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Human immunodeficiency virus (HIV-1) infection selectively downregulates PD-1 expression in infected cells and protects the cells from early apoptosis *in vitro* and *in vivo*

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ABSTRACT

Programmed Death-1 (PD-1), a member of T cell costimulatory molecules is expressed in high levels on antigen specific T cells during chronic viral infection, whereas PD-1 expression in the context of HIV-1 infected CD4+ T cells is not known. Here we report that productively infected CD4+ T cells lose PD-1, whereas bystander cells were unaffected. Additionally, p24+/PD-1 negative cells are less susceptible to apoptosis compared to bystander cells in the same infected milieu. Similar results were observed *in vivo*, as infected T cells isolated from HIV-1+ individuals have significantly low level of PD-1 and the observed loss of PD-1 *in vivo* is independent of viral load, CD4 count, and/or antiviral treatment. Together these results indicate that productively infected cells are resistant to early apoptosis by downregulating PD-1, whereas PD-1 enhances the susceptibility of effector T cells to apoptosis suggesting a dual role for PD-1 during HIV-1 infection.

Introduction

The Programmed Death-1 (PD-1) molecule, a member of the B7 family of costimulatory molecules plays a key role in the regulation of T cell activation, autoimmunity, tolerance and apoptosis (Greenwald et al., 2005; Zha et al., 2004). PD-1 is an inhibitory molecule that is induced upon TCR ligation and expressed on activated T cells, B cells, NKT cells and activated monocytes (Meng et al., 2006; Nielsen et al., 2005). Recent studies report that expression of PD-1 is high in virus specific CD8+ cells in HIV-1 and HCV positive patients and these cells exhibit an exhausted phenotype (Barber et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006; Zhang et al., 2007). Additionally, these cells are more susceptible to activation-induced and/or other apoptotic pathways suggesting that PD-1 positive cells are eliminated *in vivo* through several mechanisms (Freeman et al., 2006). Blocking the PD-1: PD-L1 (PD-Ligand 1) pathway in antigen specific CD8+ T cells using PD-L1 antibody enhanced their ability to proliferate in response to HIV-1 antigen (Day et al., 2006; Freeman et al., 2006; Grakoui et al., 2006).

During HIV-1 infection both CD4+ and CD8+ T cells are depleted in significant amounts and this loss is not specific to infected cell population (Cotton et al., 1996; Holm and Gabuzda, 2005; Mehandru et al., 2004; Varbanov, Espert, and Biard-Piechaczyk, 2006). Both viral and host cellular factors are known to play a role in T cell depletion during HIV-1 infection (Azad, 2000; Moon and Yang, 2006). More importantly, infected cells are protected from apoptosis as a means to aid virus production and dissemination, whereas, bystander cells are targeted for apoptosis (Carbonari et al., 1997; Conti et al., 1998; Finkel et al., 1995; Lelievre et al., 2004). Although role of PD-1 in HIV-1 antigen specific T cells (CD4 and CD8) is established, PD-1 expression in the context of infected T cells is not known. Using normal donor primary peripheral mononuclear cells (PBMC), and HIV-1-EGFP reporter virus, we assessed PD-1 expression on HIV-1 infected and bystander T cells. Results indicate that productively infected T cells selectively lose PD-1 expression, whereas, uninfected cells were not affected. Furthermore, PD-1−/EGFP+ cells were resistant to apoptosis compared to PD-1+/EGFP− cells.

To understand whether a similar phenomenon occurs *in vivo*, we extended our analysis in infected T cells isolated from HIV-1 positive individuals. Similar to our *in vitro* observations, HIV-1 patients' lymphocytes that are positive for HIV-1 p24 did not express detectable level of PD-1, whereas uninfected bystander cells expressed PD-1. Downregulation of PD-1 in infected cells *in vivo* is independent of viral load, basal level of host cellular PD-1 expression and/or antiviral therapy. Furthermore, the p24 positive cells also expressed a high level of anti-apoptotic protein, Bcl-2, suggesting that virus infection in early stages might protect the infected cells from virus-induced and/or activation-induced cell death through PD-1 downregulation, whereas antigen specific T cells are exhausted. Together these results indicate that HIV-1 infection differentially regulates PD-1 expression and protect the infected cells from apoptosis during early infection as a mechanism to maximize virus production.

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Results

Downregulation of PD-1 in HIV-1 infected culture

In HIV-1 infected patients, antigen specific T cells (both CD4 and CD8) are known to express a high level of PD-1. However, in the context of infection the level of PD-1 expression is unknown. Therefore, first we evaluated the PD-1 expression level in HIV-1 infected and uninfected culture from the same donor. To address the expression pattern of PD-1 within the infected culture, PHA-P or anti-CD3/anti-CD28 stimulated PBMC were infected with 0.01 MOI of HIV-1-EGFP virus as described (Venkatachari, Majumder, and Ayyavoo, 2007). Live CD3+ cells were gated (shown in Fig. 1) and PD-1 expression was evaluated on both CD4+ and CD8+ populations on day 5 postinfection. Results indicate that there is a 50% loss of PD-1 expression in CD4+ cells in the infected culture (9.7%) compared to CD4+ cells in the uninfected culture (17.2%), whereas, PD-1 level in CD8+ T cells within these two cultures are unaffected (Fig. 1A). Next, to understand the time kinetics involved in PD-1 dysregulation, we assessed PD-1 expression pre and postinfection. Expression of PD-1 in unactivated T cells on day 0 was <5% and activation with anti-CD3/anti-CD28 or PHA-P increased PD-1 up to 20% on day 3 (data not shown). Three days postinfection there was a significant loss in PD-1 within the infected culture and this loss is maintained up to 9 days. This directly correlated with the percentage of infected cells (based on EGFP+) present in the culture suggesting that productive infection is required for PD-1 loss and virus binding/entry does not alter PD-1 expression.

Next, to validate the above finding, we treated activated PBMC with AT-2 treated inactivated virus that is not defective in binding or entry and assessed PD-1 level in CD4+ cells (Fig. 1C). Results indicate level of PD-1 in CD4+ T cells remained similar to uninfected culture, whereas, a significant loss was observed in PBMC infected with replication competent virus. Collectively, these results indicate that loss of PD-1 in the infected culture is due to productive infection and not due to virus binding, entry and/or exposure to virion-associated HIV-1 proteins.

