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In vitro and in vivo differentiated effector CD8 T cells display divergent histone acetylation patterns within the *Ifng* locus

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Abstract

Epigenetic remodeling of genes encoding effector cytokines that permit accessibility to the transcriptional machinery is a central event in the differentiation of naive T cells into effectors that can attack pathogens and tumors. Covalent modifications of histones that cause a loosening of nucleosomal structures occur not only in promoter regions, but also at upstream and downstream enhancer elements that integrate various cellular stimuli to modulate the rate of transcriptional initiation. This knowledge derives mostly from the analysis of in vitro differentiated effector T cells. Here, we compared acetylation of histone H3 (AcH3) at several sites within the *Ifng* locus in CD8 T cells that underwent effector differentiation in vitro vs in vivo. While AcH3 was similar at the proximal promoter, it displayed a reciprocal pattern at two well-characterized upstream and downstream sites. These data suggest that certain epigenetic remodeling events may be artifactual consequences of in vitro culturing conditions, and indicate the importance of using in vivo models to study effector cytokine gene remodeling.

Keywords

CD8 T cells; chromatin remodeling; histone acetylation; *Ifng* gene

1. Introduction

To become competent to express the effector cytokine IFN- γ , naive T cells encountering cognate antigen for the first time must remodel the *Ifng* gene locus towards an open/permissive epigenetic configuration that allows binding of critical transcription factors [1–3]. Remodeling is initially mediated through covalent modifications of histones that cause a loosening of the nucleosomal structure [4,5], followed by demethylation of CpG dinucleotides to lock in the open chromatin configuration [6,7]. In addition to the proximal promoter, there are several upstream and downstream transcriptional enhancer elements that also undergo remodeling [8,9].

Due to the relatively large number of homogeneous T cells required to perform standard chromatin structural assays, our knowledge of chromatin remodeling events that occur within the *Ifng* gene locus during effector differentiation derives almost exclusively from studies utilizing in vitro differentiated T cells. Nevertheless, the mechanisms that drive effector

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differentiation in vivo may differ. For instance, while T-bet is not essential for CD8 T cells to gain the capacity to express IFN- γ in vitro [10], it is essential in vivo [11].

In the current study, we compared patterns of histone H3 acetylation (AcH3) within the *Ifng* loci of vitro vs in vivo primed effector CD8 T cells using a highly sensitive real-time PCR-based chromatin immunoprecipitation (ChIP) assay. Although the amount of AcH3 associated with the proximal promoter consistently correlated with IFN- γ expression potential, the relative amounts of AcH3 at two well-known sites located on either side of the proximal promoter were inverted in the two effector CD8 T cell populations. These results raise a cautionary note that the patterns of epigenetic modifications that occur during in vitro T cell priming may not fully reflect natural T cell responses.

2. Materials and Methods

2.1 TCR transgenic CD8 T cell adoptive transfers and flow cytometry

All mice were on the B10.D2 (H-2^d) genetic background. TCR transgenic (Tg) mice that express the MHC class I-restricted HA-specific clone 4 TCR [12] as well as double transgenics expressing both the clone 4 TCR and human Bcl-2 [13] expressed the congenic Thy1.1 marker. For adoptive transfer experiments, 1×10^6 TCR transgenic CD8 T cells (prepared from pooled lymph nodes (LN) plus spleen and depleted of CD4⁺ cells using magnetic beads) were transferred into Thy1.2⁺ non-transgenic (NT) recipients infected with 10^6 PFU of a recombinant vaccinia virus expressing HA (viral-HA), and subsequent functional analyses were performed as previously described [14,15]. In brief, Thy1.1⁺ TCR Tg CD8 T cells were recovered from spleens at 4 days post-transfer and analyzed for intracellular cytokine expression following 5 h in vitro restimulation with brefeldin A (BFA) and either the cognate K^d-restricted HA peptide or PMA + ionomycin (PMA + I). Total intracellular cytokine expression (expressed in arbitrary units) was calculated as previously described by multiplying the percentage of cytokine-expressing TCR Tg CD8 T cells by their mean fluorescence intensity (MFI) of cytokine staining [16].

