Cutting Edge: Prolonged Exposure to HIV Reinforces a Poised Epigenetic Program for PD-1 Expression in Virus-Specific CD8 T Cells


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Ag-specific CD8 T cells play a critical role in controlling HIV infection but eventually lose antiviral functions in part because of expression and signaling through the inhibitory programmed death-1 (PD-1) receptor. To better understand the impact of prolonged TCR ligation on regulation of PD-1 expression in HIV-specific CD8 T cells, we investigated the capacity of virus-specific CD8 T cells to modify the PD-1 epigenetic program after reduction in viral load. We observed that the transcriptional regulatory region was unmethylated in the PD-1 promoter in HIV-specific CD8 T cells, whereas it remained methylated in donor-matched naive cells at acute and chronic stages of infection. Surprisingly, the PD-1 promoter remained unmethylated in HIV-specific CD8 T cells from subjects with a viral load controlled by antiviral therapy for >2 y or from elite controllers. Together, these data demonstrate that the epigenetic program at the PD-1 locus becomes fixed after prolonged exposure to HIV virus.

Persistent Ag presentation during chronic viral infections results in a progressive deterioration in T cell function known as T cell exhaustion (1–4). Expression of the inhibitory programmed death-1 (PD-1) receptor on Ag-specific T cells has been associated with T cell dysfunction and lack of viral control in animal models, in HIV infection and other chronic viral diseases. Importantly, expression of PD-1 on virus-specific and total memory T cells correlates with disease progression in HIV-infected subjects (5). Specifically, signaling through PD-1 results in reduced cytokine production and impaired proliferation of HIV-specific CD8 T cells that can be reversed upon in vitro and in vivo exposure of HIV-specific CD8 T cells to Abs that block the PD-1–PD-1 ligand interaction (6–8). The therapeutic potential of PD-1 blockade has been demonstrated by administration of blocking Abs to SIV-infected macaques, which results in significant improvement in CTL function and reduction in SIV viral load (9). Recently, therapeutic PD-1 blockade in humans has proved to be an effective strategy for controlling several types of cancer (10, 11).

In light of the tremendous potential that PD-1 blockade strategies have to alleviate human chronic infections and cancer, current efforts are focused on understanding the mechanisms(s) that initiate and sustain PD-1 expression during chronic infection. It is now known that persistent TCR ligation during chronic viral infection maintains increased levels of PD-1 transcription in nonfunctional Ag-specific CD8 T cells, whereas PD-1 transcription is rapidly downregulated in functional Ag-specific CD8 T cells that develop during an acute infection (4, 12) or when T cells are not exposed to their cognate peptide, such as when viral escape mutations have occurred (13). We have centered our investigation on epigenetic regulation of PD-1 expression as epigenetic modification of transcriptional regulatory regions, including DNA methylation, constitutes an important mechanism for the regulation of tissue- and genespecific transcription (14–20). In this article, we provide data on DNA methylation of the PD-1 regulatory regions in HIV-
specific CD8 T cells at different stages of infection and different levels of viral control. To our knowledge, this study represents the first report on epigenetic program stability of HIV-specific CD8 T cells during the course of HIV infection in HIV progressors and elite controllers. Our results highlight the negative impact of prolonged exposure to HIV Ag on the ability of virus-specific CD8 T cells to progressively adapt their transcriptional programs to a changing environment.

Materials and Methods
Study subjects and isolation of HIV-specific T cells

Written, informed consent, approved by the University of Montreal Health Center Ethics Review Board (CRUCHM) and Partners Human Research Committee of the Massachusetts General Hospital, was provided and signed by study participants before enrollment in the study. Research conformed to ethical guidelines established by the ethics committee of the Massachusetts General Hospital, University of Montreal Health Center, and Vaccine and Gene Therapy Institute Florida Institutional Review Board. Total CD8+ T cells were purified from PBMCs by negative selection using magnetic beads separation (Stem Cell). Cells were then stained with Live/Dead aqua dye (Invitrogen) according to the manufacturer’s instructions and then labeled with PE or allophycocyanin tetramers at 37° for 15 min as described previously (6, 7). Subsequently, cells were incubated at 4°C for 20 min with cell-surface Abs: CD3 (BD), PD-1 (BioLegend), CD8 (BD), CD45RA (BD), CD27 (Invitrogen), CCR7 (BD). PBMCs were sorted for naive (CD8+CD45RA+CCR7+CD27+), central memory (CD8+CD45RA+CCR7+CD27−), effector memory (CD8+CD45RA−CCR7−CD27+), and HIV-specific cells (CD8+CD3+ tetramer+) using the BD ARIAII flow cytometer (BD Biosciences).