Productively infected CD4+ target cells lose PD-1 expression

Results above indicate that CD4+ cells lose PD-1 within the infected culture. Next to identify whether this effect is seen specifically in the infected cells, 3 days postinfection, live CD3+ cells were gated and PD-1 expression was assessed within the infected (EGFP+) and bystander (EGFP−) population by flow cytometry. Results indicate that cells that are productively infected (based on EGFP expression) with HIV-1 are negative for PD-1 expression (>95–98%), whereas uninfected bystander cells did not show loss of PD-1, suggesting that virus replication results in loss of PD-1 expression (Fig. 2A). Next, we assessed whether the amount of virus production and/or the percentage of productively infected cells present within the infected milieu alters the expression of PD-1. To address this we varied the infectivity and number of infected cells using various amounts of virus inoculum that resulted in 5–20% infection and assessed PD-1 expression (data not shown). Results indicate that infected cells lose PD-1 expression irrespective of the percent EGFP positive cells present within the culture or the amount of virus produced within an infected cell as measured by EGFP MFI level (low producers versus high producers). Using multiple donors ($n=15$), we compared PD-1 expression between the control cells (no treatment), productively infected and uninfected bystander cells present within the infected milieu (Fig. 2B). Compared to the no treatment control group, productively infected cells exhibit a significant loss of PD-1 ($p<0.0001$), whereas uninfected bystander cells showed a slight decrease in PD-1 expression (<3%) that is not statistically significant ($p=0.13$). As a specificity control we also evaluated other cell surface molecules, such as HLA-DR, and CD3 (data not shown) and results indicate no change in these molecules further confirming the specific effect of virus infection on PD-1 expression.

To rule out whether this phenomenon is not specific to the particular strain of HIV-1 (NL43) we used here, PBMC were infected with several HIV-1 isolates that are CCR5- or CXCR4-receptor utilizing as well as primary viral isolates from clade A, C and B and assessed for PD-1 expression. Using p24 (Gag) specific staining infected and uninfected cells were separated and PD-1 expression was assessed within these cells (Fig. 2C). Results indicate that >95% of p24 positive cells are negative for PD-1 expression, suggesting that virus replication results in loss of PD-1 and this property is highly conserved across all HIV-1 viral isolates.

HIV-1 regulates PD-1 expression at the transcriptional level

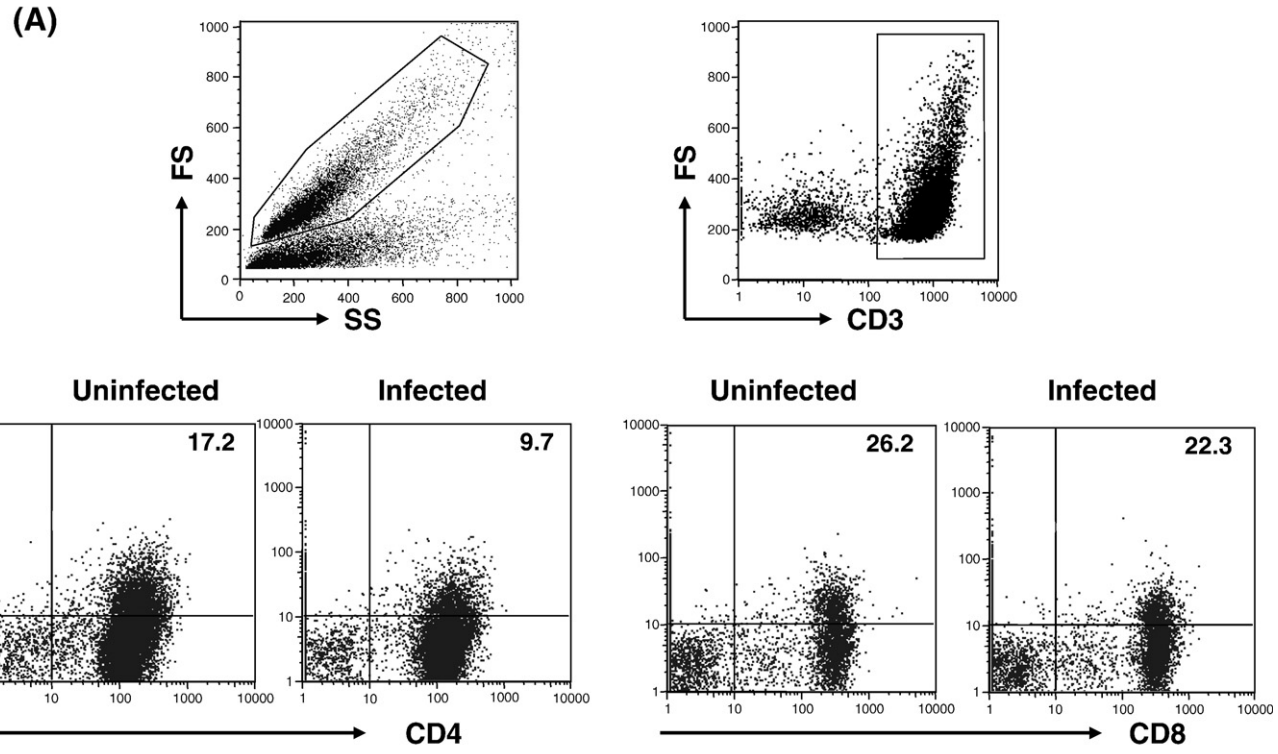
PD-1 is expressed on T cells that are targets for HIV-1 infection. HIV-1 viral proteins, Nef and Vpu are known to internalize host cell surface molecules (CD4, MHC-I) as part of an immune evasive strategy (Kamp et al., 2000; Piguet et al., 1999). To understand if PD-1 is internalized during infection and whether this could be the reason for not detecting PD-1 surface expression, we performed both surface and intracellular staining of PD-1 by flow cytometry. Results indicate PD-1 is not detected by intracellular staining within the infected cells, whereas internalized MHC-I was detected (as a positive control) suggesting that loss of PD-1 surface expression is not due to internalization (Fig. 3A). These results were further confirmed by immunofluorescence analysis using PD-1 specific antibody. While EGFP+ infected cells did not show PD-1 expression, uninfected cells exhibited a distinct cell surface expression of PD-1 (Fig. 3B) in multiple donors (Fig. 3C), suggesting that internalization is not the cause of PD-1 loss.