2.2 In vitro priming of naive polyclonal and TCR transgenic CD8 T cells

LN cells from NT B10.D2 mice or single Tg clone 4 TCR mice were immunomagnetically depleted of CD44^{high} cells and cultured for either 2 days with soluble anti-CD3 mAb (5 μ g/ml; eBioscience) (NT) or 4 days with cognate HA peptide (clone 4 TCR Tg). All cultures contained IL-2 (50 U/ml; NCI Biological Research Branch), IL-12 (2.5 U/ml; Sigma) and IFN- γ (50 ng/ml; R&D Systems), and fresh media and cytokines were added daily.

2.3 Enrichment of polyclonal and TCR transgenic CD8 T cells, chromatin immunoprecipitation (ChIP) and RT-PCR

Naive NT LN-derived CD8 T cells were enriched by depleting CD44^{high} and CD4⁺ cells using magnetic beads (Dyna). At the indicated times of in vitro culture, primed CD8 T cells were positively enriched via MACS columns using PE-conjugated anti-CD8 and anti-PE microbeads (Miltenyi Biotec), resulting in purities of at least 98% (data not shown). These purified naive or primed CD8 T cells were then either analyzed directly by ChIP or restimulated with PMA + I for RT-PCR (3×10^5 cells per sample) or intracellular cytokine staining. Adoptively transferred TCR Tg CD8 T cells recovered from spleens of viral-HA recipients at day 4 were enriched either via FACS sorting or MACS columns using the Thy1.1 marker, while polyclonal naive and effector/memory CD8 T cells were enriched via FACS sorting using the CD8 and CD44 markers (in both cases purities were 98–99%).

Quantitative real-time PCR-based ChIP analysis to measure histone H3 acetylation (AcH3) within the *Ifng* locus was performed as previously described [15,17] with the following

modifications. Sites analyzed included the proximal promoter (F: 5'–TCAGCTGATCCTTTGGACCC, R: 5'–CTCAGAGCTAGGCCGCGAGG), CNS1 (F: 5'–TCAGGAGTTCCAGGAGTTCAACCA, R: 5'–TCCTCCACCCGTTTCAGACAGATTT) and Intron 3 (F: 5'–AATGCACTGAGGAGGAGGCAGAAT, R: 5'–ACCTCCCAGCCTAAGCAAGAGTTT). Primers specific for the *Glucose 6-Phosphate Dehydrogenase* promoter (F: 5'–ACGGGGACCCAGGAGAAGT, R: 5'–TAGTTGCCGCTGCCAAACAC [18]) were used as the normalizing standard for $\Delta\Delta C_T$ calculations, with naive CD8 T cells being assigned an arbitrary value of 1.0.

Quantitative real-time PCR-based RT-PCR analysis to measure IFN- γ mRNA levels were also performed as previously described [17], with the following modification. Differences in IFN- γ mRNA levels were calculated by directly comparing ΔC_T values between samples (which all contained 4×10^2 cell equivalents) with naive CD8 T cells being assigned an arbitrary value of 1.0.

All ChIP and RT-PCR samples were run in duplicate using the cycling conditions of 95°C for 10 min, then 45 cycles of 95°C for 20 s and 59°C for 60 s. SYBR Green fluorescence was measured during the annealing/extension step.

2.4 Statistical Analyses

All quantitative data is expressed as the mean $\pm$ SEM, and p values were calculated using the unpaired two-tailed t test.

3. Results

3.1 Histone H3 acetylation within the *Ifng* locus of in vitro differentiated polyclonal effector CD8 T cells

To analyze the dynamics of histone H3 acetylation (AcH3) within the *Ifng* locus of CD8 T cells undergoing in vitro effector differentiation, lymph node (LN) suspensions prepared from non-transgenic (NT) mice were enriched for naive (CD44^{low}) CD8 T cells and primed in vitro with anti-CD3 mAb under Th1/Tc1 skewing conditions. As expected, the ability of these polyclonal CD8 T cells to express both intracellular IFN- γ protein (Fig. 1A) and mRNA (Fig. 1B) in response to restimulation with PMA + ionomycin (PMA + I) progressively increased at 24 h and 48 h post-priming. Furthermore, the amount of AcH3 bound to the *Ifng* proximal region measured by chromatin immunoprecipitation (ChIP) increased with a similar kinetics (Fig. 1C).