Genomic methylation analysis

 Sodium bisulfite–induced deamination of cytosine was performed using the Zymo Research EZ DNA methylation kit. The bisulfite-modified DNA was PCR amplified with locus-specific primers as previously described (18). Statistical significance of Cpg site methylation was determined with a two-tailed unpaired Student t test using Prism 4 software.

Results
DNA demethylation of the PD-1 locus during acute and chronic HIV infection

We sought to determine whether the PD-1 transcriptional regulatory region in HIV-specific CD8 T cells acquired an “active” epigenetic program at the acute and chronic stages of HIV infection in viremic subjects. Donor-matched naive and HIV-specific CD8 T cells were isolated at the acute (0–4 mo postinfection) and chronic stages of infection (>4 mo postinfection; Supplemental Table I). Bisulfite sequencing methylation analysis of the PD-1 conserved regulatory region was performed on purified (>95% purity) naive and HIV-specific CD8 T cell genomic DNA from donors at the acute (n = 2) and chronic (n = 3) stages of infection. Each line represents an individual clone picked for sequencing. Filled circles represent methylated cytosine; open circles represent nonmethylated cytosine. Cpg sites 18–26 are shown for clarity.

PD-1 locus demethylation persists after highly active antiretroviral therapy–mediated reduction in HIV viral load

Given the striking reduction in DNA methylation at the PD-1 locus in HIV-specific CD8 T cells relative to donor-matched naive cells, we next sought to determine whether reduction in Ag would result in remethylation of the locus because it is also known that PD-1 levels decrease upon antiviral therapy concomitantly with a decreased viral load to undetectable levels (21). Virus-specific CD8 T cells were isolated from donors before and after highly active antiretroviral therapy (HAART) after viral loads of <50 copies/ml were maintained for >1 y (Supplemental Table I). Akin to the virus-specific CD8 T cells analyzed at the acute and chronic stages from untreated donors, virus-specific CD8 T cells were unmethylated at the PD-1 locus before HAART (Fig. 2A, 2B). Methylation analysis of naive and HIV-specific CD8 T cells were performed in a longitudinal manner with cells isolated from the same donor before and after (1–4 y) HAART (Fig. 2C). Surprisingly, these cells remained unmethylated at the PD-1 regulatory region after HAART, despite undetectable levels of viral load for up to 3 y (Fig. 2A, 2B) and despite the fact that PD-1 cell-surface expression was reduced on virus-specific CD8 T cells in donors 1, 4, and 5 after HAART (Fig. 2D).

Elite controller HIV-specific CD8 T cells retain a demethylated PD-1 locus

The lack of PD-1 regulatory region DNA methylation in CD8 T cells from HAART-treated donors suggests that prolonged TCR ligation preserves the epigenetic program acquired at the acute stage of the infection even after reduction of viral load by antiviral therapy. To further examine the impact of transient high viremia on the plasticity of the PD-1 epigenetic program,
we measured the level of DNA methylation at the PD-1 regulatory region in HIV-specific CD8 T cells from elite controllers. Elite controllers can initially have increased levels of viremia, but eventually control viral infection to <50 copies of HIV RNA per milliliter (22). PD-1 expression varied among different epitope specificities within the total population of HIV-specific CD8 T cells (Fig. 3A, Supplemental Fig. 1d). DNA methylation analyses were performed on virus-specific CD8 T cells isolated from donors with undetectable viral loads for >7 y (Supplemental Table I). Again, we observed that HIV-specific CD8 T cells retained a level of methylation that is significantly higher than naive CD8 T cells (Supplemental Fig. 1f). Importantly, a population of cells within the central memory compartment of the CD8 T cells retained a level of methylation that is significantly higher than the HIV-specific CD8 T cells and also had the lowest level of PD-1 expression relative to the other polyclonal memory subset (Supplemental Fig. 1e).

