRC} subsets—Runx1, Runx3 and Cbfb are expressed in naïve CD8 T cells, but all three Runx proteins (including Runx2) are expressed in antigen-experienced CD8 T cells [64], and appear to govern T_{EFF} and T_{MEM} cell differentiation *in vivo* [59,64,130,131]. Conditional *Runx3* gene deficiency in T cells demonstrated it is essential for development of genuine CTLs, and the clearance of viral pathogens and tumors [59,64,131]. At early times after $LCMV_{Arm}$ infection, Runx3-null CD8 T cells exhibit dramatically reduced accumulation, delayed upregulation of KLRG1 and defective upregulation of multiple genes that are characteristic of T_{EFF} cells [64,131]. However, the phenotype is more complex than a simple defect in T_{EFF} cell generation. RNA interference (RNAi) of either *Runx3* or *Cbfb* in adoptively transferred LCMV-specific P14 TCR transgenic CD8 T cells during $LCMV_{Arm}$ infection demonstrated that insufficiency in either factor increases the fractions of $KLRG1^{hi}$ $CD127^{lo}$ TE-like cells, while reducing the frequencies of both $KLRG1^{hi}$ $CD127^{hi}$ DPECs and classical $KLRG1^{lo}$ $CD127^{hi}$ MP cells at the peak of the response to acute viral infection [64]. Conditional *Runx3* disruption in post-thymic T cells confirmed these phenotypes in polyclonal endogenous LCMV-specific CD8 T cells, and showed that loss of only one *Runx3* allele elicits the same phenotype without dramatically impairing cell accumulation [64]. Moreover, gene-disruption and RNAi each also demonstrated that Runx3- or Cbfb-deficient CD8 T cells form fewer normal T_{CIRC} memory cells. Runx3 deficient cells at early memory time points comprise reduced frequencies of $KLRG1^{hi}$ and $CD127^{hi}$ (T_{EM}-like) cells, increased fractions of $KLRG1^{hi}$ $CD127^{lo}$ (T_{LLE}-like) cells, and equivalent frequencies of $KLRG1^{lo}$ and $CD127^{hi}$ (T_{CM}-like) cells, compared to control cells [64]. Analogously, conditional disruption of *Runx2*

also results in reduced formation of MP cells, and impaired persistence of T_{CIRC} cells [130,132]. These results support the conclusion that Runx2 and Runx3 promote T_{MEM} cell development and negatively regulate terminal differentiation.

In contrast, Runx1 might promote terminal differentiation of T_{EFF} cells. Runx1 expression in activated CD8 T cells is lost upon T-bet depletion, but is strongly upregulated in Runx3 deficient or Cbfb-deficient cells, and reciprocally, Runx3 overexpression represses both Runx1 and T-bet expression *in vivo* [64]. However, RNAi-mediated depletion of Runx1 does not elicit clear phenotypes based on KLRG1 and CD127 staining at the peak of the response to LCMV infection [64]. Therefore, Runx1 might not be crucial for T_{EFF} cell differentiation. However, another possibility is that discerning a Runx1 loss-of-function phenotype is complicated by compensation from other Runx factors, and might only manifest in the context of Runx3 deficiency. Consistent with this viewpoint, the TE-like phenotype of cells lacking Runx3 (which overexpress Runx1) is strongly correlated with genes linked to *cis*-regulatory regions that are more accessible in Runx3 deficient cells, and that are enriched with motifs recognized by Runx1 [64]. Further experiments that analyze compound deficiency in both Runx1 and Runx3 could clarify whether the phenotype of Runx3 deficiency requires Runx1 upregulation; and, overexpression of Runx1 cDNA in activated CD8 T cells could provide gain-of-function evidence for how Runx1 might affect T_{EFF} and T_{MEM} cell formation.

Runx3 orchestrates transcription that ensures T_{MEM} cells differentiate from terminal T_{EFF} cells—Runx3 has a complex effect on gene expression during T cell activation that weaves CTL effector functions into T_{MEM} cell development, while preventing terminal differentiation (Fig. 1). At early times after infection, Runx3 deficient cells are less frequently KLRG1^{hi} and inefficiently induce multiple genes whose expression is normally upregulated in both TE and MP cells compared to naïve cells, including genes associated with T_{EFF} cells such as *Irf4*, *Prdm1*, *Id2*, *Il2ra* and *Prf1*, and this explains the failure of Runx3 deficient cells to become *bona fide* CTLs that are protective [64,131]. Simply speaking, defective expression of these genes suggests terminal differentiation should be impaired, because gene deficiency in either *Irf4, Prdm1*, *Id2,* or *Il2ra* impairs development of TE cells [9,10]. However, near the peak response to infection Runx3 deficient cells preferentially acquire a TE-like cell surface phenotype and this correlates with overexpression of *Tbx21*, *Zeb2* and *Runx1* [64] (Fig. 1). This phenotype is suppressed by *Tbx21* RNAi, indicating that the TE-like phenotype of Runx3 deficient cells is still dependent on T-bet [45]. Runx3 overexpression in wildtype cells represses both *Tbx21* and *Runx1* and the TE-like phenotype *in vivo*, whereas neither *Tbx21* deficiency nor its overexpression alters Runx3 expression [59,64]. These results suggest that Runx3 restrains terminal differentiation upstream of *Tbx21* and *Runx1* and ensures that cells with cytotoxic effector functions develop into T_{MEM} cells. It is notable that Runx3 also positively regulates Eomes expression during differentiation of CD8 T cells in cell culture, suggesting it could be important for promoting the fitness of T_{MEM} subsets [98,125].

Although Runx3 deficient cells skew toward a KRLG1^{hi} phenotype near the peak response to infection, their complex phenotype also involves overexpression of *Tcf7*, *Bach2*, and *Id3* compared to wildtype cells [64,131]. These genes encode transcriptional regulatory factors

whose expression and functions normally promote T_{CM} formation [9,53,55,133] (Fig. 1). Correlatively, Runx3 deficient cells exhibit certain features of T_{CM} cells (e.g., *Sell* (CD62L) overexpression) but are likely defective, because the effects of T_{CM}-specific regulatory factors might differ in the context of Runx3 deficiency. For example, *Bach2* encodes a TF from the bZIP family that can compete with other bZIP family factors, such as Jun-proteins, and inhibit AP-1 binding during TCR stimulation [132,133]. *Bach2*-deficient cells accumulate less efficiently after infection, do not re-express CD62L and fail to downregulate KLRG1 on some T_{EFF} cells that develop into T_{MEM} cells (i.e., 'Ex-KLRG1' cells) [48,133]. However, Runx3 deficient cells fail to establish chromatin accessibility to Bach2 binding elements during TCR stimulation [64]. Thus, it is possible that dysregulated KLRG1 expression and non-canonical T_{MEM} cell formation in Runx3 deficient cells relates to defective Bach2 dependent transcriptional control, despite its overexpression. These studies indicate that Runx-family TFs are important for properly delineating development of T_{MEM} cells from terminal differentiated T_{EFF} cells, and that the underlying transcriptional networks are complex.

Runx3 drives transcription of a core set of genes that promote tissue residency and formation of T_{RM} cells—Runx3 differentially controls the formation of T_{RM} and T_{CIRC} subsets. A role for Runx3 in T_{RM} cells was identified in a pooled RNAi screen that focused on candidate genes encoding TFs selected computationally as potential T_{RM} regulators [59,134,135]. Initially, the PageRank algorithm was used to identify potentially important TFs by ranking them according to enrichment of their binding motifs within chromatin accessible *cis*-regulatory regions annotated to a network of genes expressed in intestinal intraepithelial lymphocyte (IEL) T_{RM} cells [59,136]. The RNAi screen showed that cells carrying short hairpin RNAs in microRNA contexts (shRNAmirs) specific for Runx3 were strongly depleted from IEL T_{RM} cells compared to splenic T_{CM} cells following LCMV infection [59]. Additional loss-of-function approaches demonstrated that Runx3 deficient cells inefficiently initiate T_{RM} differentiation at early times, and also fail to sustain T_{RM} cell homeostasis *in situ* at later times, whereas enhanced Runx3 expression after retroviral transduction increased the number and differentiation of T_{RM} cells in multiple tissues, and solid tumors [59]. Runx3 deficient cells appear to initially enter non-lymphoid tissues at early times equivalently to wildtype cells, but fail to differentiate into T_{RM} cells and persist in the intestinal epithelium.

RNA-seq studies demonstrated that Runx3 drives transcription that underlies the differential development of T_{RM} and T_{CIRC} cells. Runx3 overexpression induces expression from a large fraction of genes in a 'core residency signature' that comprises genes more highly expressed in T_{RM} cells from five different non-lymphoid tissues compared to splenic T_{EM} and T_{CM} cells (T_{CIRC}); and represses expression of many genes in a 'core circulating signature' that comprises genes more highly expressed in T_{CIRC} cells relative to T_{RM} cells. Conversely, Runx3 deficient cells fail to upregulate genes in the core residency signature, and instead upregulate those in the core circulating signature [59]. Analysis of ChIP-seq data showed that Runx3 binds directly to genes whose transcription it activates, such as *Itgae* (CD103), *Tgfbr1* and *Tgfbr2*, which are functionally required for tissue residence at epithelial sites [59,113,129]. Runx3 also activates and binds directly to genes that encode TFs, such as *Prdm1*

and the *Nr4a*-family [63,137], which are required for T_{RM} cell formation [12,59]. Thus, Runx3 dependent gene activation directly accounts for the T_{RM} cell phenotype, and also positively regulates expression of additional TFs that induce T_{RM} development. The effect of Runx3 on these transcriptional programs is manifest in CD8 T cells during differentiation under reductionist conditions in cell culture [42,59], which indicates that Runx3 can promote central features of the T_{RM} gene expression program independent of tissue-specific contexts [138]. These results imply that Runx3 is an important initiator of the T_{RM} differentiation program, which might occur prior to entry into target non-lymphoid tissues. Consistent with this, Runx3 deficient cells are preferentially retained in the splenic white pulp and aberrantly manifest features of T_{CM} cells [59,64,131]. These studies suggest that Runx3 antagonizes aspects of T_{CIRC} formation by repressing transcription of genes encoding TFs that positively induce T_{CM} formation.

