Cutting Edge: Persistently Open Chromatin at Effector Gene Loci in Resting Memory CD8+ T Cells Independent of Transcriptional Status

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Memory CD8+ T cells are characterized by more rapid and robust effector function upon infection compared with naïve T cells, but factors governing effector gene responsiveness are incompletely understood. We sought to understand transcriptional control of the effector genes IFN-γ (Ifng), granzyme B (Gzmb), and perforin 1 (Prf1) in murine memory CD8+ T cells by characterizing their transcriptional profiles and chromatin states during lymphocytic choriomeningitis virus infection. Each effector gene has a distinct transcriptional profile in resting memory cells and following restimulation. Primary infection leads to reduced nucleosomal density near the transcription start sites and reduced H3K27 methylation throughout the Ifng and Gzmb loci, and these chromatin changes persist in the memory phase. Despite similarities in chromatin at the memory stage, PolII recruitment and continuous transcription occur at the Ifng locus but not the Gzmb locus. We propose that these chromatin changes poised effector genes for rapid upregulation, but are insufficient for PolII recruitment and transcription.

Vir al infection stimulates naive virus-specific T lymphocytes to undergo differentiation into effector and memory T cells. Effector CD8+ T cells clear virus from the host via cytokine production and cytotoxicity. In the lymphocytic choriomeningitis virus (LCMV) model, memory CD8+ T cells persist indefinitely after Ag clearance and respond to reinfection faster than naïve cells (1). This faster response is mediated at least partially by rapid transcription of effector genes. Three effector genes, IFN-γ (Ifng), granzyme B (Gzmb), and perforin 1 (Prf1), are critical for proper viral control and host survival (2, 3), but factors influencing their transcriptional responsiveness remain unclear.

The transcriptional status of a given gene is determined by multiple factors: recruitment of transcription factors and RNA polymerase II, as well as dynamic alterations in chromatin, including posttranslational modification of histones, DNA methylation, and nucleosome repositioning. Recently, many studies have sought to decode this mix of chromatin-related factors and determine which combinations are associated with gene activation and with poised silent genes for activation. Genomewide studies have led to models to explain the function of each chromatin feature. For example, H3K4 trimethylation and nucleosome depletion are broadly associated with gene activation (4, 5).

An epigenetic model to explain gene poising was spurred by the discovery of bivalent domains, in which silent developmental loci in human embryonic stem cells appeared to simultaneously have both positive (H3K4me3) and negative (H3K27me3) histone methyl marks (6). Further, the discovery that PolIII localized to many silent genes strengthened the poising concept (7). Bivalent domains have also been detected in CD4+ and CD8+ T cells. In CD4+ cells, bivalency at genes encoding developmental regulators has been proposed as a mechanism for plasticity among CD4+ T cell lineages (8). However, bivalent domains were not detected at effector gene loci in memory CD8+ T cells (9). We therefore aimed to understand how effector genes are poised for activation in memory CD8+ T cells.

To define epigenetic poising at effector genes, we probed naïve, effector, and memory CD8+ T cells for specific chromatin features. We found that three effector gene loci have common nucleosomal and H3K27 methylation signatures in memory CD8+ T cells despite major differences in PolIII recruitment and transcriptional status. These observations define a novel mechanism for poising genes in CD8+ T cell memory.

Materials and Methods

Mice

Mice were housed in the Wistar Institute Animal Facility. C57BL/6 mice were obtained from the National Cancer Institute Animal Production Facility.
(Frederick, MD). All experiments were carried out in accordance with the Wistar Institute Institutional Animal Care and Use Committee guidelines.

Cell generation and isolation

Spleens from P14 mice were used to isolate naive CD8+ T cells. Splenocytes (10⁶) from Ly5.1+ P14 mice were injected i.p. into C57BL/6 hosts. Host mice were infected i.p. with 2 × 10⁶ PFU LCMV Armstrong 1 to 2 d later. Spleens were harvested at day 8 (effector) or day 40+ (memory), with the average memory time point at day 80. Cell purification was accomplished by depleting spleens of CD4+ and CD19+ cells using Abs (clones GK1.5 and 1D3, respectively) and magnetic anti-rat IgG beads (Qiagen), then staining with fluorochrome-conjugated Abs against CD8α (53-6.7), CD44 (IM7), and Ly5.1 (A20), and sorting at the Wistar Institute Flow Cytometry facility on an FACSAria (BD Biosciences) or MoFlo (DakoCytomation) cell sorter. Sorted purities were routinely ≥95%.

