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Histone Acetylation at the Single-Cell Level: A Marker of Memory CD8+ T Cell Differentiation and Functionality

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Following stimulation, memory T (TM) cells rapidly express many effector functions, a hallmark feature that allows them to provide protective immunity. Recent studies suggest that genes involved in this rapid recall response may maintain an open chromatin structure in resting TM cells via epigenetic modifications. However, these studies have mostly focused on a few loci, and the techniques used required a large number of cells. We have developed a flow cytometric assay measuring histone modifications in individual murine T cells in combination with lineage-specific markers. In this study, we show that the per-cell level of a marker of open chromatin, diacetylated histone H3 (diAcH3), increases as naive CD8+ T cells develop into TM cells, demonstrating a novel correlation between the differentiation state of a CD8+ T cell and its abundance of a specific histone modification. Furthermore, our results show that TM cells defective in rapid recall ability have less diAcH3 than their fully functional counterparts, indicating that the diAcH3 level of individual TM cells is a useful marker for assessing their functionality. The Journal of Immunology, 2010, 184: 000–000.

The association between distinct chromatin landscapes and cellular identity can be studied on a gene-by-gene basis. Specifically, when a well-defined set of loci is known to be functionally important for the lineages under study, these loci can be analyzed in a targeted manner using chromatin immunoprecipitation (ChIP) followed by quantitative PCR using locus-specific primers (6). Alternatively, the advent of genome-wide methods for distinguishing functional from dysfunctional TM cells.

Materials and Methods

Mice
B6 (C57BL/6NCr) and CD4<sup>−/−</sup> (C57BL/6-Cd4tm1 Mak) mice were purchased from the National Cancer Institute (Bethesda, MD) and The Jackson Laboratory (Bar Harbor, ME), respectively. B6.P14 Thy1.1<sup>+</sup> mice were maintained as a breeding colony. All experiments were performed with adult female (6–10 wk) mice in accordance with Institutional Animal Care and Use Committee-approved protocols at the University of Pennsylvania School of Medicine Animal Facility (Philadelphia, PA).

CD8+ T cell purification
Splenocytes and lymphocytes (inguinal, brachial, axillary, and superficial cervical) were harvested from female B6 mice and pooled together. Enrichment for CD8<sup>+</sup> T cells was performed by depletion of CD4<sup>+</sup> and CD19<sup>+</sup> cells using anti-CD4 and anti-CD19 MACS beads and LS columns, followed by positive selection using anti-CD8 beads and LS columns (Miltenyi Biotec, Auburn, CA).

Immunofluorescence
Protocol was adapted from Ref. 9. Briefly, ~1 × 10<sup>6</sup> purified CD8<sup>+</sup> cells were stained with anti-CD8–PE-Texas Red (clone 5H10; 1 μg/ml), then washed and seeded on coverslips in 12-well plates. Cells were fixed with 2%...
paraformaldehyde for 10 min, and then permeabilized with Cytotox/Cytperm solution (BD Biosciences, San Jose, CA) or 0.4% Triton X-100. For staining, coverslips were transferred onto paraffin containing blocking solution (10% goat serum plus 2.5% PBS plus 0.1% Tween-20 in PBS), then anti-diAcH3 (10 μg/ml; #06-599, Millipore, Bedford, MA), then goat-anti-rabbit IgG-Alexa Fluor 488 (4 μg/ml; Invitrogen, Carlsbad, CA). All Abs were diluted in permeabilization solution, and all incubations were for 1 h at 25°C, with coverslips washed two to three times with perm solution between steps. Finally, coverslips were inverted on glass slides containing 10–20 μl Vectashield plus DAPI (Vector Laboratories, Burlingame, CA).

**Western blotting**

Aliquots of ~1 × 10^6 purified CD8^+ T cells were lysed in sample buffer (NuPAGE LDS, Invitrogen) with reducing agent (NuPAGE Sample Reducing Agent, Invitrogen), incubated at 70°C for 10 min, and sonicated. Lysates were separated on Bis-Tris gels (NuPAGE, Invitrogen). Posttransfer, membranes were incubated in blocking solution (5% w/v dry milk plus 0.1% Tween-20) for 30 min, anti-diAcH3 for 16 h (0.5 μg/ml), washed, and then incubated in anti-rabbit-IgG-HRP (Sigma-Aldrich, St. Louis, MO). All Abs were diluted in blocking solution. The HRP signal was detected using enhanced chemiluminescence (SuperSignal West Femto Kit, Thermo Fisher Scientific, Waltham, MA).

