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Inhibitory TCR Coreceptor PD-1 Is a Sensitive Indicator of Low-Level Replication of SIV and HIV-1

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Ongoing antigenic stimulation appears to be an important prerequisite for the persistent expression of programmed death 1 (PD-1), an inhibitory TCR coreceptor of the CD28 family. Although recent publications have emphasized the utility of PD-1 as a marker for dysfunctional T cells in chronic viral infections, its dependence on antigenic stimulation potentially renders it a sensitive indicator of low-level viral replication. To explore the antigenic threshold for the maintenance of PD-1 expression on virus-specific T cells, we compared PD-1 expression on virus-specific and memory T cell populations in controlled and uncontrolled SIV and HIV-1 infection. In both controlled live attenuated SIV infection in rhesus macaques and HIV-1 infection in elite controllers, elevated levels of PD-1 expression were observed on SIV- and HIV-1-specific CD8+ T cells. However, in contrast to chronic wild-type SIV infection and uncontrolled HIV-1 infection, controlled SIV/HIV-1 infection did not result in increased expression of PD-1 on total memory T cells. PD-1 expression on SIV-specific CD8+ T cells rapidly decreased after the emergence of CTL escape in cognate epitopes, but was maintained in the setting of low or undetectable levels of plasma viremia in live attenuated SIV-infected macaques. After inoculation of naive macaques with a single-cycle SIV, PD-1 expression on SIV-specific CD8+ T cells initially increased, but was rapidly downregulated. These results demonstrate that PD-1 can serve as a sensitive indicator of persistent, low-level virus replication and that generalized PD-1 expression on T lymphocytes is a distinguishing characteristic of uncontrolled lentiviral infections. The Journal of Immunology, 2010, 184: 476–487.

Programmed death 1 (PD-1) is an inhibitory member of the CD28 family of TCR coreceptors (1) and is inducibly expressed on T cells, B cells, myeloid dendritic cells, and activated monocytes (2). When engaged simultaneously with the T cell receptor by one of its two ligands, PD-L1 (3, 4) or PD-L2 (5, 6), PD-1 downmodulates TCR signaling (2) and has an inhibitory effect on T cell effector functions, including proliferation and the net production of cytokines (7, 8). Inhibition of immune responses by PD-1 and its ligands is involved in the establishment of central (9) and peripheral (10, 11) tolerance and in the maintenance of immunoprivileged sites (12). However, the persistent expression of PD-1 on T cells has also been associated with the dysfunction of CD8+ and CD4+ T cells and failure to control viral replication in various chronic infections (13–16), including HIV-1 infection in humans (17–20) and SIV infection in rhesus macaques (21–23).

CD8+ T cells play a key role in the control of retroviral infections. In HIV-1–infected humans, the emergence of an HIV-1–specific CTL response during acute infection is temporally associated with the decline of viremia to set point levels and followed by a subsequent selection of viral CTL escape variants (24, 25). Primary SIV infection of rhesus macaques results in a rapid expansion of SIV-specific CD8+ T lymphocytes, which coincides with containment of early viremia (26). More definitively, depletion of CD8+ lymphocytes with a monoclonal Ab results in impaired control of viremia in primary SIV infection and in chronically infected macaques (27, 28). CD8+ T cells also appear to play a role in the control of live attenuated SIV (LASIV) strains, such as SIVmac239Δnef (29), and in the protection elicited by LASIV against pathogenic challenge with wild-type SIV (30).

Immunization of rhesus macaques with LASIV has proven to be one of the most effective strategies to induce protection against challenge with pathogenic SIV (31–34). The prototypical LASIV strains SIVmac239Δnef and SIVmac239Δ3 have a deletion in the open reading frame for nef (SIVmac239Δnef (35)), either alone or in combination with deletions in additional genes (SIVmac239Δ3 (36)). These viruses are well-controlled in the majority of vaccinated rhesus macaques, resulting in either low-level or undetectable levels of viral replication that persist for years postinfection (p.i.), and induce robust protection from i.v. challenge with homologous wild-type SIVmac239 (31). Although safety concerns have precluded the development of a live attenuated HIV-1 vaccine (37, 38), LASIV has remained one of the leading experimental models to investigate correlates of protection against primate lentiviruses. The mechanism of protection by LASIV appears to be complex, with multiple arms of the immune system contributing to the overall protective effect.

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Abbreviations used in this paper: LASIV, live attenuated SIV; PD-1, programmed death 1; p.i., post infection; scSIV, single-cycle SIV; TcM, central memory T cell; Tem, effector memory T cell.

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Infection of rhesus macaques with LASIV strains elicits virus-specific CD8+ and CD4+ T cell responses (40), as well as SIV-specific Ab responses (33, 34, 41). It appears likely that ongoing low-level antigenic stimulation plays a key role in the distinctive efficacy of protective immunity induced by LASIV, although definitive evidence on this point is lacking. In addition to offering insights into mechanisms of protective immunity, the LASIV model also poses a unique opportunity to study SIV-specific CD8+ T cell responses in the setting of well-controlled lentiviral infection.

PD-1 is expressed in response to triggering of the TCR (42), and the presence of persistent virus-specific stimulation appears to be, among other factors (43), an important prerequisite for maintenance of its expression (22, 23, 44). Although recent publications have emphasized the utility of PD-1 as a marker to identify exhausted CD8+ T lymphocytes, but in contrast to chronic wild-type rhesus macaques and HIV-1 infection in elite controllers, we observed elevated levels of PD-1 expression on SIV– and HIV-1–specific CD8+ T cells during acute infection with nonpersisting viruses (14, 48, 49), and in cases of resolved hepatitis C virus infection (48). These observations suggest that PD-1 is not a specific marker for exhausted T cells and that further study is necessary to more precisely define the relation between antigenic stimulation and PD-1 expression.