Runx3 integrates with IL-2R signals to govern Tcf7 expression and T_{STEM} differentiation—The higher expression of *Tcf7*, *Bach2*, *Id3* and *Sell* in MP-like cells that lack Runx3 could also suggest that distinct developmental programs are invoked in the absence of Runx3 (Fig. 1). In line with this perspective, Runx3 deficient cells enter B cell follicles and are able to provide help to B cells, akin to follicular T helper (T_{FH}) cells [131]. In addition, Runx3 deficient MP-like cells overexpress *Cxcr5*, *Maf*, *Icos* and *Bcl6* genes, which are hallmarks of both T_{FH} [64,131,139] and progenitor T_{STEM}-like cells [27,28], suggesting that Runx3 deficient $CD127^{hi}$ cells in the spleen at the peak of acute infection are not canonical MP cells. The development of normal T_{STEM} cells requires the TF Tcf1 (encoded by *Tcf7*) and they are a prominent feature of chronic viral infections, where they sustain production of terminally exhausted T cells [27,28]. Enhanced *Tcf7* expression in Runx3 deficient cells could indicate that Runx3 normally represses formation of T_{STEM} cells. During acute $LCMV_{Arm}$ infection of mice with T cell specific Runx3 deficiency, the formation of follicular 'T_{STEM}-like' cells correlates with clonal deletion of NP396-reactive CD8 T cells, and failure to clear the virus [64,131], which are *de facto* features of chronic infection and T cell exhaustion [28,140,141]. Thus, one potential hypothesis is that Runx3 could be important for negatively regulating development of T cell exhaustion by repressing development of T_{STEM}-like cells.

Runx3 and its regulation of *Tcf7* could be important for controlling IL-2Rα expression, titrating the levels of Bcl6 and Blimp1 and governing differential origins of T_{RM}, T_{CM} and T_{STEM} cells (Fig. 1). Runx3 directly regulates *Bcl6* and *Prdm1* but also regulates their expression indirectly through IL-2R signals and Tcf1. Runx3 directly represses *Tcf7* and *Bcl6*, and this involves Runx3-dependent deposition of histone H3 lysine 27 trimethylation (H3K27me3) at Runx-Cbfb-binding sites in the *Tcf7* and *Bcl6* genes [131]. At the same time, Runx3 promotes transcription of *Il2ra* and *Prdm1* and increases chromatin accessibility of *cis*-regulatory regions where it binds in both loci, indicating that Runx3 activates these genes directly [64,129,131]. However, a key event in this regulatory network might be downregulation of Tcf7 by Runx3, because disruption of *Tcf7* is sufficient to restore expression of IL-2Rα and *Prdm1*, even when Runx3 is also simultaneously inactivated [131] (Fig. 1). Given that IL-2R stimulation positively regulates *Prdm1* expression and both factors negatively regulate *Bcl6* expression [42,79], the control of *Il2ra* transcription by Runx3 and Tcf1 could be a very early event that regulates diversification of circulating and non-circulating T_{EFF} and

T_{MEM} cell lineages. The regulation of *Bcl6* might be even more complicated, because its expression is more strongly reduced in cells lacking both *Runx3* and *Tcf7*, compared to those lacking only *Tcf7* [131], which suggests that both Runx3 and Tcf1 are necessary at some level for *Bcl6* expression. It is also worth noting that Bcl6 represses *Runx3* expression in CD4 T cells [142], raising the question of whether this regulation might also operate in CD8 T cells under certain circumstances. Future studies that discern the expression, epistatic relationships and kinetic binding activity of these factors at early times during T cell activation is likely to clarify how IL-2R signals integrate with these TFs to establish the differential origins of distinct T_{MEM} cell populations.

Chromatin structure provides both stability and flexibility to gene expression programs and is governed at the level of the nucleosome

Nucleosomes are the fundamental repeating subunits of chromatin structure

—Chromatin structure regulates the transcriptional control of genes by governing accessibility to its underlying DNA sequences, and by functioning as a scaffold for docking enzymatic complexes that regulate multiple genome functions. Nucleosome core particles are the fundamental repeating subunits of chromatin structure and consist of 147bp of DNA wrapped around a protein octamer formed by two copies of core histones H2A, H2B, H3 and H4 [143,144]. Adjacent nucleosomes are separated by 20–50bp of 'linker' DNA, and the DNA entering and exiting some nucleosomes is also associated with linker histone H1 [145]. Multiple amino acid residues in the N-terminal tails of the core histones, and in their globular regions, can be covalently modified by the addition or removal of various chemical groups (e.g., acetyl, methyl, ubiquitin or phosphate). Histone modifications can alter the nature of histone-DNA interactions and serve as specific docking sites of chromatin regulatory factors (CRFs) and other DNA modifying complexes that govern genome functions, including transcription [146,147]. Furthermore, 5' cytosine methylation (5mC) and hydroxymethylation (5hmC) of DNA also govern the nature of histone-DNA interactions, as well as the recruitment of protein complexes to specific chromatin regions with these modifications [148,149].

DNA from approximately 80% of the mammalian genome is wrapped in nucleosomes, thus occluding the majority of the genome. Their organization is defined by their translational location on the DNA (position), and by the frequency with which a defined DNA segment is bound by a nucleosome in a population of cells (occupancy). Nucleosomes are not randomly distributed on the genome but adopt preferred positions because the affinity of histones for particular DNA sequences varies, analogous to conventional sequence specific DNA binding TFs [150–153]. Thus, histones, TFs and other DNA-binding proteins must compete for genome occupancy. In addition, CRFs that use the energy released from hydrolysis of ATP, can mechanically alter the topology of histones on DNA and change their locations and occupancy [154,155]. The sequence of underlying DNA, the competitive binding activity of multiple proteins for overlapping DNA sequences, and the action of chromatin remodeling machines govern the nucleosome organization landscape (Fig. 2).

'Epigenetic' regulation of transcription is governed at the level of individual nucleosomes

—Individual nucleosomes are key determinants of genome function, and

both positive and negative regulation of transcription most likely occurs in the context of 'de-condensed' chromatin, rather than in the context of progressively higher-order chromatin fibers. Recent direct visualization of chromatin in fixed cells using electron microscopy tomography (EMT) has provided compelling evidence that the extent of nuclear chromatin is comprised of 5–25 nm fibers – roughly the diameter of one or two nucleosomes -- that exhibit different packing densities in 3-D space, rather than forming higher-order chromatin fibers [156]. The ordered stacking of nucleosomes and folding into hierarchically compacted, inaccessible chromatin fibers, which have been observed microscopically, and analyzed structurally under highly ionic conditions *in vitro* might not reflect the majority of chromatin *in vivo* [156,157]. One important conceptual implication from this discovery is that the roles of nucleosomes and their posttranslational modifications might relate more to controlling activity of discrete regulatory sequences, establishing the architecture of active TSSs [155,158], and governing the trafficking of RNA polymerase II (Pol II) through transcribed genes [159,160], rather than to promoting hierarchical folding of higher order chromatin fibers. From this perspective, it is notable that high resolution mapping of nucleosomes in T cells at a subset of genes that are important for T cell differentiation showed that specific nucleosomes were distinctly organized in key *cis*-regulatory regions between disparate T cell subtypes, whereas 95% of nucleosomes in non-regulatory DNA were positioned similarly between T cell subtypes [161] (and M.E. Pipkin unpublished observations). Thus, continuous competition between DNA-sequence specific TFs and nucleosomes that recruit distinct histone modifying enzymes and chromatin remodeling machines that govern the stability of nucleosomes in specific locations within *cis*-regulatory regions and transcribed regions, as well as the differential recruitment and activity of Pol II complexes at promoters and in gene-bodies, as they traverse nucleosomes, could be the important chromatin-level mechanisms that govern the 'epigenetic' control of cell differentiation [68]. The differentiation of naïve CD8 T cells into distinct antigen-experienced CD8 T cell subsets provides a great model to elucidate how chromatin regulation establishes and remodels transcriptional programs during cell differentiation *in vivo*.

Naïve CD8 T cell activation results in chromatin accessibility changes that establishes a core network of accessible *cis*-regulatory regions that are common to all antigen-experienced CD8 T cells, and a smaller number of regions that are more, or less, accessible in specific T_{EFF} or T_{MEM} subsets compared to the others. In regions where TFs bind, nucleosomes are disrupted and the local DNA is hypersensitive to cleavage by endonucleases such as DNase I, or transposase [162]. These regions can be mapped genome-wide by using high-throughput sequencing (e.g., ATAC-seq, DNase-seq) [163,164]. Inspection of these regions using computational methods has been an important method for identifying potentially functional TF binding sites [136,165]. In addition, protection of the protein-bound sequences within these regions results in DNA cleavage patterns that reveal 'footprints' which can be used to infer the occupancy of transcription factors on their cognate sites [164]. These approaches have been used to define accessible *cis*-regulatory regions in multiple purified subsets of antigen-experienced CD8 T cells, and to infer TFs that are likely critical for their function.

Pioneering chromatin accessibility during initial TCR stimulation of naïve CD8 T cells

Chromatin accessibility develops rapidly during TCR stimulation—The analysis of naïve CD8 T cells during initial activation suggests that stimulated cells undergo extensive chromatin remodeling at genes associated with mature T_{MEM} cells, and both TE and MP cells. TCR and co-stimulation induces *de novo* accessibility of ~15% of all regions that are accessible in mature T_{MEM} subsets, and similar percentages of regions that are specific to TE and MP cells, within the first 24 hours of naive CD8 T cell activation [64]. The accessibility of many of these *de novo* accessible regions appear to be maintained stably accessible in mature T_{MEM} CD8 T cells [64,166], which demonstrates that essential aspects of chromatin remodeling that is specific to T_{MEM} cells are programmed prior to the first cell division. Induction of chromatin accessibility found in TE cells at the same time also suggests many features required for terminal differentiation are co-established. A reasonable way to interpret these observations is that naïve cells initially differentiate into a state that acquires multilineage potential, and that later events reinforce the specific chromatin accessibility landscapes that are ultimately unique to T_{EFF} or T_{MEM} cell subsets. This likely includes reforming nucleosomes in regions that were initially opened upon stimulation, re-opening regions that were initially closed, and opening additional *de novo* regions that were not initially accessed. The dynamics of chromatin accessibility at these regions throughout differentiation have yet to be systematically explored.

