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Following stimulation, memory T (T_M) 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 T_M 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 T_M 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 T_M cells defective in rapid recall ability have less diAcH3 than their fully functional counterparts, indicating that the diAcH3 level of individual T_M cells is a useful marker for assessing their functionality. The Journal of Immunology, 2010, 184: 4631–4636.

The functional specialization that occurs during cellular differentiation is often accompanied by chromatin remodeling (1), a process that has been intensively studied within the hematopoietic system. For CD4+ T cells, the remodeling of Ifng and Il4 that is required for their polarization into distinct Th1 and Th2 lineages has been mapped extensively at the level of DNA methylation and histone posttranslational modifications (hPTMs) (2). In contrast, far less is known about the chromatin landscapes that establish cellular identity for CD8+ T cells and direct their differentiation into effector CTL (T_E) and long-lived, protective memory T (T_M) cells. Thus far, studies correlating specific histone modifications with CD8+ T cell identity for CD8+ T cells and direct their differentiation into effector cells have been focused largely on effector chemokine and cytokine loci (3–5). At the IFN-γ locus, diacetylated histone H3 (diAcH3), a mark of open chromatin, increases in abundance at multiple cis-acting regulatory regions during CD8+ T cell differentiation from the naïve T (T_N) cell to the T_M cell stage, and failure to acquire diAcH3 at Ifng contributes to the defect in IFN-γ protein production by CD8+ T_N cells primed in the absence of CD4+ T cell help (4).

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 genomic DNA arrays and high-throughput DNA sequencing has allowed unbiased analyses of genomic fragments recovered by ChIP (7, 8). Although these techniques have greatly facilitated genome-wide mapping of specific hPTMs, they are still dependent on large numbers of cells, which poses a challenge for studying rare populations, such as Ag-specific T_M cells. Moreover, given the polyclonal nature of T cell responses and the functional heterogeneity among T cell subsets, results can be skewed by subpopulations of cells with hyper- or hypo-modified loci.

In this report, we describe a powerful flow cytometric assay that analyzes hPTM levels on a per-cell basis in combination with lineage-specific markers, allowing for a multiparametric analysis of hPTM abundance in different T cell subsets. Our results show that CD8+ T_M cells have ~2-fold more diAcH3 than their T_N cell counterparts and that this elevated diAcH3 level is acquired by Ag-specific CD8+ T cells during a primary infection. Moreover, as compared with fully functional CD8+ T_M cells, nonprolific T_M cells generated in the absence of CD4+ T cell help have less diAcH3, correlating with their defects in cytokine production. Thus, these results associate diAcH3 abundance with T cell differentiation stage and describe a useful method for distinguishing functional from dysfunctional T_M cells.

Materials and Methods

Mice

B6 (C57BL/6NCr) and CD4-/- (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+ 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+ T cells was performed by depletion of CD4+ and CD19+ cells using anti-CD4 and anti-CD19 MACS beads and LD 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 × 10^6 purified CD8+ 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/Cytoperm solution (BD Biosciences, San Jose, CA) or 0.4% Triton X-100. For staining, coverslips were transferred onto parafilm containing blocking solution (10% goat serum plus 2.5% FBS plus 0.1% Tween-20 in PBS), then anti-diAcH3 (10 μg/ml; #06-599, Millipore, Bedford, MA), 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 onto glass slides containing 10–20 μl Vectashield plus DAPI (Vector Laboratories, Burlingame, CA).

**Western blotting**

Aliquots of ~1 × 10⁶ 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 1 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).

**Flow cytometric staining for histone modifications**

Poststaining for surface epitopes, staining of hPTMs was performed using intracellular staining kits from BD Biosciences. Cells were incubated with anti-diacyl histone H3 (K9K14; #06-599), anti-dimethyl histone H3 (K4; #07-030 and K9; #07-212), or preimmune rabbit serum as negative control, all from Millipore and used at 10 μg/ml. Cells were then washed and incubated with goat anti-rabbit–FITC (BD Biosciences). A similar protocol was performed using the BrdU staining kit (BD Biosciences), intracellular staining kit (eBioscience, San Diego, CA), or 0.1% Triton X-100, with all methods yielding similar results. All samples were analyzed on an FACSCanto cytometer (BD Biosciences).