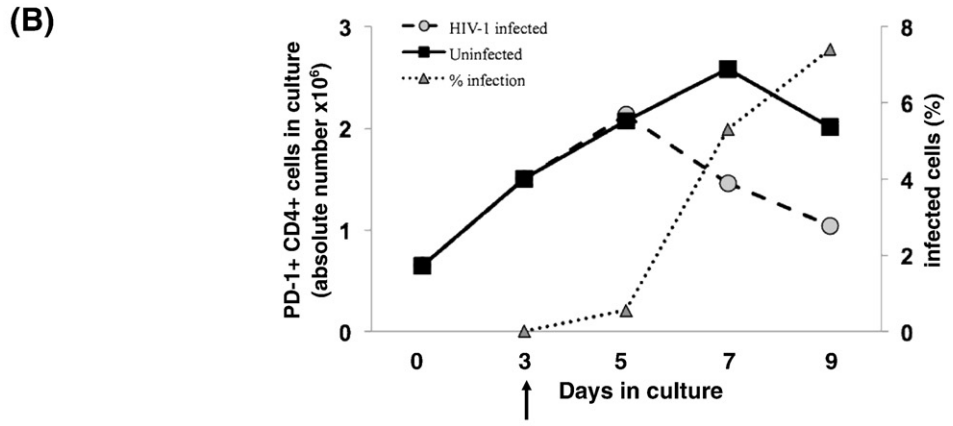
Next to answer whether virus infection regulates PD-1 expression at the transcriptional level, PD-1 positive cells were sorted and infected as described before. Forty-eight hours postinfection, an equal number of EGFP(+) and EGFP(−) cells were sorted and RNA extracted from these cells were used in a real-time RT-PCR assay. PD-1 transcripts were quantitated in comparison to RLPLO level (Fig. 3D). Results indicate that EGFP(+) infected cells did not show PD-1 transcripts, whereas an equal number of uninfected cells from the same culture expressed PD-1 mRNA, suggesting that PD-1 expression is regulated at the transcriptional level by HIV-1. Additionally, the internal control, RLPLO did not show any difference within the two populations (Fig. 3D) suggesting that PD-1 downregulation is specific and is not due to cell death or lack of RNA in these cells. Similar results were observed in multiple donors ($n=6$).

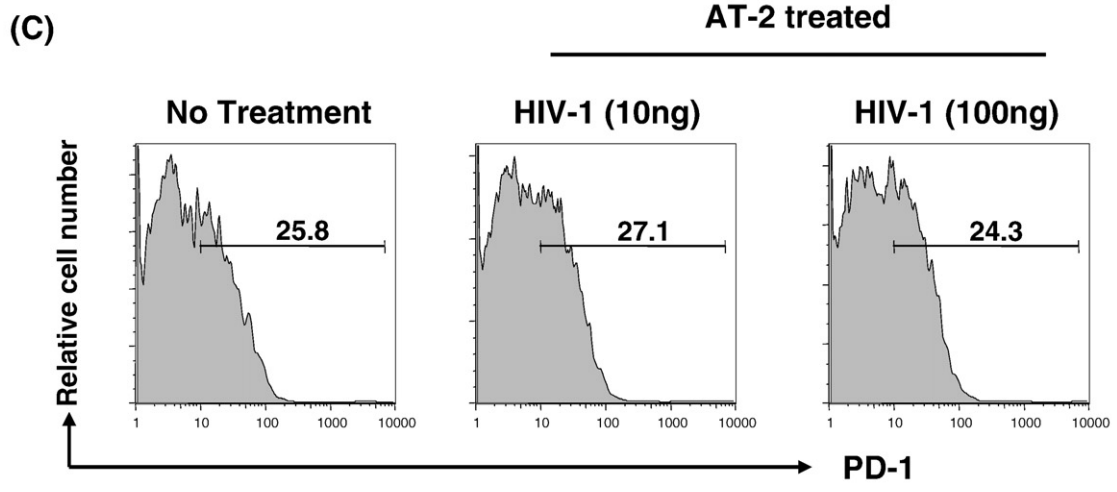
PD-1 positive cells are highly susceptible to HIV-1 infection

Results presented above indicate that PD-1 expression is fully abolished in productively infected cells. This led us to ask whether HIV-1 preferentially infects PD-1 negative cells or infection causes loss of PD-1. PD-1 positive and PD-1 negative cells were sorted to purity (shown in Fig. 4A) and infected with 0.01 MOI of virus and assessed for infectivity (Fig. 4B). Results indicate that 3 days postinfection, 10% of the cells in PD-1 positive group are infected, whereas a significantly reduced (20-fold less) infection (<0.67%) was observed in PD-1 negative cells. Similar results were observed in PBMC isolated from six different donors with varying level of PD-1 expression (Fig. 4C), suggesting that PD-1 positive cells are preferentially infected by HIV-1. This could be due to their activation status of target cells as PD-1 is expressed in activated T cells.

HIV-1 infects activated T cells and virus replication is higher in activated T cells compared to resting T cells (Chun et al., 2003). To address whether preferential infection of PD-1 positive cells is due to the activation status of these cells, we assessed expression of early and late activation markers such as CD69, CD25 and HLA-DR within the


(A)
FS
SS
FS
CD3
Uninfected
Infected
17.2
9.7
PD-1
CD4
Uninfected
Infected
26.2
22.3
CD8


(B)
HIV-1 infected
Uninfected
% infection
PD-1+ CD4+ cells in culture (absolute number x10^6)
infected cells (%)
Days in culture


(C)
AT-2 treated
No Treatment
HIV-1 (10ng)
HIV-1 (100ng)
25.8
27.1
24.3
Relative cell number
PD-1

PD-1 positive and PD-1 negative population (Fig. 4D). Results indicate that PD-1 positive cells are also highly positive for several activation markers, suggesting that stimulation increases the expression of PD-1 in CD4+ T cells and these cells are more susceptible to infection compared to unactivated or resting cells *in vitro* as reported previously (Arlen et al., 2006; Yamashita and Emerman, 2006). Additionally, we also tested the level of viral co-receptors CCR5 and CXCR4 expression in the PD-1 positive and negative populations and our results indicate that both populations express viral co-receptors, and that there was no significant difference in the surface expression of CXCR4 in either PD-1+ or PD-1− cells, when evaluated in multiple donors, though few donors had higher expression of CCR5 in PD-1 negative cells (Fig. 4E).

