T cells with high PD-1 expression are associated with lower HIV-specific immune responses despite long-term antiretroviral therapy

Bernard J.C. Macatangay[a,f], Rajesh T. Gandhi[b], Richard B. Jones[c], Deborah K. Mcmahon[a,f], Christina M. Lalama[d], Ronald J. Bosch[d], Joshua C. Cyktor[a], Allison S. Thomas[e], Luann Borowski[f], Sharon A. Riddler[a,f], Evelyn Hogg[g], Eva Stevenson[c], Joseph J. Eron[h], John W. Mellors[a,f], Charles R. Rinaldo[f,i], for the ACTG A5321 Team

Objective: We evaluated frequencies of T cells with high PD-1 expression (PD-1^{HI}) before and after long-term effective antiretroviral therapy (ART), and determined if frequencies on-ART correlated positively with measures of HIV persistence and negatively with HIV-specific responses.

Methods: We enrolled individuals who started ART during chronic infection and had durable suppression of viremia for at least 4 years ($N = 99$). We assessed PD-1^{HI} T-cell frequencies at timepoints pre-ART and on-ART using flow cytometry, and evaluated how frequencies on-ART are associated with measures of HIV persistence, HIV-specific immune responses, and immune activation levels.

Results: Pre-ART, PD-1^{HI} $CD4^+$ T cells correlated positively with viremia and negatively with $CD4^+$ T-cell count. At year 1 on-ART, %PD-1^{HI} $CD4^+$ T cells decreased but then remained stable at 4 and 6–15 years on-ART, whereas %PD-1^{HI} $CD8^+$ T cells on-ART remained similar to pre-ART. PD-1^{HI} $CD4^+$ T cells correlated positively with HIV DNA pre-ART and on-ART, and with $CD4^+$ T-cell activation on-ART. PD-1^{HI} $CD4^+$ T cells negatively correlated with HIV Gag-specific and Env-specific T-cell responses but not with CMV-specific or EBV-specific responses. PD-1^{HI} $CD8^+$ T cells trended towards a negative correlation with responses to Gag and Env, but not to CMV and EBV.

Conclusion: PD-1^{HI} T cells persist in blood despite prolonged suppression on ART, correlate with HIV DNA levels, and are associated with lower HIV-specific T-cell responses but not CMV-specific or EBV-specific responses, suggesting that these cells are HIV-specific. The findings support evaluating PD-1 blockade strategies for their effect on HIV persistence and HIV-specific immunity.

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[a]Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, [b]Division of Infectious Diseases, Massachusetts General Hospital, Boston, Massachusetts, [c]Division of Infectious Diseases, Weill Cornell Medicine, New York, New York, [d]Center for Biostatistics in AIDS Research, Harvard T.H. Chan School of Public Health, [e]Department of Microbiology, Boston University School of Medicine, Boston, Massachusetts, [f]Department of Infectious Diseases and Microbiology, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania, [g]Social & Scientific Systems, Inc., Silver Spring, Maryland, [h]Department of Medicine, University of North Carolina, Chapel Hill, North Carolina, and [i]Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA.

Correspondence to Bernard J.C. Macatangay, MD, S827 Scaife Hall, 3550 Terrace Street, Pittsburgh, PA 15261, USA.

Tel: +1 412 3831272; fax: +1 412 6488455; e-mail: macabj@upmc.edu

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Introduction

Persistent antigenic stimulation during chronic viral infection is associated with T-cell expression of inhibitory receptors that downregulate immune responses [1–5]. A specific inhibitory receptor, programmed cell death protein 1 (PD-1), was initially isolated in a T-cell hybridoma [6] and later described in mice with immune exhaustion from chronic lymphocytic choriomeningitis virus infection [7]. It is associated with disease progression and reversible impairment of antigen-specific T-cell function in other chronic viral infections, including HIV [8–12]. In HIV animal models, PD-1 receptor blockade increases antigen-specific immunity, reduces immune activation, and decreases plasma viremia [13–15]. In a clinical trial evaluating blockade of the PD-1 ligand (PD-L1) in individuals on effective ART, a subset (two of six participants) showed enhancement of HIV-specific immune responses [16]. PD-1 expression on $CD4^+$ T cells in blood also marks cells that are more likely to carry HIV provirus [17]. Specifically, $CD4^+$ T cells with high PD-1 expression and those co-expressing other inhibitory receptors are enriched for proviral DNA [18–20] and have greater permissiveness to HIV infection [21,22]. These findings suggest that $CD4^+$ T cells expressing PD-1 contribute to the HIV reservoir and that targeting these cells could be an important strategy for decreasing HIV-infected cells.

Although total PD-1 expression on T cells has been used to define an exhausted T-cell phenotype, T cells rapidly express PD-1 during antigen activation [23], with expression levels either decreasing once antigen is cleared or persisting in chronic infections and cancer [3]. As such, total PD-1 expression alone is not necessarily a specific marker for a subset of exhausted T cells in the setting of persistent antigen stimulation. Instead, in certain malignancies, high expression of PD-1 (PD-1^{HI}) on flow cytometry, rather than total PD-1 expression, is associated with a more exhausted phenotype and worse clinical outcome [24,25]. Similarly, PD-1^{HI} expression is related to impaired immunologic function in chronic simian immunodeficiency virus (SIV) and HIV infection [26,27]. Indeed, PD-1^{HI} HIV-specific $CD8^+$ T cells have decreased expression of multiple cytokines following ex-vivo stimulation with HIV peptides [9].

