Histone Acetylation Facilitates Rapid and Robust Memory CD8 T Cell Response through Differential Expression of Effector Molecules (Eomesodermin and Its Targets: Perforin and Granzyme B)

Yasuto Araki, Monchou Fann, Robert Wersto and Nan-ping Weng

*J Immunol* 2008; 180:8102-8108; doi: 10.4049/jimmunol.180.12.8102

http://www.jimmunol.org/content/180/12/8102

**References** This article cites 26 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/180/12/8102.full#ref-list-1

**Why The JI?** Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

*average

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2008 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Histone Acetylation Facilitates Rapid and Robust Memory CD8 T Cell Response through Differential Expression of Effector Molecules (Eomesodermin and Its Targets: Perforin and Granzyme B)\textsuperscript{1}

Yasuto Araki,* Monchou Fann,* Robert Wersto,† and Nan-ping Weng\textsuperscript{2*}

To understand the mechanism regulating the effector function of memory CD8 T cells, we examined expression and chromatin state of a key transcription factor (eomesodermin, EOMES) and two of its targets: perforin (PRF1) and granzyme B (GZMB). Accessible chromatin associated histone 3 lysine 9 acetylation (H3K9Ac) was found significantly higher at the proximal promoter and the first exon region of all three genes in memory CD8 T cells than in naive CD8 T cells. Correspondingly, EOMES and PRF1 were constitutively higher expressed in memory CD8 T cells than in naive CD8 T cells at resting and activated states. In contrast, higher expression of GZMB was induced in memory CD8 T cells than in naive CD8 T cells only after activation. Regardless of their constitutive or inducible expression, decreased H3K9Ac levels after treatment with a histone acetyltransferase inhibitor (Curcumin) led to decreased expression of all three genes in activated memory CD8 T cells. These findings suggest that H3K9Ac associated accessible chromatin state serves as a corner stone for the differentially high expression of these effector genes in memory CD8 T cells. Thus, epigenetic changes mediated via histone acetylation may provide a chromatin “memory” for the rapid and robust transcriptional response of memory CD8 T cells. The Journal of Immunology, 2008, 180: 8102–8108.

During the process of naive cell to memory cell differentiation, memory CD8 T cells acquire two unique properties: long life and enhanced effector function (1–3). This enhanced effector function is associated with a low threshold for activation and a rapid production of effector molecules of memory CD8 T cells, and is controlled in part by transcription factors such as Eomesodermin (EOMES) and T-box transcription factor 21 (TBX21) (4–6). It has been shown that EOMES directly regulates expression of perforin (PRF1), granzyme B (GZMB), and IFNγ (IFNG), and promotes effector function in CD8 T cells (5). However, the mechanisms that differentially regulate the expression of EOMES and its targets in memory CD8 T cells are not fully understood.

Activation of gene transcription is controlled by the availability of transcription factors and the accessibility of chromatin structure at the gene locus. Recent studies suggest that an “accessible” or “repressed” chromatin structure is regulated in part by the modifications of histone amino-terminal tails such as acetylation, methylation, phosphorylation, and other posttranslational modifications (7–9). Histone acetylation occurs at a lysine residue of histone tails and is modified by two opposing enzymes: histone acetyltransferases (HATs)\textsuperscript{3} and histone deacetylases (HDACs) (10, 11). An enhanced level of histone acetylation is generally correlated with accessible chromatin structure, which is seen not only in active gene transcription but also in nontranscribed genes poised for response (12). Furthermore, a decrease in histone acetylation using an inhibitor of HAT activity, such as curcumin, results in a decrease in gene expression (13). Thus, histone acetylation could provide a means of rapid activation of specific gene expression in a cell in response to stimulation.

Accumulating evidence suggests that acetylation of histone H3 lysine 9 (H3K9Ac) and lysine 14 (H3K14Ac) are markers for accessible chromatin or chromatin that is transcriptionally active while methylation of H3 lysine 9 (H3K9me) is associated with gene silencing (14–18). In naive CD4 T cells, histones H3 and H4 in the Il4 and Ifng loci were unacylated but were rapidly acetylated after activation (19). In activated CD8 T cells, hyperacetylation of histone H3 at the Ifng promoter and enhancer was maintained through memory T cells (20). Our recent findings demonstrate that the differentially higher expressed genes have high levels of H3K9Ac in their gene loci in memory CD8 T cells and increased H3K9Ac levels after an HDAC inhibitor (Trichostatin A) treatment increases gene expression in naive CD8 T cells (21), providing evidence that histone acetylation may regulate gene expression and function of memory CD8 T cells. In addition, changes in DNA methylation in some of these gene loci have also been reported (20, 22, 23). There is an inverse correlation between the methylation of the 5’UTR and EOMES expression (24) and DNA hypomethylation in the PRF1 promoter increases PRF1 expression (25). However, it is unknown whether epigenetic changes such as histone modifications play