In this study, we investigated the antigenic threshold for the maintenance of PD-1 expression on virus-specific CD8+ T cells by comparing controlled and uncontrolled lentiviral infections of rhesus macaques and humans. In controlled LASIV infection in rhesus macaques and HIV-1 infection in elite controllers, we observed elevated levels of PD-1 expression on SIV– and HIV-1–specific CD8+ T lymphocytes, but in contrast to chronic wild-type SIV infection and untreated HIV-1 infection, not on total CD4+ and CD8+ memory T cells. PD-1 expression on SIV-specific CD8+ T cells rapidly decreased after the emergence of CTL escape mutations in the cognate epitope but was maintained in LASIV-infected rhesus macaques, even in the setting of undetectable levels of plasma viremia. PD-1 was initially expressed on SIV-specific CD8+ T cells induced by inoculation with single-cycle SIV (scSIV), a strain of SIV that is limited to one cycle of infection, but was downregulated within weeks thereafter. Elite controllers of HIV-1 infection maintained elevated PD-1 expression levels on HIV-1–specific CD8+ T cells, despite control of viremia to levels of <50 copies/ml. These findings show that high levels of PD-1 are not only expressed on dysfunctional T cells during uncontrolled viral infections but may also be seen under conditions of low-level antigenic stimulation.

**Materials and Methods**

**Rhesus study cohort**

A total of 82 rhesus macaques of Indian origin (Macaca mulatta) were selected for this study, including animals infected with LASIV strains (n = 6), 8 SIV strains (n = 14) with SIVmac239, or scSIV trans-complemented with the vesicular stomatitis virus glycoprotein (n = 8), and uninfected, healthy rhesus macaques as a control group (n = 16). A subset (n = 39) of animals infected with pathogenic SIV strains had been vaccinated with either scSIV (n = 6 (50)), a multigenic SIV DNA prime/modified vaccinia Ankara boost vaccine regimen (n = 4), or either of the following LASIV strains (n = 29): SIVmac239ΔV1V2 (n = 12 (51, 52)), SIVmac239Δnef (n = 11), or SIVmac239A3 (n = 6). Ten of the LASIV-vaccinated, wild-type challenged animals remained healthy without evidence for wild-type SIV infection or progression to simian AIDS.

All of the rhesus macaques used in this study were housed at the New England Regional Primate Research Center and maintained in accordance with institutional and federal guidelines mandated by the Animal Care and Use Committee of Harvard Medical School. Animal experiments were approved by the Harvard Medical Area Standing Committee on Animals and conducted according to the principles described in the Guide for the Care and Use of Laboratory Animals (53).
For human samples, the following reagents were used: Blue LIVE/DEAD Fixable Dead Cell Staining reagent (Molecular Probes/Invitrogen, Eugene, OR), CD38-FITC (HT2), HLA-DR PerCP (L243), CD23-PECy7 (CD28.2), CD8-Alexa Fluor 700 (RPA-T8), CD14-allophycocyanin H7 (MoP9), CD19- allophycocyanin H7 (SJ25C1), and CD3-Pacific Blue (UCHT1) (all Abs from BD Biosciences). The PD-1 Ab (clone EH12.2H7) was custom conjugated to Alexa Fluor 647. All Abs were titrated for determination of optimal concentrations.

Determinations of plasma viral loads

Plasma for the determination of viral loads was obtained from venous blood drawn into EDTA tubes. Viral RNA loads for animals infected with a single SIV strain were determined using a standard quantitative real-time RT-PCR assay based on amplification of conserved sequences in gag and gagpol products (61). The nominal threshold for this assay was between 10 and 30 viral RNA copy equivalents/ml plasma, dependent on the input volume of plasma. For animals initially infected with SIVmac239 and subsequently challenged with either SIVmac239 or SIVmac251, highly specific, real-time RT-PCR assays were developed to discriminate and accurately determine the viral loads of the respective virus species, following the methods referenced above. An assay specific for SIVmac239Δ nef was developed by designing a reverse primer that uniquely recognizes the sequence generated by the deletion of nef coding sequences in SIVmac239 (35). This primer, dnefR02.10L3 (CCCTTTTTTTAATAGTGAACATGAGACACGCCC), was combined with a forward primer, dnefF02 (GAGAAGAGAGGTCGATGCGAATGCTACG), and a fluoro-orescently labeled fluorescence resonance energy transfer probe, dnefP02 (5′-CAL FluorOrange560)CAATCCCCAGGAGGATTGACAGAGGCGTTG-3′ (BHQ1) (Biosan Technologies, Novato, CA). The assay was optimized with particular attention to the discrimination priming potential of the reverse primer sequence to yield an assay able to reliably detect and discriminate five copies of SIVmac239Δ nef template in the presence of a minimum 100,000-fold excess of unmodified template. Assay specific for the unmodified challenge viruses, SIVmac239 and SIVmac251, were developed targeting the nef coding sequences absent from SIVmac239Δ nef. The assay for SIVmac239 consisted of the primers−probe combination of dnefF03 (GAATATCCCTGAGGAAACCCAGC), dnefR03 (ATTGCGAAATTGATCATCTGCTAC), and neP03 (5′−Guanar670)CTTTTGCCCTGCTACTGACACCCCTAC−3′ (BHQ2). Viral load results obtained with this assay and with the SIVmac239Δ nef assay on specimens predicted to contain only SIVmac239 or SIVmac251Δ nef, respectively, were validated by comparison with test results obtained using the gag-based assay noted above. Application of the SIVmac239 assay to samples predicted to contain only SIVmac251, however, yielded discrepant results when compared with results from the standard gag-based assay. Subsequent sequence analysis (data not shown) of the nef region derived from plasma specimens from multiple animals infected with SIVmac251 confirmed sequence variation from SIVmac239 and sequence diversity consistent with the uncloned SIVmac251 inoculum. An assay for SIVmac251 was developed and optimized consisting of the following primers−probe combination: 251 nef (GAATATCCCTGAGGAAACCCAGC), 251 ren (GTTCAATTGTGAAACCCATCGATGATG), and neP03 (5′−Guanar670)CTTTTGCCCTGCTACTGACACCCCTAC−3′ (BHQ2), where P and K refer to degenerate bases mimicking a C/T and A/G mix, respectively (GenenResearch, Sterling, VA). Performance of this assay on samples predicted to contain only SIVmac251 was similarly validated against the standard gag-based assay.