In addition to the proximal promoter, there are several other sites within the *Ifng* locus that associate with acetylated histones in T cells that have become competent to transcribe this gene. Two well-characterized sites located on either side of the proximal promoter are the upstream conserved noncoding sequence 1 (CNS1, –5.7 kb) and the enhancer located in the third intron +2 kb downstream of the promoter [2,8,9] (Fig. 1D). Following in vitro CD8 T cell priming, AcH3 also progressively increased at both CNS1 and Intron 3. In comparing the relative amounts of AcH3 at the three genomics sites, AcH3 was greatest at Intron 3, intermediate at the proximal promoter and the least at CNS1 (Fig. 1C). This pattern is consistent with that previously observed by Shnyreva et al. who also found in in vitro primed CD8 T cells the greatest amount of AcH3 at Intron 3 and the least at CNS1 [9].

3.2 Histone H3 acetylation within the *Ifng* locus of in vivo differentiated adoptively transferred TCR transgenic effector CD8 T cells

To characterize histone acetylation within the *Ifng* locus in in vivo differentiated effector CD8 T cells, we utilized a model in which naive TCR transgenic (Tg) CD8 T cells specific for

influenza hemagglutinin (HA) [12] are adoptively transferred into NT recipients infected with a recombinant vaccinia virus that expresses HA (viral-HA) [14,15]. Although our real-time PCR-based ChIP assay is sufficiently sensitive to analyze as few as 100,000 T cells [17], the number of viral-HA-primed TCR Tg effectors recovered from the adoptive transfer recipients was maximized by using TCR Tg CD8 T cells that overexpress the anti-apoptotic molecule Bcl-2. The Bcl-2-overexpressing virally-primed TCR Tg CD8 T cells developed a similarly robust capacity to express the effector cytokines IFN- γ and TNF- α compared to wild type counterparts (Fig. 2A), consistent with a previous study showing that Bcl-2 overexpression increases CD8 T cell clonal expansion without altering effector functions [19].

In striking contrast to in vitro primed effector CD8 T cells where AcH3 was 5-fold greater at Intron 3 compared to CNS1 ($p=0.004$) (Fig. 1C), TCR Tg effector CD8 T cells that were virally-primed in vivo displayed 4-fold greater AcH3 at CNS1 compared to Intron 3 ($p=0.002$) (Fig. 2B).

Taken together, these data suggested that the patterns of histone acetylation within the *Ifng* locus can differ markedly in CD8 T cells that undergo in vitro vs in vivo effector differentiation. It was possible, however, that these divergent patterns resulted from either differences in the duration of priming (2 days for in vitro vs 4 days for in vivo), the TCR ligand (anti-CD3 mAb for in vitro vs cognate MHC-peptide complex for in vivo) or the selective overexpression of Bcl-2 in the in vivo differentiated effector CD8 T cells. To simultaneously test these possibilities, we compared wild type TCR Tg CD8 T cells that had undergone effector differentiation for 4 days in vitro in response to HA peptide stimulation vs 4 days in vivo in response to viral-HA infection (Fig. 3).

Both the in vitro and in vivo differentiated TCR Tg effector CD8 T cells developed robust capacities to express IFN- γ and TNF- α (Fig. 3A). Importantly, the in vitro differentiated TCR Tg effector CD8 T cells displayed 2-fold greater AcH3 at Intron 3 compared to CNS1 ($p=0.002$), while in vivo differentiated counterparts displayed 3-fold greater AcH3 at CNS1 compared to Intron 3 ($p=0.02$) (Fig. 3B). Thus, this reciprocal pattern where TCR Tg effector CD8 T cells primed for 4 days with cognate MHC-peptide complex contain greater AcH3 at Intron 3 under in vitro conditions but greater AcH3 at CNS1 under in vivo conditions (Fig. 3B) mirrored the difference between in vitro anti-CD3 mAb-primed day 2 polyclonal effector (Fig. 1C) and in vivo day 4 virally-primed Bcl-2-overexpressing TCR Tg effector CD8 T cells (Fig. 2B).