\textsuperscript{1} Laboratory of Immunology and \textsuperscript{2} Flow Cytometry Unit, National Institute on Aging, National Institutes of Health, Baltimore, MD 21224

Received for publication November 13, 2007. Accepted for publication April 14, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \textit{advertisement} in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{3} This work was supported by the Intramural Research Program of the National Institute on Aging and National Cancer Institute, National Institutes of Health.

\textsuperscript{2} Address correspondence and reprint requests to Dr. Nan-ping Weng, 5600 Nathan Shock Drive, Baltimore, MD 21224. E-mail address: wengn@mail.nih.gov

www.jimmunol.org

\textsuperscript{3} Abbreviations used in this paper: HAT, histone acetyltransferase; PRF1, perforin 1; GZMB, granzyme B; HDAC, histone deacetylase; ChIP, chromatin immunoprecipitation.
a role in the regulation of the effector function of memory CD8 T cells.

In this study, we examined the expression and H3K9Ac of EOMES, PRF1, and GZMB in human naive and memory CD8 T cells before and after stimulation. EOMES and PRF1 were expressed at higher levels in memory cells than in naive cells at rest and after activation while GZMB was expressed at higher levels in memory cells than in naive cells only after activation. The levels of H3K9Ac in the EOMES, PRF1, and GZMB loci were higher, particularly at the transcription start site, in memory cells than in naive cells. Induced hypoacetylation in these gene loci by a HAT inhibitor (curcumin) resulted in decreased expressions of EOMES, PRF1, and GZMB in memory cells in response to in vitro stimulation. These findings suggest that histone acetylation is essential in determining the level of EOMES, PRF1, and GZMB expressions in memory CD8 T cells.

Materials and Methods

Abs and reagents

The Abs and reagents used in this study are as follows: FITC-conjugated anti-CD45RA mAb was purchased from eBioscience. Tri-color-conjugated anti-CD8 mAb was purchased from Invitrogen. Anti-acetyl-Histone H3 (Lys9) and purified rabbit IgG were purchased from Upstate Biotechnol- ogy. Anti-GZMB mAb (2CSF5) was purchased from BD Biosciences. Anti-perforin mAb (P1–8) was purchased from Kamiya Biomedical. Anti-ZAP70 Ab was a gift from Dr. Ronald Wange (Food and Drug Adminis- tration, Rockville, MD). HRP-linked anti-mouse, anti-rabbit, or anti-rat Abs were purchased for GE Healthcare. Curcumin was purchased from Sigma-Aldrich.

Isolation and stimulation of naive and memory CD8 T cells

Peripheral blood was obtained from healthy adults by the National Institute on Aging Pheresis Unit (Institutional Review Board-approved protocol MR12003-054). The procedure for isolation and stimulation of naive and memory CD8 T cells was previously described (26). In brief, PBMC were isolated by Ficoll (GE Healthcare) gradient centrifugation. Naive and memory CD8 T cells were then enriched by removing other types of cells through incubation with a panel of mouse mAbs against human CD4, CD19, CD11b, CD14, CD16, MHC class II, erythrocytes, platelets, and CD45RO (for the enrichment of naive cells) or CD45RA (for memory cells). Ab-bound cells were subsequently removed by incubation with anti- mouse IgG-conjugated magnetic beads (Qiagen). These enriched naive and memory CD8 T cells were further purified into CD8 C D45RA naïve T cells and CD8 C D45RA memory T cells by a cell sorter (MoFlo; Dako). The purity of sorted naive and memory CD8 T cells was over 96%. The sorted naive and memory CD8 T cells were either used for experiments right away or incubated with anti-CD3 plus anti-CD28 Ab (anti-CD3/CD28) coupled magnetic beads (Invitrogen) at the cell: bead ratio of 1:1 in RPMI 1640 with 10% FBS and penicillin (10 U/ml)/streptomycin (10 µg/ml) (Invitrogen). The stimulated cells were harvested at the specified time for analyses of mRNA, protein, and chromatin immunoprecipitation (ChIP) assay.