Determination of peripheral CD4+ T cell counts

PBMCs were isolated and stained with monoclonal Abs specific for CD3, CD4, and CD8 as described above. Absolute cell counts for each subset were calculated by multiplying the percentage of total CD3+CD4+ or CD8+ T cells by the number of total lymphocytes at the corresponding time points as determined by complete blood count analysis.

Determination of CTL escape in viral isolates

Viral RNA was isolated from frozen EDTA plasma samples using the Qiagen QIAmp MinElute Virus Spin Kit (Qiagen, Valencia, CA), at 8 wk p.i. with SIVmac239. Briefly, plasma samples were thawed at room temperature, and 160 plasm was centrifuged at 1500 × g for 10 min to remove residual cells and cellular fragments. Viral RNA was isolated from 140 pl of this cell-free plasma and used directly in a RT-PCR (Qiagen OneStep RT-PCR kit) using the following sequence-specific primers, to obtain complementary DNA encoding the A20 Nef59_160Y9 (Nef YY9), A0v1 Gag81_189CM9 (Gag CM9), or A01 Tat25_38SL8 (Tat SL8) epitope. To amplify the Nef YY9 epitope, 9273-F (5′-TGGAGGCTTGTGAACTTTGAGACAG-3′) and 9046-R (5′-GGGAAAGGTGGATTCTGACG-3′) were used. Amplification was performed for 45 cycles (98°C for 30 s, 67°C for 30 s, and 72°C for 1 min) after a reverse transcription step (50°C for 30 min and 95°C for 1 min) and followed by a final elongation step (68°C for 2 min). Resulting amplicons were analyzed by agarose gel electrophoresis and photographed. 

Statistical analysis and statistics

Statistical analysis was performed using GraphPad Prism version 5.0 for Mac OSX (GraphPad Software, La Jolla, CA) or StatView 5.01 (SAS Institute, Cary, NC). Differences in expression levels (mean fluorescence intensity (MFI)), frequencies of marker expression, or cell populations were calculated using the Mann-Whitney, Wilcoxon signed rank or, when multiple populations were compared, the Kruskal-Wallis or Friedman tests. Correlations were calculated using Spearman Rank test. p-values of ≤0.05 were considered statistically significant.

Results

Differential distribution of PD-1 on memory T cell subsets in SIV-seronegative rhesus macaques

Recent publications have reported elevated PD-1 expression on T cells in the setting of chronic viral infections in mice and humans (14, 16–23, 46, 62). To characterize expression of PD-1 on CD8+ T cells in normal rhesus macaques and the distribution of its expression among memory subsets, we analyzed the expression of PD-1 on peripheral blood CD8+ and CD4+ T cells in a cohort of 15 rhesus macaques, identifying naive and memory T cell subsets based on the expression of CD28 and CD95 (63). Naive T cells were defined as CD28intmediate/CD95−, central memory T cells (CD28intCD95−), effector memory T cells (CD28int/CD95+), and effector memory T cell subsets based on the expression of CD28 and CD95 (63). Naive T cells were defined as CD28int/CD95−, central memory T cells (CD28int/CD95−), effector memory T cells (CD28int/CD95+), and effector memory T cell subsets based on the expression of CD28 and CD95 (63).

In healthy, SIV-seronegative rhesus macaques, PD-1 was almost exclusively expressed on memory CD8+ T cells. CD8+ T cells had a slightly higher, though not significant, expression of PD-1 compared with CD8+ TEM (Fig. 1B). Mean PD-1 expression was 29% ± 5% in CD8+ TEM; 20% ± 7% in CD8+ TEM. PD-1 expression was negligible on both naive CD8+ T cells and naive CD4+ T cells. Both CD4+ memory T cell subsets were found to express PD-1 more frequently compared with their corresponding CD8+ T cell subset, with CD4+ TEM showing the highest frequency of PD-1+ cells (Fig. 1C; mean PD-1 expression 45% ± 5% in CD4+ TEM; 30% ± 7% in CD4+ TEM, p = not significant).
Wild-type SIV infection causes upregulation of PD-1 on memory T cell subsets, which is not seen in LASIV infection