The impact of long-term ART on PD-1-expressing T cells, specifically the PD-1^{HI} subset, has not been characterized. We, therefore, obtained serial samples from participants in the AIDS Clinical Trials Group (ACTG) Study A5321, a noninterventional longitudinal cohort study [28], to assess ART-related changes in the frequencies of $CD4^+$ and $CD8^+$ T cells with any PD-1 expression (total PD-1^+), as well as the PD-1^{HI} T cell subset, and determine how their frequencies are associated with measures of persistence, and HIV-specific responses. Given findings on PD-1 expression in chronic viral infections [5,29,30], we hypothesized that with persistent HIV, even after stable suppression of plasma viremia, frequencies of PD-1^{HI} T cells will positively correlate with the different measures of viral persistence and T-cell activation, and will be negatively associated with HIV-specific T-cell immune responses.

Methods

Participants and samples

We determined frequencies of PD-1 expression, measures of HIV persistence, and different immunologic markers in samples obtained from participants in ACTG study A5321, a longitudinal cohort of participants who initiated ART during chronic infection and had well documented consistent suppression of HIV viremia (Table 1). Samples were obtained prior to ART initiation, and at years 1, 4, and 6–15 on-ART [28]. Institutional review boards at the institutions of the investigators conducting ACTG A5321 approved the study. All participants provided written informed consent.

Evaluation of programmed cell death protein 1 expression

Peripheral blood mononuclear cells (PBMC) obtained from four time points (pre-ART, 1, 4, and, where available, 6–15, years on-ART) were stained with the following mAbs: CD3 APC-H7, CD4 PC5, CD8 V450, PD-1 (clone M1H4) A488 (all from BD Biosciences, San Diego, California, USA), and Live/Dead Aqua (Invitrogen, Grand Island, New York, USA). Cells were fixed in 1% paraformaldehyde, and analyzed using a BD LSR Fortessa (FACSDiva) within 24 h after staining. Fluorescence minus one (FMO) controls were used in setting

Table 1. Study population demographics.

Age in years at initiation of ART, median (Q1–Q3); $N=99$	39 (31–47)
Sex at birth – female	21 (21%)
Race/ethnicity	
White non-Hispanic	50 (51%)
Black non-Hispanic	19 (19%)
Hispanic (regardless of race)	28 (28%)
American Indian, Alaskan Native	2 (2%)
Pre-ART plasma HIV-1 RNA (log_{10} copies/ml), median (Q1–Q3)	4.6 (4.2–4.9)
Pre-ART $CD4^+$ T cell count (cells/μl), median (Q1–Q3)	294 (162–384)
ART regimen (initial/at time of last sample collection)	
NNRTI-based	42%/41%
PI-based	40%/35%
INSTI-based	19%/24%
Years on therapy at time of last sample collection, median (Q1–Q3)	7 (4–8)

ART, antiretroviral therapy; INSTI, integrase strand transfer inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor; PD-1, programmed cell death protein 1; PD-1^{HI}, high PD-1 expression; PI, protease inhibitor.

gates for positive events. Cluster and population analysis of flow cytometry plots with a mean fluorescence intensity (MFI) of at least five-fold the FMO control MFI determined the PD-1^{HI} T-cell subset (Supplemental Figure 1, http://links.lww.com/QAD/B552). Non-specific cellular staining by the PD-1 antibody was confirmed by analyzing HEK-293 cell lines $\pm$ transfection with plasmid pBudCE4.1/iRFP-hPD-1 as published [31] (Supplemental Figure 2, http://links.lww.com/QAD/B552).

Virologic assays

PBMCs from all time points were tested for HIV DNA and cell-associated RNA using published methods in batches that included all sample time points for each participant [32,33]. Levels of cell-free HIV RNA were measured in plasma samples obtained after 4 or more years on-ART by single-copy assay (iSCA) using published methods [33,34].

Peptide pools and IFNγ ELISPOT

Interferon (IFN) γ ELISPOT was performed as previously described [35]. Peptide pools used for HIV-specific immune response assays consisted of consensus HIV Clade B 15 amino acid peptides overlapping by 11 amino acids, obtained from the NIH AIDS Research and Reference Reagent Program, and included: Gag (cat #8117), Pol (cat #6208), Nef (cat #5189), consensus B Tat (cat #5138), Vif (cat #6446), Rev (cat #6445), Vpr (cat #6447), Vpu (cat #6444), Env (cat #9480). All peptides were dissolved at 20 mg/ml in 100% DMSO (Hybri-Max, Sigma-Aldrich, St. Louis, Missouri, USA). A cytomegalovirus (CMV)-pp65 PepMix peptide pool and an Epstein Barr virus (EBV) BZLF-1 PepMix peptide pool (JPT Peptide Technologies, Acton, Massachusetts, USA) were tested for responses. PHA at 2 μg/ml served as a positive control. Positive responses were defined as having met the following criteria: more than 50 spot-forming units/million PBMC after background subtraction and more than two times above background.