Transcription factors involved in establishing chromatin accessibility of T_{EFF} and T_{MEM} cells—The mechanisms by which nucleosome-occupied chromatin is remodeled to a stable accessible state is incompletely understood but is thought to involve specialized transcription factors that can invade nucleosome-bound DNA [167,168]. In naïve CD8 T cells, the *de novo* chromatin opening of *cis*-regulatory regions during TCR stimulation likely depends on the concerted activity of multiple TFs (Fig. 3). DNA sequences within the regions that gain accessibility during initial TCR stimulation are enriched with motifs that can be bound by TFs from multiple different families. Those that are recognized by the RUNX, ETS, bZIP, T-BOX, IRF, RHD, PRDM1 and KLF family of TFs are likely to be of particular importance because their binding motifs are not only statistically enriched, but also highly frequent among all of the *de novo* accessible regions [64,166,169,170]. These motifs fall into three general categories in terms of the pattern of their frequencies within accessible regions at different times after TCR stimulation [64]. The first are those whose motif frequencies are greatest within regions that become accessible in the first two hours of TCR stimulation but that are comparatively less frequent at later times in mature differentiated CD8 T cell subsets. These mainly include TF motifs recognized by basic leucine zipper (bZIP, e.g., Fos and Jun [AP-1]) proteins, and Rel homology domain (RHD) family (e.g., NFAT or NFκB) of proteins, which are transiently activated upon TCR stimulation [171]. The second category includes motifs that are frequent throughout initial TCR stimulation and whose high frequencies are maintained among accessible regions of mature differentiated CD8 T cell subsets. Notably, these motifs increase in absolute number with increasing time after stimulation, because progressively more *cis*-regulatory regions become accessible during this time (Fig. 3A, right), and likely reflect positive feedback from cytokine pathways activated by TCR stimulation. These regions mainly include RUNX and ETS motifs [123,124,172]. A third category comprises motifs that increase in frequency

later during TCR stimulation, and are maintained in mature T_{EFF} and T_{MEM} cells, but are comparatively less frequent overall compared to the previous two categories (Fig. 3A and B). These include interferon regulatory factor (IRF) motifs, and their composite derivatives with ETS or bZIP factors [173,174], and positive regulatory domain (PRDM1) motifs [175]. Multiple members of the TF families that recognize these DNA motifs have established requirements for the formation of T_{EFF} and T_{MEM} cells [10–12], which argues these TFs are critical within the first few hours of naïve CD8 T cell activation for initiating and then sustaining multiple avenues of T cell differentiation.

Runx3 and AP-1 are essential for initial chromatin accessibility during TCR stimulation of naïve cells—Initial chromatin opening of many *cis*-regulatory sites during TCR stimulation of naïve CD8 T cells involves concerted actions of NFAT, AP-1, RUNX and ETS TFs. In both CD4 and CD8 T cells, TCR stimulation acutely activates NFAT and AP-1 TFs, and chromatin accessibility develops transiently in *cis*-regulatory regions encoding composite NFAT/AP-1 binding sites. Many of these inducible sites reside adjacent to *cis*-regulatory regions enriched with RUNX and ETS binding sites that also develop accessibility, but which remain persistently accessible after cessation of TCR signals [64,176–178]. Exactly how these TFs modify nucleosomes to establish accessibility is unresolved, but regulatory regions bound by AP-1 or Runx3 that normally gain chromatin accessibility during initial TCR stimulation are not remodelled when AP-1 TF activity is blocked using a dominant negative FOS protein [179] or if cells are Runx3 deficient [64]. Runx3 occupancy substantially overlaps with binding of the bZIP TFs BATF, Jun and Jund [64,88,129]. Regions of chromatin accessibility in Runx3 deficient cells following TCR stimulation almost entirely lack those that harbor NFAT, AP-1 and other bZIP motifs, (among several others) [64]. These results suggest that NFAT and bZIP TFs are insufficient for establishing chromatin accessibility in most of their sites in the absence of Runx3. Thus, multiple classes of factors are critical for early chromatin accessibility, although it is still unclear how each TF is important, whether they act simultaneously at the same sites, or whether there is a specific order of operations.

Concerted action of AP-1, other bZIP dimers and Runx3 TFs during TCR stimulation are likely to initiate chromatin accessibility at a large number of *cis*-regulatory regions that remain accessible in mature T_{MEM} cells (Fig. 4). A substantial fraction of Runx3 protein in naive cell nuclei is not liberated into nuclear extracts unless salt concentrations that completely extract histones are applied, which indicates Runx3 strongly associates with bulk chromatin prior to TCR stimulation [64]. In addition, ChIP-seq analyses indicate that a substantial number of Runx3 binding sites are present in *ex vivo* CD8 T cells from naïve mice, which suggests Runx3 could be pre-loaded at some target regulatory regions in naïve cells that become stably remodelled following TCR stimulation [129]. Consistent with this, the intensity of binding at many of these sites increases after cells differentiate in response to TCR stimulation and culture with IL-2 [129]. Nuclear Fos and Jun (AP-1) expression in CD4 T cells is induced by CD28-mediated co-stimulation during TCR activation, and is required for chromatin accessibility at AP-1 sites [179,180]. Thus, TCR and co-stimulation dependent signals induce increased Runx3 binding and chromatin remodelling, which are likely stimulated by AP-1 activity (Fig. 4), at a large number of *cis*-regulatory regions in

naïve CD8 T cells. It will be interesting to determine the requirement of NFAT proteins in coordinating chromatin opening at AP-1 and Runx3 controlled sites [181].

Potential mechanisms for de novo TF access into nucleosomes in activated CD8 T cells—Multiple mechanisms could account for the ability of Runx3 and other TFs to gain access to nucleosomes during TCR stimulation (Figs. 2 and 4). Certainly, this process could involve the activity of ATP-dependent chromatin remodelling machines that alter histone-DNA contacts and mobilize nucleosomes [154] (Fig. 2A). Consistent with this possibility, multiple essential subunits of two ATP-dependent chromatin remodelling complexes (SWI/SNF- and CHD-families) are highly represented in co-immunoprecipitates after tandem-affinity purification of an epitope-tagged version of Runx3 expressed after stimulation and transduction of naïve CD8 T cells cultured *in vitro* (D. Wang and M.E. Pipkin, unpublished observations). Thus, chromatin remodelling machines could be recruited to Runx3 during TCR stimulation in response to increased expression or posttranslational modifications of remodeller subunits that increases their affinity for Runx3 in stimulated cells [182], or by interactions with additional TFs, that deliver them to Runx3 bounds sites.

However, enzymatically manufactured chromatin accessibility might not be the incipient event that facilitates access of these TFs to their binding sites. Nucleosomes located in *cis*-regulatory regions turn over rapidly *in vivo* and are exchanged many times within a cell generation [183], which suggests that TFs might have multiple opportunities to sample sequences embedded in otherwise nucleosome-occupied DNA. This process could be the result of spontaneous topological transitions that nucleosomes undergo wherein the DNA partially unwraps (~ 4 times per second), and suddenly rewraps [160] (Fig. 4). The rewrapping kinetic is relatively fast, occurring in ~ 20 ms, but the binding of TFs to their sites is known to be fast enough (i.e., nearly instantaneous) to achieve occupancy on their sites prior to the rewrapping event, given sufficient TF concentration and affinity [160]. In this way, TFs might first gain access to their binding sites passively according to thermodynamic parameters, and then employ recruited chromatin remodeling machines to enzymatically aid the competing nucleosome(s) to reform on adjacent lower affinity sequences that are otherwise non-preferred [151–153].

Specificity and stability of chromatin accessibility at Runx-TF dependent regulatory regions—Cooperative recognition of *cis*-regulatory regions by Runx-TFs and several additional TF families are likely to enhance competition with nucleosomes and contribute both to the specificity and stability of *cis*-regulatory regions that undergo remodeling at Runx-bound sites. Both direct and indirect cooperative mechanisms might account for how Runx-TFs could gain access to their binding sites during TCR stimulation [184]. Direct interactions between the Runt-domain of Runx- and ETS-family TFs facilitate their dimerization on DNA and could be important for the specificity and/or increased binding affinity of Runx-TFs at particular sites during TCR stimulation [185]. Sequences encoding ETS-family binding sites are some of the most highly enriched motifs in Runx3 ChIP-seq binding sites of T_{EFF} CD8 T cells after differentiation in cell culture [129]. In addition, Runx3 deficiency differentially affects the frequency of *cis*-regulatory regions

encoding distinct ETS-family motifs during TCR stimulation. Thus, multiple different TF-TF interactions between different Runx- and ETS-family TFs are likely to shape the accessible chromatin landscape upon TCR stimulation [64]. The ETS-family of TFs is encoded by 27 different genes in mice [186], which implies that extensive TF-TF diversity could account for specificity of Runx-dependent gene expression. In addition, Runx-proteins are also known to indirectly activate transcription with bZIP-family TFs on adjacent elements [124,187]. The accessibility of *cis*-regulatory regions encoding motifs recognized by multiple bZIP-family TFs, including BATF and Bach2, are strongly reduced in Runx3 deficient cells during TCR stimulation, and Runx3 binding strongly overlaps BATF and Bach2 ChIP-seq binding sites [64,129]. Thus, specificity in Runx3-dependent transcription is likely also conferred by distinct bZIP family TFs. Finally, cooperation between Runx and T-box TFs appear to account for stable remodelling of *cis*-regulatory regions. Runx and T-box motifs co-occur frequently in sequences that are stably remodelled in memory CD8 T cells, many of which are co-occupied by Runx-Cbfb, T-bet and Eomes proteins [166]. The accessibility of *cis*-regulatory regions encoding T-box motifs are strongly impaired in Runx3 deficient CD8 T cells during TCR stimulation [64], and both sets of TFs cooperatively drive the transcriptional program of effector and memory CD8 T cells after naive cell stimulation [99,125].

Extensive differences in chromatin accessibility develop in T_{EFF} and T_{MEM} cells after naïve CD8 T cell activation—ATAC-seq analyses of bulk T_{EFF} cells, day 35 KLRG1^{lo} CD127^{hi} cells (i.e., a mixture of T_{EM} and T_{CM}, with T_{LLE} excluded), as well as TE and MP CD8 T cell populations that arise following acute infection with the $LCMV_{Arm}$ have shown that each of these subsets develop accessibility in a large number of putative *cis*-regulatory regions that are distinct from those that are accessible in naïve CD8 T cells [136,169,170]. The majority of regions that become accessible after naïve cell activation are shared between both T_{EFF} and T_{MEM} cells. There is also extensive overlap between the accessible regions from bulk T_{EFF} cells from acute infection, and T_{EX} cells that develop following chronic infection with the clone 13 strain of LCMV ($LCMV_{Cl13}$) [169,170,188–190]. Differences also develop between each of these subsets (discussed below). However, the extensive commonality of accessible chromatin regions that develops after naïve cell activation in all antigen-experienced CD8 T cell subsets suggests all cells might initially share a common developmental history.