Elevated PD-1 expression on CD8⁺ and CD4⁺ T lymphocytes has been observed in uncontrolled retroviral infections in humans (17–20) and in rhesus macaques (21–23). We sought to examine expression of PD-1 in response to nonpathogenic infection with a virus that replicates in the host at low levels, as well as in macaques infected with wild-type SIV. To this end, we compared expression of PD-1 on CD8⁺ and CD4⁺ T cells in the peripheral blood of animals infected with LASIV (n = 8) with those of animals infected with wild-type SIV strains (n = 24) and SIV-seronegative animals of matched age (Fig. 2A,2B, n = 14). We found a significantly elevated frequency of CD8⁺ and CD4⁺ T cells expressing high levels of PD-1 in the cohort infected with wild-type SIV compared with the uninfected control group (mean PD-1⁺ CD8⁺ T cells 29% ± 10% in wild-type SIV, TCM 43% ± 10% in controls, p ≤ 0.0005; TEM 35% ± 12% in wild-type SIV, TEM 20% ± 7% in controls, p ≤ 0.0005). PD-1 expression in wild-type SIV infection correlates with markers of disease progression

Upregulation of PD-1 expression on T cells has been associated with failure to control viral replication and disease progression in chronic HIV infection (17–19), and increased PD-1 expression has been observed in SIV-infected rhesus macaques with high viral loads compared with macaques with lower viral loads (21). To examine the relationship among PD-1 expression, viral load, and disease progression in pathogenic wild-type SIV infection, we analyzed PD-1 expression on CD8⁺ and CD4⁺ T cell subsets in a large cohort of rhesus macaques chronically infected with pathogenic SIV strains (n = 24), which exhibited a wide range of plasma viral loads (10³ to 2.3 × 10⁶ viral RNA copies/ml plasma) and total CD4⁺ T cell counts (199–1913 CD4⁺ T cells/μl). Some animals in this cohort received vaccines prior to a pathogenic challenge with either SIVmac239 or SIVmac251. We found a significant positive correlation between the viral load and the frequency of PD-1⁺ cells in total CD8⁺ T cells (data not shown), as well as in CD8⁺ TCM and CD8⁺ TEM cells, irrespective of the vaccination status of the animals (Fig. 2C, p = 0.02 for CD8⁺ TCM
and \( p = 0.04 \) for CD8\(^+\) TCM). In addition, the frequency of PD-1\(^+\) CD8\(^+\) T cells was inversely correlated with total CD4\(^+\) T cell counts (data not shown). For CD4\(^+\) T cells, the frequency of PD-1\(^+\) cells positively correlated with viral loads and negatively correlated with total CD4\(^+\) T cell counts (Fig. 2D, \( p = 0.004 \); Fig. 2E, \( p = 0.0005 \)). No difference was observed in viral load, total CD4\(^+\) T cell count, or PD-1 expression in animals challenged with SIVmac239 compared with animals challenged with SIVmac251 (data not shown). These results in a large cohort of pathogenic wild-type SIV-infected rhesus macaques demonstrate that, as previously reported in HIV-1–infected humans (17–20), expression of PD-1 on memory CD4\(^+\) and CD8\(^+\) T cells correlates with markers of disease progression.

**Downregulation of PD-1 expression on SIV-specific CD8\(^+\) T cells following the emergence of CTL escape mutations**

Recent reports have supported a causal link between PD-1 upregulation and antigenic stimulation of T cells through recognition of cognate Ag by the TCR during acute and chronic viral infections (17, 23, 44, 62, 64). To determine whether similar findings could be observed in chronically wild-type SIV-infected animals, we determined the frequency of PD-1\(^+\) T cells specific for three immunodominant epitopes with different propensities for CTL escape: the stable Mamu-A\(^+\)restricted Nef159–167 epitope (Nef YY9) (which shows intermediate kinetics of CTL escape) (58), Epitope-specific CD8\(^+\) T cells were identified using MHC class I tetramers presenting the respective viral epitopes.

During the chronic phase of SIV infection, we observed a significantly increased frequency of PD-1\(^+\) Gag CM9-specific CD8\(^+\) T cells compared with total CD8\(^+\) T cells (mean PD-1\(^+\) Gag CM9-specific CD8\(^+\) T cells 75% \( \pm \) 15% versus 29% \( \pm \) 10% total CD8\(^+\) T cells, \( p = 0.0005 \)) that exceeded the frequency of PD-1\(^+\) cells within the TCM and TEM CD8\(^+\) T cell subsets (Fig. 3A). The frequency of PD-1 expression within the Nef YY9-specific CD8\(^+\) T cell population in the Mamu-A\(^+\)02\(^+\)+ animals was also significantly increased and reached levels comparable to those seen within the Gag CM9-specific population (71% \( \pm \) 13%). In contrast, the frequency of PD-1\(^+\) Tat SL8-specific CD8\(^+\) T cells in the chronically SIV-infected Mamu-A\(^+\)01\(^+\) animals was low (23% \( \pm \) 11%, \( p = 0.0005 \) compared with Gag CM9-specific CD8\(^+\) T cells). The frequency of PD-1\(^+\) Tat SL8-specific cells was lower than the frequency of PD-1\(^+\) cells in the TCM or TEM CD8\(^+\) T cell subsets (40% \( \pm \) 8% in TCM and 31% \( \pm \) 9% in TEM) and did not exceed the percentage of PD-1\(^+\) cells in the total CD8\(^+\) T cell population, even though PD-1 was expressed on the majority of the contemporaneous Gag CM9-specific CD8\(^+\) T cells in the same animal.