**Discussion**

Persistent exposure to Ag drives T cells toward a state of functional impairment and terminal differentiation (23, 24). Our previous report on epigenetic regulation of PD-1 expression in Ag-specific CD8 T cells differentiating in response to acute and chronic viral infections in mice and humans revealed that the transient upregulation of PD-1 expression in (Fig. 3C), specifically IWB9-specific CD8 T cells isolated from donor 10 for the DNA methylation analysis were only ~4% positive for PD-1 expression (Fig. 3b). Median fluorescence intensity (MFI) analysis of PD-1 expression on the HIV-specific CD8 T cells from HAART-treated donors and elite controllers also demonstrated that the degree of PD-1 downregulation was variable among different populations of Ag-specific CD8 T cells. The heterogeneity in PD-1 expression among different virus-specific CD8 T cell populations in low viremic conditions may suggest that the Ag-specific CD8 T cells have lost the ability to fully suppress PD-1 transcription.

To further explore the relationship of PD-1 downregulation and Ag persistence, we next assessed the level of PD-1 expression on virus-specific CD8 T cells from donors who recognized a conserved HIV epitope in the persisting virus versus PD-1 expression on virus-specific CD8 T cells from donors who have a mutated HIV epitope. As expected, the level of PD-1 expression (median MFI from four responses) was lower on the Ag-specific CD8 T cells in the presence of the mutated epitope (Fig. 3c). Interestingly, PD-1 expression (MFI) on the mutated epitope-specific CD8 T cells was still significantly higher than the donor-matched naive cells. These data suggest that the mechanism for downregulation of PD-1 expression is altered after TCR ligation.

To assess the association between PD-1 expression and DNA methylation at the PD-1 regulatory region before and after exposure to HIV Ag, we compared the average percentage of DNA methylation from the cumulated sites in the PD-1 transcriptional regulatory region relative to the percentage of PD-1 expression from all cells used for the DNA methylation study. All naive CD8 T cells isolated from HIV-infected donors retained a clear inverse correlation between DNA methylation and PD-1 expression. In contrast, the level of PD-1 expression was uncoupled from remethylation of the PD-1 regulatory region (Fig. 3d) in HIV-specific T cells from elite controllers. Taken together, our data reveal that significant reduction in HIV viral load is not sufficient to further modify the DNA methylation program at the PD-1 locus in Ag-specific CD8 T cells. To further determine the impact of Ag-induced T cell differentiation on PD-1 transcriptional programming, we proceeded to measure the methylation status at the PD-1 promoter in the polyclonal memory populations in HIV-infected individuals. Indeed, the level of DNA methylation at all CpG positions in each of the memory subsets was significantly lower than naive CD8 T cells (Supplemental Fig. 1f). Importantly, a population of cells within the central memory compartment of the CD8 T cells retained a level of methylation that is significantly higher than the HIV-specific CD8 T cells and also had the lowest level of PD-1 expression relative to the other polyclonal memory subset (Supplemental Fig. 1e).
both mouse and human functional virus-specific CD8 T cells was coupled to acquisition of an unmethylated transcriptional regulatory region during the peak of viremia (18). After clearance of the acute viral infection, the PD-1 transcriptional regulatory region regained the DNA methylation program in functional memory CD8 T cells from both the murine LCMV model system of acute viral infection and yellow fever vaccination of humans. Importantly, the repressive transcriptional program was not reacquired in human EBV and CMV-specific CD8 T cells (18). In this study, analysis of the PD-1 epigenetic program was performed on virus-specific CD8 T cells from HIV-infected individuals that are well defined in the date of initial exposure, peak viremia, and reduction in viral load. The results from our current study suggest that the pliable quality of epigenetic mechanisms in naive and functional memory CD8 T cells is lost during chronic HIV infection, even in HIV-specific CD8 T cells from elite controllers. Although the exact mechanism for fixation of epigenetic programs is not well understood, it is clear that it involves altered expression of de novo DNA methyltransferase variants, as well as lineage-defining transcription factors (25).