**Statistical analyses**

Mean fluorescence intensity (MFI) values are presented as the mean ± SD, calculated from multiple mice over at least three experiments. Statistical significance was assessed by the unpaired Student t test (performed in GraphPad Prism, GraphPad, San Diego, CA), and only p values < 0.05 were considered significant.

**Results**

A flow cytometric assay for measuring hPTMs in individual cells

To examine the abundance of specific histone modifications in CD8+ T cells, we developed a novel flow cytometric assay that combines intracellular staining for acetylated histones with surface staining for T cell phenotypic markers. Several control experiments were performed to verify the specificity of the anti-diAcH3 sera used in our studies. Western blotting of whole cell lysates from CD8+ T cells with anti-diAcH3 sera yielded a single band at the expected size of histone H3, and the abundance of this band increased when CD8+ T cells were pretreated with the histone deacetylase inhibitor TSA (Supplemental Fig. 1 and data not shown). Furthermore, immunofluorescence experiments verified the nuclear localization of the target epitope 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 diAcH3⁹⁹ 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 naive (CD44⁺) and memory (CD44⁺) 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⁺, and ~15% were diAcH3⁻ (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⁻ and diAcH3⁺ populations had a memory phenotype. Several control experiments were performed using specific monoclonal antibodies (mAbs) directed against the following markers: CD44 (clone 125.16; 0.5 μg/ml), K9; #07-212), or preimmune rabbit serum as negative control, all from Millipore and used at 10 μg/ml. Cells were then washed and incubated with goat anti-rabbit–FITC (BD Biosciences). A similar protocol was performed using the BrdU staining kit (BD Biosciences), intracellular staining kit (eBioscience, San Diego, CA), or 0.1% Triton X-100, with all methods yielding similar results. All samples were analyzed on an FACSCanto cytometer (BD Biosciences).

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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 CD62Lhi, indicating that neither contained large numbers of effector cells, which are commonly CD127lo and CD62Llo. 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+ Tγ and Tδ 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+ Tγ cells that can be detected at the single-cell level by FACS.

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

To analyze well-defined, Ag-specific CD8+ Tγ cell populations, we infected B6 mice with LCMV. LCMV-specific CD8+ Tγ 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 Tγ cells (Fig. 2C). In addition, CD8+ Tγ cells generated in response to *L. monocytogenes* infection were uniformly diAcH3hi (Fig. 2D), showing that this is a common feature of CD8+ Tγ 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 Tγ cells had detectable levels of dimethylated H3K4 and H3K9 (Fig. 2E). However, in contrast to diAcH3, the per-cell levels of methyl histone marks in Tγ 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 Tγ, Ag-specific CD8+ cells into Tγ 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 Tγ cells (>60 d postinfection) were diAcH3hi (Fig. 2F), similar to polyclonal Tγ cells identified by MHC I tetramers. Together, these results show that CD8+ Tγ cell differentiation from Tδ to Tγ cells is associated with an increase in the per-cell diAcH3 level and that the diAcH3hi phenotype is a common feature of Tγ cells, independent of their specificities and origins.

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

Because diAcH3 positively regulates several loci highly expressed by Tδ 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+ Tδ cells were diAcH3hi, independent of their specificity for the GP33 or NP396 epitopes. In addition, the total frequency of diAcH3hi cells increased dramatically from ~15 to ~85% of CD8+ Tδ 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 D3′gp33 and D3′np396-negative, but diAcH3hi, cells represent CD8+ Tδ cells specific for other LCMV epitopes. This conclusion is further supported by the fact that the expansion and contraction of the diAcH3hi CD8+ population occurred with the same kinetics as LCMV-specific CD8+ T cells identified 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+ Tδ cell diAcH3 level occurs concomitantly with Tδ differentiation and is maintained in Tδ cells.