To further investigate the link between TCR stimulation and PD-1 expression, we analyzed the temporal association between the occurrence of CTL escape mutations and the downregulation of PD-1 expression on SIV-specific CD8\(^+\) T cells. We focused on the acute phase of SIV infection, taking advantage of the different escape kinetics of the Gag CM9, Tat SL8, and Nef YY9 epitopes. Whereas CTL-mediated escape within the Gag CM9 epitope is rare (66),
Tat SL8 has a high propensity to escape as early as 2–4 wk p.i. (65, 67). Nef YY9 has been reported to accumulate sequence heterogeneity within the first 16 wk after SIV infection, followed by an increase in the frequency of amino acid substitutions at later time points, which exhibit variable impact on peptide binding and CTL recognition (58). We studied a cohort of eight Mamu-A*01 or Mamu-A*02, SIVmac239-infected rhesus macaques, six of which had been vaccinated with an experimental scSIV vaccine regimen (50). Starting at week 4 p.i., we monitored PD-1 expression on CD8+ T cells specific for the three SIV epitopes noted above for 24 wk. At 4 wk p.i., PD-1 was expressed at high levels on the vast majority of Gag CM9-, Tat SL8- (Fig. 3B), and Nef YY9-specific CD8+ T cells (Fig. 3B, 3C, left panel). Although the frequency of PD-1+ cells within the Nef YY9-specific CD8+ T cells did not differ significantly from that within the Gag CM9-specific CD8+ T cells at 4 wk p.i., downregulation of PD-1 on Tat SL8-specific CD8+ T cells was already detectable at that time point (Fig. 3B, 3C, p < 0.005). This initially subtle difference increased over the following 4-wk period, resulting in a significantly decreased population of PD-1+ Tat SL8-specific CD8+ T cells by week 8 p.i.. At this time point, Nef YY9-specific CD8+ T cells began to exhibit PD-1 downregulation (Fig. 3C, right panel, p < 0.005 for Tat SL8-specific), and the Gag CM9-specific cells increased PD-1 expression to form a more homogenous PD-1+ population. The different kinetics of CTL escape within the three epitopes was also reflected in the magnitude of the three epitope-specific CD8+ T cell responses. Although we observed a sharp decline in the magnitude of the Tat SL8-specific CTL responses between 4 and 8 p.i., Gag CM9 responses were maintained at constant levels throughout the duration of the study, and Nef YY9-specific responses declined with less abrupt kinetics (Fig. 3D).

To confirm the temporal association of PD-1 downregulation with CTL escape, we analyzed the sequences of the Gag CM9, Tat SL8, and Nef YY9 epitopes in viral RNA isolated 8 wk p.i.. This time point lies well within the period of initial manifestation of sequence variability within the Nef YY9 epitope and complete escape of Tat SL8. We compared the week 8 sequences to the epitope sequences in SIVmac239 (Fig. 3E). As expected, sequencing of the Gag CM9 epitope did not reveal any variation from the wild-type SIVmac239 sequence in any macaque. In the five Mamu-A*01+ animals, 38 of 39 week 8 Tat SL8 sequences diverged from the wild-type SIVmac239 sequence and contained one or more published CTL escape mutations (65). Viral isolates from three out of four Mamu-A*02+ rhesus macaques showed complete divergence from the original Nef YY9 epitope sequence, with a majority of the mutations resulting in amino acid replacements that have been confirmed to reduce either epitope presentation or CTL recognition (58). Interestingly, the only Mamu-A*01*/Mamu-A*02+ animal in the cohort showed complete CTL escape within the Mamu-A*01-restricted Tat SL8 epitope, whereas the Mamu-A*02-restricted Nef YY9 sequences were 100% identical to the wild-type SIVmac239 sequence. No sequence variation in the Tat SL8 or Nef YY9 epitopes was observed in animals that did not express the restricting MHC class I allele.

CTL escape of the Tat SL8 epitope might be associated with a phenotypic shift of Tat SL8-specific CD8+ T cells from an effector memory (CD28+CD69+) toward a predominantly central memory (CD28+CD69-) phenotype, and PD-1 is differentially expressed on these two memory subtypes. We therefore analyzed the expression of CD28 on Gag CM9-, Tat SL8-, and Nef YY9-specific CD8+ T cells in our cohort of chronically SIV-infected macaques. We detected an equal distribution of Gag CM9-, Tat SL8-, and Nef YY9-specific cells in the TCM and TEM CD8+ T cell subsets (Fig. 3F).

We subsequently determined PD-1 expression on Gag CM9-, Tat SL8-, and Nef YY9-specific TCM and TEM subsets. Consistent with our observations in the total CD8+ memory populations, PD-1 was expressed more frequently and at higher levels in Gag CM9-specific TCM CD8+ T cells compared with the Gag CM9-specific TEM CD8+ T cells (Fig. 3G, p = 0.01). The Tat SL8- and Nef YY9-specific CD8+ T cells, however, showed low frequencies and levels of PD-1 expression irrespective of their memory phenotype, indicating that PD-1 downregulation on Ag-specific T cells is not simply a covariant of memory differentiation.

Taken together, these results strongly support the notion that the upregulation and maintenance of high PD-1 expression levels on Ag-specific T cells during viral infections is dependent on direct stimulation of the TCR by its cognate Ag.

Low levels of persistent antigenic stimulation are sufficient for the maintenance of PD-1 expression on SIV-specific CD8+ T cells

Our results demonstrate clearly that loss of antigenic stimulation of the TCR has a negative impact on the maintenance of PD-1 expression on virus-specific CD8+ T cells. This observation is consistent with reports of PD-1 downregulation on virus-specific T cells in nonpersistent viral infections (14, 48, 64) in which TCR–Ag interactions are abrogated by clearance of the virus from the host. However, high levels of PD-1 expression on virus-specific cells have been described in the setting of chronic viral infections with low viral loads (23, 62), or even in the absence of detectable virus, for example, in cases of spontaneously resolved hepatitis C virus infection in chimpanzees (48). Similarly, we failed to detect a correlation between PD-1 expression on Gag CM9-specific CD8+ T cells and plasma viral loads in our cohort of Mamu-A*01+ animals chronically infected with SIVmac239 or SIVmac251, because they showed uniformly high PD-1 expression levels despite a wide range of viral loads (10 × 10^4 to 2.3 × 10^6 copies/ml plasma, data not shown).