**In vitro activation and trichostatin A treatment**

Splenocytes were activated with soluble anti-CD3 (clone 145-2C11; 1.0 μg/ml) and anti-CD28 (clone 37.51; 0.5 μg/ml) Abs for 3 d. For trichostatin A (TSA) treatment, CD8^+ T cells were purified with anti-CD8 MACS beads and MACS LS columns (Miltenyi Biotec) on day 2 and recultured in the presence of 5 ng/ml TSA for 16 h.

**Adoptive transfers and infections**

Naive (CD44^lo) Thy1.1^+ CD8^+ T cells were purified from 5–8-wk-old female C57BL/6 (B6) mice by flow cytometric sorting. To examine the abundance of specific histone modifications in CD8^+ T cells, in addition to its absence from DAPI-dense regions of heterochromatin, which is consistent with the localization of acetylated histone H3 (Supplemental Fig. 1) (10). Lastly, treatment of CD8^+ T cells with TSA resulted in an ~3-fold increase in their average di-AcH3 level, measured as MFI using the flow cytometry assay (Fig. 1A).

**The population of diAcH3hi cells in specific pathogen-free mice has a memory phenotype**

In our initial studies, we used specific pathogen-free B6 mice, which contain T cells of both naïve (CD44^lo) and memory (CD44^hi) phenotype. All CD8^+ T cells from these mice had levels of diAcH3 that were above background, with a bimodally distributed population of positive events (Fig. 1B). Staining of cells from the spleen, lymph nodes, and bone marrow of many B6 mice showed that ~85% of CD8^+ T cells were diAcH3^lo, and ~15% were diAcH3^hi (Fig. 1B and data not shown). To determine if the two populations corresponded with known T cell subsets, we combined diAcH3 staining with that of a panel of cell surface markers. Both the diAcH3^lo and diAcH3^hi populations expressed CD44^hi, costained for diAcH3 and a panel of surface markers. Dot plots show expression of CD44 (x-axes) and three individual hPTMs (y-axes), with the numbers indicating the percentages (mean ± SD) of diAcH3^lo and diAcH3^hi CD8^+ T cells. C, B6 splenocytes were costained for diAcH3 and a panel of surface markers. Dot plots show expression of CD44 (x-axis) and three individual hPTMs (y-axis) for CD8^+ T cells. MFI values shown in A are representative of two independent experiments. MFI and percentage (± SD) values in B and C were calculated from 15 mice analyzed over five independent experiments with representative plots shown.
populations were negative for the activation markers CD25 and CD69 (Fig. 1C and data not shown), indicating that both populations consisted largely of resting cells. Furthermore, the majority of cells in both populations were CD127hi and CD62Llo, indicating that neither contained large numbers of effector cells, which are commonly CD127lo and CD62Lhi. Interestingly, diAcH3hi cells were CD44hi and CD122hi, whereas diAcH3lo cells were CD44lo and CD122lo (CD122 MFIs of 338 ± 13 and 1700 ± 322, respectively). These results indicated that the diAcH3hi and diAcH3lo populations corresponded with CD8+ T N and TM cells, respectively. Notably, such a correlation was not found for other H3 modifications, such as dimethylation of lysine 4 (K4me2) and dimethylation of lysine 9 (K9me2), because we observed a single population of positively staining events and similar levels of K4me2 and K9me2 between CD44hi and CD44lo CD8+ T cells (Fig. 1B, 1D). Together, these results demonstrate an increased level of diAcH3 in CD8+ T M cells that can be detected at the single-cell level by FACS.

