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Cutting Edge: Chromatin Remodeling as a Molecular Basis for the Enhanced Functionality of Memory CD8 T Cells

John K. Northrop,* Andrew D. Wells,†‡ and Hao Shen2*

Memory CD8 T cells, unlike their naive precursors, are capable of rapidly producing high levels of cytokines, killing target cells, and proliferating into numerous secondary effectors immediately upon Ag encounter. This ready-to-responsiveness of memory CD8 T cells makes them capable of immediate cytokine production and providing protective immunity. These results demonstrate that CD4 T help-dependent chromatin remodeling provides a molecular basis for the enhanced responsiveness of memory CD8 T cells.

The CD8 T cell plays a critical role in immune responses against intracellular pathogens. Following an acute infection, Ag-specific naive CD8 T cells expand and differentiate into a large population of effector CD8 T cells that contribute to the control of infection (1). After clearance of the pathogen, the majority of effector CD8 T cells die (2); however, a small percentage survive and further differentiate into mature memory CD8 T cells (CD8 T M)3 (3–5). Unlike naive CD8 T cells, which require days to differentiate into cytokine-producing effectors, CD8 T M are immediately able to produce high levels of effector cytokines and kill target cells upon Ag recognition, even before rapid proliferation into large numbers of secondary effectors (6–9). The molecular basis that confers this poised, ready-to-respond state to memory CD8 T cells remains largely unknown.

Under certain conditions, such as the absence of adequate CD4 T cell help (CD4 T H), a poor immune response is induced that often leads to persistent viral infection and/or generation of CD8 T M that are defective and unable to provide protective immunity (10–13). Although CD4 T cells are needed during priming for programming the differentiation of CD8 T M (14), they have also been shown to play a role in the maintenance of CD8 T M (15). The precise nature of CD4 T H during the priming and maintenance of CD8 T M is still unclear. Furthermore, the molecular basis that underlies the defective phenotype of “unhelped” CD8 T M remains to be elucidated. Our recent results show that defects in unhelped CD8 T M are not due to aberrations in the TCR repertoire nor to impairment in functional avidity maturation (16). Instead, our results suggest a correlation between diminished histone acetylation and poor functionality in CD8 T M generated in the absence of CD4 T H (16). In this report, we demonstrate a direct role for CD4 T H-dependent histone modifications in contributing to the enhanced functionality of memory CD8 T cells.

Materials and Methods

Mice

C57BL/6J (B6) and B6.129S2-Cd4ΔIIIΔIV/J (B6-Cd4−/−) mice were purchased from The Jackson Laboratory. B6-P14 Thy1.1 mice were obtained from in-house breeding colonies crossing B6;D2-Tg(TcrLCMV)327Sdz/J (B6-TCR) with B6.PL-Tg(Thy1.2/CyJ). All experiments were performed with female age-matched (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).

In vitro activation and trichostatin A (TSA) treatment

P14 CD8 T cells (~20% of total) in coculture with B6 or B6-Cd4−/− spleenocytes were activated with soluble anti-CD3 (1.0 μg/ml) and anti-CD28 (0.5 μg/ml) Abs for 3 days. For TSA treatment, a final concentration of 5 ng/ml (17 nM) TSA was added to the above in vitro cocultures on day 2. This concentration was based on pilot experiments in which we determined the optimal concentration and duration of TSA treatment that resulted in increased histone acetylation with minimal toxicity.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) with an anti-acetyl histone-H3 Ab was performed as described previously, and the quantity of DNA was determined by quantitative PCR (16). Values for specific enrichment of immunoprecipitated DNA from each sample are normalized to the mean value for the

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3 Abbreviations used in this paper: CD8 T M, memory CD8 T cell; CD4 T H, helper CD4 T cell; CD4 T M, CD4 T cell help; ChIP, chromatin immunoprecipitation; GP, gycropeptin; HDAC, histone deacetylase; P+1, PMA and ionomycin; TSA, trichostatin A.

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expressed lower levels of the high affinity IL-7R could be detected 1 wk posttransfer (Fig. 2), and these cells
duction by the unhelped CD8 T cells further indicates that the
CD8 T cells, consistent with our in vivo results observed in the
IFN-γ response of unhelped P14 cells transferred into B6-CD4
levels to at least 45 days posttransfer. However, only low num-
the lymphocyte choriomeningitis virus for which P14 CD8 T cells are specific.

Results and Discussion
To further define the role of CD4 T cells and the molecular basis of the defective phenotypes in unhelped CD8 T cells, we used an experimental system of in vitro activation followed by adoptive transfer into naive hosts (4, 17). A population of homogeneous, monoclonal CD8 T cells from P14 TCR transgenic mice were purified and stimulated with anti-CD3 and anti-CD28 Abs in the presence of splenocytes from either B6 or B6-CD4 knockout mice. Three days after in vitro activation, P14 CD8 T cells were adoptively transferred into congenic naive hosts to examine whether the presence of CD4 T cells during activation influences the development of CD8 T cells.