Quantitative PCR

PCR primers were designed using Primer Express 2.0 software (Applied Biosystems), and amplification of a single product was confirmed by dissociation curve analysis. Quantitative PCR was carried out on the 7000HT Fast Real-Time PCR system (Applied Biosystems) using SYBR green (Sigma-Aldrich). Absolute quantification was performed by generating a standard curve for each primer set on each plate. For RT-PCR, RNA was isolated using the RNeasy kit (Qiagen) and reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems) prior to quantitative PCR analysis.

Chromatin immunoprecipitation

Sorted cells were cross-linked in 1% formaldehyde for 15 min, then quenched with 125 mM glycine for 5 min. Cell pellets were lysed and sonicated using a Bioruptor (Diagenode) for 20 min at high output. Total protein was quantified by Bradford assay. Abs used for chromatin immunoprecipitation (ChIP) are as follows: anti-H3 (Ab1791; Abcam), anti-PolII (clone 8WG16, Ab187; Abcam), anti-H3K27me3 (07-449; Millipore), anti-H3K27me2 (07-452; Millipore), and anti-H3K4me3 (39159; Active Motif). Abs were bound to Protein A or Protein G Dynabeads (Invitrogen), then immunoprecipitations were set up using 20 μg total protein from each lysate and 4 μg for input controls. Because we had limited memory material and had to do low-input ChIP assays, the quantitative nature of the assay was tested and confirmed (Supplemental Fig. 1). After immunoprecipitation, beads were washed, protein was digested with protease K, and DNA was purified by phenol extraction and ethanol precipitation. DNA was amplified with primer sets covering the promoter regions, the transcriptional start site (TSS), and the gene bodies.

Results and Discussion

Distinct transcriptional responses of three effector genes in restimulated memory CD8+ T cells

To investigate mechanisms of gene poising in representative effector genes, we used the P14 transgenic TCR system (10). CD8+CD44hi naive T cells were sorted directly from Ly5.1+ P14 transgenic mice. Effector and memory CD8+ T cell populations were generated by transferring P14 cells into C57BL/6 Ly5.2+ congenic host mice and infecting hosts with LCMV. CD8+Ly5.1+CD44hi effector and memory T cells were harvested at days 8 and 40+, respectively. Supplemental Fig. 1A shows the postsorting purity of each population used for further analyses.

We first characterized the transcriptional responsiveness of effector genes in naive and memory CD8+ T cells after TCR stimulation. Sorted cells were cultured with plate-bound anti-CD3 and anti-CD28, harvested at various time points, and then analyzed for transcript levels of Ifng, Gzmb, and Prf1 using quantitative RT-PCR (qRT-PCR) (Fig. 1A). Consistent with previous reports, transcripts from the Ifng locus were 100-fold higher in memory cells compared with naive cells at 0 h and underwent a further 100-fold increase by 3 h post-stimulation (Fig. 1A) (11). In contrast, the increase in Ifng transcripts in naive cells at this time point was <10-fold. A 100-fold difference between naive and memory cells persisted throughout the time course. The Gzmb gene also had more robust transcriptional response in memory CD8+ T cells than naive CD8+ T cells, although it was not as dramatic as Ifng. Prf1 transcription was not appreciably activated in either naive or memory cells within the 3-d time course (data not shown), which is consistent with published data (9, 12).

These results establish a transcriptional basis for rapid Ifng and Gzmb function in memory CD8+ T cells.

Ifng is continuously transcribed in resting memory CD8+ T cells

We noticed in the restimulation experiment that resting memory CD8+ T cells had increased transcripts at both the Ifng and Gzmb loci compared with naive cells. To compare transcript levels in resting memory cells with those of effector cells, we performed qRT-PCR on freshly sorted cells (Fig. 1B). The Prf1 gene underwent a 4-fold induction at the effector stage and had moderately elevated transcripts in memory CD8+ T cells compared with naive CD8+ T cells. The Gzmb locus showed a 950-fold induction of transcription by day 8 of infection, but transcript levels fell significantly in resting memory CD8+ T cells. In contrast, Ifng transcripts increased 100-fold in effector cells and remained at that level in resting memory cells. These results are consistent with previously reported microarray data (13, 14).