**Pathogen-specific CD8+ T M cells have an elevated level of diAcH3**

To analyze well-defined, Ag-specific CD8+ T M cell populations, we infected B6 mice with LCMV. LCMV-specific CD8+ T M cells had high levels of diAcH3, and this was true for both GP33- and NP396-specific cells, identified by either intracellular cytokine staining (ICS) or MHC class I (MHC I) tetramers (Fig. 2A, 2B). Short in vitro stimulation with viral-derived peptides during ICS did not change the diAcH3 level of LCMV-specific T M cells (Fig. 2C). In addition, CD8+ T M cells generated in response to *L. monocytogenes* infection were uniformly diAcH3hi (Fig. 2D), showing that this is a common feature of CD8+ T M cells generated by different infections. We also analyzed the abundance of methylated histone marks in virus-specific memory cells. When compared with a control Ab, all tetramer-positive T M cells had detectable levels of dimethylated H3K4 and H3K9 (Fig. 2E). However, in contrast to diAcH3, the per-cell levels of histone marks in T M cells were not greater than those for naive (CD44lo)CD8+ T cells (Fig. 2E), consistent with our results from specific pathogen-free mice (Fig. 1D). Next, we used P14 TCR transgenic cells, which recognize the gp33 epitope from the LCMV glycoprotein (11), to follow the differentiation of T N, Ag-specific CD8+ cells into T M cells. Purified naive (CD44hi) P14 T cells were transferred to congenic recipients, who were infected 16 h later with LCMV. Although naive P14 cells had low diAcH3 levels, P14 T M cells (>60 d postinfection) were diAcH3hi (Fig. 2F), similar to polyclonal T M cells identified by MHC I tetramers. Together, these results show that CD8+ T cell differentiation from T N to T M cells is associated with an increase in the per-cell diAcH3 level and that the diAcH3hi phenotype is a common feature of T M cells, independent of their specificities and origins.

**Elevation of the per-cell diAcH3 level occurs during T E differentiation**

Because diAcH3 positively regulates several loci highly expressed by T E cells (12), we next tested whether elevation of the CD8+ T cell diAcH3 level occurred during a primary LCMV infection. As early as 8 d postinfection, we found an increase in the average diAcH3 level (measured by MFI) of pathogen-specific cells (Fig. 3A). At this time point, all tetramer-positive CD8+ T cells were diAcH3lo, independent of their specificity for the GP33 or NP396 epitopes. In addition, the total frequency of diAcH3lo cells increased dramatically from ~15 to ~85% of CD8+ T cells (Fig. 3A, 3B), which is equivalent to the estimated frequency of total LCMV-specific CD8+ T cells at the peak of the primary response (13). Therefore, it is likely that these D7/gp33 and D9/np396-negative, but diAcH3lo, cells represent CD8+ T cells specific for other LCMV epitopes. This conclusion is further supported by the fact that the expansion and contraction of the diAcH3lo CD8+ population occurred with the same kinetics as LCMV-specific CD8+ T cells iden-

**FIGURE 2.** Viral and bacterial-specific CD8+ T M cells are diAcH3hi. B6 mice were infected with LCMV (A–C and E) or *L. monocytogenes* (D) and analyzed >40 d postinfection. Epitope-specific CD8+ T cells were identified by ICS (A) following stimulation with GP33 (top panels) or NP396 (bottom panels) peptides, and by D7/gp33 (B) (top panels) or D9/np396 (bottom panels) MHC I tetramers. Histograms display diAcH3 levels of the gated CD8+INF-γ+ or tetramer+ cells (shaded) overlaid with that of CD8+INF-γ− or CD8+tetramer− cells (unfilled). C, Histograms display diAcH3 levels of D7/gp33+ or D9/np396+ T M cells (black line, unfilled) cells immediately ex vivo, overlaid with IFN-γ+ T M cells identified after peptide stimulation and ICS (shaded). D, The dot plot displays gating of D7/gp33+ T M cells and histogram displays diAcH3 levels of D7/gp33+ (shaded) and total CD8+ T cells (unfilled). E, Dot plots display gating of naive (top panel) and D7/gp33+ T M cells (bottom panel). Histograms display the levels of three different histone modifications in these naive (unfilled, solid line) and T M (shaded) cells. Staining of isotype controls for each population are shown by dashed lines. F, P14 Thy1.1+ CD8+ T cells were adoptively transferred to B6 mice followed by infection with LCMV and analysis on day 75 postinfection. Histogram displays anti-diAcH3 staining of donor P14 TM (shaded) and P14 TN (unfilled) cells. Plots in A–D and F are representative of four independent experiments and in E are representative of two independent experiments.

tifed by MHC I tetramers (Fig. 3B). Lastly, we found that the diAcH3 level was not significantly different between LCMV-specific CD8+ T cells at days 8 and 30 postinfection (Fig. 3C). Together with results described above, these data demonstrate that elevation of the CD8+ T cell diAcH3 level occurs concomitant with T E differentiation and is maintained in T M cells.