To explore the minimal threshold of Ag necessary to maintain PD-1 expression on virus-specific CD8+ T cells in vivo, we determined the frequency and level of PD-1 expression on cells specific for the stable Gag CM9 epitope in Mamu-A*01+ rhesus macaques chronically infected with SIVmac239Δnef. The animals controlled viral loads below the limit of detection (≤10–20 copies/ml plasma) while maintaining distinct Gag CM9 responses ranging between 0.19% and 0.55% of total CD8+ T cells throughout the period of observation (data not shown). Although viral loads were undetectable, PD-1 was expressed on a vast majority of the Gag CM9-specific CD8+ T cells in the SIVmac239Δnef-infected animals (88% ± 8% versus 43% ± 12% in the total CD8+ T cell population (Fig. 4A, 4B and data not shown). These frequencies were comparable to those observed during the acute phase of wild-type SIV infection in a cohort of Mamu-A*01+ animals (92% ± 6% versus 51% ± 11% in total CD8+) and slightly, but not significantly, lower than those seen during the chronic phase of infection in the same cohort (98% ± 2% versus 54% ± 11% in total CD8+). The frequencies of PD-1+ cells were mirrored in the expression levels per Gag CM9-specific cell, for which PD-1 expression during chronic SIVmac239Δnef infection was equal to the levels seen in acute wild-type SIV infection (geometric MFI in SIVmac239Δnef 1819 ± 468 versus 2163 ± 730 in acute wild-type SIV infection). However, we observed a significant increase in PD-1 expression in the chronic phase of SIV infection (geometric MFI 3236 ± 804, p ≤ 0.005 compared with acute wild-type SIV infection and p = 0.05 compared with SIVmac239Δnef infection), confirming previously published observations (22, 23).

To further investigate the relationship between PD-1 expression and antigenic stimulation, we studied the kinetics of PD-1
FIGURE 3. Sustained high levels of PD-1 expression are found on CD8+ T cells specific for SIV epitopes that do not develop CTL escape mutations. A, Summary of data showing the frequency of PD-1+ cells (%) in naive, TCM, TEM CD8+ T cell subsets, in total CD8+ T cells, and within CD8+ T cells specific for the Mamu-A*01-restricted epitopes Gag 181–189CM9 and Tat 28–35SL8 and the Mamu-A*02-restricted epitope Nef 159–167YY9 in Mamu-A*01+ (n = 10) and Mamu-A*02+ (n = 7) animals chronically infected with either SIVmac239 or SIVmac251. Horizontal bars represent mean values. B, Representative flow cytometry plots from one scSIV-vaccinated, Mamu-A*01+ rhesus macaque at the indicated times post challenge with SIVmac239, showing PD-1 expression levels on TatSL8-specific CD8+ T cells (upper panels, orange filled histograms) and GagCM9-specific CD8+ T cells (lower panels, blue filled histograms) in an overlay with a fluorescence-minus-one control (black open histograms). C, Summary of data showing the frequency of PD-1 expression (%) on CD8+ T cells specific for the SIV epitopes GagCM9, TatSL8, and NefYY9 at 4 wk (left panel) and 8 wk (right panel) p.i. with SIVmac239 in scSIV-vaccinated (colored circles) and unvaccinated (open and black circles) Mamu-A*01+ (n = 5) and Mamu-A*02+ (n = 4) rhesus macaques. Horizontal bars represent mean values. A and C, ***p ≤ 0.0005; **p ≤ 0.005; *p ≤ 0.05. p Values were calculated using the Kruskal-Wallis test with Dunn’s post test. D, Frequency of CD8+ T cells specific for the SIV epitopes GagCM9, NefYY9, and TatSL8 in the peripheral blood of Mamu-A*01+ (n = 5) and Mamu-A*02+...
expression in three different settings in which the stable Gag CM9 epitope was presented in the context of infection with wild-type SIVmac251 (sustained high viral loads, n = 5), SIVmac239 nef (initial peak of high viral replication, followed by sustained low-level viremia, n = 3), or scSIV (n = 5), which leads to clearance of viremia from the host within 1–2 wk (50). We compared PD-1 expression on Gag CM9-specific CD8+ T cells in these cohorts during acute versus chronic infection, or 1 versus 16 wk post-inoculation with scSIV. As expected, the frequency of PD-1+ cells within the Gag CM9-specific CD8+ T cell subset in the five Mamu-A01+, SIVmac251-infected rhesus macaques was high during acute infection (weeks 4–7, plasma viral loads between 1.5 × 10^5 and 1.5 × 10^6 copies/ml) and increased with progression into the chronic phase of infection (weeks 21–29, p = 0.04; plasma viral loads between 9 × 10^5 and 3.9 × 10^6 copies/ml, data not shown and Fig. 4C). Similarly, PD-1 expression on Gag CM9-specific CD8+ T cells in seven Mamu-A01+ animals 2–4 wk p.i.; with SIVmac239 nef (plasma viral loads 1.5 × 10^5 to 5.2 × 10^5 copies/ml) started out at comparably high frequencies. These high frequencies of PD-1+ Gag CM9-specific CD8+ T cells were maintained in two of seven and increased slightly in the remaining five animals with progression into the chronic phase of infection, even though viral loads were controlled to undetectable limits by that time (p = 0.018; limit of detection ≤ 20 copies/ml plasma, data not shown). The increase in the frequency of PD-1+ Gag CM9-specific CD8+ T cells in animals infected with SIVmac251 and SIVmac239 nef was also reflected in an increase in the MFI of PD-1 on Gag CM9-specific CD8+ T cells. One week after administration of the scSIV vaccine, which typically results in a transient peak of viremia between 10^4 and 10^5 copies/ml plasma at that time (50), PD-1 was expressed on Gag CM9-specific CD8+ T cells at frequencies comparable to those seen during the acute phase of SIVmac239 nef infection. However, by 16 wk after scSIV inoculation, PD-1 expression on Gag CM9-specific CD8+ T cells decreased significantly, as reflected both by a decrease in the percentage of PD-1+ cells and in the MFI of PD-1 to levels seen in the bulk CD8+ T cell population (Fig. 4C, right panels, p = 0.04). At this time point single-cycle viral RNA could no longer be detected in plasma (50).