Real-time quantitative RT-PCR

The procedure was standard as previously described (21). In brief, total RNA was extracted from fresh and stimulated naive and memory CD8 T cells by Stat 60 (Tel-Test). The quantity of total RNA was measured by NanoDrop (Agilent) and 500 ng of total RNA was used to synthesize cDNA by reverse transcriptase (SuperScript II; Invitrogen). PCR was conducted in 20 µl of total volume with 0.125 µM primers using a SYBR GREEN kit (Applied Biosystems) for 40 cycles on ABI Prism 7500 (Ap- plied Biosystems). The specific amplification of RT-PCR products was confirmed by agarose (2%) gel electrophoresis. The threshold cycle Ct of PCR was calculated using the ABI Prism 7500 software. The specific amplification of RT-PCR products was confirmed by agarose (2%) gel electrophoresis. The threshold cycle Ct of PCR was calculated using the ABI Prism 7500 software.

Cells were washed once in PBS, resuspended in lysis buffer (50 mM Tris- HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease inhibitors (Roche), and incubated on ice for 30 min. Lysates were spun for 15 min at 14,000 rpm at 4°C, mixed with NuPage LDS sample buffer (Invitrogen), and loaded onto a NuPAGE 4–12% gradient Bis-Tris gel for GZMB or a 7.5% SDS-PAGE gel (non- reducing condition) for PRF1 detection. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) using an XCell II Blot Module (NoveX) in Transfer buffer (25 mM bicine, 25 mM Bis-Tris, 1 mM EDTA, 8% methanol, and 0.375% SDS). The membranes were first incubated with a blocking buffer (PBS, 5% BSA, and 0.1% Tween 20) at room temperature for 1 h, and followed by the incubation with the primary Ab for 1 h at room temperature. Anti-GZMB mAb (2CSF5) was used at a 1/10,000 dilution and anti-perforin mAb (P1–8) at a 1/10,000 dilution. The membranes were washed three times in PBS, 0.1% Tween 20, incubated with an HRP-labeled anti-lg secondary Ab at a 1/10,000 dilution, washed three times, developed for 1 min in ECL Western Blotting Detection Reagents (GE Healthcare), and exposed to Hyperfilm (GE Healthcare). The film images were digitized by Alphalmage (Alpha Innotech) and quantified by AlphaEase FC software (Alpha Innotech).

GZMB (Sigma-Aldrich), a HAT inhibitor, was dissolved in ethanol at 10 mM. Memory CD8 T cells were cultured in the presence or absence of 20 µM curcumin for 2 h, and followed by stimulation with anti-CD3/28 Abs for the specified time. Cells were then harvested for the analyses of mRNA, protein, and ChIP assay.

ChIP assay

The procedure of ChIP was previously described (21). In brief, cross-link- ing of chromosomal DNA of naive or memory CD8 T cells were conducted by incubating with 1% formaldehyde at 37°C for 10 min, quenching by 125 mM glycine, and washing twice with cold PBS containing a mixture of protease inhibitors (Roche). The treated cells were then lysed in three se- quential lysis buffers 1–3 containing protease inhibitors (lysis buffer 1: 50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and 0.25% Triton X-100; lysis buffer 2: 10 mM Tris- HCl (pH 8), 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA; lysis buffer 3: 10 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, and 0.5% N-lauroylsarcosine). The chromo- somal DNA was then sheared by sonication (Model 100; Fisher Scientific), and the average length of sonicated chromosomal DNA was around 200 bp. A total of 1/10 volume of sonicated lysates was used for DNA isolation as INPUT while the remaining lysates (9/10 vol- umes) were used for ChIP with anti-H3K9Ac and control Abs. For the ChIP, cell lysates were first incubated with protein-A conjugated agarose (GE Healthcare) and 3 µg of anti-H3K9Ac (Upstate Biotechnology) or 3 µg control rabbit IgG overnight at 4°C, followed by washing with RIPA buffer (50 mM HEPES-KOH (pH 7.5), 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, and 0.7% sodium deoxycholate) and TE buffer containing 50 mM NaCl sequentially, and then immunoprecipitated histone complexes were eluted at 65°C for 15 min. DNA was reverse-crosslinked by incubating at 65°C for 16 h and RNA and proteins were digested by treatment of proteinase K and proteinase inhibitor cocktail. The immunoprecipitated DNA was purified by phenol/chloroform extraction and eth- anol precipitation, and was used for analysis by real-time PCR. The same PCR reagents, instrument, and verification of the amplification product were used here as described in above section. The formula for quantitation of ChIP results is INPUT % = chIP / INPUT × 100.