Measurement of T-cell activation and cell cycling

Levels of T-cell activation were measured as the percentage of $CD4^+$ and $CD8^+$ T cells co-expressing HLA-DR and CD38. Cryopreserved PBMC were thawed, washed, and stained with the following antibodies: CD3 APC-H7, CD4 A488, CD8 V450, HLA-DR PE, CD38 APC (all BD Biosciences), and Live/Dead Aqua (Invitrogen). Cell cycling was measured by intracellular expression of Ki-67. Samples were permeabilized (Permeabilizing Solution 2, BD Biosciences) and stained with Ki-67 PerCP Cy5.5 (BD Biosciences). Cells were fixed in 1% paraformaldehyde and were analyzed using a BD LSR Fortessa within 24 h of staining. FMO controls were used to gate positive events.

Measurement of soluble markers

Frozen plasma samples were thawed and analyzed in batches that included samples from all time points for each participant. Plasma concentrations of interleukin (IL)-6, C-reactive protein (CRP), soluble (s) CD14 and sCD163 were quantified using an ELISA per manufacturer's instructions (R&D, Minneapolis, Minnesota, USA). Duplicates of 20% of the samples were included in each ELISA plate. Results were analyzed using a BioTek ELx800 ELISA reader and KCjunior software (version 1.10).

Statistical analyses

Data are presented as medians and interquartile ranges. The signed-rank test was used to assess changes between pre-ART and on-ART time points, and differences between PD-1 expression measures. *P* values less than 0.05 were considered significant. Rank-based correlations (Spearman) evaluated associations between PD-1 and PD-1^{HI} expression and the different immunologic and virologic parameters. Partial Spearman correlations were used for associations with pre-ART $CD4^+$ T cells and measures of HIV persistence, adjusted by pre-ART plasma HIV RNA. Correlations at least 0.20 or −0.20 or less with *P* values less than 0.05 were considered significant. Wilcoxon rank-sum test was used to assess associations with binary demographic variables.

Results

Study population

Study participants had a median age of 39 years at ART initiation and were mostly male (79%), with a median pre-ART plasma HIV RNA of 4.6 $\log_{10}$ copies/ml and $CD4^+$ T-cell count of 294 cells/μl (Table 1). At the time of last sample collection, the participants had a median (min, max) of 7 (4, 15) years on ART and a median $CD4^+$ T-cell count of 681 cells/μl.

Total programmed cell death protein 1 and PD-1^{HI} T-cell populations pre-antiretroviral therapy and on- antiretroviral therapy

PD-1^{HI} $CD4^+$ T cells had a 14-fold higher median MFI (Q1 8.5-fold, Q3 50.5-fold) than the PD-1-negative $CD4^+$ T cells ($P < 0.0001$), whereas PD-1^{HI} $CD8^+$ T cells had an 11-fold higher median MFI (Q1 7.2-fold, Q3 51.4-fold) than PD-1-negative $CD8^+$ T cells ($P < 0.0001$). We also compared the MFI of the PD-1^{HI} population with the PD-1^+ nonhigh (PD-1^{LO}) population to confirm that PD-1^{HI} cells are a distinct population based on intensity of PD-1 surface expression. PD-1^{HI} $CD4^+$ T cells had a 2.5-fold higher median MFI (Q1 1.8-fold, Q3 4.7-fold) compared with the PD-1^{LO} $CD4^+$ T cells ($P < 0.0001$). PD-1^{HI} $CD8^+$ also had a median of 2.5 higher median MFI (Q1 1.9-fold, Q3 5.5-fold) than PD-1^{LO} $CD8^+$ T cells ($P < 0.0001$) (Supplemental Figure 3A, http://links.lww.com/QAD/B552). There were no differences in the MFI of the PD-