3.3 Histone H3 acetylation within the *Ifng* locus of naturally formed polyclonal effector/memory CD8 T cells

The reciprocal patterns in the amounts of AcH3 associated with CNS1 and Intron 3 in in vitro vs in vivo primed effector CD8 T cells suggested that histone acetylation patterns observed in in vitro differentiated cells might not reflect epigenetic remodeling as it occurs during natural T cell responses. Alternatively, given that the functional response of adoptively transferred TCR Tg CD8 T cells can be influenced by the number transferred [20,21], it might have been possible that the AcH3 pattern in the TCR Tg CD8 T cells that were adoptively transferred into virally-infected recipients at supra-physiological numbers (1×10^6) was artifactual. To resolve this issue, we compared AcH3 within the *Ifng* locus of naturally formed effector/memory (CD44^{high}) vs naive (CD44^{low}) polyclonal CD8 T cells that were FACS-sorted from NT mice. Relative to naive CD8 T cells (which formed the baseline), effector/memory CD8 T cells displayed the greatest amount of AcH3 at CNS1 and the least at Intron 3 (Fig. 3B). In fact, the 3-fold greater amount of AcH3 at CNS1 relative to Intron 3 in naturally formed polyclonal effector/memory CD8 T cells ($p=0.02$) (Fig. 4B) closely matched the 3–4-fold difference observed in virally-primed TCR Tg effector CD8 T cells (Figs. 2B & 3B). Taken together,

these data suggest that the histone acetylation patterns observed in in vitro primed effector CD8 T cells might not be entirely reflective of natural formed effector/memory CD8 T cells.

4. Discussion

Epigenetic remodeling of effector cytokine genes is a central event in the differentiation of effector T cells. Within the *Ifng* locus, histones are covalently modified and DNA demethylated at the proximal promoter as well as several upstream and downstream sites. Remodeling of the proximal promoter is of obvious importance since this is where RNA polymerase II and core transcription factors bind to initiate transcription. Remodeling of upstream and downstream sites is also important since these enhancer elements modulate the rate of transcription by binding a variety of specialized transcription factors that are induced through a combination of TCR and cytokine-induced signals [22]. Sites of epigenetic remodeling within the *Ifng* locus have been studied almost exclusively using in vitro differentiated effector T cells. This is largely because the assays to analyze chromatin structure, such as ChIP to measure histone modifications, generally require large numbers of homogeneous T cells.

In the current study, we used a sensitive real-time PCR-based ChIP assay to compare the patterns of histone H3 acetylation (AcH3) at the *Ifng* proximal promoter as well as two other well-characterized upstream and downstream sites in CD8 T cells that underwent effector differentiation in vitro vs in vivo. Although only a few of the epigenetic events that can occur during effector differentiation were examined, we nevertheless observed a striking difference in the relative degrees to which different sites are bound by acetylated histones. Thus, while the proximal promoter similarly bound AcH3 in both in vitro and in vivo differentiated effectors, Intron 3 associated with a greater amount of AcH3 compared to CNS1 in in vitro differentiated effector CD8 T cells, as where the opposite pattern was observed in effector CD8 T cells that had been primed by vaccinia virus in vivo.

Remodeling of the *Ifng* locus is a complex process driven by the integration of TCR, cytokine and other signals [22]. Given that the precise immune response elicited by a particular pathogen is programmed by its unique combination of pathogen-associated inflammatory mediators [23], it might be possible that the *Ifng* locus histone acetylation pattern produced during in vitro CD8 T cell priming might reflect patterns produced in vivo in response to certain pathogens. Nevertheless, when AcH3 was examined in naturally formed polyclonal effector/memory CD8 T cells (which presumably represent the “average” of responses to environmental microbes), the pattern resembled that of in vivo vaccinia virus-primed effector CD8 T cells rather than in vitro primed counterparts. This suggests that to the extent that *Ifng* locus histone acetylation patterns observed in in vitro differentiated effector CD8 T cells might be reflective of certain in vivo CD8 T cell responses, such responses are unlikely to be typical. Although a more thorough analysis will be required to gain a comprehensive understanding of epigenetic remodeling events that occur within the *Ifng* locus during T cell effector differentiation, our current study highlights the necessity to incorporate the use of in vivo models to develop this understanding.