Loss of PD-1 in infected cells protects the cells from early apoptosis

Several studies have reported that antigen specific CD8+/PD-1+ cells are more susceptible to spontaneous and induced apoptosis (Dong et al., 2002; Ishida et al., 1992; Petrovas et al., 2006). To further understand, whether infected cells (that are PD-1 positive before infection) are resistant to apoptosis, we measured apoptosis in the infected and uninfected cells by Annexin V staining (Fig. 5A). Results indicate that only 5.6% of virus-infected (EGFP+/PD-1 negative) cells are positive for Annexin, whereas 23.7% of PD-1 positive bystander cells are positive for Annexin. A similar scenario was observed in the case of the uninfected cells also (21.6% in PD-1+ cells) further correlating with the previously published reports (Ishida et al., 1992; Petrovas et al., 2006).

Viruses are known to protect infected cells from cell death through upregulation of the anti-apoptotic gene, Bcl-2 (Bahbouhi, Landay, and Al-Harthi, 2004; Marshall et al., 1999). As infected cells exhibit 50% less staining for Annexin V, we evaluated for levels of Bcl-2 in infected and bystander cells (Fig. 5B). Results show that ~65% of infected cells express detectable level of Bcl-2, compared to 37.7% in bystander cells in the infected culture, whereas 63% of the cells are positive for Bcl-2 in the uninfected control. Similar two-fold difference was observed in multiple donors, suggesting that infected cells express high level of Bcl-2 during early infection.

PD-1 expression is downregulated within the infected cells in vivo

In HIV-1 infected individuals viral antigen specific CD8 effector T cells express high level of PD-1 that are characteristic of an exhausted phenotype. This leads to loss of CTL function and directly correlates with disease progression (Day et al., 2006; Petrovas et al., 2006; Trautmann et al., 2006). However, expression of PD-1 within the infected cells *in vivo* is not known. To further correlate the *in vitro* results with *in vivo*, PBMC were isolated from ten HIV-1 positive donors and were stained for HIV-1 p24 gag and PD-1 and assessed by flow cytometry (Fig. 6A; Table 1). HIV-1 positive patients were selected randomly and this population includes patients with high and low viral load, varying CD4 counts and/or antiviral therapy. Consistent with our *in vitro* results, infected cells (based on p24+) did not express PD-1 *in vivo*. Similar results were observed in both freshly isolated and activated lymphocytes. Results presented in Fig. 6A represent donors with varying levels of p24 positive cells indicating that p24 positive cells did not express PD-1, whereas bystander cells exhibit normal PD-1 level. As PD-1 expression is increased in activated cells, we also tested the expression of PD-1 from the same donors before and after activation (Fig. 6A). Together these results for the first time indicate that productively infected T cells *in vivo* lose PD-1 as part of their survival strategy. Next, we assessed Bcl-2 expression within the p24+ cells (infected) obtained from HIV infected donors PBMC (Fig. 6B). Results indicate that live p24+ cells are also positive for Bcl-2, whereas p24+ Bcl-2 negative cells are very minimal suggesting that productively infected cells are protected during virus replication.

Discussion

Viruses utilize several strategies to protect infected cells from being eliminated by immune responses and other cell-mediated apoptosis in order to maximize virus replication and survival. HIV-1 is known to induce both apoptotic and anti-apoptotic signaling in infected and uninfected bystander lymphocytes (mainly CD4 and CD8 cells) thereby counteracting the antiviral immune response and effector cell functions (Ameisen, 2001; Gougeon, 2003; Majumder et al., 2007). In draining lymph nodes of HIV-1 infected individuals, apoptosis is evident mainly in uninfected bystander cells suggesting that infected cells may be protected from apoptosis early on as a way to maximize virus production (Finkel et al., 1995; Ji et al., 2007). HIV-1 gene products such as Nef, Tat and Env are known to play dual roles in host cell apoptosis by inducing apoptosis in exposed cells, whereas infected cells are protected from apoptosis (McCloskey et al., 1997; Moon and Yang, 2006; Seelamgari et al., 2004). Here we report that HIV-1 infection selectively abolishes PD-1 expression in productively infected cells thus protecting them from apoptosis, whereas exposed and/or non-productively infected bystander cells are unaffected in the *in vitro* infection model. These results identify an important role for the PD-1 molecule in virus-infected CD4+ cells and how HIV-1 protects the infected cells from apoptosis during early infection before lysis. Although, infected cells eventually die or undergo necrosis as a result of virus infection, our results suggest that PD-1 down regulation in productively infected cells might protect the cells from spontaneous and/or activation-induced apoptosis during early infection to increase virus production.

PD-1 molecule has gained attention in HIV-1 pathogenesis as increased level of PD-1 is reported in antigen specific CD8+ and CD4+ T cells (D'Souza et al., 2007; Petrovas et al., 2007; Trautmann et al., 2006; Zhang et al., 2007). Although it is not well established how antigen specific CD4 and CD8 T cells express high level of PD-1, it is well accepted that the PD-1 molecule has a role in effector cell survival and blocking PD-1 interaction with PD-L1 restoring the cell function. As PD-1 is constitutively expressed in all activated T cells and these activated cells are major producers of HIV-1, we assessed PD-1 expression in virus-infected cells. Results from this study suggest that activated cells are preferentially infected by HIV-1, and virus replication specifically downregulates PD-1 expression. Additionally, PD-1 downregulation by HIV-1 is not due to internalization or degradation, but is at the transcriptional level. It is interesting to note that virus binding, entry or virion-associated proteins do not alter PD-1 expression (as AT-2 treated virus did not show any change in PD-1 level) suggesting that the viral transcription machineries might control PD-1 expression. Though the exact mechanism(s) are unknown it is reasonable to predict that redirection of transcription factors from host cellular protein promoters to HIV-1 LTR could be a possibility.