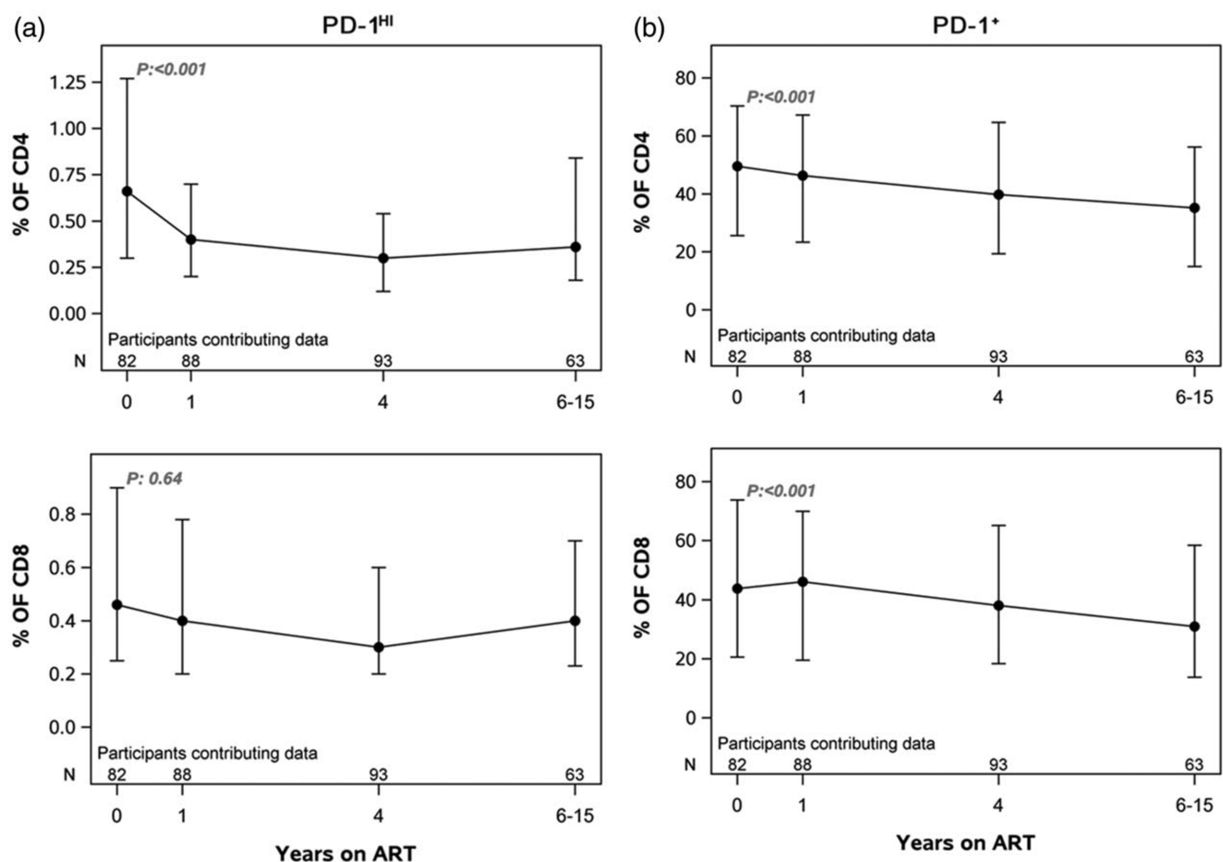

Fig. 1. (a) PD-1^{HI} and (b) PD-1^{+} T cell frequencies following antiretroviral therapy initiation. The frequencies of PD-1^{HI} and total PD-1^{+} CD4^{+} and CD8^{+} T cells prior to ART (0) and at 1, 4, and 6–15 years following ART initiation. *P*-values are for the change in frequencies between pre-ART (year 0) and year 1 on-ART. ART, antiretroviral therapy; PD-1, programmed cell death protein 1; PD-1^{HI}, high PD-1 expression.

1^{HI} T cells at the different timepoints (CD4^{+} PD-1^{HI} $P=0.40$, CD8^{+} PD-1^{HI} $P=0.54$; Supplemental Figure 3B, http://links.lww.com/QAD/B552). Pre-ART (day 0), PD-1^{HI} cells constituted only a small proportion of CD4^{+} and CD8^{+} T cells [median (Q1–Q3) 0.7% (0.3–1.3) and 0.5% (0.3–0.9), respectively] compared with total PD-1^{+} CD4^{+} and PD-1^{+} CD8^{+} T cells [50% (26–70) and 44% (21–74), respectively] (Fig. 1).

There were modest correlations observed between PD-1^{HI} T cells and total PD-1^{+} T cells (Supplemental Figure 4, http://links.lww.com/QAD/B552) but these disappeared on-ART. Pre-ART, PD-1^{HI} T-cell populations were not different between men and women, and there were no correlations with participant age (data not shown). Pre-ART PD-1^{HI} CD4^{+} cells correlated positively with the pre-ART level of plasma HIV-1 RNA ($r=0.28$, $P=0.01$) and negatively with pre-ART CD4^{+} T-cell count ($r=-0.28$, $P=0.01$). Similarly, pre-ART PD-1^{HI} CD8^{+} T cells correlated positively with pre-ART plasma HIV-1 RNA ($r=0.24$, $P=0.03$), but not with CD4^{+} T-cell count.

Within the first year of ART, PD-1^{HI} CD4^{+} T-cell frequency decreased by an absolute median of 0.2% from pre-ART ($P<0.001$; Fig. 1a). This relative decline in PD-1^{HI} frequencies following 1 year of ART was proportionally greater compared with the relative decline in total PD-1^{+} CD4^{+} T cells (43% relative reduction in PD-1^{HI} vs. 9% reduction in total PD-1^{+}; $P=0.01$; Fig. 1b). There were no further significant decreases in the PD-1^{HI} CD4^{+} T-cell frequency after year 1 of ART. PD-1^{HI} CD8^{+} T-cell frequencies did not decline significantly over a median of 7 years on-ART (Fig. 1a).

Pre-ART levels of both PD-1^{HI} CD4^{+} and CD8^{+} T-cell frequencies correlated with on-ART levels at years 1 and 4 ($r=0.40$–0.52, $P<0.001$; Fig. 2a). At 6–15 years on ART, PD-1^{HI} T-cell frequencies continued to correlate with pre-ART frequencies, albeit modestly ($r=0.28$; $P=0.04$). Pre-ART total PD-1^{+} CD4^{+} and CD8^{+} T-cell frequencies correlated very strongly with on-ART levels at years 1, 4, and 6–15 ($r=0.92$–0.95, $P<0.001$; Fig. 2b).

These results indicate that PD-1^{HI} T cells persist in the peripheral blood of ART-treated people with HIV at similar frequencies after years of durable viral suppression, and that these on-ART frequencies are correlated with pre-ART frequencies.