The primer sequences were used for EOMES: forward 5′- TGTAAGCTCCACGGACCAAGGCGAG-3′ and backward 5′- GAAAAGCCGGAGTTGTGC-3′; forward 5′- ACTATGGACCATG-3′ and backward 5′- GGTTTCTGCTTCTCCCTCC-3′; forward 5′- CTCTTTGCTTCTCCTCCCTCC-3′; forward 5′- CTCTTTGCTTCTCCTCCCTCC-3′; forward 5′- TGGGAGGGGGACGCACAAAG-3′ and backward 5′- CACCAAGGCACCACCTACATAC-3′; GZMB: forward 5′- GACGGAAGGAAACACACACAC-3′ and backward 5′- AATGTGGAGGAAAGGGCGAACCC-3′; GAPDH: forward 5′- GCAGCCAGCTGGTGCAG-3′ and backward 5′- GGGCCGCACTGGTGGTTC-3′. The specific primers for detection of GZMB, PRF1, and EOMES are as follows:

```
EOMES:
forward- 5′-AGCTCAAGAGTTGTCGTA-3′ and reverse- 5′-CGCAACAAATCTCC-3′;
PRF1:
forward- 5′-GAGTTGGAGCTGCCTCTACAGTT-3′ and reverse- 5′-GGTCCGATTGTGGATT-3′;
GZMB:
forward- 5′-GGTTGCTTCTTGATACAAAGCAGG-3′ and reverse- 5′-GGTGGCTTCTCGTCTTGTGAT-3′;
```
HISTONE ACETYLATION, GENE EXPRESSION IN CD8 CELLS

Mean by quantitative real-time PCR at each time point. The data are presented as the relative levels of EOMES in naive and memory CD8 T cells. Although the overall kinetic pattern of EOMES expression was similar between naive and memory cells, memory cells consistently expressed higher levels of EOMES mRNA from 2.1-3.0-fold than did naive cells throughout the course of 72-h stimulation (Fig. 1B). The higher expression of EOMES in resting and activated memory CD8 T cells provides a plausible mechanism for their rapid and robust effector response compared with naive cells.

The EOMES gene locus contains higher levels of H3K9Ac in memory CD8 T cells than in naive CD8 T cells

To determine the mechanism underlying the differential expression of EOMES in memory CD8 T cells, we analyzed the levels of H3K9Ac in the promoter, the coding region, and the 3′UTR of the EOMES gene locus in naive and memory CD8 T cells by ChIP and quantitative real-time PCR assays. To provide good spatial resolution, we designed nine pairs of primers covering the promoter (2000 bp of upstream region from the transcription start site), six pairs of primers in the coding region, and one pair of primers at the 3′UTR (data not shown). As high levels of H3K9Ac are found in the proximal promoter and the first exon in the EOMES locus in naive and memory CD8 T cells, the data are presented as the average ratio of the sum of these products at each time point to freshly isolated naive cells in mean ± SEM (n = 7).

**Results**

**Memory CD8 T cells express higher levels of EOMES than do naive CD8 T cells before and after in vitro stimulation**

As memory cells exhibit a faster and more robust effector response than do naive cells and EOMES regulates expression of several key cytolytic effector molecule genes (4), we compared EOMES expression in human naive and memory CD8 T cells from peripheral blood of normal donors before and after activation in vitro with anti-CD3/CD28 Abs. Resting memory CD8 T cells expressed significantly higher levels of EOMES than did resting naive cells (2.5-fold, p < 0.05) (Fig. 1A). After activation, EOMES mRNA level peaked around 4–8 h and then gradually decreased in both naive and memory cells. Although the overall kinetic pattern of EOMES expression was similar between naive and memory cells, memory cells consistently expressed higher levels of EOMES mRNA (2.1-3.0-fold) than did naive cells throughout the course of 72-h stimulation (Fig. 1B). The higher expression of EOMES in resting and activated memory CD8 T cells provides a plausible mechanism for their rapid and robust effector response compared with naive cells.