Differential chromatin accessibility in subset-specific T_{EFF} and T_{MEM} cells—Differential chromatin accessibility of *cis*-regulatory regions in antigen-experienced CD8 T cell subsets partly explains their subset-specific gene expression profiles. After an acute $LCMV_{Arm}$ infection, bulk T_{EFF} cells from the peak response exhibit a larger number of accessible *cis*-regulatory regions compared to T_{MEM} cells at the beginning of the memory phase. Depending on the study, which each used distinct criteria for calling accessible regions, ~6,000–13,000 regions are more accessible in T_{EFF} cells on day 8 post infection compared to naïve cells, whereas ~3,000–9,000 regions are more accessible in T_{MEM} cells, compared to naïve cells [169,170]. These patterns of chromatin accessibility develop progressively, because only ~2,000 regions were more accessible in T_{EFF} cells from day 4 after infection compared to naïve cells [169]. Bulk T_{EFF} and T_{MEM} cells after $LCMV_{Arm}$

infection are more similar to each other than either is to naïve cells. Only ~1,100 regions are uniquely accessible in T_{EFF} cells, and only ~140 regions are uniquely accessible in T_{MEM} cells, because many of the regions that are more accessible in either subset compared to naïve cells are accessible in both T_{EFF} and T_{MEM} cells [170]. The smaller number of memory-specific regions is because T_{MEM} cells are more closely related to naïve cells and 're-develop' accessibility in regions that are initially accessible in naïve cells, which are inaccessible in T_{EFF} cells. Thus, bulk circulating T_{EFF} and T_{MEM} cells are similar at the level of chromatin accessibility.

Chromatin accessibility in TE and MP cells from the peak response is more similar between each other than MP cells are with mature T_{MEM} cells [170]. This emphasizes overall developmental similarity between TE and MP cells, even though more than 1,000 genes are differentially expressed between the two [64]. However, consistent with the propensity of MP cells to give rise to circulating T_{MEM} cells, the regions that are accessible in MP cells are slightly biased toward those found in T_{MEM} cells [170]. In addition, the regions that are more accessible in mature T_{MEM} cells compared to T_{EFF} cells also show increased accessibility in MP cells compared to TE cells, suggesting that cis-regulatory regions underlying T_{MEM} cell gene expression are more active in MP cells compared to TE cells. In contrast, the regions of accessibility in both TE and MP cells isolated from the spleen differ substantially from putative T_{RM} precursors isolated from non-lymphoid tissues near the peak response to $LCMV_{Arm}$ infection [59]. Differential chromatin accessibility in these different T cell subsets positively correlates with the specific gene expression patterns in the different subsets [59,136,169,170]. Thus, there are cell type specific patterns in chromatin accessibility that develop and correlate with transcriptional changes which drive gene expression profiles specific to distinct T_{EFF} and T_{MEM} cell subsets, and there is substantial similarity between T_{EFF} subpopulations, compared to naïve cells. It is still unknown exactly how these differential patterns of accessibility develop, however, a substantial portion of *de novo* accessible sites found in both T_{EFF} and T_{MEM} subsets are induced rapidly upon TCR stimulation [64], which suggests they are selectively stabilized in one or the other subset at later times.

Differential enhancer activity correlates with the differentiated states of T_{EFF} and T_{MEM} CD8 T cells—The specific transcriptional activity that defines TE, MP and T_{MEM} cells positively correlates with increased chromatin accessibility, histone modifications that are indicative of active enhancers, and three-dimensional contacts between distal putative *cis*-acting regulatory regions and target gene promoters within each specific cell subset. Accessible ATAC-seq regions that are shared between all antigen-experienced subsets of CD8 T cells are located in distal regions and near gene transcription start sites (TSSs) [170]. In contrast, ATAC-seq regions that are specifically accessible in T_{EFF} or T_{MEM} cells are located in regions distal to TSSs, suggesting that distal *cis*-regulatory sequences account for lineage-specific gene expression [170]. Many of these distal regions appear to be transcriptional enhancers as judged by ChIP-seq analyses of histone modifications (H3K4me3, H3K4me1, H3K27me3, and H3K27Ac) and their specific patterns that can be used to distinguish TSSs from enhancers, and predict their activity [136,191–195]. Based on these criteria, distinct T_{EFF} and T_{MEM} subsets manifest specific

enhancer repertoires, and TE cells gain nearly 2-fold more active enhancers relative to naive cells, as compared to MP and T_{MEM} cells [136,192,195]. This suggests that TE cells might develop from T_{EFF} cells that are more transcriptionally active than MP cells, and is consistent with greater expression of genes in TE cells compared to MP cells that are otherwise upregulated in both cell populations after naïve cell activation. Conversely, MP cells re-engage multiple enhancers that were active in naïve cells and give rise to T_{MEM} cells.

The apparent activity of distal enhancers correlates with increased binding activity of specific sets of TFs and is defined by the enhancer's motif composition. TE-specific enhancer sequences are enriched with motifs recognized by both T-box and bZIP family TF motifs (as well as others), and are more strongly occupied by T-bet and BATF TFs, compared to putative enhancers that are more active in MP or T_{MEM} cells [88,136,170]. This coincides with the requirement for both TFs in terminal differentiation [45,88]. However, certain T_{MEM} (T_{EM} and T_{CM})-specific enhancers are also enriched with T-box motifs, but are less enriched with bZIP motifs [136,170]. This correlates with observations that regions of stable chromatin accessibility are bound by T-bet and Eomes [166], and that both TFs are necessary to establish and maintain T_{MEM} cells, in addition to their requirements for the development of terminally differentiated cells [99,136]. Notably, a significant fraction of these sites is also occupied by Runx-Cbf complexes [166]. Reciprocally, motifs recognized by distinct sets of TFs are enriched in enhancers that are specifically active in T_{CM} cells relative to TE cells [136,192], and include Tcf1, Foxo1, Foxp1, Eomes, Gabpa, Gfi1 and Nr3c1 (as well as others), several of which have essential roles in promoting either MP cell or T_{CM} formation by inducing T cell quiescence, lymphoid tissue homing, and homeostatic self-renewal potential [40,55,98,196–198]. Therefore, concerted activity of TFs that are expressed in a lineage or cell type-specific fashion appear to drive the activity of accessible *cis*-regulatory regions and transcription of genes that promote the respective cellular phenotype.

Differential chromatin modifications at these cell type specific *cis*-regulatory regions argues that their associated cell types are 'epigenetically' distinct. However, maintaining the lineage-specific accessibility and active histone modification profiles probably requires ongoing binding by the relevant TFs to their cognate sites in these regions, as opposed to being maintained passively. For example, the stability of gene expression and the phenotypic characteristics of TE cells and T_{LLE} memory cells depends on the continued function of Zeb2 and Id2 [93,199], and probably T-bet (H. Diao and M.E. Pipkin unpublished observations). Conditional disruption of either *Zeb2* or *Id2* in the memory phase results in KLRG1^{hi} T_{LLE} cells 'converting' into those resembling T_{CM} cells [93,199]. It is possible that part of this maintenance involves IL-2R signals, because IL-2R stimulation promotes Id2 expression [53], and the persistence of KLRG1^{hi} T_{LLE} cells after viral infection is severely impaired in cells that lack IL-2Rα [56], resulting in enhanced frequencies of T_{CM}-like cells [56,57]. These examples reaffirm that the differentiated states of cells at both the epigenetic and transcriptional level are actively, rather than passively, maintained by the continued actions of cell type specific TFs [68]. Thus, chromatin accessibility landscapes that are established in human T_{EFF} CD8 T cells during the first week of viral infection, which are maintained for a decade or more in the resulting T_{MEM} populations [5], are most likely the result of continued activity of specific TFs in those cells rather than latent epigenetic modifications. These

observations emphasize the importance of the basic mechanics of transcriptional regulation itself, in addition to alterations in chromatin structure that produce 'epigenetic' effects.

Basic transcriptional mechanics account for differential gene expression in distinct T_{EFF} cell subsets.

Regulation of transcriptional elongation by pre-recruited Pol II is a major regulatory mechanism—A key feature of T_{MEM} cells is differential chromatin remodeling that creates stable chromatin accessibility in lineage-specific genes that are only transcribed at basal levels in resting cells, as in the case of cytokine genes in T helper cells [200], and the *Prf1* gene in T_{CM} cells [5,42,201,202]. However, additional transcriptional mechanics account for the induced transcriptional output of these genes. In CD8 T cells, the TSSs of the *Ifng*, *Prf1*, and *Gzmb* genes are persistently accessible in both T_{EFF} and T_{MEM} cells after LCMV infection of mice, and yellow fever virus vaccination of humans [5,203]. Pol II is present at the *Ifng* and *Prf1* TSSs, but not at the *Gzmb* TSS, and this generally correlates with the level of basal expression of mRNA from each of these genes [203]. The *cis*-regulatory regions that control the *Prf1* locus are similarly accessible in both T_{EFF} and T_{MEM} cells; however, *Prf1* is highly expressed in T_{EFF} cells, but not in T_{CM} cells [42,116,170,202]. IL-2R stimulation enhances *Prf1* gene expression during the differentiation of activated CD8 T cells in cell culture, and this positively correlates with increased Pol II occupancy at the TSS, but not within the gene body. In contrast, restimulation of T_{CM}-like cells with TCR like signals induces increased Pol II occupancy at the *Prf1* TSS and in the gene body (M.E. Pipkin unpublished observations), which correlates with dramatically increased *Prf1* mRNA expression [42]. Thus, initial establishment of chromatin accessibility, RNA Pol II loading at TSSs and subsequent transcriptional elongation of genes are each steps of transcription that are regulated in CD8 T cells.

There is extensive overlap in the TSSs of genes that become accessible in both T_{EFF} and T_{MEM} cells following initial TCR stimulation [136,170], and this most likely correlates with loading of Pol II at these TSSs and basal transcription. In resting CD4 T cells, the promoters of many genes are chromatin accessible and loaded with Pol II, but their gene bodies lack Pol II and the genes are not expressed highly [204]. In contrast, increased lineage-specific gene expression correlates with specific activity of distal enhancers in defined T_{EFF} and T_{MEM} subsets, and predicted interactions with their cognate promoters [192]. One hypothesis is that lineage-specific enhancer-promoter interactions activate transcriptional elongation of 'poised' Pol II that has already been recruited (Fig. 5).

Although it has yet to be analyzed directly in T cells, initiated Pol II complexes stall very quickly after departing the promoter, generally within ~100bp of the transcription start site (TSS), and only some paused Pol II complexes elongate successfully [205]. Photobleaching studies using fluorescently-tagged RNA Pol II in a transgenic system suggests that 99% of promoter-recruited Pol II complexes do not successfully elongate [206], implying that Pol II elongation is the limiting step in high-level gene expression. Thus, regulation of Pol II pausing and transcriptional elongation is a central mechanism that determines a gene's rate of transcription in many developmental and acute stimulation conditions, a revised view

compared to the original perspective that RNA Pol II recruitment and initiation were the major determinants [205].