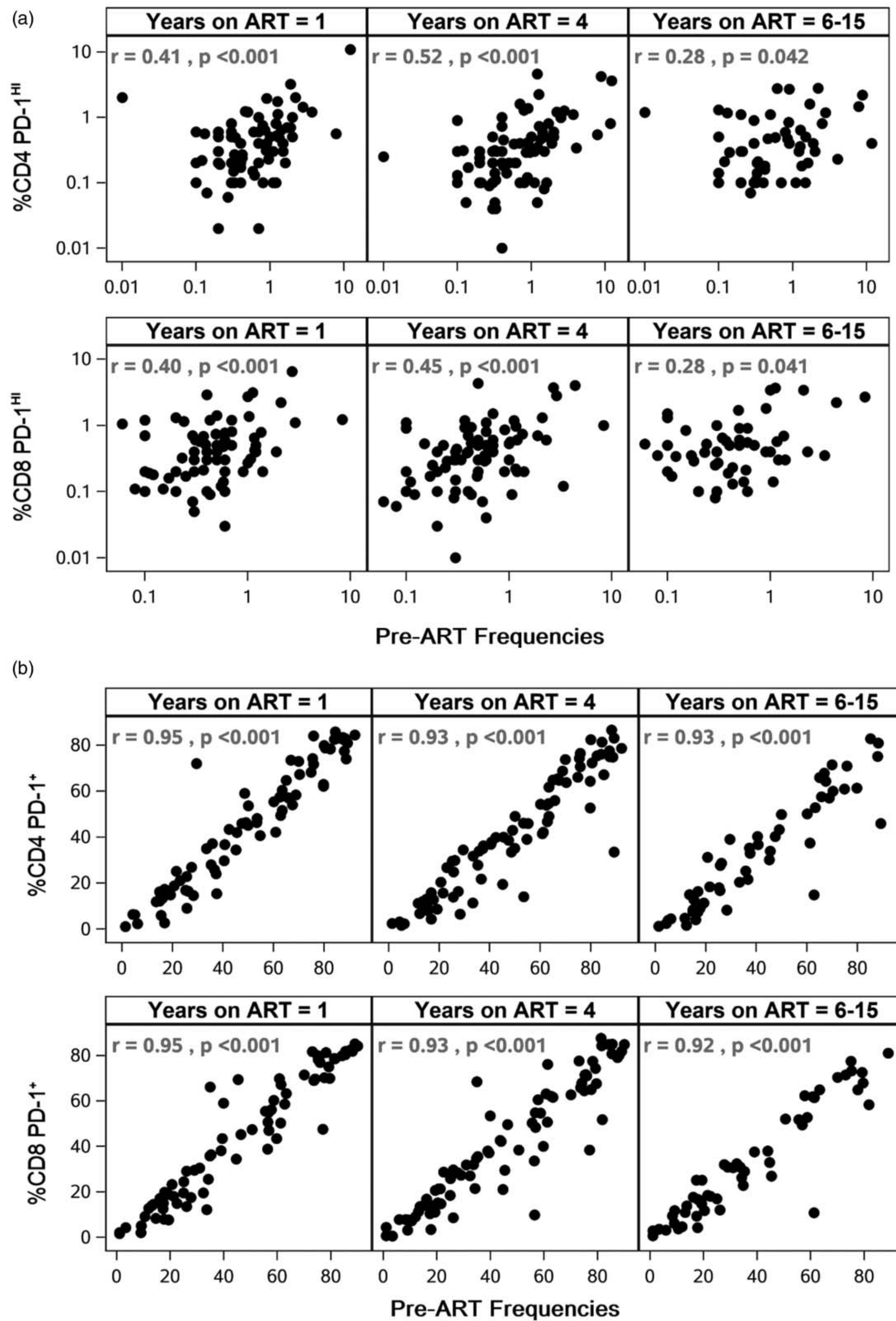

Fig. 2. Correlations of on-ART (a) PD-1^{HI} and (b) total PD-1^{+} T-cell frequencies with pre-ART percentages.

PD-1^{HI} T-cell frequencies and measures of viral persistence

We next assessed whether frequencies of PD-1^{HI}-expressing T cells were associated with different measures of HIV persistence on ART, specifically, residual viremia measured by SCA, HIV DNA, and cell-associated HIV RNA. There was no significant association observed between the level of residual viremia measured at the date