Recently Velu et al. (2007) reported that a high level of PD-1 expression in antigen specific CD8+ T cells is observed only during chronic infection, but not in the case of vaccination-induced effector

Fig. 1. PD-1 expression on CD4+ and CD8+ lymphocytes during HIV-1 infection: (A) PBMC were infected with HIV-1 EGFP reporter virus and was analyzed by flow cytometry for PD-1 expression. Viable lymphocytes were gated based on side scatter and forward scatter dot plot. Using the CD3 gating, PD-1 expression on total CD4+ and CD8+ lymphocytes were assessed by flow cytometry using directly conjugated antibodies or isotype controls. Numbers in quadrants indicate percent positive cells. (B) PD-1 expression on CD4+ lymphocytes was evaluated following activation (using α-CD3 and α-CD28) and during the course of HIV-1 infection. On day 3 of activation, the cells were washed and infected with HIV-1 EGFP reporter virus and PD-1 expression on total CD4+ cells was evaluated at regular intervals. Arrow indicates the day of infection. The black squares indicate the absolute number of cells expressing PD-1 in uninfected culture and the grey circles the absolute number of cells expressing PD-1 in infected culture from the same donor. The solid triangles indicate the percentage of infected cells present at the indicated time points. (C) Effect of AT-2 treated virus on PD-1 expression. Activated PBMC were treated with AT-2 virus (10 and 100 ng) and assessed for PD-1 expression. Results represent one of four independent experiments.

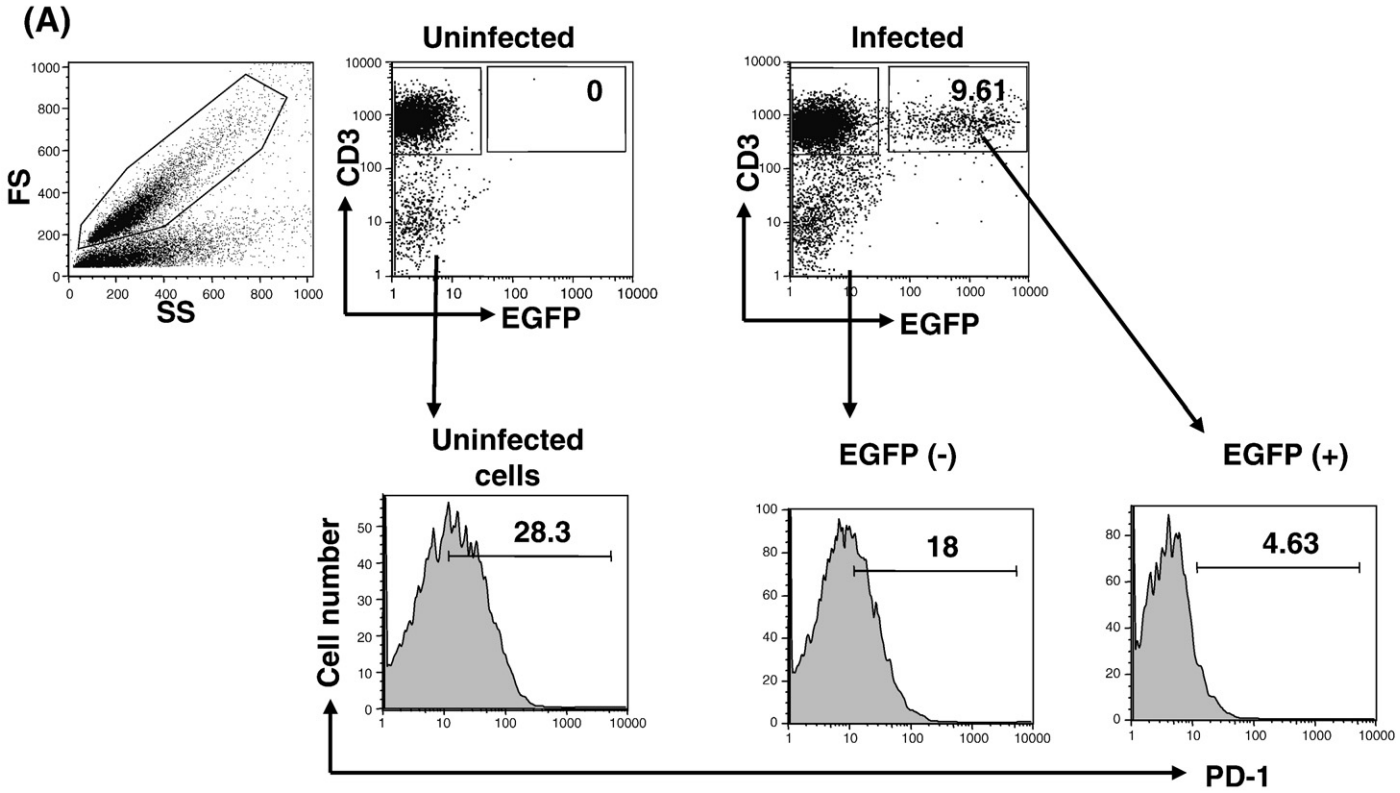
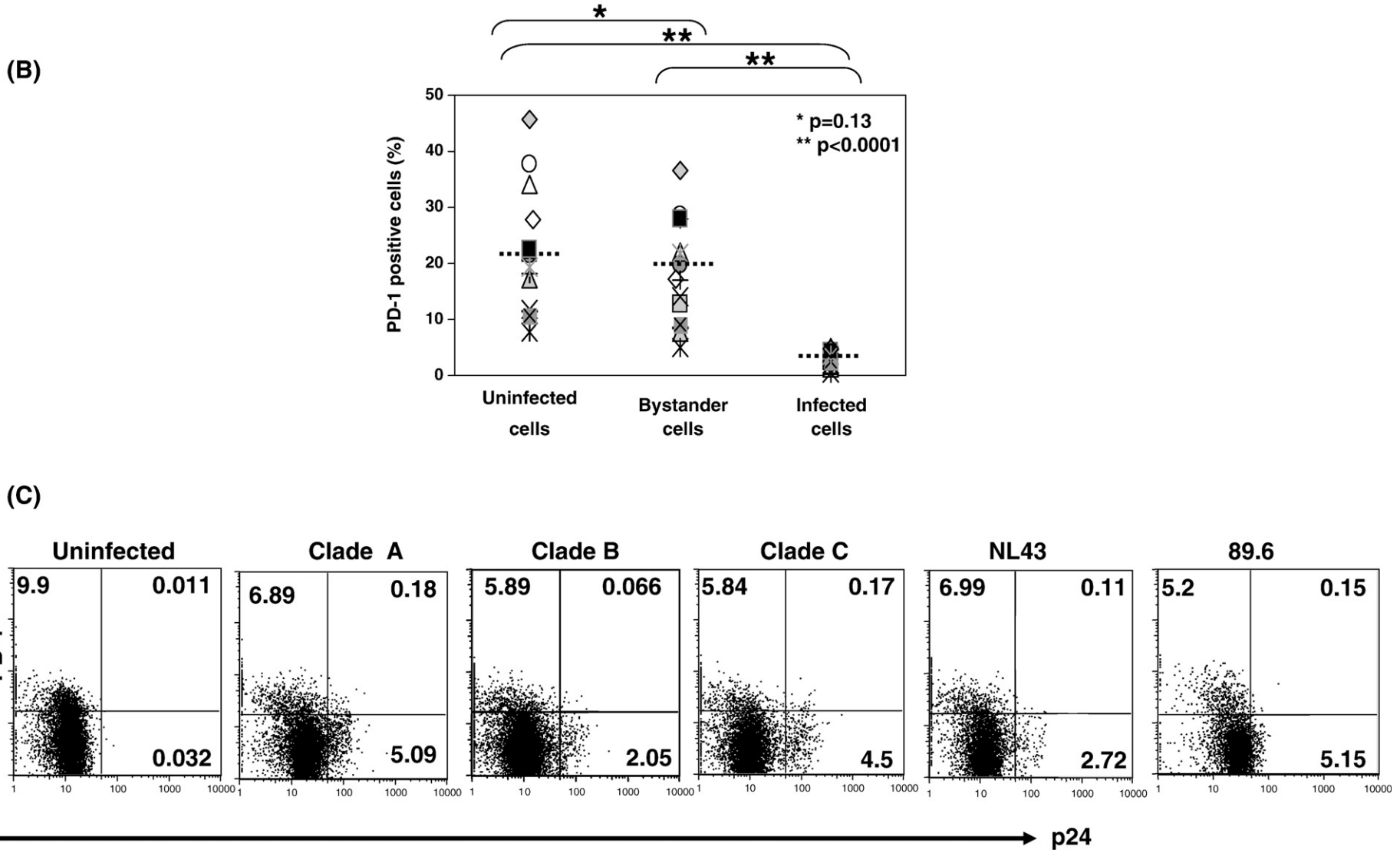


