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# PD-1 contributes to the establishment and maintenance of HIV-1 latency

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## Abstract

**Objective—**In HIV-infected individuals on antiretroviral therapy (ART), latent HIV is enriched in $CD4^+$ T-cells expressing immune checkpoint molecules (ICs), in particular programmed cell death-1 (PD-1). We therefore assessed the effect of blocking PD-1 on latency, both in vitro and in vivo.

**Methods—**HIV latency was established in vitro following co-culture of resting $CD4^+$ T-cells with myeloid dendritic cells. Expression of PD-1 was quantified by flow cytometry, and latency assessed in sorted PD-$1^{high}$ and PD-$1^{low/-}$ non-proliferating $CD4^+$ memory T-cells. The role of PD-1 in the establishment of latency was determined by adding anti-PD-1 (pembrolizumab) to co-

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cultures before and after infection. Additionally, a single infusion of anti-PD-1 (nivolumab) was administered to an HIV-infected individual on ART with metastatic melanoma, and cell-associated (CA) HIV DNA and RNA, and plasma HIV RNA were quantified.

**Results—**HIV latency was significantly enriched in PD-1$^{high}$ compared to PD-1$^{low/-}$ nonproliferating, CD4$^+$ memory T-cells. Sorting for an additional IC, T-cell immunoglobulin domain and mucin domain-3 (Tim-3), in combination with PD-1 further enriched for latency. Blocking PD-1 prior to HIV infection, in vitro, resulted in a modest but significant decrease in latently infected cells in all donors (n=6). The administration of anti-PD-1 to an HIV-infected individual on ART, resulted in a significant increase in CA HIV RNA in CD4$^+$ T-cells, without significant changes in HIV DNA or plasma HIV RNA, consistent with reversal of HIV latency.

**Conclusions—**PD-1 contributes to the establishment and maintenance of HIV latency and should be explored as a target, in combination with other ICs, to reverse latency.

### Keywords

HIV-1; virus latency; CD4-positive T-lymphocytes; PD-1 receptor; pembrolizumab; nivolumab; ipilimumab

## Introduction

Long-lived latently infected resting memory CD4$^+$ T-cells persist in HIV-infected individuals on antiretroviral therapy (ART), and are a major barrier to HIV cure[1]. Latent infection is enriched in CD4$^+$ T-cells expressing immune checkpoint molecules (ICs), including programmed cell death-1 (PD-1)[2–4]. ICs regulate the immune response via suppression of T-cell activation[5, 6], following ligation of the T-cell receptor with a specific antigen peptide-major histocompatibility complex molecule displayed on antigen presenting cells[7]. We hypothesized that through this process, IC signalling may inhibit productive HIV infection, thereby facilitating the establishment of latent HIV infection.

If the expression of ICs is important in establishing and/or maintaining latency, then the administration of a blocking antibody could potentially reverse latency. Here, we investigate the effects of anti-PD-1 on HIV latency both in vitro, using a co-culture model of myeloid dendritic cells (mDC) and resting CD4$^+$ T-cells[8], and in vivo following administration of anti-PD-1 to a single HIV-infected individual on suppressive ART.

## Methods

### Isolation of cellular subsets

Peripheral blood mononuclear cells (PBMC) were isolated from HIV-uninfected donors (Australian Red Cross Blood Service, Melbourne, Australia). Resting (CD69$^-$HLA-DR$^-$) CD4$^+$ T-cells and HLA-DR$^+$CD11c$^+$CD123$^-$ mDC were isolated as previously described[8].

### Plasmids, virus production and infection

CCR5-tropic, replication and Nef competent EGFP-reporter virus, NLAD8-E, was kindly provided by Yasuko Tsunetsugu-Yokota (National Institute of Infectious Diseases, Tokyo,

Japan)[9]. HIV stocks were generated by FuGene (Promega, Madison, WI) transfection of 293T cells[8]. Cells were infected at 37°C for 2hrs (MOI=0.5, determined by limiting dilution on PBMC[10]), and then washed to remove unbound virus.