Before transfer, levels of histone H3 acetylation (AcH3) at the Ifng promoter and enhancer were analyzed by ChIP. P14 cells from coculture with B6 splenocytes ("helped") were observed to have a significant increase in AcH3 at the Ifng locus relative to unstimulated naive CD8 T cells (Fig. 1, A and B). In contrast, P14 cells cocultured with splenocytes from B6-CD4 knockout mice ("unhelped") failed to substantially increase AcH3 at the Ifng enhancer (Fig. 1, A and B). Similar results were observed when splenocytes from B6 mice depleted of CD4 T cells by MACS beads (<1% CD4 T cells; Miltenyi Biotec) were used in the cocultures (data not shown). Irrespective of the presence of CD4 T cells, activated P14 cells restimulated with PMA and ionomycin (P+I) produced equal amounts of IFN-γ (Fig. 1C) and exhibited similar activation phenotypes (Fig. 1D). Thus, the difference in AcH3 levels is not merely due to sub-
optimal activation of P14 cells in the absence of CD4 T cell.

Following transfer into B6 hosts, the in vitro activated, helped P14 cells contracted within 1 wk and survived at stable levels to at least 45 days posttransfer. However, only low numbers of unhelped P14 cells transferred into B6-CD4 knockout mice could be detected 1 wk posttransfer (Fig. 2A), and these cells expressed lower levels of the high affinity IL-7Rα (CD127) compared with the helped P14 cells (Fig. 2B). Furthermore, significantly less IFN-γ was produced by fewer unhelped P14 cells following GP33–41 peptide restimulation or with P+I (Fig. 2, C and D). The failure of P+I to even partially restore IFN-γ production by the unhelped CD8 T cells further indicates that the IFN-γ defect in these cells lies downstream of TCR signaling. Together, these results from a well-defined in vitro experimental system clearly show that CD4 T cells influence epigenetic modifications in CD8 T cells and the development of functional CD8 T cells, consistent with our in vivo results observed in the context of an infection (16).

Importantly, the in vitro system allowed us to use chemical inhibitors to alter AcH3 levels and ask whether the AcH3 level at the Ifng locus is simply correlative or plays a direct role in regulating IFN-γ production. TSA is a chemical inhibitor of histone deacetylase (HDAC) activity that increases the total cell

Adoptive transfer and infectious challenge
In vitro activated P14 cells were transferred into B6 or B6-CD4 knockout recipient mice (1–2 × 10⁶ per mouse). At different time points, splenocytes were harvested for FACS analysis and intracellular cytokine staining. To assess protective immunity, recipient mice were infected i.p. with 1.5 × 10⁵ CFU of a recombinant strain of Listeria monocytogenes expressing glycoprotein (GP)-derived peptide GP33–41, of the lymphocyte choriomeningitis virus for which P14 CD8 T cells are specific. Bacterial loads were determined on day 3 postinfection.

![FIGURE 1. Diminished histone H3 acetylation at the Ifng enhancer of CD8 T cells activated without CD4 T cells. A, Specific enrichment of DNA from the Ifng enhancer of P14 cells from helped (H) or unhelped (U) cocultures. Individual replicates from B6 (▲) and B6-CD4 knockout (◇) cocultures are normalized to values from naive P14 cells (X), and a horizontal bar indicates the mean for each group. The dashed line shows the average background signal from control ChIP assays with polyclonal rabbit IgG (0.006). B, A 250-bp region of the Ifng enhancer was amplified by conventional PCR from total cell extract (left band) or ChIP fraction (right band) from control Ab (◇), naive (N), helped (H), and unhelped (U) CD8 T cells. C, Ifng γ production by restimulated P14 cells. Dots plots are gated on CD8 T cells and the percentage of IFN-γ+ cells is shown in the upper right corner (mean ± SD). D, Expression of activation markers CD69 and CD44 (mean fluorescence intensity of each at the respective axes). ChIP data are from three experiments with 6–9 repeat samples per group. Flow cytometric data are representative of five independent experiments. D, Day.

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production upon restimulation was also increased in P14 cells from the TSA-treated, unhelped group (data not shown). Thus, TSA treatment during priming had a positive impact on the ability of unhelped CD8 T cells to produce effector cytokines.

Additionally, TSA treatment had a positive effect on the ability of unhelped P14 cells to survive in the adoptive host (Fig. 3C). On day 7 after transfer, significantly fewer numbers of P14 cells were present in mice that received unhelped P14 than those that received helped P14 cells (Fig. 3C). TSA treatment of unhelped P14 cells resulted in a significant increase in the number of P14 cells surviving in vivo, with frequencies and cell numbers similar to those of helped P14 cells at 7 (Fig. 3C) and 30 days posttransfer (data not shown). Thus, TSA treatment of unhelped CD8 T cells had a positive effect on the subsequent numbers of cells recovered from host mice after 1 wk of resting. These results suggest that defective survival of unhelped CD8 T cells may also be due to impaired histone acetylation, possibly at loci involved in prosurvival, anti-apoptosis, and/or homeostatic proliferation.