Taken together, these results strongly support the notion that even very low levels of antigenic stimulation are sufficient to maintain PD-1 expression on virus-specific CD8+ T cells.

Elevated levels of PD-1 expression on HIV-1–specific CD8+ T cells in elite controllers

Studies of PD-1 in HIV-1 infection have demonstrated that PD-1 expression on HIV-1–specific CD8+ T cells correlates well with plasma viral loads (17, 19, 20). In addition, it has been shown that PD-1 expression is significantly lower in long-term nonprogressors, who have durable plasma viral loads of ≥500 copies/ml in the absence of antiretroviral treatment, than that in typical progressors, who fail to control viral load (20).

We were interested in establishing whether the sensitivity of PD-1 expression as a marker for ongoing low-level viral replication that we observed in SIV-infected rhesus macaques would also be observed in HIV-1–infected individuals. To this end, we studied a cohort of 12 elite controllers of HIV-1 infection, who spontaneously controlled viral load below 50 copies/ml plasma in the absence of antiretroviral therapy, and 9 untreated individuals during chronic HIV-1 infection, who failed to control viral replication (2.3 × 10^2 to 6 × 10^5 copies/ml plasma, data not shown). We determined PD-1 expression on the total CD8+ T cell population and on a total of 30 tetramer-specific CD8+ T cell responses, which were specific for 10 immunodominant HIV-1 epitopes and restricted by 7 different HLA class I molecules (Fig. 5). On the total CD8+ T cell population, both frequencies and levels of PD-1 expression were significantly lower in the elite controllers than those in the chronic untreated individuals (Fig. 5B; mean frequency in elite controllers 26% ± 12% versus 40% ± 11% in chronic untreated (p = 0.008), geometric MFI in elite controllers 95 ± 50 versus 222 ± 92 in chronic untreated, p = 0.0016). Within the HIV-1–specific CD8+ T cell population, PD-1 was expressed at variable levels, but expression was significantly elevated on virus-specific CD8+ T cells compared with the total CD8+ T cell population in both elite controllers and chronic untreated individuals (p = 0.0002). Although the frequency of PD-1+ HIV-1–specific CD8+ T cells in elite controllers did not differ significantly from the high frequency seen in chronic untreated individuals (64% ± 23% versus 79% ± 16%, respectively, p = 0.07), PD-1 expression levels per HIV-1–specific CD8+ T cell were significantly lower in elite controllers than those in chronic untreated individuals (geometric MFIs in 393 ± 237 versus 669 ± 297, respectively, p = 0.0087).

These results indicate that PD-1 expression on HIV-1–specific CD8+ T cells is maintained in the presence of very low levels of antigenic stimulation, consistent with the results obtained in SIV-infected rhesus macaques.

Discussion

Utilizing the distinctive advantages of the SIV/macaque model, we examined the upregulation and maintenance of PD-1 expression on CD8+ T cell responses in the setting of controlled and uncontrolled lentiviral infection. To our knowledge, our study is the first cross-species comparison of PD-1 expression in lentiviral infections and enabled us to identify shared features of the cellular immune response against immunodeficiency viruses in the rhesus macaque model and in humans.

Expression of PD-1 was primarily restricted to memory T cell subsets in both healthy, uninfected and SIV-infected rhesus macaques. PD-1 expression on naive CD8+ and CD4+ T cells in healthy macaques was negligible and not upregulated in response to SIV infection, supporting the notion that stimulation of the TCR through cognate Ag is an important requirement for PD-1 expression. In contrast to previous reports (22, 23), however, we found that despite substantial heterogeneity, PD-1 was...
significantly upregulated on memory CD8+ and CD4+ T cell populations during chronic, pathogenic SIV infection and that PD-1 expression correlated with markers of disease progression. The correlation between PD-1 expression on memory T cells and plasma viremia was underscored by the absence of PD-1 upregulation on total memory T cells during nonpathogenic, controlled LASIV infection, which indicates a potential link between high PD-1 expression and generalized immune activation in pathogenic wild-type SIV infection (68).