### In vitro HIV latency model

HIV latency was established in vitro as previously described[8]. Briefly, eFluor®670-labelled resting $CD4^+$ T-cells were cultured with syngeneic mDC (DC:T-cell ratio 1:10) and infected with NLAD8-E. At day 5 post-infection, productive infection ($EGFP^+$ cells) was determined. The eFluor670$^{high}$EGFP$^-$ T-cells were sorted and cultured for 72hrs (interleukin (IL)-7+Raltegravir, kind gift from Merck), with or without stimulation (anti-CD3/CD28). Post-integration latency was defined as the number of $EGFP^+$ cells in stimulated minus unstimulated cultures.

### Phenotyping and sorting

PD-1 ligand expression was determined using anti-PD-L1-PE or PD-L2-PE, combined with HLA-DR-perCP (BD, Franklin Lakes, NJ). PD-1 expression was determined using anti-PD-1-PE/isotype control (BioLegend, San Diego, CA), in combination with anti-CD3-PB, CD45RA-V500 and HLA-DR-APC-Cy7 (BD). At day 5 post-infection eFluor670$^{high}$EGFP$^-$CD45RA$^-$ T-cells were sorted into PD-1$^{high}$ and PD-1$^{low/-}$ populations. Some donors were labelled with both anti-PD-1-PE and anti-Tim-3-PE-Cy7 (BioLegend), and cells positive for both or either IC (PD-1/Tim-3$^{high}$), or cells negative for both IC (PD-1$^{low/-}$Tim-3$^{low/-}$) were sorted.

### Blocking experiments

Resting $CD4^+$ T-cells were incubated with anti-PD-1 (pembrolizumab) or isotype control (anti-respiratory syncytial virus (RSV); IgG4; 10ug/mL; Merck, Kenilworth, NJ), at 37°C for 30mins prior to co-culture with mDC. Following infection with NLAD8-E, anti-PD-1 (10ug/mL) was re-added to cell cultures. At day 5 post-infection, IFNγ levels were quantified by ELISA (MAX Deluxe kit, BioLegend), and pembrolizumab binding saturation determined following staining with anti-CD45RA-V500 (BD) and anti-PD-1-PE (BioLegend).

### PCR assays

RNA and DNA were extracted (Allprep isolation kit, Qiagen) from total blood $CD4^+$ T-cells (MACS; Miltenyi; purity >95%). Cell-associated unspliced (CA-US) RNA (lower limit of detection (LLOD)=1 copy/well), and total HIV DNA was quantified as previously described[11]. Plasma HIV RNA was quantified using both a standard Roche (LLOD=20 copies/mL) and an ultra-sensitive single copy assay (SCA; LLOD=0.13 copies/mL), as previously described[11, 12].

### Flow cytometry

Cell sorting was performed using a FACSAria (BD) or MoFlo® Astrios (Beckman Coulter, Brea, CA). Analysis was performed using a FACSCalibur or LSRII (BD), and Weasel software (Walter and Elisa Hall Institute, Melbourne, Australia).

### Statistical analysis

Statistical analyses were performed using Prism Software (GraphPad 6.0). A paired t test was used where n<6; a Wilcoxon signed-rank test where n≥6[13].

## Results

### Expression of PD-1 and its ligands within a DC-T-cell model of latency

Expression of PD-1 and its ligands PD-L1 and PD-L2 were determined within our previously described mDC-T-cell co-culture model of HIV latency[8].

PD-1 expression was low on resting $CD4^+$ T-cells isolated from HIV-neg donors (mean 2.4%, n=4). However, co-culture with mDC for 2–5 days significantly increased PD-1 expression on the $eFluor670^{high}EGFP^-$ (non-proliferating, non-productively infected) memory $CD4^+$ T-cells, when compared to T-cells cultured alone (Figure 1A, mean fold change (MFC)=2.9 at day 5 post-infection, p=0.01). Similar results were observed in uninfected and HIV-infected cultures (Figure 1A). Cell viability was comparable in the presence and absence of mDC (data not shown).

PD-L1 and PD-L2 expression was low on freshly isolated mDC (mean 0.4% and 0.2% respectively), as previously reported[14]. However, following co-culture with resting T-cells, and infection with NLAD8-E, mDC significantly up-regulated PD-L1 (Figure 1B, mean 13.5%, p=0.01) and PD-L2 (mean 2.2%, p=0.03). Similar expression levels were observed in uninfected co-cultures (data not shown).