Fig. 2. Loss of PD-1 expression on HIV-1 infected lymphocytes: (A) PBMC infected with HIV-1 EGFP reporter virus was analyzed by flow cytometry for PD-1 expression. Viable lymphocytes were gated based on side scatter and forward scatter dot plot. Using CD3 gating, infected (EGFP+) and bystander (EGFP−) lymphocytes were identified based on EGFP expression. Expression of PD-1 was assessed by flow cytometry using directly conjugated specific antibodies or isotype controls. The expression of PD-1 was analyzed on the infected (EGFP+) and bystander (EGFP−) cells. (B) Loss of PD-1 on infected cells in multiple donors ($n=15$). p value was derived using paired t test analysis comparing uninfected cells and bystander cells (*), bystander cells and infected cells (**) or uninfected and infected cells (**). (C) PBMC were infected with primary isolates from clade A, B, C or with lab strains NL43, 89.6. Postinfection (6 days) viable infected cells were identified by intracellular p24 and expression of PD-1 was analyzed by flow cytometry. Figure represents one of three independent experiments.



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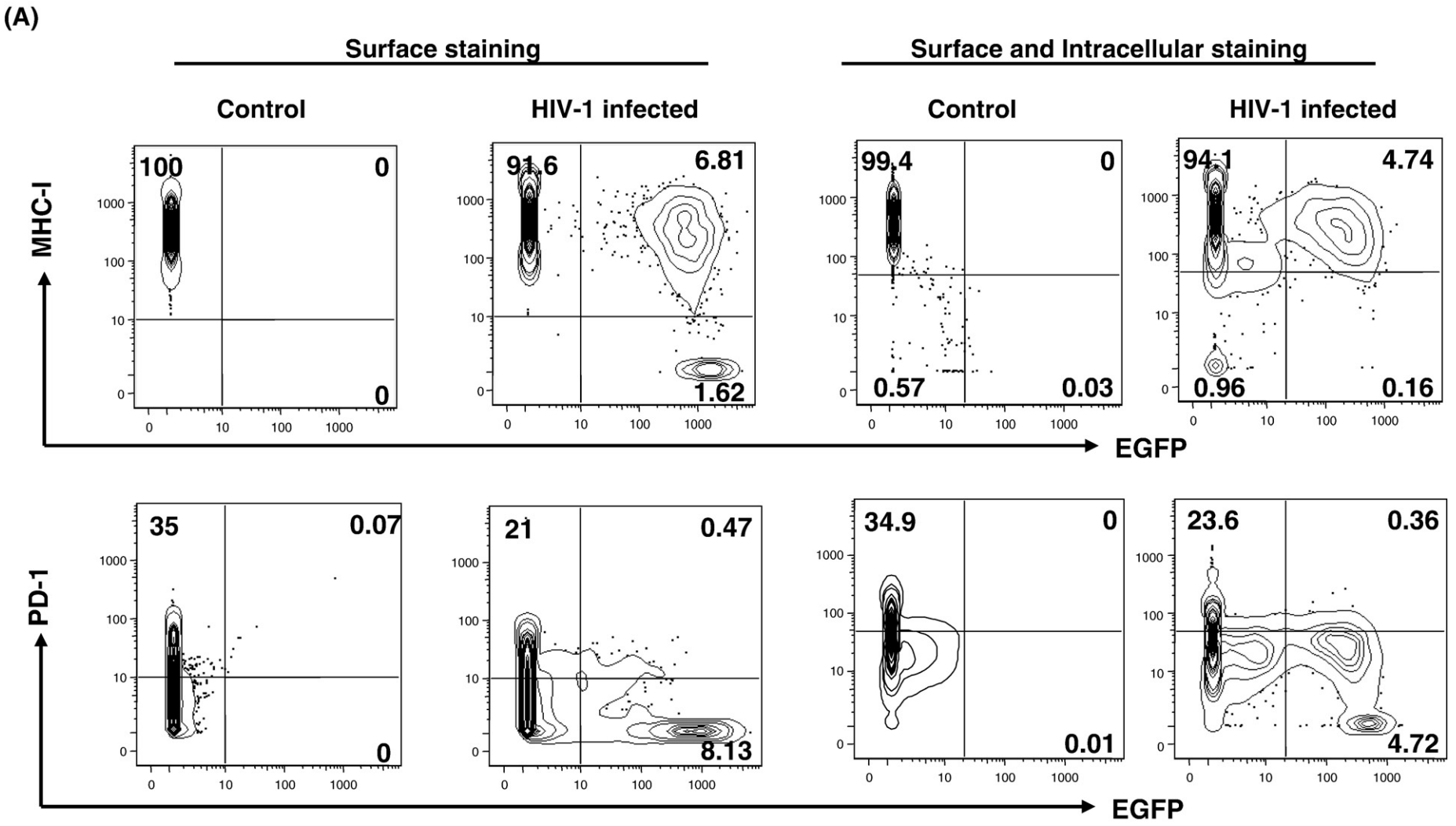