Reduced histone acetylation (H3K9Ac) levels decrease EOMES expression in memory CD8 T cells

To further determine the role of H3K9Ac levels in EOMES expression, we pretreated memory CD8 T cells with a HAT inhibitor, curcumin (20 μM), for 2 h before stimulation with anti-CD3/CD28 Abs. A significant decrease of the H3K9Ac levels in the EOMES locus was observed in curcumin-treated memory CD8 T cells after stimulation for 6 h compared with curcumin-untreated activated memory T cells (Fig. 3A). The H3K9Ac level of the EOMES locus in curcumin-treated memory CD8 T cells after stimulation was only slightly higher than in resting naive CD8 T cells (Fig. 3A). Consequently, a significant decrease of EOMES mRNA levels (63% reduction) but not a control gene GAPDH mRNA levels was observed in curcumin-treated activated memory cells compared with curcumin-untreated activated memory T cells (p < 0.001) (Fig. 3B). These results suggest that the level of H3K9Ac in the EOMES locus may regulate the expression of EOMES.

Memory CD8 T cells express high levels of perforin (PRF1) and contain high levels of H3K9Ac in the PRF1 locus

Next, we wanted to determine whether the target gene of EOMES, PRF1, is expressed and regulated in a similar manner to EOMES in memory CD8 T cells. The mRNA levels of PRF1 were significantly higher in resting memory CD8 T cells than in

**Statistical analysis**

Two-tailed Student’s t test was used in analysis of the levels of EOMES, PRF1, and GZMB mRNA expression and the levels of H3K9Ac in these gene loci between naive and memory CD8 T cells. The significance was defined by a value of p < 0.05.

**FIGURE 1.** EOMES is expressed at higher levels in memory CD8 T cells than in naive CD8 T cells. A, The levels of EOMES expression in naive and memory CD8 T cells before and after stimulation in vitro. Sorted naive and memory CD8 T cells were stimulated with anti-CD3/CD28 Abs for 0, 4, 8, 16, 32, and 72 h. The level of EOMES expression was determined by quantitative real-time PCR at each time point. The data are presented as mean ± SEM (n = 9). B, The relative expression levels of EOMES in naive and memory CD8 T cells. The data are presented as the ratio of each time point to freshly isolated naive cells in mean ± SEM (n = 9). Values of p are indicated by asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001) used in all figures.

**FIGURE 2.** High levels of H3K9Ac are found in the EOMES locus of memory CD8 T cells. The relative levels of H3K9Ac in the proximal promoter and the first exon in the EOMES locus in naive and memory CD8 T cells at rest and after stimulation were presented. Sorted naive and memory CD8 T cells were stimulated with anti-CD3/CD28 Abs and were used for ChIP assay. The primer pairs 9 and 10 were used for quantitative real-time PCR (same conditions used in the Fig. 1), and the data are presented as the average ratio of the sum of these products at each time point to freshly isolated naive cells in mean ± SEM (n = 7).
resting naive cells (6.1-fold, p < 0.05) (Fig. 4A). After activation, mRNA levels were up-regulated consistently higher in memory cells than naive cells until 72 h after stimulation. Corresponding to the mRNA levels, perforin protein levels were also higher in memory cells than in naive cells before and after activation (Fig. 4, B and C). We then examined H3K9Ac levels and found significant higher levels of H3K9Ac at the PRF1 locus in resting memory CD8 T cells than in resting naive cells (Fig. 4D). After in vitro stimulation (at 6, 24, and 72 h), increase of H3K9Ac levels was observed mainly in memory cells but their differences did not reach statistical significance (Fig. 4D). Thus, high levels of H3K9Ac in the PRF1 locus are also associated with high PRF1 expression in resting and activated memory CD8 T cells.