### HIV latency is enriched in PD-1 expressing $CD4^+$ T-cells within a DC-T-cell model of latency

At day 5 post-infection, $eFluor670^{high}EGFP^-$ memory $CD4^+$ T-cells were sorted into $PD\text{-}1^{high}$ and $PD\text{-}1^{low/-}$ populations (Figure 1C). Inducible post-integrated latency was significantly enriched in cells expressing PD-1 (MFC=6.6, p=0.007, n=7) compared to cells with little to no PD-1 (Figure 1D). These findings are consistent with what has been demonstrated ex vivo using $CD4^+$ T-cells from HIV-infected individuals on ART[3, 4], and support the use of this in vitro model to test the effect of IC blockade on HIV latency.

### Blocking PD-1 modestly inhibits HIV latency establishment in non-proliferating $CD4^+$ T-cells

We next determined whether blocking PD-1 could inhibit the establishment of latency within our DC-T-cell model. Anti-PD-1 (pembrolizumab), approved for the treatment of advanced melanoma[15] and other malignancies, was added to resting $CD4^+$ T-cells prior to co-culture with mDC, and again following infection with NLAD8-E, to block interactions between PD-1 and its ligands.

At 10μg/mL, anti-PD-1 was both functional, inducing a significant increase in supernatant interferon-gamma (IFNγ; Figure 1E), and saturating, preventing binding of anti-PD-1-PE (Figure 1F).

In the presence of anti-PD-1, we observed a modest, but statistically significant, decrease in latent infection (number of $EGFP^+$ cells following anti-CD3/28 stimulation of sorted

eFluor670$^{high}$EGFP$^{-}$ cells) in all donors compared to isotype control (MFC=1.6, p=0.01, n=6; Figure 1G). There was no significant difference in productive infection (Figure 1G). This was unexpected, and might be explained by death of the productively infected cells, or possibly, a small increase may have been masked by the higher absolute numbers of EGFP$^{+}$ cells in the productively infected population. Additionally, we observed a 1.5 mean fold decrease in the ratio of latent to productive infection (n=6; Figure 1H), although, this did not reach significance (p=0.1) due to one donor where the ratio increased. Excluding this donor resulted in a significant decline in this ratio (p=0.03, MFC=1.6).

Together, this data suggests that while HIV is enriched in PD-1$^{high}$ cells, blocking PD-1 alone was not sufficient to completely prevent the establishment of latency. Other ICs may also contribute to the establishment of mDC-induced latency[4]. Indeed, sorting for the IC, T-cell immunoglobulin domain and mucin domain-3 (Tim-3), in addition to PD-1 (including both single (majority) and double positive cells), within our DC-T-cell latency model, resulted in a further enrichment of latency (MFC=15, p=0.02, n=6; Supplementary Figure 1, http://links.lww.com/QAD/B276), as compared to experiments where cells were sorted for PD-1 alone (MFC=6.6; Figure 1 D). However, further experiments are required to determine whether blocking other ICs, in combination with PD-1, will prevent the establishment of HIV latency.

### Administration of nivolumab to an HIV-infected individual on suppressive ART leads to an increase in CA-US HIV RNA

In a previous case report of an HIV-infected individual on ART, we demonstrated a 20-fold increase in CA-US HIV RNA in CD4$^{+}$ T-cells following multiple infusions of anti-CTLA-4 (ipilimumab), consistent with reversal of HIV latency[16]. This same individual received a single intravenous infusion of anti-PD-1 (nivolumab, 3mg/kg; Figure 2A) for the treatment of metastatic melanoma. Seven days post-administration of nivolumab, we observed a significant increase in CA-US HIV RNA (fold change (FC) =24.3; p=0.0001; Figure 2B) and an increase in the ratio of CA-US HIV RNA:HIV DNA (FC=7.9; Figure 2C), with no significant change in plasma HIV RNA (Figure 2D), as compared to pre-nivolumab levels. The individual died shortly after, due to complications of metastatic melanoma. Although only a single case report, these data demonstrate a significant change in CA-US HIV RNA, consistent with latency reversal, following the administration of nivolumab *in vivo*.