A growing body of literature has linked the emergence of CTL escape mutations to PD-1 downregulation on virus-specific T cells in several chronic viral infections, showing that the upregulation and maintenance of high levels of PD-1 expression on virus-specific CD8+ T cells is mediated, among other factors (43), by the stimulation of the TCR through interaction with its cognate Ag (23, 44, 62, 64). By comparing PD-1 expression on CD8+ T cells specific for three SIV epitopes with different kinetics of CTL escape in a large cohort of acutely and chronically SIV-infected animals, we were able to comprehensively study this relationship and to confirm the link between the loss of TCR stimulation through CTL escape and PD-1 downregulation. In addition, we showed that CTL escape was associated with a modest, but not statistically significant, tendency...
FIGURE 5. High levels of PD-1 expression on HIV-1–specific CD8+ T cells in elite controllers. A, Representative flow cytometry plots showing the identification of total CD8+ and HIV-1–specific CD8+ T cells using MHC class I tetramers (left panel, gated on CD3+ lymphocytes) and the subsequent determination of PD-1 expression on both subsets in an overlay (right panels; HIV-1–specific cells depicted by solid blue line, total CD8+ T cells depicted by red dashed line). Data are from an untreated individual during chronic HIV-1 infection, following staining of cryopreserved PBMCs with tetramers specific for the HLA-A*0301–restricted QK10 epitope. B, Summary of data from elite controllers (elite, n = 12) and individuals during chronic, untreated HIV-1 infection (chronic untreated, n = 9), showing PD-1 expression (frequency, left panel; MFI, right panel) on total CD8+ T cells and cells specific for 10 immunodominant HIV-1 epitopes as represented by the colors in the legend. Each symbol represents one measurement on total CD8+ T cells or one epitope-specific response. Horizontal bars represent mean values. p Values were calculated using the Mann-Whitney nonparametric test for comparisons between elite controllers and chronic, untreated individuals and Wilcoxon signed rank test for comparisons within the two groups.

of Tat SL8-specific T cells to exhibit a central memory phenotype but that this change in memory differentiation was not independently associated with the downregulation of PD-1.

The ability of LASIV strains to persist and induce ongoing low-level antigenic stimulation is a unique feature that is likely to play a key role in their remarkable ability to induce protective immunity against homologous wild-type SIV challenge strains. However, the nature of this persistent antigenic stimulation has not been well-defined. Two lines of evidence support the notion of active replication resulting in low-level, chronic antigenic stimulation. First, depletion of CD8+ cells in SIVmac239Δnef-infected animals with low baseline levels of plasma viremia resulted in a rapid rebound of plasma viremia, suggesting that SIV-specific CD8+ T cell responses are involved in suppression of low levels of viral replication (29). Second, macaques infected with SIVmac239Δnef and controlling viral loads to low levels develop deletions in upstream U3 long terminal repeat sequences, implying active viral replication as a prerequisite of viral sequence evolution (31, 35, 69). Our study provides additional indirect evidence that LASIV strains actively replicate, even under circumstances in which plasma viremia is not detectable using sensitive RT-PCR assays.

We found sustained high PD-1 expression levels on virus-specific CD8+ T cells during controlled LASIV infection but only transient expression on virus-specific CD8+ T cells generated in response to infection with scSIV, a virus that is limited to one round of replication. These findings suggest that PD-1 is expressed in response to very low levels of antigenic stimulation. This result implies that PD-1 expression on virus-specific CD8+ T cells may reflect viral replication below the threshold of detection of conventional assays and may therefore serve as a sensitive immunological complement to molecular techniques of viral RNA quantification. Our results may also have implications for reports of sustained PD-1 expression levels in the absence of apparent virus replication, for instance, in cases of spontaneously resolved hepatitis C virus infection (48, 62).

By undertaking a cross-species comparison of PD-1 expression in the setting of controlled and uncontrolled chronic viral infections, we found substantial similarities in PD-1 expression on total and virus-specific CD8+ T cells in SIV-infected rhesus macaques and HIV-1–infected individuals. In both macaques and humans, PD-1 expression on total memory CD8+ T cells was significantly higher under conditions of uncontrolled, chronic SIV or HIV-1 infection than in controlled LASIV or HIV-1 infection. This finding indicates that general upregulation of PD-1 on the total population of CD8+ T cells versus the targeted, exclusive upregulation of PD-1 on lentivirus-specific T cells may be a characteristic that distinguishes pathogenic and uncontrolled infection from controlled lentiviral infections.

Similar to our findings in LASIV-infected macaques, we found that elite controllers of HIV-1 infection showed increased frequencies and elevated levels of PD-1 expression on virus-specific CD8+ T cells. Although CTL responses appear to play an important role in the control of viral load in elite controllers of HIV-1 infection (70–73), the overall contribution of the cellular arm of the immune system varies among individuals and remains controversial (73, 74). Our results support evidence for ongoing antigenic stimulation and T cell–mediated control of HIV-1 infection in the cohort of elite controllers included in this study (75). In both LASIV-infected macaques and elite controllers of HIV-1 infection, the frequency of PD-1+ virus-specific CD8+ T cells did not differ significantly from the high frequencies seen in acute or chronic uncontrolled infection. However, the levels of PD-1 on virus-specific CD8+ T cells were significantly lower in macaques and humans controlling the infection than in those not controlling. The level of PD-1 expression thus appears to reflect the magnitude of antigenic burden much more sensitively than the frequency of
PD-1+ virus-specific T cells. Our results therefore support previous reports associating high PD-1 expression levels with greater impact on PD-1-regulated functions, such as cellular survival or propensity for apoptosis (18, 22).

Our study demonstrates that PD-1 is a sensitive indicator of viral replication in lentiviral infection of both rhesus macaques and humans. The finding of sustained high levels of PD-1 expression in the setting of controlled low viral load during nonpathogenic LASIV infection and in the setting of elite control of HIV-1 infection challenges the notion that PD-1 is a specific marker for T cell exhaustion. Our results imply that the identification of truly dysfunctional T cells might be improved by evaluating PD-1 expression in the context of other coinhibitory and costimulatory cell surface molecules (49, 76).

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Disclosures
G.J.F. has patents and receives patent royalties on the PD-1 pathway.

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