**Memory CD8 T cells express high levels of GZMB and contain high levels of H3K9Ac in the GZMB locus**

GZMB is another key target of EOMES in regulation of effector function. We found low levels of GZMB mRNA (Fig. 5A) and no detectable levels of granzyme B protein (Fig. 5, B and C) in resting naive or memory CD8 T cells. In vitro stimulation increased the mRNA levels of GZMB in both naive and memory cells. Activation-induced GZMB expression increased significantly faster and higher in memory cells than in naive cells for the first 24–32 h (Fig. 5, A–C). At 72 h after stimulation, naive cells expressed higher levels of GZMB than did memory cells. Unlike the differential expression of EOMES and PRF1 in resting memory cells, the differential expression of GZMB in memory cells occurred only after activation. We then examined H3K9Ac levels and found that the resting memory CD8 T cells had significant higher levels of H3K9Ac than did resting naive cells (Fig. 5D). Like the EOMES and PRF1 loci, H3K9Ac levels increased in the GZMB locus mainly in memory CD8 T cells after stimulation (Fig. 5D). However, the control of the GZMB locus is mediated by EOMES.
expression differs from that of the EOMES and PRF1 expression in resting memory cells.

Decreasing acetylation (H3K9Ac) in the PRF1 and GZMB loci in memory CD8 T cells decreases PRF1 and GZMB expression

To determine whether the H3K9Ac levels play an essential role in activation-induced rapid up-regulation of PRF1 and GZMB in memory CD8 T cells, we pretreated resting memory CD8 T cells with curcumin (20 μM) for 2 h followed by stimulation with anti-CD3/CD28 Abs. Like the EOMES gene, H3K9Ac levels in the PRF1 and GZMB loci significantly decreased after curcumin treatment in memory cells (Fig. 6A and 7A). The levels of H3K9Ac in the PRF1 and GZMB loci in curcumin-treated memory cells after stimulation were close to the H3K9Ac levels in resting naive cells. Strikingly, significant decreases of PRF1 and GZMB mRNA and protein were observed in curcumin-treated and curcumin-untreated memory CD8 T cells. Interestingly, the levels of mRNA of PRF1 and GZMB in curcumin-treated memory cells after activation were comparable with their levels in resting naive cells while the control gene GAPDH did not change significantly between curcumin-treated and curcumin-untreated memory cells. These findings support the notion that higher levels of histone acetylation (H3K9Ac) in memory CD8 T cells is

FIGURE 5. Rapid increase of GZMB expression after stimulation is associated with high levels of H3K9Ac in the GZMB locus in memory CD8 T cells. A, The mRNA levels of GZMB expression in naive and memory CD8 T cells before and after stimulation. The levels of GZMB expression are presented as mean ± SEM (n = 9). B, The protein levels of GZMB expression in naive and memory CD8 T cells before and after stimulation. C, The quantified data of B are presented as mean ± SEM (n = 4). D, The relative levels of H3K9Ac in the GZMB locus in naive and memory CD8 T cells at rest and after stimulation. The primer pairs 8 and 9 were used for quantitative real-time PCR (same conditions used in the Fig. 7) and the data are presented as the average ratio of the sum of these two products at each time point to freshly isolated naive cells (n = 5).

FIGURE 6. Inducing hypoacetylation of H3K9 in the PRF1 locus results in decreased PRF1 expression in memory CD8 T cells. A, The relative levels of H3K9Ac in the PRF1 locus in curcumin-treated and curcumin-untreated memory CD8 T cells after stimulation, and resting naive CD8 T cells. The data are presented as the average ratio to curcumin-untreated memory CD8 T cells (n = 4). B, The relative levels of PRF1 expression of curcumin-untreated and curcumin-treated memory CD8 T cells, and resting naive CD8 T cells. The levels of PRF1 and GAPDH expression are presented as the average ratio to curcumin-untreated memory CD8 T cells (n = 5). C, Perforin protein levels in curcumin-treated and curcumin-untreated memory CD8 T cells after stimulation.
we demonstrated that reduced levels of H3K9Ac in the EOMES, PRF1, and GZMB loci by a HAT inhibitor (curcumin) resulted in decreased expression of all three genes in activated memory CD8 T cells. Together, these results indicate that histone acetylation is tightly correlated with the rapid and robust increase in expression of effector function genes in memory CD8 T cells.