It is important to note that although reduction in PD-1 expression is likely due to lack of TCR engagement, virus-specific cells could recirculate from other anatomical sites where HIV replication is still persistent at levels higher than those detected in plasma. Thus, it is possible that limited or intermittent TCR ligation resulting from low-level persistent viremia may reinforce an unmethylated state through periodic activation of the virus-specific cells. Interestingly, our data and those of others demonstrated that PD-1 expression is quite heterogeneous among different tetramer+ CD8 T cells after the reduction in viral load. Recently, it has been reported that viral escape mutations can result in reduced PD-1 expression on CD8 T cells specific to the WT epitope (13). Thus, mutation in viral epitopes may contribute to the wide range of PD-1 expression observed. Indeed, in this article, we observed that there was a trend for lower PD-1 expression on Ag-specific CD8 T cells targeting a mutated epitope compared with responses targeting conserved viral sequences. However, because the absence of methylation at the PD-1 locus leaves it poised for transcriptional activation, non-TCR stimulatory signals may be sufficient to induce PD-1 expression, including γ-C receptor cytokines and type I IFNs (26–30). Such alternative mechanisms are strongly suggested by the broad range in PD-1 expression seen on HIV-specific CD8 T cells targeting conserved epitopes in individuals with similar viral loads. It will be of great interest to determine whether the inflammatory environment that is characteristic of HIV infection can induce PD-1 expression on the poised PD-1lo HIV-specific CD8 T cells.

This study on epigenetic regulation of PD-1 reveals that even though HIV-specific CD8 T cells have reduced or no PD-1 expression when isolated from donors who have undergone successful HAART treatment or from elite controllers, the cells retain a transcriptional program that is poised for PD-1 expression. This first analysis of epigenetic programs in HIV-specific CD8 T cells during infection represents a basic model for acquisition of heritable transcriptional regulation of PD-1 in the context of chronic HIV infection that invokes preservation of the demethylated regulatory regions during persistent stimulation throughout naïve to effector to memory differentiation (Supplemental Fig. 1G). In the proposed model, acquired transcriptional programs are maintained during cell
division via duplication of the epigenetic modifications from the parental DNA onto the newly synthesized strand of DNA. Therefore Ag-experienced PD-1hi HIV-specific CD8 T cells are poised to rapidly upregulate PD-1 expression and are then susceptible to PD-1–mediated termination of an effective recall response. Further, these results emphasize the need for PD-1 signaling blockade in conjunction with therapeutic strategies that attempt to restore the immune response to purge the latent HIV reservoir, and indicate that an alternative approach to PD-1 Ab blockade may involve directed reprogramming of epigenetic modifications.

Disclosures
R.A. holds a patent for the PD-1 inhibitory pathway. The other authors have no financial conflicts of interest.

References
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**Supplemental Table 1:** The study population included 10 HIV-infected individuals at various stages of infection. HIV-infected patients were categorized into 5 subgroups: subjects with acute HIV infection (n=2) analyzed within 3 months of infection; chronically infected subjects (n=3) infected for more than 6 months, untreated with HIV viral loads of >5,000 HIV RNA copies/ml; subjects HIV infected, before any HAART treatment with viral loads of >5,000 HIV RNA copies/ml; post HAART treated (n=3) participants with viral loads of <50 HIV RNA copies/ml on fully suppressive HAART; and elite controllers (n=4) infected for more than 7 years with undetectable viremia and naïve to antiretroviral therapy at the time of testing.
Supplemental Figure 1. Compiled methylation analyses and PD-1 expression from HIV infected untreated donors, HAART treated donors, and elite controllers. A) Graphical summary of CpG methylation from 2 acute donors and 3 chronic donors. B) Graphical summary of CpG methylation from 3 donors pre and post-HAART. C) Graphical summary of CpG methylation from 5 independently sorted tetramer+ CD8 T cell populations. D) Graph of PD-1 mean fluorescence intensity on HIV-specific and donor matched CD8 T cells from donors that had high viremia at the chronic stage of infection, HAART treated, or elite controllers. Different HIV-specific CD8 T cells from a common donor are indicated by the same color. E) Graph of PD-1 mean fluorescence intensity on polyclonal memory CD8 T cell subsets from chronically infected donors. F) Graphical summary of DNA methylation of Tcm, and Tem memory CD8 T cells subsets (n=4) relative to naïve and HIV-specific DNA methylation form chronically infected individuals. G) Model for persistence of PD-1 locus demethylation; Naïve cells have an epigenetically silenced PD-1 locus that becomes accessible following TCR ligation during the acute stage of HIV infection. PD-1 expression (blue line) remains elevated during chronic infection and persistence of a high viral load. PD-1 expression is reduced when the infection is controlled during HAART therapy or in elite controllers. The unmethylated status acquired in the effector cells at the acute stage of the infection is retained despite reduction in viral load and T-cell activation (methylation = black lollipops, chromatin = green cylinders).