Fig. 3. HIV-1 regulates PD-1 at the transcriptional level: (A) PBMC infected with HIV-1 EGFP reporter virus were stained for surface expression of CD3, PD-1 or MHC class I molecules and evaluated by flow cytometry. To evaluate further the internalized molecules, the surface stained cells were permeabilized and intra cellular staining of PD-1 and MHC class I was performed as mentioned in Materials and methods, and evaluated by flow cytometry. The figures are representative of one of the multiple donors ($n=6$) evaluated. (B) Infected PBMC were fixed with 2% paraformaldehyde and cytospun on to slides, and stained for PD-1, as described in Materials and methods. Infected lymphocytes were detected by direct fluorescence of EGFP (Green), and the PD-1 was detected using a monoclonal antibody (Red). Nuclei were identified by DAPI staining (Blue). Arrows in panel (overlay) indicate the presence of PD-1 in the cells. As indicated, the upper panel shows the uninfected control lymphocytes, and the lower panels show the cells infected with HIV-1 EGFP virus. Results represent one of four independent experiments. (C) Infected cells were identified by presence of EGFP florescence and bystander cells by the absence of EGFP fluorescence. Presence of PD-1 on infected, bystander cells and uninfected control cells was evaluated in fifteen random fields (50 cells) in multiple donors ($n=6$). The percentage of cells expressing PD-1 is presented. (D) Following activation PD-1(+) cells were sorted and infected with HIV-1-EGFP reporter virus as described. Equal number of infected cells and uninfected cells were sorted and assessed for PD-1 and RLPLO transcripts. Green line represents EGFP(−) cells and Red line represents EGFP(+) cells. Figure represents one of six experiments using different donor PBMC.

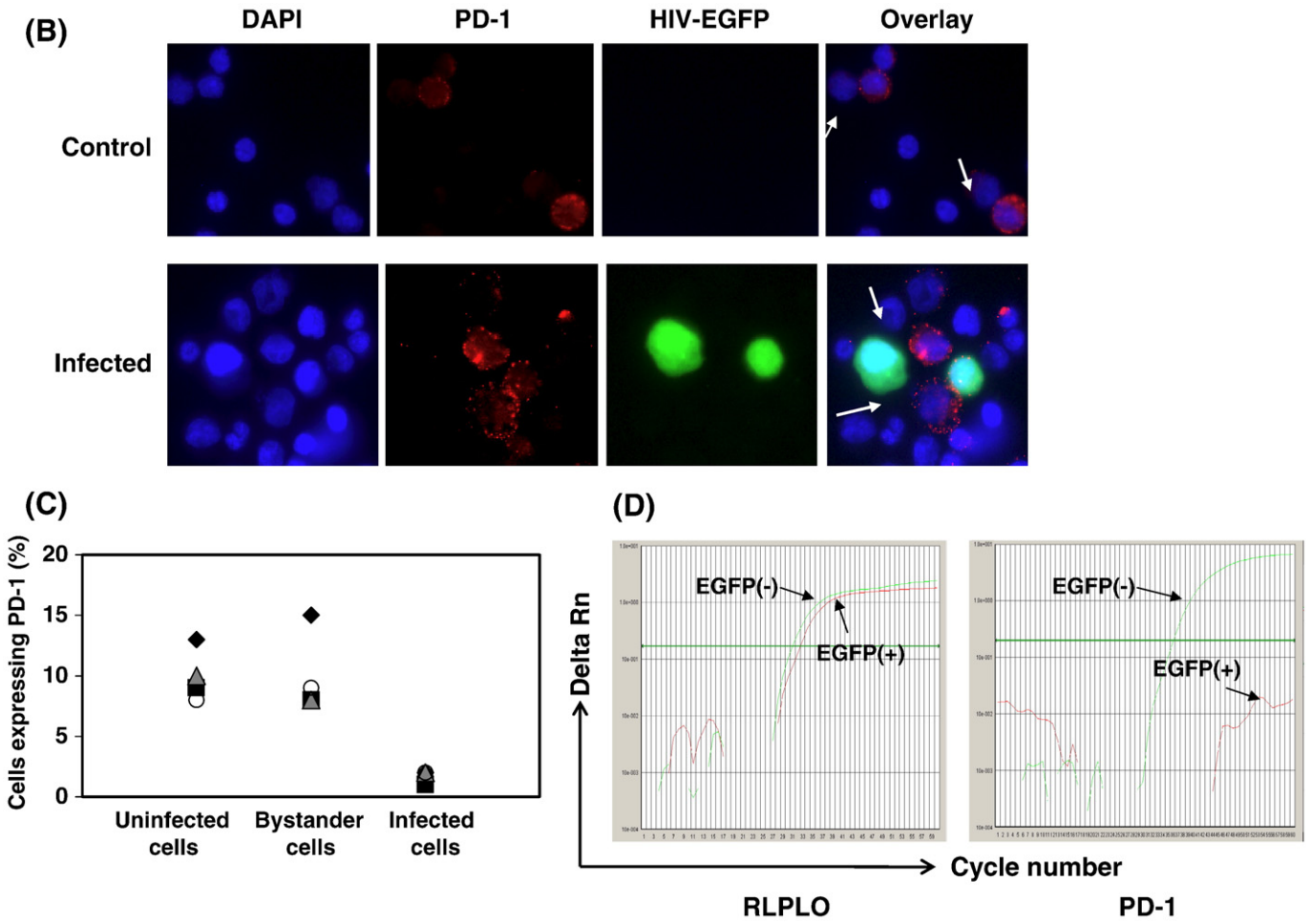

Fig. 3 (*continued*).