Regulating transcription by relieving Pol II pausing fits logically into the biology of T_{MEM} cells which harbor remodeled chromatin structure at many genes that are poised for rapid transcriptional activation. Multiple mechanisms cause Pol II to arrest after it initiates. These include the intrinsic properties of RNA Pol II and its interaction with the underlying DNA, as well as, extrinsic factors including nascent RNA processing, abutment against positioned nucleosomes, and two regulatory protein complexes, NELF (Negative Elongation Factor) [207] and DSIF (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole Sensitivity Inducing Factor; encoded by *Spt4* and *Spt5*) [208] (Fig. 5). The Spt5 subunit of DSIF captures the 3' end of nascent transcripts, recruits NELF and both factors trap Pol II within its pause sites [209]. Both NELF and DSIF are sufficient to induce Pol II pausing in purified *in vitro* systems, and each are necessary for Pol II pausing in mammalian cells [205,209,210] (Fig. 5). Recruitment of the positive transcription elongation factor b (P-TEFb), induces the release of paused Pol II into productive elongation. Canonical P-TEFb is composed of Cyclin T1 and Cdk9, and its recruitment induces DSIF phosphorylation, which causes dissociation of NELF from the stalled Pol II complex [205,209,210] (Fig. 5), converts DSIF into a positive elongation factor, and induces Pol II elongation [205,209]. These events also prime Pol II for intense phosphorylation of its C-terminal domain (CTD), which becomes the binding scaffold that recruits multiple additional RNA-processing and chromatin modifying complexes that facilitate transcription through nucleosomes, mRNA splicing and polyadenylation [205,209,211], resulting in mature mRNAs (Fig. 5).

P-TEFb positively regulates formation of TE cells and Th1 cells during viral infection. Parallel *in vivo*, pooled RNAi screens of candidate transcriptional regulators identified Cyclin T1 (*Ccnt1*) as one factor that was required for Th1 cell formation in CD4 T cells, and TE ($KLRG1^{hi}CD127^{lo}$) cell formation in CD8 T cells, during LCMV infection [134]. CD8 T cells depleted of either Cyclin T1 or Cdk9 accumulate normally during LCMV infection, but inefficiently differentiate into TE cells and preferentially acquire a MP phenotype. These cells do not upregulate Prf1 or Gzmb expression and less efficiently control viral titers in hosts after adoptive transfer [134]. These results suggest that P-TEFb could be required to increase transcription of genes that encode effector CTL capabilities, and that drive terminal differentiation. In line with this, Cyclin T1-depleted cells inefficiently upregulate T-bet and Blimp1 expression, which are normally expressed in both TE and MP cells, but are more highly expressed in TE cells and required for their terminal differentiation [45,87,99,136,212]. One possibility is that activation of distal TE-specific enhancers recruits P-TEFb to a wide array of poised promoters that harbor stalled RNA Pol II at genes that define TE and Th1 cells (Fig. 5), or only at key positive regulators, such as *Tbx21* and *Prdm1*. Conversely, enhancers and TFs that promote Pol II pausing in T_{EFF} cells and do not recruit P-TEFb could favor the differentiation of MP and T_{MEM} cells by maintaining chromatin accessibility at effector-type genes while restricting high rates of their transcription. It is possible that transcription of genes necessary to generate MP cells might not require P-TEFb or might be less dependent on its activity.

Although many of these processes are considered ‘general transcriptional mechanisms’, they are employed in context-dependent fashions, as in the case of lineage-specific enhancer repertoires that are controlled by specific assemblies of TFs [136,192,195]. For example, an interaction between Runx1 and P-TEFb is necessary for CD4 silencing during thymic T cell development [213]. Thus, regulation of transcriptional elongation has specific developmental consequences. Notably, P-TEFb is incorporated into four related super elongation complexes (SECs) defined biochemically by distinct subunit compositions that each possess P-TEFb catalytic activity against the Pol II CTD *in vitro*, but exhibit distinct transcriptional effects and gene-specific binding patterns in cells [214]. Future studies that map the occupancy of P-TEFb and the distributions of unmodified relative to CTD-phosphorylated forms of Pol II in developing T_{EFF} cell subsets will likely resolve how differential enhancer landscapes specifically control gene activity by regulating P-TEFb recruitment and transcriptional elongation during differentiation of T_{EFF} and T_{MEM} cells.

Chromatin modifications that regulate transcriptional elongation and enforce terminal differentiation—Naïve CD8 T cells express multiple genes that are considered pro-memory (e.g. *Il7ra*, *Sell,* and *Tcf7*) that are initially downregulated following activation and stably repressed in TE cells, but are re-expressed in some T_{EFF} cells that develop into T_{MEM} cells. The initial and stable repression of these genes in T_{EFF} cells is the result of several CRFs with enzymatic activities that methylate specific residues in histones and DNA. CD8 T cells lacking the H3K9 methyltransferase Suppressor Of Variegation 3–9 Homolog 1 (*Suv39h1*) [215], the H3K27 histone methyltransferase subunits enhancer of zeste subunit 2 (Ezh2) and embryonic ectoderm development (Eed) [72,216], or the *de novo* DNA methyltransferase 3 *Dnmt3a* [47,217] each exhibit defects in repression of pro-memory genes in developing T_{EFF} cells.

Normally, naïve CD8 T cells develop islands of H3K9 trimethylation (H3K9me3) at pro-memory genes including *Il7r* and *Sell* within 3 days of initial TCR stimulation *in vitro* [215], but *Suv39h1*-deficient CD8 T do not develop H3K9me3 density at these and other T_{CIRC} and T_{STEM} cell-expressed genes in T_{EFF} cells *in vivo* [215]. Likewise, DNA sequences in a similar set of pro-memory genes develop *de novo* 5mC in T_{EFF} cells by 4 days after viral infection, but these regions are much less methylated in cells lacking the *de novo* methyltransferase *Dnmt3a*, and these genes are reexpressed with faster kinetics as the infection resolves compared to control cells [47]. In addition, deposition of H3K27me3 at pro-memory genes (e.g., *Bach2*, *Id3*, *Tcf7*) appears to ensure commitment to terminal differentiation by preventing their re-expression near the end of the effector phase [72,216]. Initially, downregulation of these genes is independent of H3K27me3 deposition. Locus-specific H3K27me3 deposition is relatively low in both $KLRG1^{hi}$ and $KLRG1^{lo}$ T_{EFF} cells on day 4.5 after infection, but is pronounced in these genes in TE cells on day 10 after infection [216]. CD8 T cells in which *Ezh2* is conditionally ablated using CD4-Cre are devoid of bulk H3K27me3 in cells after 3 days of TCR stimulation [216]. These cells accumulate less and have higher fractions of CD62L positive cells by day 4.5 after infection, despite normal frequencies of $KRLG1^{hi}$ cells at this time [72,216]. However, Ezh2-deficient CD8 T cells on day 8 after infection comprise increased frequencies of MP-like cells and reduced frequencies of TE cells compared to wildtype cells. Accordingly, the bulk

Ezh2-deficient CD8 T cell population more highly expresses pro-memory genes, and these genes exhibit reduced H3K27me3 deposition [72,216]. Thus, Suv39h1 and Ezh2 appear to function sequentially to initiate and then stabilize repression of pro-memory genes, which promotes terminal differentiation.

H3K9me3 and H3K27me3 are found in both promoter proximal and distal *cis*-acting regions in T cells [215,216,218]. However, both modifications are most heavily concentrated in the transcribed aspects of repressed genes, skewed mainly toward the 5' ends of genes [219]. This suggests that the main effects of these histone modifications in activated CD8 T cells could be to govern transcription itself, rather than directly controlling the accessibility or activity of *cis*-regulatory regions. In other cell types, both H3K9me3 and H3K27me3 in gene bodies negatively regulate transcription by reducing transcriptional elongation and inducing Pol II pausing [220–222]. For example, RNAi of the histone H3K9 methyltransferase SETDB1 in preadipocytes reduces H3K9me3 deposition and causes premature release of Pol II from promoter proximal regions and elongation into its target genes, prior to when transcription normally engages in definitive adipocytes [221]. Analogously, RNAi of the H3K27me3 de-methylase JMJD3/KDM6B in HL-60 cells increases H3K27me3 at its target genes, which impairs Pol II occupancy in gene bodies, but not at promoter regions [220]. This also impairs recruitment of the positive elongation factors SUPT6, which normally associates with elongating Pol II and DSIF, and SUPT16H, a member of the FACT complex that facilitates Pol II elongation by unraveling nucleosomes and re-assembling them in the wake of Pol II [159]. Thus, one hypothesis is that recruitment of Suv39h1 and Ezh2-EED and deposition of H3K9me3 and H3K27me3 at pro-memory genes in T_{EFF} cells counteracts the activity of P-TEFb at these genes in TE cells. Reciprocally, because T-bet and JMJD3/KDM6B have been shown to interact in CD4 T cells [223], it stands to reason that T-bet-dependent recruitment of demethylase activity that prevents accumulation of H3K27me3 could account for increased P-TEFb activity at T-bet activated genes that drive the differentiation of Th1 and TE cells.

Concluding remarks

A model for the differential development of T_{EFF} and T_{MEM} cells after naïve cell activation

During hematopoiesis, progenitor cells promiscuously express intermediate amounts of lineage-determining factors and manifest mixed gene expression programs that account for multilineage developmental potential [224]. This variability arises within the progenitor population at the level of individual transcriptomes and leads some cells to transiently manifest increased expression of specific regulatory factors that creates lineage-bias and the propensity to activate one developmental pathway instead of another [69]. Activated CD4 T cells manifest analogous behavior as they undergo lineage-determination into particular effector T helper cell lineages [225]. A similar regulatory logic might also explain how cell fate determination of T_{EFF} and T_{MEM} cell subsets occurs, whereby activation of naïve CD8 T cells induces a general programming phase that initially generates progenitor cells with fluctuating transcriptomes and distinct metastable states that create lineage-bias in some cells (Fig. 6). Extracellular signals that favor differentiation along one or another pathway 'captures' these early stochastic deviations and reinforces them. Because CD8

T cells are not sessile and readily migrate through different tissues or microanatomic locales composed of distinct tissue-specific extracellular signals [138], the differentiation pathways that are initially selected can be more or less reinforced in a spatially formatted pattern. Terminal differentiation most likely involves cells that develop TE cell bias and then positively regulate transcriptional elongation of TE-specific genes while preventing transcription elongation of pro-memory genes. Conversely, cells whose regimes favor Pol II pausing at TE-related genes, and that fail to repress pro-memory genes retain the capacity to reactivate aspects of the naïve cell program and develop into T_{MEM} cells. At any stage, cells are likely to retain some potential to revert or revise their developmental program (often referred to as 'plasticity'), but these revisions are likely to be constrained by their developmental history and their capacity to experience signals that would reinvest in a distinct developmental program. Thus, T_{MEM} CD8 T cell ontogeny might be arranged in an initial common differentiation phase that generates uncommitted progenitors with multilineage potential, followed by stochastic sampling of distinct differentiation paths toward alternative T_{EFF} and T_{MEM} cell subsets that are ultimately reinforced in some cells, as opposed to developing in an exclusively linear continuum, or divergent paths that are specified deterministically [11,35,38]. Given that virtually all chromatin modifications are fully reversible, the apparent 'fates' of differentiated T_{EFF} cells can be 'undone', which explains the reexpression of genes such *Sell* in MP cells as they transition into the memory phase [47], and the repression of genes such as *Klrg1* and *Gzmb* that are initially expressed but then de-activated in some T_{EFF} cells that become T_{MEM} cells [48,67].