**Levels of diAcH3 correlate with CD8+ Tγ cell functionality**

CD8+ Tγ 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 Tδ cell model, we tested whether a correlation exists between the functionality of a Tδ cell and its diAcH3 level. Naive (CD44lo) P14 T cells were transferred to congenic B6 or CD4−/− recipients who were then infected with LCMV. At >60 d postinfection, the unhelped P14 Tδ cell population

![FIGURE 2. Viral and bacterial-specific CD8+ Tγ cells are diAcH3hi. B6 mice were infected with LCMV (A–C and E) or rLMgp33 (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 D6′gp33′ (B) (top panels) or D6′np396′ (bottom panels) MHC I tetramers. Histograms display diAcH3 levels of the gated CD8+IFN-γ+ or tetramer+ cells (shaded) overlaid with that of CD8+IFN-γ− or CD8+ tetramer− cells (unfilled). C, Histograms display diAcH3 levels of D6′gp33′ and D6′np396′ Tδ cells (shaded) and total CD8+ Tδ cells (unfilled). C, Dot plots display gating of naive (top panel) and D6′gp33′ Tδ cells (bottom panels). Histograms display the levels of three different histone modifications in these naive (unfilled, solid line) and Tδ (shaded) cells. Staining of isotype controls for each population are shown by dashed lines. F, P14 Thy1.1+ CD8+ Tδ cells were adoptively transferred to B6 mice followed by infection with LCMV and analysis on day 75 postinfection. Histograms display anti-diAcH3 staining of donor P14 Tδ (shaded) and P14 Tγ (unfilled) cells. Plots in A–D and F are representative of four independent experiments and in E are representative of two independent experiments.](http://www.jimmunol.org/)

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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 TM cells had an ~2.5-fold lower per-cell level of diAcH3 than helped TM cells, correlating with their defects in cytokine production (Fig. 4B). Moreover, unhelped TM cells expressed lower levels of the IL-7Rα-chain (CD127), which is important for receiving IL-7 signals that promote TM cell survival, but did not have a global defect in protein expression, as levels of surface CD8 were similar between helped and unhelped TM cells (Fig. 4B). Taken together, these results show that the diAcH3 level of individual CD8+ TM 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 cytometric 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 TM 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 epidermis 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 monomethylation 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+ TM cells contain a higher level of diAcH3 because they are actively transcribing more genes than TN cells. This would be consistent with the observation that resting Ag-specific TM cells contain more RNA than TN 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+ TM cells is simply that these cells are more transcriptionally active than their TN precursors, one might expect to find that TM 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 TN and TM cells (Fig. 1D).

Although a difference in the number of actively transcribed genes may not explain the diAcH3 imbalance between TN and TM cells, our results do not discount an important role for diAcH3 in the gene expression of resting TM cells. The diminished per-cell level of surface IL-7Rα found in dysfunctional, unhelped TM cells immediately ex vivo could be due, at least in part, to suboptimal diAcH3 at this locus (Fig. 4B). Thus, further experiments are needed to look at the role of diAcH3 in regulating the expression of IL7Rα and other related genes involved in TM cell homeostatic proliferation. However, even if diAcH3 positively regulates such genes in TM 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+ TM 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 TM cells and their ability to produce IFN-γ protein poststimulation (4). By showing that the per-cell diAcH3 level is elevated in T-E cells and maintained in resting TM cells, our new results suggest that the acquisition and maintenance of appropriate diAcH3 levels may be important for TM 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+ TM 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 TM cells.

As compared with naive cells, the ability of TM 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 TM 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 TM cell differentiation, suggesting that this modification may mark a large number of genes whose expression is responsible for executing Treg rapid recall. Moreover, our results suggest that the positive correlation between TM cell functionality and diAcH3 level could be useful as a measure of the ability of different vaccine platforms to induce highly functional TM 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.

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