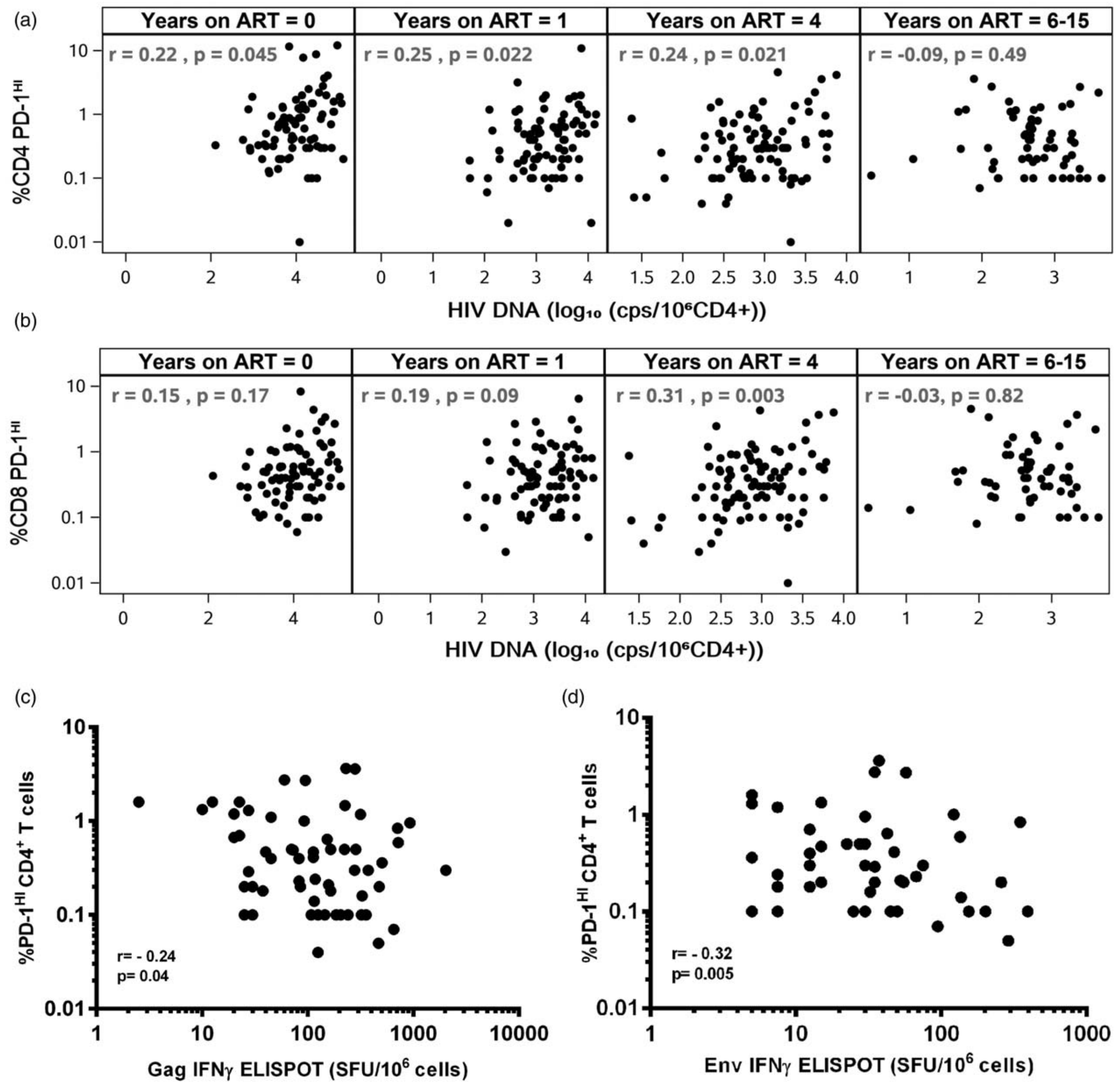

Fig. 3. Association between PD-1^{HI} (a) CD4$^+$ and (b) CD8$^+$ T-cell frequencies with levels of HIV-1 DNA pre-ART and on-ART and with interferon gamma ELISPOT responses to (c) Gag and (d) Env peptide pools. ART, antiretroviral therapy; PD-1, programmed cell death protein 1; PD-1^{HI}, high PD-1 expression.

of last sample collection (at four or more years on-ART), and the frequencies of CD4$^+$ and CD8$^+$ PD-1^{HI} T cells at the same time point (data not shown). Modest correlations were observed between levels of HIV DNA and PD-1^{HI} CD4$^+$ T-cell frequencies pre-ART ($r = 0.22$, $P = 0.045$; Fig. 3a). The positive correlations between HIV DNA and PD-1^{HI} CD4$^+$ T-cell frequencies remained significant, albeit modest, at 1 and 4 years on-ART ($r = 0.25-0.24$; $P \leq 0.022$). No significant correlations were seen between the %CD4$^+$ PD-1^{HI} T cells and cell-associated HIV RNA at any time point. PD-1^{HI} CD8$^+$ T-cell frequencies correlated with both HIV DNA ($r = 0.31$, $P = 0.003$; Fig. 3b) and cell-associated HIV RNA ($r = 0.27$, $P = 0.022$; data not shown) at year 4 on-ART but not at any other time point. In contrast, no correlations were observed between HIV DNA on-ART and frequencies of total PD-1$^+$ CD4$^+$ T cells (data not shown). These results suggest that the PD-1^{HI} subset of CD4$^+$ T cells could contain a higher frequency of HIV-infected cells before and after ART compared with CD4$^+$ T cells with any PD-1 expression.

PD-1^{HI} T-cell frequencies and HIV-specific T-cell immune responses

Given that loss of IFNγ secretion is observed in later stages of exhaustion in persistent viral infection [30,36], we determined whether PD-1^{HI} T-cell frequencies negatively associated with HIV-specific T-cell immune

Table 2. Association of PD-1^{HI} and total PD-1^{+} T-cell frequencies with interferon γ ELISPOT responses to HIV-1, CMV, and EBV peptide pools.

	Gag		Pol		Env		Nef, Tat, Rev		Vpr, Vpu, Vpf		EBV		CMV	
	r[a]	*P*	*R*	*P*	*r*	*P*	*R*	*P*	*R*	*P*	*r*	*P*	*r*	*P*
CD4^{+} PD-1^{+}	−0.03	*0.80*	0.15	*0.20*	−0.03	*0.82*	−0.03	*0.77*	−0.08	*0.51*	−0.14	*0.25*	−0.04	*0.71*
CD4^{+} PD-1^{HI}	**−0.24**	***0.04***	**−0.20**	***0.08***	**−0.32**	***0.005***	−0.10	*0.42*	**−0.22**	***0.07***	−0.05	*0.65*	0.04	*0.71*
CD8^{+} PD-1^{+}	−0.02	*0.89*	0.14	*0.26*	−0.02	*0.88*	−0.02	*0.87*	−0.08	*0.52*	−0.16	*0.18*	−0.04	*0.72*
CD8^{+} PD-1^{HI}	**−0.22**	***0.06***	−0.16	*0.19*	**−0.21**	***0.07***	−0.10	*0.51*	−0.19	*0.10*	−0.08	*0.49*	0.01	*0.42*