Future perspectives

Deciphering how transcriptional regulation drives cell differentiation *in vivo* is complicated technically because the fates of individual cells are not easily traceable, which prevents discerning how the historical experiences of individual cells influence their transcriptional programs and that of their descendants. A key future challenge that will partially overcome this complication is applying single cell genomics and computational strategies to link the genealogies of individual cells to their respective transcriptomes and chromatin structure states. Furthermore, work to date has mainly focused on defining regulatory factors using bulk mRNA analyses in comparative analyses of phenotypically defined subsets and inferring potentially important factors based on differential expression [9,10,12]. Dozens of regulatory factors have been defined in this manner, but the mammalian genome encodes ~ 2,000 conventional sequence specific DNA-binding TFs [226], and more than 300 chromatin regulatory factors [146,227]. Many of these are likely essential for controlling the differentiation of activated CD8 T cells but are not appreciated because of minor deviations in expression that are not discernable in bulk mRNA analyses. Still others might not be differentially expressed, but are modified posttranslationally, or function specifically due to combinatorial assembly with other factors that are differentially expressed. These are biological themes epitomized by how distinct subunits of CRFs are assembled in unique fashions to provide regulatory specificity [182], and how TFs cooperatively recognize specific *cis*-regulatory sequences [165,184]. Thus, the majority of regulatory factors which establish and maintain the differentiated states of antigen-experienced CD8 T cells might yet to be discovered. The application of both RNAi and CRISPR-based tools in strategies to individually perturb the expression of hundreds or thousands of genes in parallel while

analyzing their resulting phenotypes individually in an unsupervised fashion are likely to enable rapid and comprehensive definition of gene functions in myriad of *in vivo* settings [135,228].

Finally, future studies that map TF binding and nucleosome organization at very high-resolution will be instrumental in determining how competition between TFs and histones might drive chromatin reorganization that underlies CD8 T cell differentiation during immune reactions. One could imagine that *cis*-regulatory networks are organized in affinity landscapes that sense fluctuations in TF binding activities and TF partnerships to engage distinct T_{MEM} cell differentiation outputs in response to specific T cell stimulation conditions. In this way, specific *cis*-regulatory region ensembles that become occupied under particular TF activity regimes would respond accordingly with defined transcriptional responses. Nucleosomes can now be mapped at sufficient resolution to detect reliable changes in their occupancy and positioning [161,229], and ChIP-seq methods can map TF-protected DNA at near base-pair resolution [230,231]. When these assays are combined with competitive ChIP [232], and the analyses incorporate experimental time series, it is likely that both architectural and kinetic understanding of how specific sets of TFs engage chromatin remodelling and alter transcription during CD8 T cell differentiation. By compiling functional atlases of these factors in T cells, entirely new avenues in which to engineer durable immunity against chronic infections and tumors are likely to be devised.

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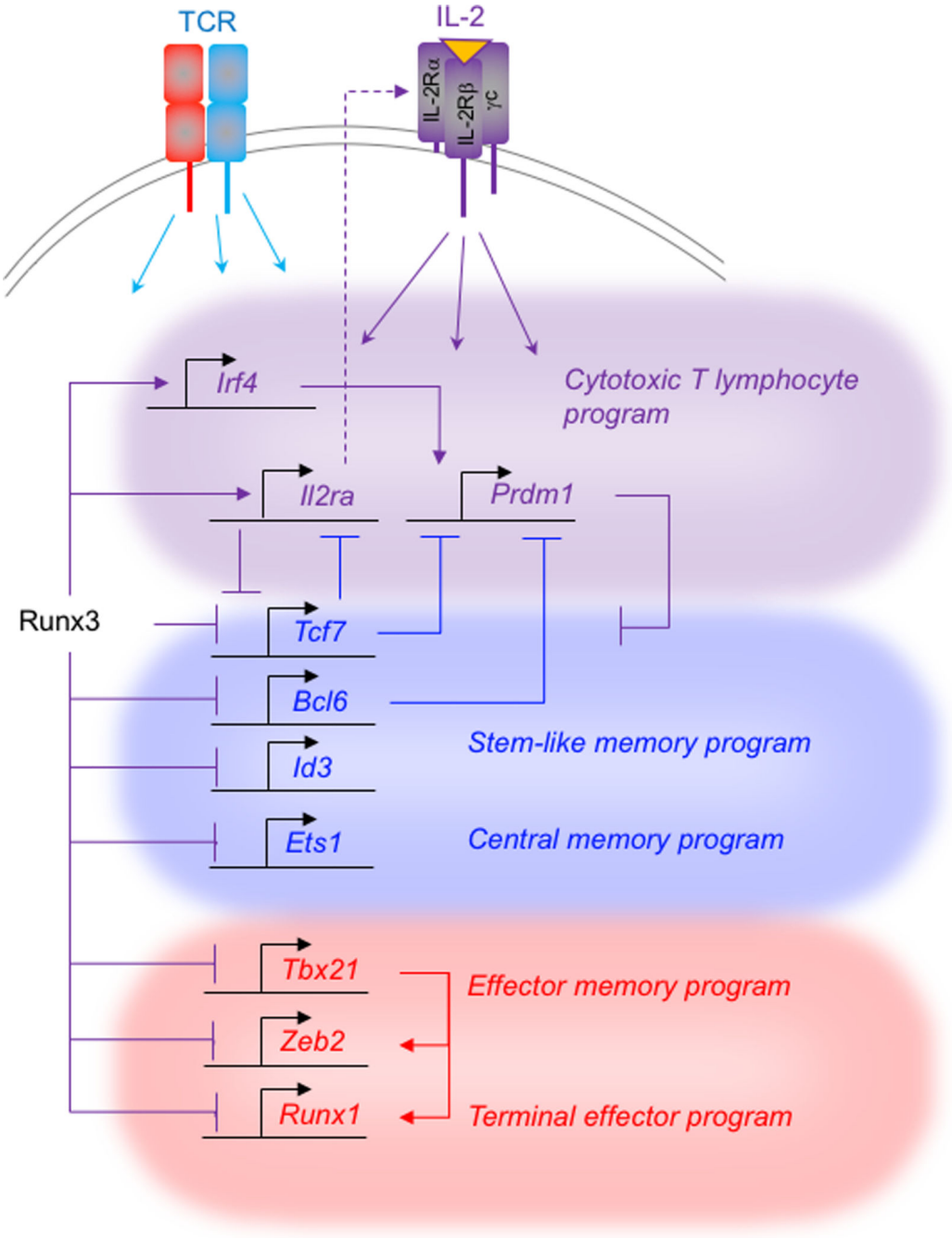

Fig. 1. Runx3 and Tcf1 titrate IL-2R expression and regulate multiple transcriptional circuits that promote development of distinct T_{EFF} and T_{MEM} cell programs.

Runx3 activates transcription and binds to multiple genes encoding regulatory factors that drive transcription of genes underlying the core cytotoxic T lymphocyte differentiation program (purple). IL-2 receptor driven feedback resulting from increased or sustained *Il2ra* (IL-2Rα) expression creates signals that activate and repress multiple genes encoding regulatory factors. Runx3 directly represses *Tcf7* and multiple additional genes that promote differentiation of T_{CM} and T_{STEM} cells (blue). Tcf1 negatively regulates *Il2ra* expression. Reduced *Il2ra* expression in *Runx3 deficient* cells is restored upon concomitant ablation

of *Tcf7*, and this partially restores certain IL-2R-dependent gene expression events. Runx3 reduces expression of multiple TFs that promote terminal differentiation (red).