The analysis of expression of EOMES, PRF1, and GZMB in naive and memory CD8 T cells before and after activation showed that memory cells rapidly and greatly increased the levels of EOMES, PRF1 and GZMB regardless of their initial expression levels in the first 32 h after activation. Interestingly, EOMES expression peaked at 4–8 h and declined at 32 h after stimulation, slightly ahead of the peak of expression of PRF1 and GZMB. Although the kinetic pattern of PRF1 expression was similar to EOMES in memory CD8 T cells before and after stimulation with PRF1 levels slightly behind EOMES, GZMB expression exhibited a different pattern before and after stimulation. GZMB mRNA level was similar between naive and memory CD8 T cells at rest and remained stably high through 72-h stimulation in memory CD8 T cells. This difference in PRF1 and GZMB expression suggests that the regulation of PRF1 and GZMB expression is different, even though similarly high levels of H3K9Ac are present at these genes in memory CD8 T cells. It is intriguing that the expression of these two key effector molecules functioning in the same cytotoxic pathway is regulated differently. Further study will be needed to determine how PRF1 and GZMB expression are precisely regulated in naive and memory CD8 T cells and the relative contributions of EOMES and other transcription factors as well as other histone modifications associated with accessible chromatin structure.

Although histone acetylation at the cytokine gene loci facilitates their expression in effector T cells (19, 20), its role in effector function-related genes in memory T cells has not been reported. Our analysis showed that the acetylation levels of one specific residue on histone H3 (H3K9Ac) were higher in resting memory CD8 T cells than in resting naive CD8 T cells in the EOMES, PRF1, and GZMB loci, providing a chromatin basis for the high level of expression of EOMES, PRF1, and GZMB in memory CD8 T cells. Equally interesting is that the levels of H3K9Ac in these gene loci do not appear to increase dramatically after activation. Thus, activation-induced changes in gene expression may be mediated by acetylation of other histone residues and/or by different types of histone modification.

Based on these findings, we propose a transcription and chromatin model for the effector functions of memory CD8 T cells (Fig. 8). In naive CD8 T cells, the EOMES, PRF1, and GZMB loci are in a repressed chromatin state as shown by low levels of H3K9Ac and of their mRNA levels. By contrast, in memory CD8 T cells, these three gene loci are in an active chromatin state as shown by high levels of H3K9Ac with either high levels of EOMES and PRF1 mRNA ("primed") or no increase of GZMB mRNA ("poised"). Upon activation, both naive and memory CD8 T cells increase expression of EOMES, PRF1 and GZMB. However, the accessible chromatin structure of these gene loci in memory CD8 T cells leads to a much more rapid and vigorous transcriptional response than in naive cells. Thus, the memory CD8 T cell response at the transcription level is controlled at two stages: 1) differential expression of the key effector regulators such as EOMES (through regulation of the chromatin structure of the gene locus and of transcription activators); and 2) differential expression of the effector targets (PRF1 and GZMB) through two modes. PRF1 has accessible chromatin at its locus and differential transcription ("primed" status), whereas GZMB has accessible chromatin at its locus without increased expression ("poised" status).
light on the molecular nature of memory T cells but also provide new targets and strategies for improving vaccines.

Acknowledgments
We thank Drs. Dan Longo and Mike Pazin for critical reading of the manuscript. We thank Francis J. Chrest and Cuong Nguyen of the Flow Cytometry Unit for sorting cells, and Karen Madara and her staff at the National Institute on Aging Apheresis Unit for collecting blood samples.

Disclosures
The authors have no financial conflict of interest.