Because TSA treatment rescued the ability of unhelped P14 cells to survive and form functional memory fully capable of producing immediate effector cytokines upon restimulation, we next tested whether TSA treatment could also restore the ability of unhelped CD8 T cells to provide protective immunity. Equal numbers of in vitro activated P14 CD8 T cells and naive P14 cells were transferred into B6 recipients. One week following transfer, mice were challenged with a high dose of recombinant *L. monocytogenes* expressing the GP$_{33-41}$ epitope. Three days following challenge, bacterial loads in the spleen were assessed. The transfer of helped CD8 T cells afforded increased protection compared with the transfer of naive P14 cells (41-fold fewer CFU; Fig. 4). Unhelped CD8 T cells offered significantly less protection compared with mice that received helped CD8 T cells (36-fold difference in CFU; Fig. 4). Importantly, transfer of TSA-treated, unhelped CD8 T cells provided substantially greater protection than the transfer of untreated, unhelped CD8 T cells (62-fold decrease in CFU, $p = 0.004$; 7.0-fold (7.0 ± SD) are from 4–5 individual host mice representative of four independent experiments.

**FIGURE 3.** TSA treatment increases AcH3 of in vitro activated CD8 T cells and the survival and IFN-$\gamma$ production of these cells rested in vivo. A, Specific enrichment for AcH3 at the Ifng enhancer in P14 cells from B6 (○), B6-CD4$^{-/-}$ (⊗), and TSA-treated B6-CD4$^{-/-}$ (△) cocultures compared with naive (X) controls. B, In vitro activated P14 cells were rested in congeneric B6 hosts for 1 wk and IFN-$\gamma$ production was measured by intracellular cytokine staining upon restimulation with GP$_{33-41}$ peptide. Numbers in the dot plots indicate a percentage of P14 cells producing IFN-$\gamma$, and the bar graphs show mean fluorescence intensity of IFN-$\gamma$ (E3, ×1,000). There are significant differences in percentage ($p = 0.001$) and mean fluorescence intensity (MFI) ($**$, $p = 0.0006$) between TSA-treated unhelped and untreated, unhelped P14 cells. C, Survival of P14 cells in vivo. Numbers in dot plots indicate a percentage of total CD8 T cells that are P14 cells (○) vs ⊗+TSA; $p = 0.00006$, and bar graph show the total numbers of donor P14 cells recovered per spleen (§§ per spl; t4, ×10,000) (○) vs ⊗+TSA; 7.0-fold (7.0×) increase; ***, $p = 0.0006$. ChIP data are compiled from 4–9 repeat samples from two independent TSA experiments. Transfer data (mean ± SD) are from 4–5 individual host mice representative of four independent experiments.
Ifng regulating gene expression for a broad range of biological phenomena. In vitro activated P14 cells were transferred into congenic B6 hosts. One week posttransfer, mice were recombined with reconstituted L. monocytogenes expressing the GP33-41 epitope, and bacterial numbers in the spleen were enumerated on day 3 after challenge. Bacterial load is shown on a log scale bar graph of CFU per gram of spleen. The lower limit of detection (LOD) is 230 CFU per gram of spleen. A group of mice that received naive P14 cells 1 day before challenge was included as a control. Mice receiving no P14 cells had ~8 x 10^6 CFU per gram of spleen (data not shown). Protection data are from 6–9 individual mice and representative of four independent experiments.

Fig. 4), and the level of protection was similar to that seen in mice receiving helped CD8 T cells (Fig. 4).

Our previous in vivo studies have shown that the absence of CD4 T cells in virus-infected mice is associated with decreased CD4 T cell proliferation (21–24). In CD4 T cells, chromatin remodeling at the Ifng locus and H4H1 or H4H2/12 polarized effectors (25–28). In addition to histone acetylation, other epigenetic modifications such as DNA methylation likely play a role in maintaining long-term persistence of host immunity, immediate effector cytokine production, and, most importantly, the ability of CD8 T cells to induce IFN-γ. TSA treatment at priming rescues key functional defects of the unhelped CD8 T cells and restores long-term persistence in vivo, immediate effector cytokine production, and, most importantly, protective immunity. HDAC inhibition was effective in overcoming defects normally impaired on unhelped CD8 T cells. These results establish chromatin remodeling as a molecular basis for the efficient function of CD8 T cells and provide direct evidence for a role of CD4 T cells in this process.

Heritable epigenetic modifications play an essential role in regulating gene expression for a broad range of biological processes (21–24). In CD4 T cells, chromatin remodeling at the Ifng and H4H1 loci occurs during differentiation into Th1 or Th12-polarized effectors (25–28). In addition to histone acetylation, other epigenetic modifications such as DNA methylation likely play a role in rapid expression of effector functions by CD8 T cells upon restimulation (16, 29). Although our analyses have focused on IFN-γ as a representative effector cytokine, our results show the rescue of multiple functions by TSA treatment and thus suggest a model in which the transition of naive CD8 T cells into memory involves epigenetic modifications of numerous genes involved in T cell survival, proliferation, and effector functions. Consistent with this model, increased histone acetylation has been observed in other loci in CD8 T cells (30). Thus, histone acetylation likely plays a role in maintaining a broad range of genes in an accessible state, which represents an important aspect of CD8 T cell functionality critical to its ability to mediate immune protection.

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Disclosures
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