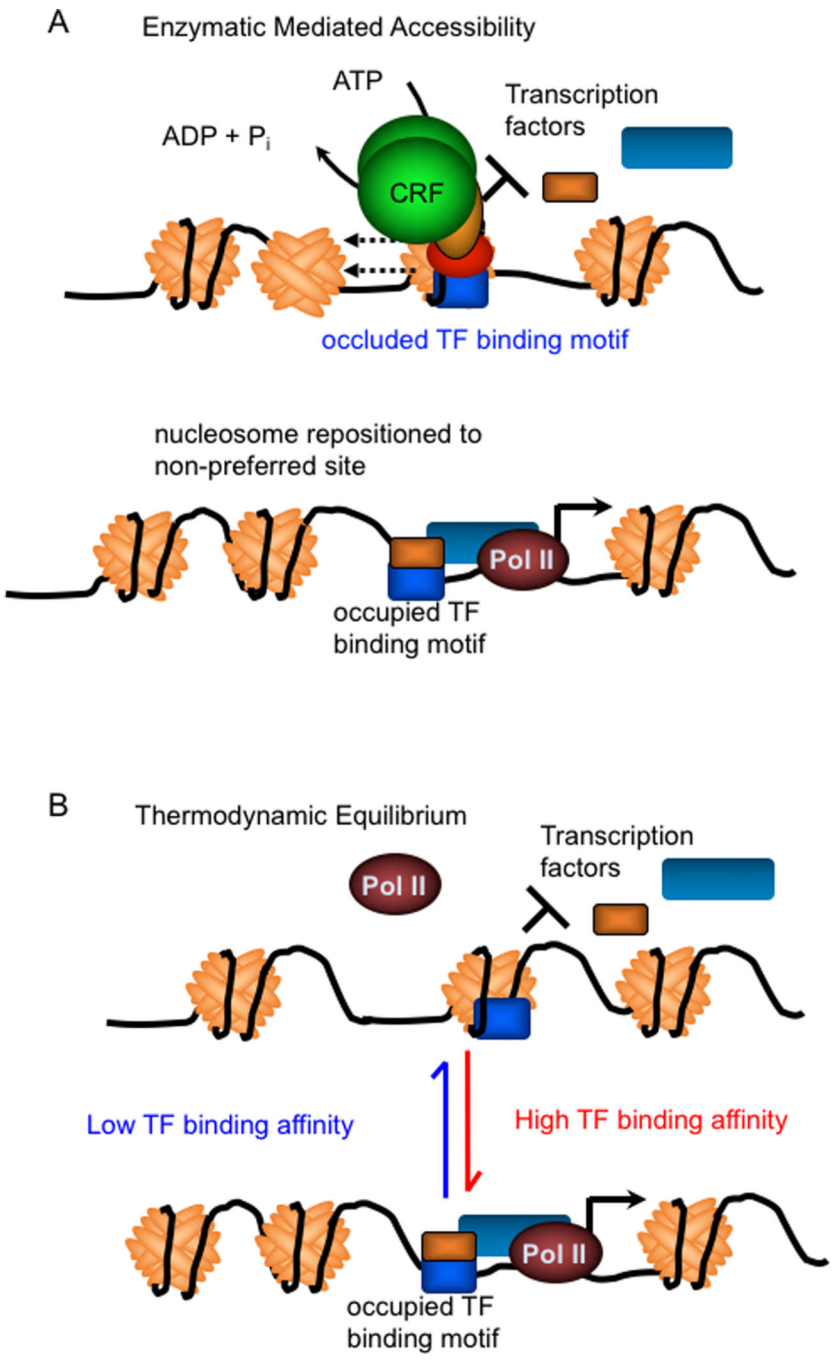

Fig. 2. Nucleosome organization in the genome is established by chromatin remodelling machines and competition with other DNA-binding regulatory factors.

(**A**) Nucleosome organization, especially near TSSs and other *cis*-regulatory regions is constrained by chromatin remodelling factor (CRF) enzymes that use the energy released from hydrolysis of ATP to catalyze repositioning of nucleosomes on DNA. Diverse assemblies of chromatin remodelling complexes built around one of four families of ATPases provide specificity. However, most subunits with these chromatin remodelling complexes lack DNA-binding domains that provide sequence-specificity, and require

conventional sequence specific TFs to guide CRFs to their appropriate targets. (**B**) The histones in nucleosomes bind to DNA with varying affinities, and nucleosomes prefer to occupy certain sequences more than others. Competition between nucleosomes and other DNA-binding factors influences the positioning and occupancy of nucleosomes on the genome. TF-directed recruitment of ATPase-dependent remodellers to specific genes probably facilitates formation of nucleosomes on non-preferred sequences instead of regulatory sequences actively bound by their cognate TFs.

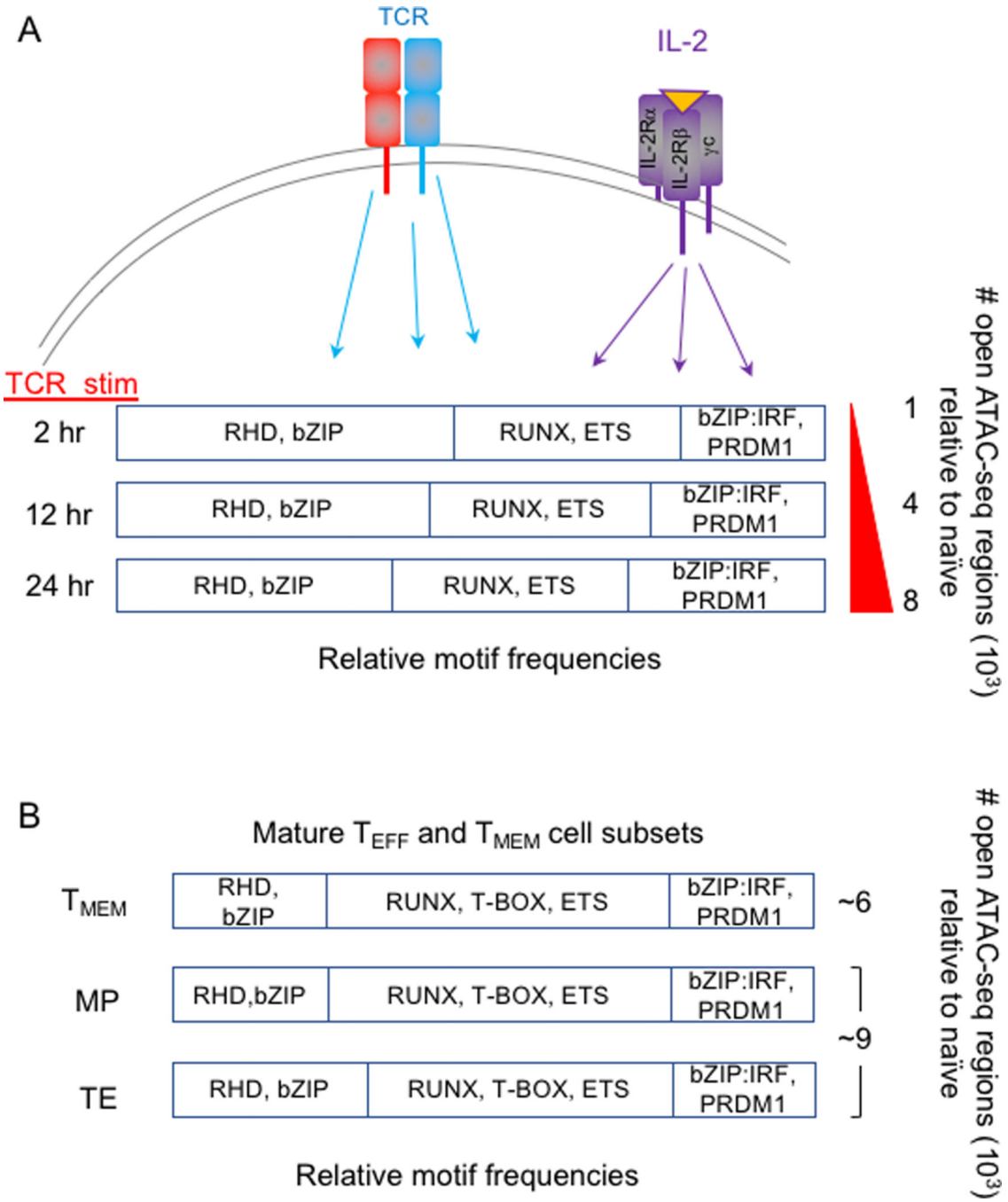

Fig. 3. TCR stimulation induces stable chromatin accessibility of *cis*-regulatory regions encoding binding sites for multiple TF families.

(**A**) TCR signals and co-stimulation (not depicted) progressively increases the number of accessible ATAC-seq regions before naïve cells undergo their first cell division. The relative proportions of the most frequently occurring enriched TF motifs within accessible ATAC-seq regions at each time point are depicted. (**B**) The relative proportions of the most frequently occurring enriched TF motifs within accessible ATAC-seq regions (number of regions shown to right) of mature T_{EFF} and T_{MEM} cell subsets are depicted. Runx, ETS and

bZIP motifs are highly frequent in all cases, suggesting their importance for initiating and maintaining the differentiated states of T_{EFF} and T_{MEM} cells.

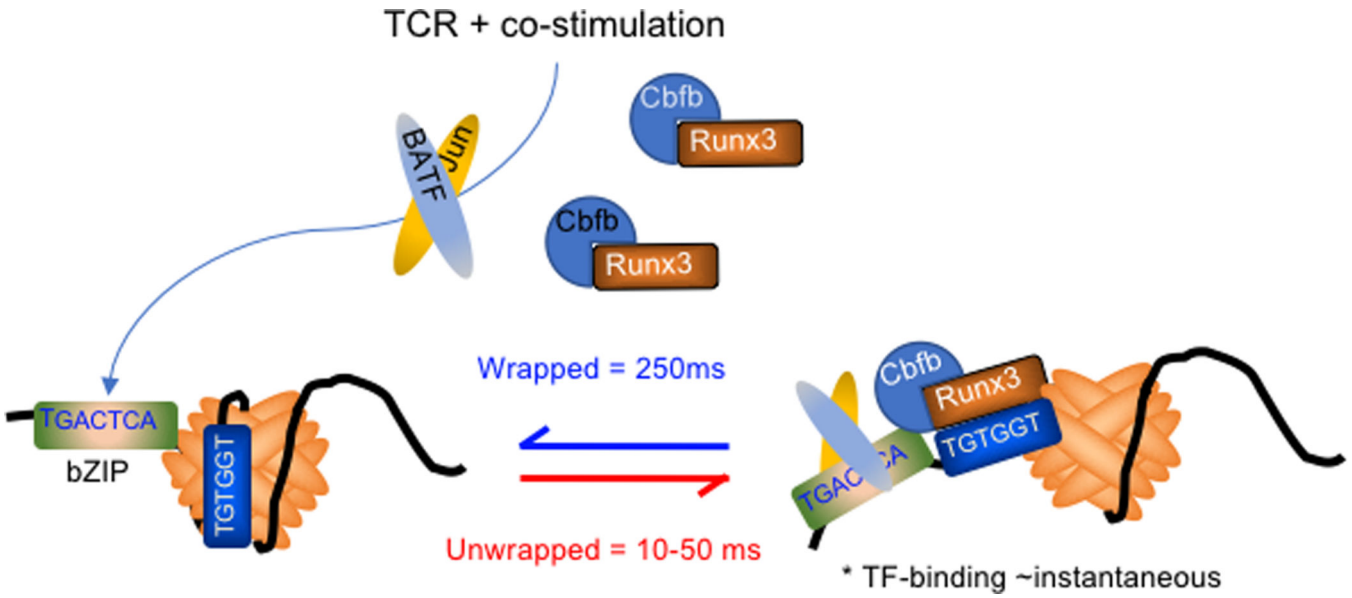

Fig. 4. Spontaneous topological deformations of nucleosomes might facilitate passive entry of TFs into occluded *cis*-regulatory DNA.

(**A**) The fully wrapped state of DNA in a nucleosome is transient (left). Spontaneous unwrapping that begins at the DNA entry and exit locations on the nucleosome occurs ~ 4 times per second. The DNA rewraps very fast, as the unwrapped lifespan is only 10–50 ms. However, transcription factor binding is considered nearly instantaneous, allowing TFs to have many opportunities to access their recognition sites in otherwise nucleosome-occluded sequences (right). Transient activation of bZIP family member TFs (and other TFs, e.g., RHD) in response to TCR stimuli facilitates bZIP and Runx-family TFs to capture transiently accessible nucleosome-DNA and prevent rewrapping. Stable remodelling might require additional chromatin remodelling activities that are delivered by the TFs, or might only depend on cooperative binding by multiple TFs.