Correlations with *P* values 0.08 or less are bolded. *P* values are italicized. CMV, cytomegalovirus; EBV, Epstein Barr virus; PD-1, programmed cell death protein 1; PD-1^{HI}, high PD-1 expression
[a]Spearman *r* values.

responses by assessing IFNγ ELISPOT response to HIV peptide pools (15 amino acid overlapping by 11) for Gag, Pol, Env, Nef, Tat, Rev, Vpr, Vif, and Vpu [35] (Table 2). Specimens used for the ELISPOT assay were obtained at the date of last sample collection, that is, at 4 or more years on-ART. Both CD4^{+} and CD8^{+} PD-1^{HI} T-cell frequencies showed negative correlations with IFNγ responses to all HIV peptides, but only CD4^{+} PD-1^{HI} correlations with Gag ($r = -0.24$; $P = 0.04$) and Env ($r = -0.32$; $P = 0.005$; Table 2; Fig. 3c–d) responses reached significance. A modest negative trend was observed with Pol ($r = -0.2$; $P = 0.08$) and the combined Vpr, Vif, and Vpu peptide pool ($r = -0.22$, $P = 0.07$).

The frequency of CD8^{+} PD-1^{HI} T cells showed a trend for a negative correlation with the same HIV peptide responses (Gag, $r = -0.22$, $P = 0.06$; and Env, $r = -0.21$, $P = 0.07$, respectively). In contrast, no consistent trends or significant positive or negative correlations were found between total PD-1^{+} T-cell frequencies and HIV-specific responses. Similarly, no correlations were observed with PD-1^{HI} T-cell frequencies and CMV-specific or EBV-specific responses. These data support the conclusion that PD-1^{HI} T cells exhibit a more exhausted phenotype, and that this functional impairment is associated with decreased immune responses for specific HIV viral proteins (Gag and Env).

PD-1^{HI} T-cell frequencies and markers of inflammation and immune activation

We next evaluated whether PD-1^{HI} T-cell frequencies were associated with cellular and soluble immunologic markers. No associations were noted pre-ART between PD-1^{HI} T-cell frequencies and levels of T-cell activation (HLA-DR^{+}CD38^{+}, Supplemental Figure 5, http://links.lww.com/QAD/B552). However, correlations on-ART were observed between PD-1^{HI} CD4^{+} T cells and CD4^{+} T-cell activation at 1 and 4 years [Supplemental Figure 5.9, http://links.lww.com/QAD/B552 ($r = 0.40$, $P < 0.001$) and Supplemental Figures 5.11, http://links.lww.com/QAD/B552 ($r = 0.46$, $P < 0.001$)]. Modest correlations were observed between PD-1^{HI} CD8^{+} T cells and CD8^{+} T-cell activation at similar timepoints on-ART ($r = 0.20$–0.27, $P \leq 0.06$; Supplemental Figures 5.22 and 5.24, http://links.lww.com/QAD/B552). No consistent correlations were observed between percentage of total PD-1^{+} T cells and T-cell activation (Supplemental Figure 5.1–5.6 and 5.13–5.18, http://links.lww.com/QAD/B552). Finally, neither the PD-1^{HI} nor the total PD-1^{+} T-cell populations correlated with levels of T-cell cycling on-ART (data not shown). PD-1^{HI} T-cell frequencies were not associated with levels of the different soluble markers [IL-6, CRP, sCD14] apart from sCD163, the marker of macrophage activation, for which pre-ART %PD-1^{HI} CD4^{+} and CD8^{+} T cells correlated with pre-ART plasma levels ($r = 0.35$, $P = 0.001$ and $r = 0.28$, $P = 0.01$, respectively, data not shown). These results confirm the association between T-cell immune activation and PD-1^{HI} frequencies, suggesting that persistent immune activation could be driving T-cell exhaustion.

Discussion

We longitudinally evaluated the impact of ART on peripheral blood PD-1-expressing T cells and the subset of T cells with high PD-1 expression, and determined whether PD-1 expression correlated positively with measures of viral persistence and negatively with HIV-specific immune responses. Our results show that although PD-1^{HI} T cells constitute a small fraction of T cells in peripheral blood (median of 0.30–0.40% of T cells on ART), their frequencies remain stable despite prolonged viral suppression on ART. PD-1^{HI} CD4^{+} T-cell frequencies decreased only during the first year of ART, whereas PD-1^{HI} CD8^{+} T cells did not decline significantly with ART initiation. This stable persistence of PD-1^{HI} T cells on-ART is in contrast to total PD-1^{+} T cells that have much higher frequencies (median of 39.8% of CD4^{+} T cells after 4 years on-ART) with a very broad range (Q1–Q3 = 19.4–64.7) across individuals, and continuously decline in frequency with ART. Importantly, it was in the PD-1^{HI} subset that correlations, although mostly modest, were observed with measures of viral persistence, levels of immune activation, and HIV-specific immune responses.