**Levels of diAcH3 correlate with CD8+ T M cell functionality**

CD8+ T M cells primed in the absence of CD4+ T cell help have defects in long-term survival, secondary proliferation, and recall of effector functions, resulting in suboptimal protection of hosts from subsequent infections (14, 15). Using the unhelped T M cell model, we tested whether a correlation exists between the functionality of a T M cell and its diAcH3 level. Naive (CD44hi) P14 T cells were transferred to congenic B6 or CD4−/− recipients who were then infected with LCMV. At >60 d postinfection, the unhelped P14 T M cell population contained fewer cells capable of producing both IFN-γ and IL-2 after
5 h of ex vivo GP33 peptide stimulation (29.5 ± 3.1% versus 59.6 ± 3.2%, respectively; Fig. 4A). They also produced lower levels of IFN-γ and TNF-α on a per-cell basis, as determined by comparing the MFI of each cytokine-producing population (Fig. 4A). Interestingly, unhelped T<sub>M</sub> cells had an ~2.5-fold lower per-cell level of diAcH3 than helped T<sub>M</sub> cells, correlating with their defects in cytokine production (Fig. 4B). Moreover, unhelped T<sub>M</sub> cells expressed lower levels of the IL-7Rα-chain (CD127), which is important for receiving IL-7 signals that promote T<sub>M</sub> cell survival, but did not have a global defect in protein expression, as levels of surface CD8 were similar between helped and unhelped T<sub>M</sub> cells (Fig. 4B). Taken together, these results show that the diAcH3 level of individual CD8<sup>+</sup> T<sub>M</sub> cells positively correlates with their expression of a prosurvival marker and predicts their functional capacity following stimulation.

**Discussion**

It is remarkable that memory lymphocytes exist in a functionally quiescent state for long periods of time, yet maintain the ability to reactivate gene expression programs tailored to the elimination of specific pathogens. Recent studies suggest that some memory of the effector T cell differentiation program, encoded epigenetically, remains in resting memory cells, contributing to their enhanced...
functionality (3, 7). Although histone modifications are numerous and an essential component of chromatin structure, relationships between the abundance and/or pattern of specific marks and CD8+ T cell differentiation state are largely unknown. In this study, using a novel flow cytometry assay, we provide the first description of a positive correlation between the per-cell level of a specific histone modification and T cell differentiation state and show that this modification also predicts the functional capacity of TSC cells.

Currently, a major question facing CD8+ T cell biology involves understanding how chromatin landscapes work together with lineage-specific transcription factors to establish the distinct fates and functions of naive, effector, and memory cells. In other fields, it has been shown that precursor and progeny cells can be distinguished by their total cellular levels of specific hPTMs. For example, immunofluorescence microscopy of human basal epidermis explants showed that epidermal stem cells had dramatically lower levels of acetylated histone H4 than their neighboring, terminally differentiated sebaceous cells (16). In addition, ELISA analysis of purified mononucleosomes demonstrated that human embryonic stem cells could be distinguished from embryonic fibroblasts by 2-fold higher levels of diAcH3 (K9K14) and lower levels of di- and trimethylated H3K9 (17). Importantly, not all modifications showed this imbalance, as levels of certain modifications, such as monoacetylation of H3K9 and trimethylation of H3K4, were equivalent between the stem cells and fibroblasts (17). Given that several different chemical groups can be added to multiple residues of each core histone, our FACS-based assay comparing levels of individual hPTMs between cells at different developmental stages can focus costly, labor-intensive studies seeking to globally map hPTMs, including those combining ChiP with high-throughput DNA sequencing.

Diacetylation of histone H3 on lysines 9 and 14 is associated with actively transcribed loci (18, 19). Thus, it is possible that CD8+ TSM cells contain a higher level of diAcH3 because they are actively transcribing more genes than TNS cells. This would be consistent with the observation that resting Ag-specific TSM cells contain more RNA than TNS cells (20), although it is possible that differences in other regulators of steady-state transcript levels, such as RNA stability, also contribute to this phenotype. However, if the explanation for the higher diAcH3 level in resting CD8+ TSM cells is simply that these cells are more transcriptionally active than their TNS precursors, one might expect to find that TSM cells contain a higher level of other modifications associated with active transcription, such as methylation of lysine 4 of histone H3 (21). Yet we found no difference in the level of this modification between TNS and TSM cells (Fig. 1D).