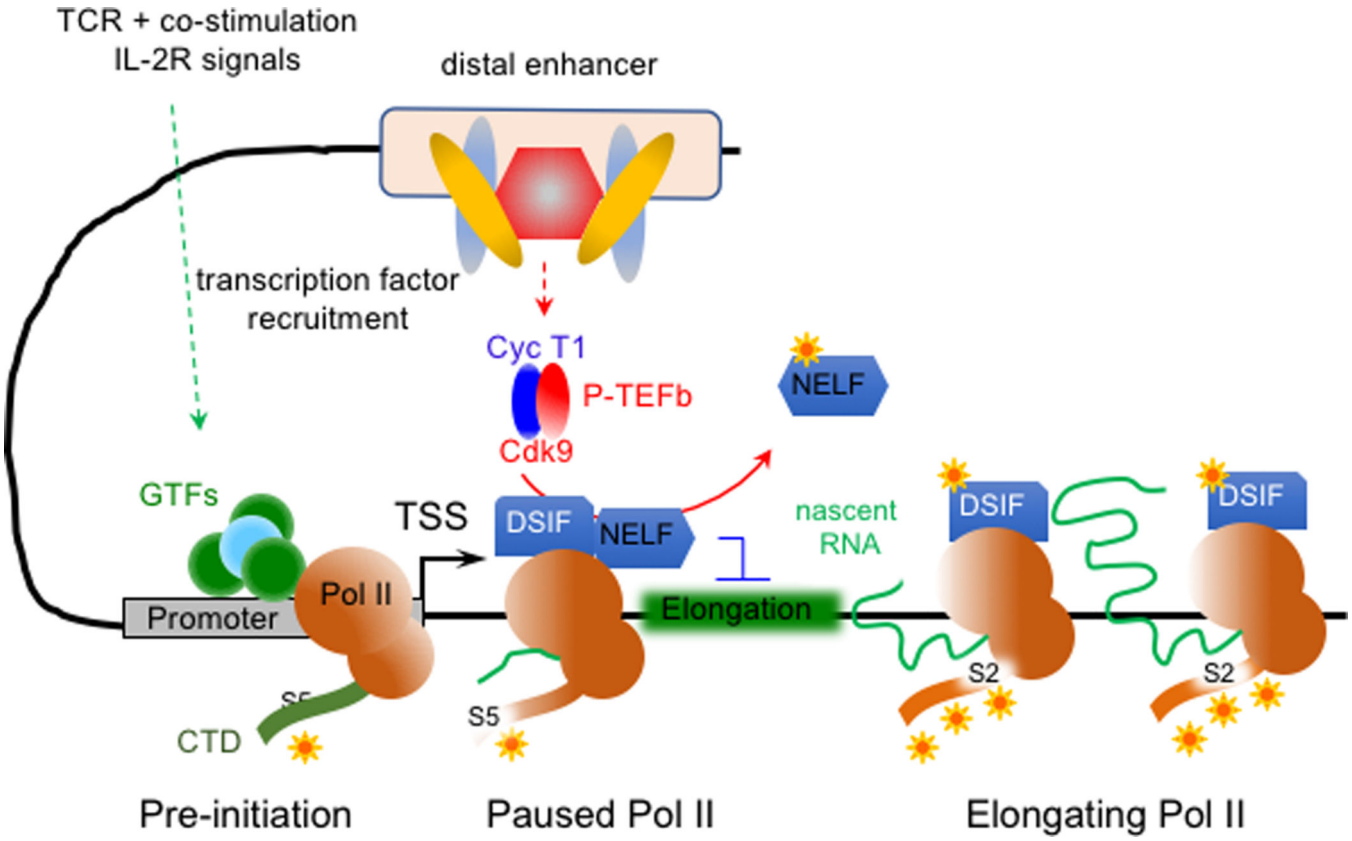

Fig. 5. Terminal differentiation of T_{EFF} CD8 T cells might require induced positive regulation of transcriptional elongation.

Initial TCR, co-stimulatory and IL-2R dependent signals might drive chromatin remodelling that promotes binding of general transcription factors (GTFs), Pol II recruitment and pre-initiation to generally activate promoters of genes that are important in both T_{EFF} and T_{MEM} cells. However, Pol II that escapes the promoter stalls downstream of the transcription start site (TSS) because its processivity is inhibited by negative elongation factors NELF and DSIF. Successful elongation of Pol II depends on P-TEFb. TE-specific distal enhancers could recruit P-TEFb, which phosphorylates DSIF and NELF, dissociating NELF and converting DSIF into a positive elongation factor. Pol II elongates full length nascent mRNAs (green). This process also induces extensive phosphorylation of the Pol II CTD, which recruits additional mRNA processing activities, and CRFs (e.g., the Set2 family) that deposit histone modifications which demarcate transcriptionally active genes (e.g., H3K36me3) (not depicted). Differential association of lineage-specific enhancers with distinct super elongation complexes that incorporate P-TEFb could explain differential dependence of TE and MP cells on P-TEFb.

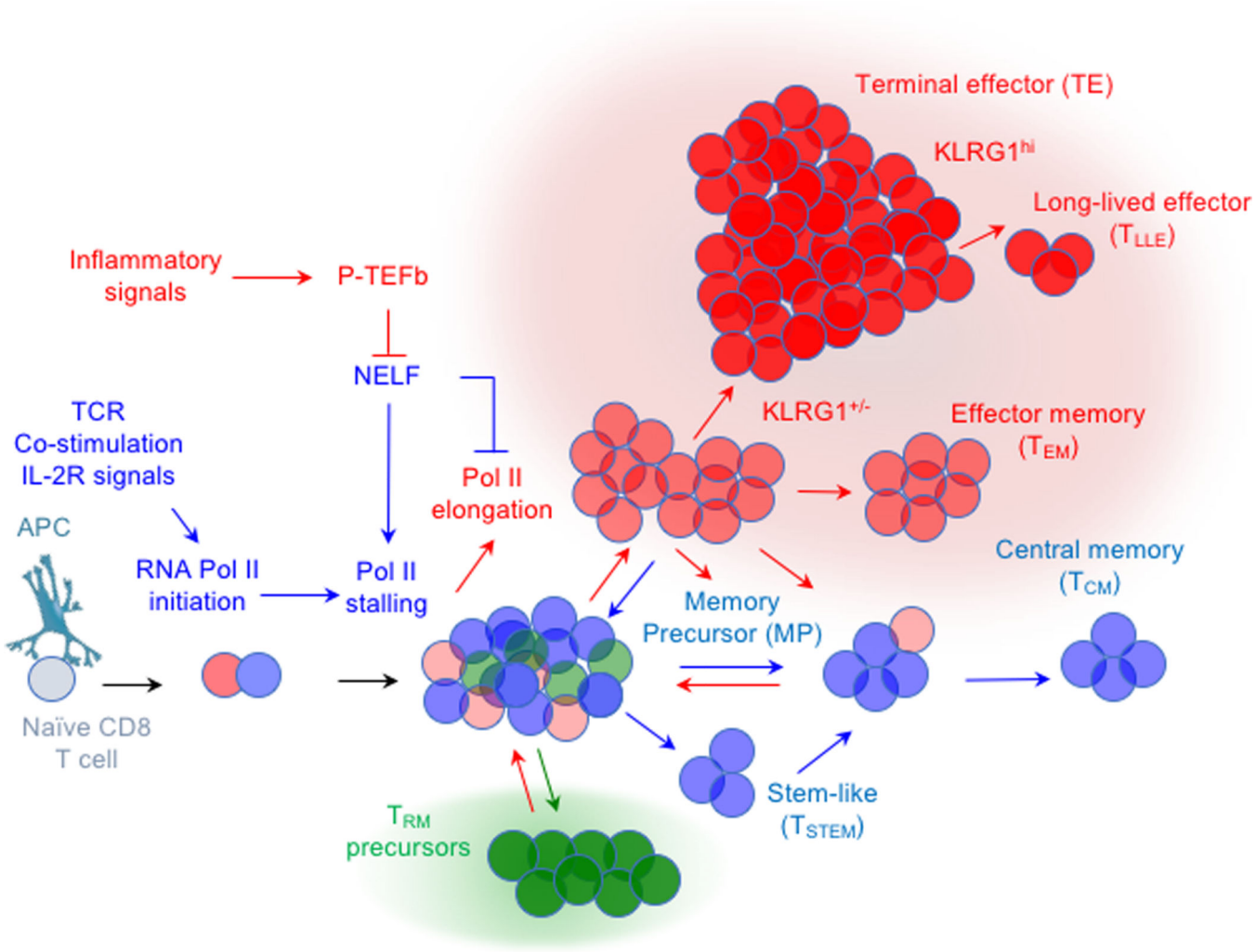

Fig. 6. A multilineage priming model to conceptualize the origins of distinct T_{EFF} and T_{MEM} cells.

Activation of naïve CD8 T cells might initially induce a common programming phase that establishes competence at genes necessary for both T_{EFF} and T_{MEM} cell formation, followed by divergence into distinct T_{EFF} and T_{MEM} pathways that are self-reinforcing. In such a scenario, initial TCR, co-stimulation and integration of multiple extracellular signals induces widespread chromatin remodeling and a population of T_{EFF} cells comprising heterogeneous transcriptomes that develop lineage-bias because of persistent variations in TF protein concentrations in some cells (differently colored cells). These fluctuations activate cell-type specific enhancers that act on previously poised genes, which reinforces and extends the biased transcriptional states. For example, TFs that activate TE-specific enhancers which cause enhanced transcription elongation of genes encoding TFs that also bind TE-specific enhancers creates a feed-forward loop that promotes TE cell differentiation. Simultaneous deposition of chromatin modifications that prevents Pol II elongation in pro-memory genes reduces the survival potential of certain T_{EFF} cells, ensuring terminal differentiation. Conversely, cells that favor TFs and chromatin modifications that enforce Pol II pausing in genes that otherwise drive TE cell differentiation, preserves the potential to develop into

distinct T_{MEM} cell subsets. Arrows imply the overall differentiation framework. However, the developmental changes are likely open to revision given the appropriate environmental signals and capacity to receive the signals (bi-directional arrows), because transcriptional states are dynamic, continuously influenced by TF binding activity and virtually all chromatin modifications are known to be reversible.