Because our primers for Ifng transcript detection were 5′ biased, it was possible that abortive transcription was occurring at the memory stage. Primers targeting the 3′ end of the transcript confirmed the presence of full-length transcripts (Fig. 1C). The superior inducibility of Ifng at early time points may be related to the fact that this gene is continuously transcribed at high levels in resting memory CD8+ T cells.

Nucleosome-depleted regions persist in resting memory CD8+ T cells

Active genes typically have a nucleosome-depleted region near the TSS that provides accessibility for chromatin binding
factors and promotes transcription (15). We wanted to understand how such accessibility might influence effector gene transcriptional responsiveness in memory CD8+ T cells. To that end, we carried out ChIP in freshly sorted cells, with Abs specific for the core histone H3 and primers that spanned each gene locus (Fig. 2). We used Foxp3 as a negative control gene, because it is not induced during CD8+ T cell differentiation.

Foxp3 did not show any nucleosomal changes at the TSS or downstream regions in naive, effector, or memory CD8+ T cells. At the Ifng, Gzmb, and Prf1 loci, we detected clear areas of reduced nucleosomal density in effector CD8+ T cells surrounding the TSS, but not in downstream areas of the genes (Fig. 2B). These nucleosome-depleted regions persisted in memory CD8+ T cells. This is particularly notable for the Gzmb locus, which markedly shuts down transcription in resting memory CD8+ T cells (Fig. 1B). Similarly, open chromatin at the Gzmb locus without accompanying transcription was also recently described in activated CD4+ T cells (16). Thus, nucleosomes are not the only barriers to PolII recruitment and transcription. Furthermore, these results imply that TCR signaling can set up open chromatin at the promoter without necessarily dictating actual transcription. We also detected some degree of nucleosome depletion in Gzmb and Prf1 in naive CD8+ T cells, consistent with the observation that these genes are expressed during thymocyte differentiation.

**FIGURE 2.** Depletion of nucleosomes and H3K27me occurs at the Ifng and Gzmb loci in effectors and persists in memory cells. A, Schematics (not to scale) showing the genomic organization of effector genes Ifng, Gzmb, and Prf1 and the inactive Foxp3 gene. Exons are shown as filled rectangles, and the location of PCR amplicons are depicted as dashes marked with lowercase letters. Amplicon size averaged 50 bp. B, ChIP using Abs specific for histone H3 was used to detect nucleosomal density at indicated locations across effector genes. Data were normalized by dividing ChIP-PCR signal by input signal. Data are pooled from nine independent experiments. C, H3K27me3 was detected by ChIP at indicated locations across effector genes. Data were normalized by dividing the H3K27me3 ChIP-PCR signal by the H3 ChIP-PCR signal to normalize the methyl mark to total histone levels. Data are pooled from four independent experiments. *Significant differences between naive and effector or naive and memory samples at the same genomic sites; # significant differences between indicated sites within the Gzmb and Prf1 loci. p < 0.05.

**FIGURE 3.** PolII recruitment reflects transcriptional status of effector genes. ChIP-PCR on freshly sorted naive, effector, and memory CD8+ T cells using the Ab 8WG16, which is specific for the RNA polymerase II C-terminal domain. Data were normalized by dividing ChIP-PCR signal by input signal and are pooled from four independent experiments. *Significant enrichments compared with the same genomic site in naive cells. p < 0.05.
development (17, 18). The presence of nucleosome-depleted regions therefore appears to be more indicative of past gene expression or signaling history than current transcriptional status. However, our data and those of others show that active transcription correlates with the appearance of the nucleosome-depleted region in T cells (5, 16). Together, these results suggest that the presence of a nucleosome-depleted region is not sufficient for transcription, but it may contribute to increased inducibility in memory CD8+ T cells.