## Discussion

We have provided evidence that PD-1 contributes to the establishment and maintenance of HIV latency, and that antibodies to ICs, including anti-PD-1, should be further explored as a strategy to disrupt or reverse HIV latency.

We have developed a physiologically relevant *in vitro* model of DC-induced primary T-cell latency[8], that will enable us to now screen multiple IC blockers for their ability to disrupt HIV latency, alone and in combination. Within this model, latency was enriched in PD-1$^{high}$ CD4$^{+}$ T-cells. PD-1 blockade prior to infection, reduced, but did not eliminate, the establishment of latency. The persistence of other ICs, which are also enriched for latent HIV, as demonstrated in CD4$^{+}$ T-cells from HIV-infected individuals on ART[4], may

explain why anti-PD-1 alone did not abrogate latency in our model. Co-expression of these ICs is minimal[17, 18], therefore, it is likely that a combination IC blockade approach will be necessary. Given the successful clinical trials with concurrent administration of anti-CTLA-4 and anti-PD-1 in individuals with malignancy[19, 20], this is potentially feasible, although toxicity remains a significant concern with this combination[21]. Next generation engineered IC blockers, such as afucosylated anti-CTLA-4 (BMS-986218), may potentially have lower toxicities [ClinicalTrials.gov *identifier* **NCT03110107].**

Administration of IC blockers in HIV-infected individuals on ART may not only reverse latent infection, but could also have the added benefit of enhanced immune clearance of latently infected cells [recently reviewed in[22]]. Blocking interactions between PD-1 and PD-L1, as well as CTLA-4 and its ligands (CD80 and CD86), has been shown to increase HIV-specific $CD4^+$ and $CD8^+$ T-cell function both *ex vivo*[23], and *in vivo* in SIV-infected macaques[24]. In a recent case report, the administration of multiple repeat doses of anti-PD-1 (nivolumab) to an HIV-infected individual on ART was well tolerated and resulted in a dramatic increase in HIV-specific $CD8^+$ T-cells, a modest and delayed increase in plasma HIV RNA and a 3 log decrease in HIV DNA[25]. The mechanism of how HIV DNA decreased in this case was unclear, but could potentially be a consequence of T-cell proliferation, which would dilute unintegrated HIV DNA that is also included in a total DNA assay.

Increases in HIV-specific $CD8^+$ T-cells have also been observed in two of six otherwise healthy HIV-infected individuals following a single dose of anti-PD-L1 (BMS-936559). In this study, however, there was no effect on plasma HIV RNA or CA HIV RNA or DNA[26]. The different virological findings between this clinical trial and our case report may have several explanations. First, a very low dose of 0.3mg/kg anti-PD-L1 was given, while in our case report, the full dose of 3mg/kg of nivolumab was administered. Second, following the administration of anti-PD-L1, ligation of PD-1 is still possible through PD-L2. PD-L2 has multiple functions in addition to binding to PD-1 and, in some settings, can enhance rather than suppress T-cell function[27, 28]. Surprisingly, we were unable to find any publications on the relative expression of PD-L1 and PD-L2 in HIV-infected individuals on ART. Third, the level of PD-1 expression and signalling may potentially differ in the setting of disseminated malignancy compared to otherwise healthy HIV-infected individuals on ART. Fourth, the activity of anti-PD-1 (or anti-PD-L1) on latency reversal could potentially vary significantly in different recipients, as well described in responses to both antibodies for the management of malignancy[29]. Finally, it is unclear whether the degree of latency reversal observed here was in any way related to the prior administration of ipilimumab[16]. In clinical trials of individuals with metastatic melanoma, a clinical response to ipilimumab may predict a subsequent response to nivolumab[30].