Although a difference in the number of actively transcribed genes may not explain the diAcH3 imbalance between TNS and TSM cells, our results do not discount an important role for diAcH3 in the gene expression of resting TSM cells. The diminished per-cell level of surface IL-7Rα found in dysfunctional, unhelped TSM cells immediately ex vivo could be due, at least in part, to suboptimal diAcH3 at this locus (Fig. 1B). Thus, further experiments are needed to look at the role of diAcH3 in regulating the expression of IL7Rα and other related genes involved in TSM cell homeostatic proliferation. However, even if diAcH3 positively regulates such genes in TSM cells, it is likely not limited to these loci, because we observed the diAcH3 increase in LCMV-specific T cells found at days 8 and 15 postinfection, which have not yet gained the ability to undergo homeostatic proliferation (22).

Because diAcH3 is a mark of open loci (those accessible to the transcriptional machinery but not actively transcribed), the elevated diAcH3 level in resting CD8+ TSM cells may also represent certain genes poised for rapid and robust transcriptional activation following stimulation. Our previous results demonstrated this phenomenon for a single locus, where we found a correlation between diAcH3 abundance at Ifng in resting TSM cells and their ability to produce IFN-γ protein poststimulation (4). By showing that the per-cell diAcH3 level is elevated in TSC cells and maintained in resting TSM cells, our new results suggest that the acquisition and maintenance of appropriate diAcH3 levels may be important for TSM cell functionality on a more global scale, with diAcH3 essentially serving as a memory of the effector cell gene expression program. Consistent with this idea, unhelped CD8+ TSM cells, defective in the recall of effector functions (cytokine production and secondary proliferation), had less diAcH3 on a per-cell basis than their fully functional counterparts (Fig. 4B). Moreover, we have previously shown that treatment of unhelped CD8+ T cells with a histone deacetylase inhibitor, which effectively increases total histone acetylation levels, results in the rescue of unhelped cells’ cytokine production and protective capacity (15). Together, these results provide a solid foundation for future experiments that harness the power of high-throughput DNA sequencing to compare diAcH3 abundance on a genome-wide scale between functional and dysfunctional TSM cells.

As compared with naive cells, the ability of TSM cells to rapidly and robustly accumulate cytokine and chemokine transcripts following stimulation is a hallmark feature of their superior protective capacity. During effector CD8+ T cell differentiation, changes in locus accessibility that involve the addition of hPTMs associated with open chromatin occur at a handful of genes (3, 4) and may contribute to keeping these loci accessible to the transcriptional machinery in resting TSM cells. In this study, we show that the total cellular level of one such modification, diAcH3, is low in naive CD8+ T cells and increases during TSM cell differentiation, suggesting that this modification can mark a large number of genes whose expression is responsible for executing TSM rapid recall. Moreover, our results suggest that the positive correlation between TSM cell functionality and diAcH3 level could be useful as a measure of the ability of different vaccine platforms to induce highly functional TSM cells, and they provide a rationale for efforts focused on manipulating T cell histone acetylation levels in immunotherapies.

Disclosures
The authors have no financial conflicts of interest.

References

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Figure S1: The target of α-diAcH3 sera has the appropriate size and localization in CD8+ T cells. A, Lysates of purified CD8+ T cells were separated by SDS-PAGE, run in duplicate and blotted with α-diAcH3 followed by Goat-α-Rabbit-HRP. B, Purified CD8+ T cells were seeded on poly-L-lysine coated coverslips followed by α-CD8 staining, fixation, permeabilization, α-diAcH3 and DAPI staining and analysis by indirect immunofluorescence. C, Purified CD8+ T cells were seeded on poly-L-lysine coated coverslips followed by fixation, permeabilization and α-diAcH3 and DAPI staining. diAcH3 staining does not co-localize with DAPI-dense areas of heterochromatin, (arrows) consistent with reports showing that heterochromatin is depleted of acetylated histones.