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Abbreviations

AcH3	acetylated histone H3
ChIP	

chromatin immunoprecipitation

CNS1

conserved noncoding sequence 1

NT

non-transgenic

viral-HA

recombinant vaccinia virus expressing hemagglutinin

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Fig. 1.

Histone H3 acetylation (ACh3) within the *Ifng* locus of in vitro differentiated polyclonal effector CD8 T cells. (A) Naive (CD44^{low}) CD8 T cells from non-transgenic (NT) LN were primed in vitro with anti-CD3 mAb plus cytokines for the indicated times, restimulated with PMA + I and analyzed for total expression of intracellular IFN- γ protein. Data is expressed in arbitrary units (mean $\pm$ SEM), $n=6$. (B) Expression of IFN- γ mRNA in in vitro primed and PMA + I-restimulated CD8 T cells. Data is expressed as the fold-difference between day 1 or 2 and the day 0 (naive) samples, $n=4$. (C) Non-restimulated in vitro primed CD8 T cells were directly analyzed by ChIP to measure ACh3 at the *Ifng* proximal promoter, CNS1 and Intron 3. For each site, data is expressed as the fold-difference between day 1 or 2 and the day 0 samples, $n=5$. Note that the proximal promoter data was used to generate part of a figure presented in a previous study [15]. (D) Diagram of the *Ifng* gene locus drawn approximately to scale, with the location of the four exons (thick vertical lines identified by roman numerals) as well as CNS1, the proximal promoter and Intron 3 indicated.

Fig. 2. Histone H3 acetylation within the *Ifng* locus of in vivo differentiated adoptively transferred TCR Tg effector CD8 T cells. One million naive Bcl-2-overexpressing TCR Tg CD8 T cells were adoptively transferred into viral-HA-infected recipients and recovered from spleens on day 4. (A) Intracellular IFN- γ vs. TNF- α expression following restimulation with cognate HA peptide, representative of $n=3$ per group. (B) AcH3 within the *Ifng* locus of non-restimulated purified TCR Tg CD8 T cells. For each site, data is expressed as the ratio between effector and corresponding naive (pre-transfer) samples, $n=4$. The proximal promoter data was presented in a previous study [15].

Fig. 3.

Histone H3 acetylation within the *Ifng* locus of in vitro vs in vivo differentiated wild type TCR Tg effector CD8 T cells. Naive TCR Tg CD8 T cells from three clone 4 Tg mice were enriched (by depleting CD44^{high} cells) and portions from each were individually cultured for 4 days with HA peptide plus cytokines (in vitro). The remaining cells were pooled with one portion being retained as the naive (day 0) baseline, and the rest were adoptively transferred into three separate viral-HA-infected recipients (1×10^6 TCR Tg CD8 T cells per transfer) and purified from spleens on day 4 (in vivo). (A) Intracellular IFN- γ vs. TNF- α expression following restimulation with PMA + I, representative of $n=3$ per group. (B) ACh3 within the *Ifng* locus of non-restimulated purified TCR Tg CD8 T cells. For each site, data is expressed as the ratio

between individual in vitro and in vivo primed TCR Tg effector CD8 T cell samples ($n=3$ per group) and the pooled naive (day 0 pre-transfer) sample.

Fig. 4. Histone H3 acetylation within the *Ifng* locus of naturally formed polyclonal effector/memory CD8 T cells. (A) CD44 vs intracellular IFN- γ expression in anti-CD3 mAb-stimulated polyclonal CD8 T cells prepared from NT LN, representative of $n=3$ per group. (B) ACh3 within the *Ifng* locus of FACS-sorted non-stimulated polyclonal effector/memory CD8 T cells. For each site, data is expressed as the ratio between individual effector/memory (CD44^{high}) samples ($n=3$) and the pooled naive (CD44^{low}) samples.