Here, we report that anti-PD-1 led to an immediate, marked increase in CA-US HIV RNA, with no observable change in HIV DNA, in an HIV-infected individual on ART followed for 7 days after a single dose of nivolumab. Together, these data suggest that anti-PD-1 can have a direct effect on latently infected $CD4^+$ T-cells and should be further explored as a clinical intervention in HIV-infected individuals on ART.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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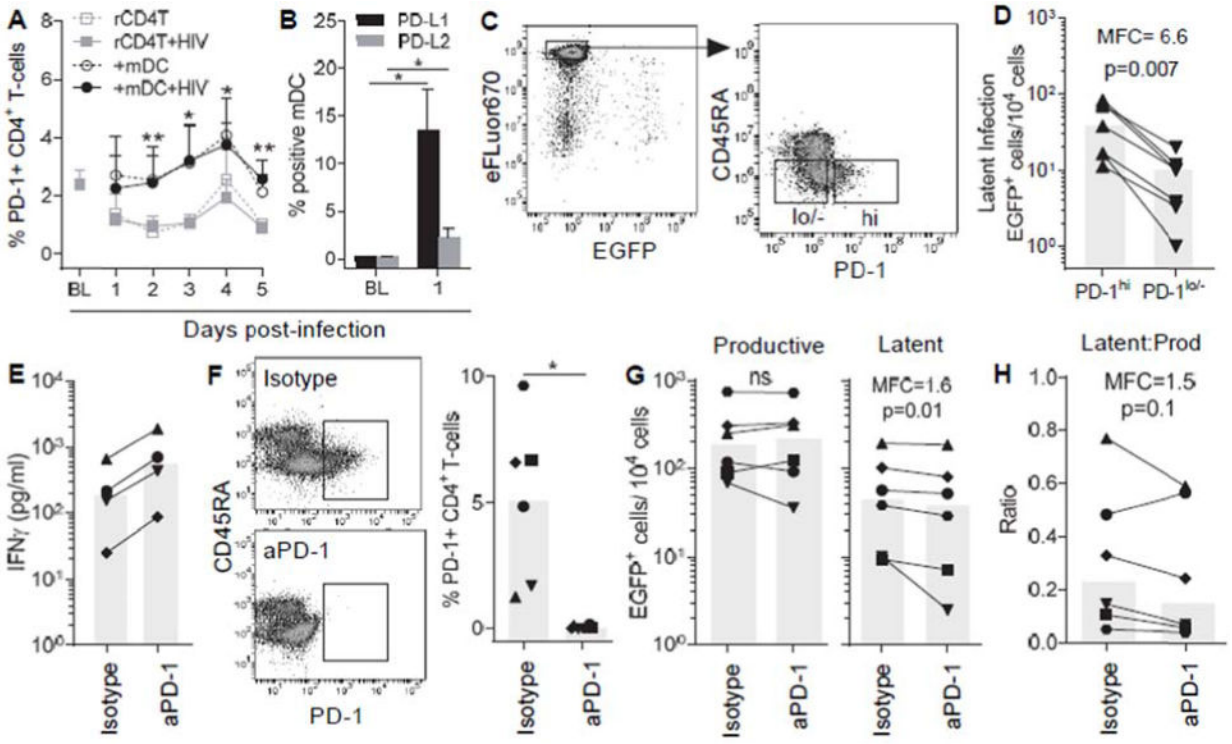


**Figure 1. Role of PD-1 in an in vitro model of HIV latency**

Resting CD4$^+$ T-cells, labelled with eFluor670, were cultured alone or with syngeneic mDC for 24h, infected with NLAD8-E and cultured for a further 5 days. (A) PD-1 expression was determined on resting CD4$^+$ T-cells at baseline (BL), and on eFluor670$^{high}$EGFP$^-$ CD4$^+$ T-cells during culture. Points represent the mean of 4 donors and error bars the standard deviation (SD). (B) PD-L1 and PD-L2 expression was determined on mDC at BL, and 1 day post-infection of mDC-T-cell co-cultures with NLAD8-E (day 1). (n=6; mean (SD) is shown). (C) At day 5 post-infection, eFluor670$^{high}$EGFP$^-$ memory CD4$^+$ T-cells were sorted into PD-1$^{high}$ and PD-1$^{low/-}$ populations; (D) inducible latency was determined in the sorted cells, measured by EGFP expression. Each symbol represents a different donor; MFC=mean fold change (E-H). Anti-PD-1 (10ug/mL; pembrolizumab, Merck), or isotype control, was added to resting CD4$^+$ T-cells prior to co-culture with mDC, and re-added following infection. Each donor is represented by the same symbol; columns represent the median. (E) IFNγ production was determined in supernatants at day 5 post-infection. (F) To confirm a saturating level of neutralising antibody, cells treated with isotype or anti-PD-1 were labelled with anti-CD45RA-V500 and anti-PD-1-PE at day 5 post-infection. (G) Productive infection was determined at day 5 post-infection and inducible latency was determined in sorted memory eFluor670$^{high}$EGFP$^-$ CD4$^+$ T-cells. (H) The ratio of latent to productive infection is shown. For all panels, p-values were determined by paired t test (n<6) or Wilcoxon signed-rank test (n≥6); *p<0.05, **p<0.01.