References
1. Dutton, R. W., L. M. Bradly, and S. L. Swain. 1998. T cell memory. Annu. Rev. Immunol. 16: 201–223.
2. Kaech, S. M., E. J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. Nat. Rev. Immunol. 2: 251–262.
3. Williams, M. A., and M. J. Bevan. 2007. Effector and memory CTL differentiation. Annu. Rev. Immunol. 25: 171–192.
4. Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zedek, M. Banica, C. B. Y. Delicato, D. A. Gross, C. A. Miao, et al. 2003. Control of effector CD8+ T cell function by the transcription factor Eomesodermin. Science 302: 1041–1043.
5. Inflekofer, A. M., N. Takezomo, E. J. Wherry, S. A. Longworth, J. T. Northrup, V. F. Fialanuel, A. C. Mullen, C. R. Gassink, S. M. Kaech, J. D. Miller, et al. 2005. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat. Immunol. 6: 1236–1244.
6. Sullivan, B. M., A. Juedes, S. J. Szabo, m. Herrath, and L. H. Glimcher. 2003. Antigen-driven effector CD8 T cell function regulated by T-bet. Proc. Natl. Acad. Sci. USA 100: 15818–15823.
7. Bernstein, B. E., A. Meissner, and E. S. Lander. 2007. The mammalian epigenome. Cell 128: 669–681.
8. Turner, B. M. 2002. Cellular memory and the histone code. Cell 111: 285–291.
9. Kouzarides, T. 2007. Chromatin modifications and their function 3. Cell 128: 693–705.
10. Roth, S. Y., J. M. Denu, and C. D. Allis. 2001. Histone acetyltransferases 1. Annu. Rev. Biochem. 70: 81–120.
11. Nusinson, L., and C. M. Horvath. 2005. Histone deacetylases as transcriptional activators: role reversal in inducible gene regulation 4. Sci. STKE 2005: re11.
12. Clayton, A. L., C. A. Hazzalin, and L. C. Mahadevan. 2006. Enhanced histone acetylation and transcription: a dynamic perspective 1. Mol. Cell. 23: 289–296.
13. Balasubramanyam, K., J. A. Varier, M. Alfai, V. Swaminathan, N. B. Siddappa, U. Ranga, and T. K. Kundu. 2004. Curcumin, a novel p300/CREB-binding protein-specific inhibitor of acetyltransferase, represses the acetylation of histone nonhistone proteins and histone acetyltransferase-dependent chromatin transcription 1. J. Biol. Chem. 279: 51163–51171.
14. Zhao, K., W. Wang, O. J. Rando, Y. Xue, K. Swiderek, A. Kuo, and G. R. Crabtree. 1998. Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. Cell 95: 625–636.
15. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 9: 765–775.
16. Roh, T. Y., S. Cudkaph, and D. Zhao. 2005. Active chromatin domains are defined by acetylation islands revealed by genome-wide mapping. Genes Dev. 19: 542–552.
17. Bernstein, B. L., M. Kamal, K. Linfield-Thomas, D. S. Reinert, D. D. J. Huebert, S. McMahon, E. K. Karlsson, E. J. Kulbokas III, T. R. Gingeras, et al. 2005. Genomic maps and comparative analysis of histone modifications in human and mouse. Cell 120: 169–181.
18. Korn, Y., L. Shen, P. C. Li, T. H. Huang, and J. P. Issa. 2004. Chromatin immunoprecipitation microarrays for identification of genes silenced by histone H3 lysine 9 methylation. Proc. Natl. Acad. Sci. USA 101: 7398–7403.
19. Fields, P. E., S. T. Kim, and R. A. Flavell. 2002. Changes in histone acetylation at the IL-4 and IFN-g loci accompany Th1/Th2 differentiation. J. Immunol. 169: 647–650.
20. Northrop, K. J., R. M. Thomas, A. D. Wells, and H. Shen. 2006. Epigenetic remodeling of the IL-2 and IFN-g loci in memory CD8 T cells is influenced by CD4 T cells. J. Immunol. 177: 1062–1069.
21. Paik, M., J. M. Godlove, M. Catalano, W. H. Wood Iii, F. J. Chrest, N. Chun, L. Granger, R. Wersto, K. Madara, K. Becker, et al. 2006. Histone acetylation is associated with differential gene expression in the rapid and robust memory CD8+ T cell response. Blood 108: 3363–3370.
22. Ansell, K. M., D. U. Lee, and A. Rao. 2003. An epigenetic view of helper T cell differentiation. Nat. Immunol. 4: 616–623.
23. Lee, G. R., S. T. Kim, C. G. Spiliakinas, P. E. Fields, and R. A. Flavell. 2006. T helper cell differentiation: regulation by cis elements and epigenetics 2. Immunity 25: 359–379.
24. Ivancu, C., R. Wasserkort, R. Lesche, J. Dong, H. Stein, A. Thiel, and F. Eckhardt. 2007. DNA methylation profiling of transcription factor genes in normal lymphocyte development and lymphomas 1. Annu. Rev. Immunol. 25: 253–1538.
25. Lu, Q., A. Wu, D. Ray, C. Deng, J. Attwood, S. Hanash, M. Pippkin, M. Lichtenheld, and B. Richardson. 2003. DNA methylation and chromatin structure regulate T cell perforin gene expression 17. J. Immunol. 170: 5124–5132.
26. Chiu, W. K., M. Fann, and N. P. Weng. 2006. Generation and growth of CD8+ memory T cells mediated by IL-15 and its induced cytokines. J. Immunol. 177: 7802–7810.