Histone methylation at effector gene loci

We asked whether histone methyl marks also changed at effector gene loci in resting memory CD8+ T cells. We performed ChIP in naive, effector, and memory CD8+ T cells using Abs specific for H3K27me3, H3K27me2, H3K4me3, H3K36me3, H3K79me3, H3K9me3, and H2K20me1. We did not detect any consistent or significant changes for most of these marks among the three differentiation stages (data not shown). We did detect minor H3K4me3 peaks near the Ifng TSS in effector and memory T cells (Supplemental Fig. 2). In contrast, we did not detect appreciable H3K4me3 enrichment at the Gzmb locus in effector or memory cells. This result differs from a previously published study in which H3K4me3 increased at Gzmb after CD8+ T cell activation (16). This discrepancy might be due to the differing T cell activation conditions between the two studies. Low H3K4me3 has previously been observed at some active genes in memory CD8+ T cells (9).

The repressive marks H3K27me3 and H3K27me2 were reduced throughout the Ifng and Gzmb loci in memory CD8+ T cells, with the greatest reductions near the TSS (Fig. 2C and data not shown). In naive CD8+ T cells, Prf1 already had reduced H3K27 methylation near the TSS compared with downstream regions. Similarly to nucleosome depletion, these results indicate that the loss of H3K27 methylation persists at effector loci in memory CD8+ T cells regardless of transcriptional status.

PollII is not poised on effector genes, and its recruitment reflects transcriptional status

We next asked whether RNA polymerase II poised could be contributing to rapid Ifng and Gzmb transcriptional responsiveness in memory CD8+ T cells. Previous work has indicated that PollII localizes to a large number of silent genes and poises them for transcriptional induction during differentiation (7). We assayed for the presence of PollII at effector genes by ChIP (Fig. 3). As expected, no change was detected in PollII signal at the TSS or within the body of the silent Foxp3 gene in effector cells. In contrast, relative to naive CD8+ T cells, effector CD8+ T cells had increased PollII throughout the Ifng and Gzmb loci, and, to a lesser extent, at the Prf1 locus. PollII enrichment was consistent with the level of transcriptional induction at the effector stage, with Prf1 showing less enrichment and less robust transcriptional induction. At the memory stage, PollII remained enriched at the active Ifng locus (and, to a lesser extent, the Prf1 locus) but not at the relatively silent Gzmb locus. These results indicate that PollII poised does not occur on silent effector genes in resting memory CD8+ T cells and that effector gene responsiveness cannot be explained by preloaded polymerase. Instead, PollII recruitment reflects the transcriptional status of each gene.

Previous studies have shown increased H3K9 acetylation at the IFNG, GZMB, and PRF1 loci in human resting memory CD8+ T cells (19, 20). Our results are similar to these reports in that resting memory CD8+ T cells also maintain nucleosome-depleted regions and low H3K27me levels. The stability of these three chromatin features at Ifng, Gzmb, and Prf1 may explain why the expression of these genes steadily increases during successive rounds of memory generation in vivo (21). However, these chromatin features do not explain why continuous transcription occurs at the Ifng locus but not the Gzmb locus in resting memory CD8+ T cells. Regulation of the expression of these loci in resting memory CD8+ T cells appears to occur at the level of PollII recruitment, which may be influenced by the differential levels of H3K4me3 at the TSS (22). Additionally, these genes may have different requirements for active TCR signaling and/or transcription factor recruitment.

Multiple factors likely contribute to the transcriptional component of rapid memory responses. Our data show that each of the effector genes we studied had a common chromatin signature of nucleosome-depleted regions and low H3K27 methylation in memory cells. However, PollII recruitment, H3K4me3 levels, ongoing transcription, and acute gene inducibility in memory CD8+ T cells are distinct for each of the three effector loci studied, indicating that there is not a single, uniform program of gene regulation governing effector genes in memory CD8+ T cells. This observation indicates that H3K4me3/H3K27me3 bivalency, although important for genes encoding developmental regulators and downstream cell fate, is not the only epigenetic poised mechanism operative in T lymphocytes (8, 23). It is likely that categories of combinatorial chromatin features exist among effector genes. Such combinations may provide flexibility in the gene expression programs of memory T cells generated under different vaccination or infection conditions. Future genome-wide descriptive studies coupled with functional studies will determine if this is the case.

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Disclosures

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References