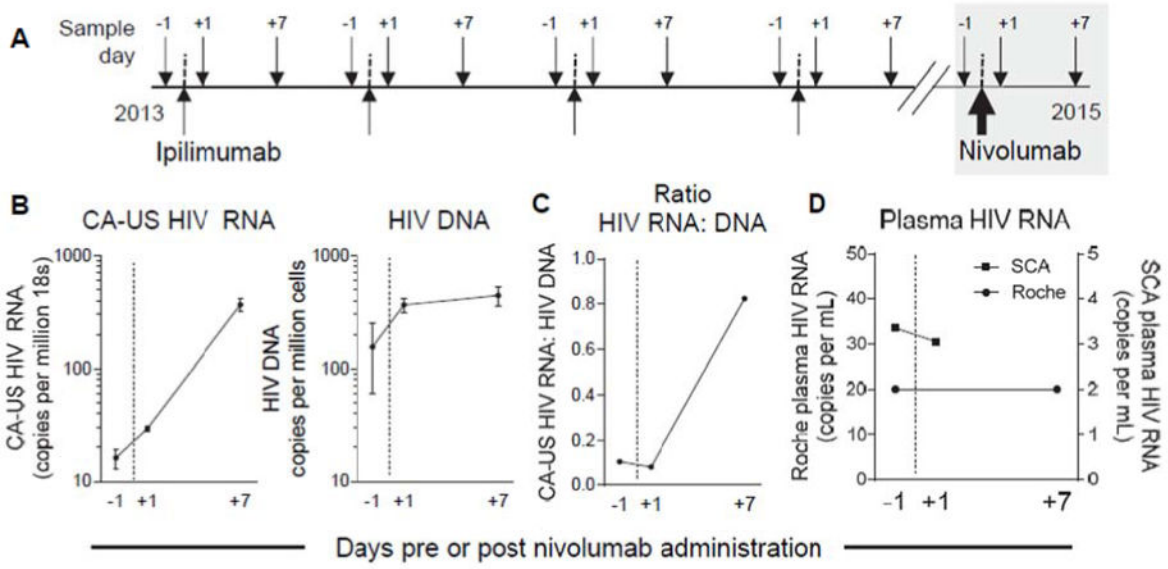


**Figure 2. Increase in cell associated HIV RNA following anti-PD-1 in an HIV-infected individual on suppressive ART**

(A) A single intravenous administration of anti-PD-1 (nivolumab, BMS-936558, 3mg/kg; bold arrow) was given to an HIV-infected individual on suppressive ART, following multiple infusions of anti-CTLA-4 (ipilimumab). (B) Prior to, and following, administration of nivolumab, CA-US HIV RNA and HIV DNA were quantified in sorted $CD4^+$ T-cells, and (C) the ratio of CA-US HIV RNA to HIV DNA calculated. CA-US HIV RNA and HIV DNA assays were performed in quadruplicate and triplicate respectively, the mean +/− standard error of the mean is shown. (D) Plasma HIV RNA levels were determined using a standard commercial assay (Roche; LLOD = 20 copies/mL) and an ultrasensitive single copy assay (SCA; LLOD = 0.13 copies/mL). HIV RNA measured by SCA was quantified in triplicate and the mean is shown.