Previous studies in both cancer and nonhuman primate (NHP) models of HIV infection have shown the significance of distinguishing PD-1^{HI} expressing T cells

from any level of PD-1 expression[24,25,29,37–39] and of the immunotherapeutic targeting of this subset in HIV infection [27]. Results of these studies offer insight into possible explanations for our findings. First, we found no changes in the frequencies of PD-1^{HI} T-cell subsets despite prolonged ART therapy. In the NHP model, PD-1^{HI} $CD4^{+}$ T cells significantly expand in the rectal mucosa in uncontrolled SIV infection [37]. PD-1^{HI} $CD4^{+}$ T cells retain survival potential as evidenced by higher expression of levels of Bcl-2, an antiapoptotic protein, and have enhanced proliferation, with increased Ki-67 expression. The enhanced proliferation, therefore, could contribute to viral persistence. Petrovas *et al.* [39], showed that SIV-specific $CD8^{+}$ T cells with PD-1^{HI} expression have increased generation rates, allowing them to sustain their frequencies. The enhanced proliferation of infected PD-1^{HI} $CD4^{+}$ T cells could, therefore, contribute to viral persistence. Although we did not see any correlation between PD-1^{HI} T-cell frequencies and overall T-cell cycling, it is possible that the PD-1^{HI} cells had higher Ki-67 expression, but their low frequencies in the blood would be unlikely to contribute significantly to overall levels of T-cell cycling. Second, PD-1^{HI} $CD4^{+}$ T-cell frequencies pre-ART and on-ART correlated with levels of HIV DNA, suggesting that PD-1^{HI} cells are more likely to carry proviruses than other PD-1 positive or negative cell subsets. In contrast with findings from Hatano *et al.* [17], we did not find correlations with total PD-1^{+} T cells. This could be because of differences in the demographics of study population as our cohort had a longer median duration of viral suppression and had higher levels of pre-ART $CD4^{+}$ T-cell counts. Our findings are consistent with NHP models showing that a significant proportion of PD-1^{HI} cells are infected in the rectal mucosa [37]. PD-1^{HI} $CD4^{+}$ T cells in virally suppressed individuals [18,19] also are enriched for proviral DNA HIV-infected PD-1^{HI} $CD4^{+}$ T cells, which could represent an important component of the HIV reservoir that is difficult to eliminate, given the long-term survival of PD-1^{HI} cells. If PD-1^{HI} cells are indeed enriched for HIV DNA, then the increased proliferation could also be because of clonal proliferation of infected cells [40,41], which contributes to viral persistence.

The significant negative association between PD-1^{HI} $CD4^{+}$ T cells and Gag and Env peptide-IFNγ ELISPOT responses suggests that exhausted $CD4^{+}$ T cells provide less helper function, resulting in decreased HIV-specific $CD8^{+}$ T-cell immune responses. Indeed, trends towards significant negative correlations between PD-1^{HI} $CD8^{+}$ T cells and the ELISPOT responses to Gag and Env were also observed. Lower levels of IFNγ and IL-2 production by PD-1^{HI} $CD4^{+}$ T cells [37] could lead to diminished $CD8^{+}$ T-cell proliferation and differentiation, hence lower IFNγ responses upon stimulation with HIV antigen. The fact that PD-1^{HI} $CD4^{+}$ frequencies correlated negatively with HIV-specific responses but not with EBV or CMV responses suggests but does not prove that PD-1^{HI} T cells are enriched for HIV-specific $CD4^{+}$ T cells. Evaluation of T cells using HIV-specific multimer staining could address this hypothesis. If confirmed, then this finding supports a previous study that show HIV DNA is maintained in antigen-specific $CD4^{+}$ T cells among individuals on ART [42]. Taken together, our findings of PD-1^{HI} CD4 lymphocyte persistence despite long-term ART, modest negative correlations between %PD-1^{HI} $CD4^{+}$ T-cell frequencies, and modest positive correlations with HIV DNA levels, not only support previous findings but also suggest that these cells could contribute to viral persistence as they are enriched for infected cells and are functionally exhausted, thus unable to provide help to HIV-specific $CD8^{+}$ lymphocytes.

Our study has limitations. Given issues with sample availability at certain timepoints, we were only able to evaluate PD-1 expression on all T cells rather than HIV-specific T cells. We were also unable to do functional studies on the PD-1^{HI}-expressing population and thereby unable to confirm functional impairment of the PD-1^{HI} cells apart from negative associations with HIV-specific immune responses. In addition, differences in the number of participant specimens analyzed for the PD-1 expression per time point, specifically the lower number of participants at the 6–15-year time point (63 vs. 82–93 in the earlier time points), could have affected the power to detect associations for the later time point. Third, because of the lack of seronegative controls in this study, we were unable to evaluate whether our results were specific to people living with HIV. Finally, co-expression of other inhibitory receptors not characterized in our study is also an important hallmark of T-cell exhaustion.

In conclusion, our study indicates that high expression of PD-1 persists in a small percentage of T cells despite prolonged viral suppression on ART. Frequencies of these cells in blood are stable despite prolonged ART, and are positively associated with pre-ART frequencies, levels of HIV DNA, and levels of T-cell activation. Negative correlations between PD-1^{HI} cells and HIV-specific immune responses suggest functional impairment of HIV-specific PD-1^{HI} cells. The contribution of PD-1^{HI} T-cell subset to HIV persistence as long-lived infected cells and as immune cells with impaired HIV-specific immune responses underscores the importance of evaluating whether interventions blocking PD-1 reduce HIV persistence and improve HIV-specific immunity.

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Conflicts of interest

There are no conflicts of interest.

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