CD8+ T cells. This important finding suggests that virus infection per se has a role in PD-1 dysregulation and clearly distinguishes that expression of viral antigens by vaccine does not have a role in PD-1 dysregulation. Results from our *in vivo* data further support this observation where p24 positive cells did not show PD-1 expression, whereas bystander cells were not affected.

One possible explanation for this specific downregulation would be that HIV-1 might alter PD-1 expression as a method to avoid activation-induced cell death and maintain the cells in an altered activated state which assist in virus replication. Though virus-infected cells are eventually exhausted and die due to the cytopathic effect of the virus and the lack of host cellular protein transcription, losing PD-1 on productively infected cells might protect the cells from apoptosis during early infection and enhance their survival longer thus increasing virus replication and dissemination. Data from our *in vivo* analysis using HIV-1 patients with varying amount of viral load further support that productively infected cells specifically lose PD-1 expression, whereas PD-1 level is not affected in bystander cells that includes both exposed and unaffected cells. This virus-induced effect is independent of viral strains, clades and co-receptor utilization indicating that this might a basic function of virus infection. It is not known whether this phenomenon is specific to HIV-1 or if other viruses that target lymphocytes also utilize similar mechanisms.

Published results indicate that an increased level of PD-1 expression in lymphocytes is accompanied by the loss of anti-apoptotic protein, Bcl-2. Conversely, the loss of PD-1 in infected cells exhibit elevated the level of Bcl-2 suggesting that a similar apoptotic pathway is utilized in infected cells. Together, these findings for the first time indicate a role of PD-1 in infected T cells during HIV-1 infection and pathogenesis. Identifying the mechanism(s) involved in the differential regulation of PD-1 in T cells will provide information to further understand the multifaceted functions of HIV-1 during pathogenesis and disease progression.

Materials and methods

Cell culture

PBMC were isolated by Ficoll–Hypaque gradient centrifugation and stimulated with anti-CD3 antibody (10 μg/ml) and anti-CD28 antibody (1 μg/ml) or with PHA-P (5 μg/ml) for 3 days as described (Venkatachari et al., 2007) and cultured in growth media containing rIL-2 (5 U/ml). HEK293T cells were maintained in DMEM containing 10% FBS, 1% L-glutamine and 1% penicillin–streptomycin (GIBCO).

Virus preparation and infectivity

A full length HIV-1 proviral DNA containing Enhanced Green Fluorescence Protein (EGFP) reporter was constructed as described (Venkatachari et al., 2007). HEK293T cells (2×10^6 per plate) were transfected with 10 μg of HIV-1-EGFP proviral plasmid and virus preparation was done as described (Venkatachari et al., 2007). Virus titer and multiplicity of infection (MOI) was calculated by flow cytometry using the HIV-1 reporter cell line cMAGI (AIDS Research and Reference Reagent Program [RRRP], National Institutes of Health [NIH]). PBMC were infected with the HIV-1-EGFP reporter virus at a MOI of 0.01.

Six hours following infection, unbound virus was washed off and cells were maintained in growth media containing rIL-2 (5 U/ml).

Flow cytometry

Surface staining of the cells was done with CD3, CD4, PD-1, MHC-I, CCR5, CXCR4, activation markers CD25, CD69, HLA-DR or with isotype controls as mentioned (Venkatachari et al., 2007). For the detection of intracellular p24, fixation and permeabilization were carried out using the CytoFix–CytoPerm kit (BD Biosciences, Mountainview, CA) and intracellular p24 staining was performed at room temperature for 1 h using 5 µl of anti-p24-FITC antibody (Coulter, Miami, FL; clone KC47) per 10^6 cells, followed by two washes in Perm-Wash buffer, and finally resuspended in FACS buffer. Samples were analyzed using Epics-XL (Beckman Coulter, Miami, FL) with 20,000 to 50,000 gated events acquired for each sample, and the results were analyzed using FlowJo software (Tree Star, Inc., OR). Apoptosis was measured using Annexin V-PE-Cy5 antibody (MBL, Wobourn, MA) and Bcl-2 level was measured using Bcl-2 specific antibody according to the manufacturer's suggestions (BD Biosciences).

Live cell sorting

Normal donor PBMC were stimulated as described before and CD4+ cells were isolated by negative selection using CD4 isolation Kit II (Miltenyibiotec, Auburn, CA). Untouched CD4 cells were stained for PD-1 and, stained cells were sorted using the high-speed live cell sorter (FACS Aria, BD Biosciences or MoFlo, Dako cytomation) in IDM or UPCI biocontainment facility according to the standard protocols. Sorted cells were tested for their purity and cells that are >95% positive or negative for PD-1 was used for further infection studies. Infected cells (based on EGFP positive) were sorted using the MoFlo sorter at UPCI Flow facility with the purity of >98%.

Immunofluorescence

Forty-eight hours postinfection, PBMC were washed with PBS, and attached onto slides using cytospin. Cells were fixed in 3.7% formaldehyde at room temperature for 10 min, and washed and permeabilized with 0.5% Triton X-100 for an additional 10 min. After washing 3 times with PBS, cells were blocked with 5% BSA at room temperature for 1 h followed by incubation with primary antibody (PD-1; 1:200 dilution, BD Biosciences) for 1 h at room temperature and incubated with rabbit anti-mouse IgG Rhodamine (RRX) (1:400; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Cells were mounted with VECTASHIELD mounting media containing DAPI (Vector Laboratories, Burlingame, CA). Immunofluorescence analysis was performed using a fluorescence microscope with Nikon SPOT camera (Fryer, Huntley, IL) and images were processed using MetaMorph software (Universal Imaging Corporation, Downington, PA).

Real-time RT-PCR analysis

Equal number (2000 or 5000) of infected and uninfected cells was sorted using MoFlo cell sorter as described before. Total RNA was extracted by cell lysis as described (Esumi et al., 2006) and used to

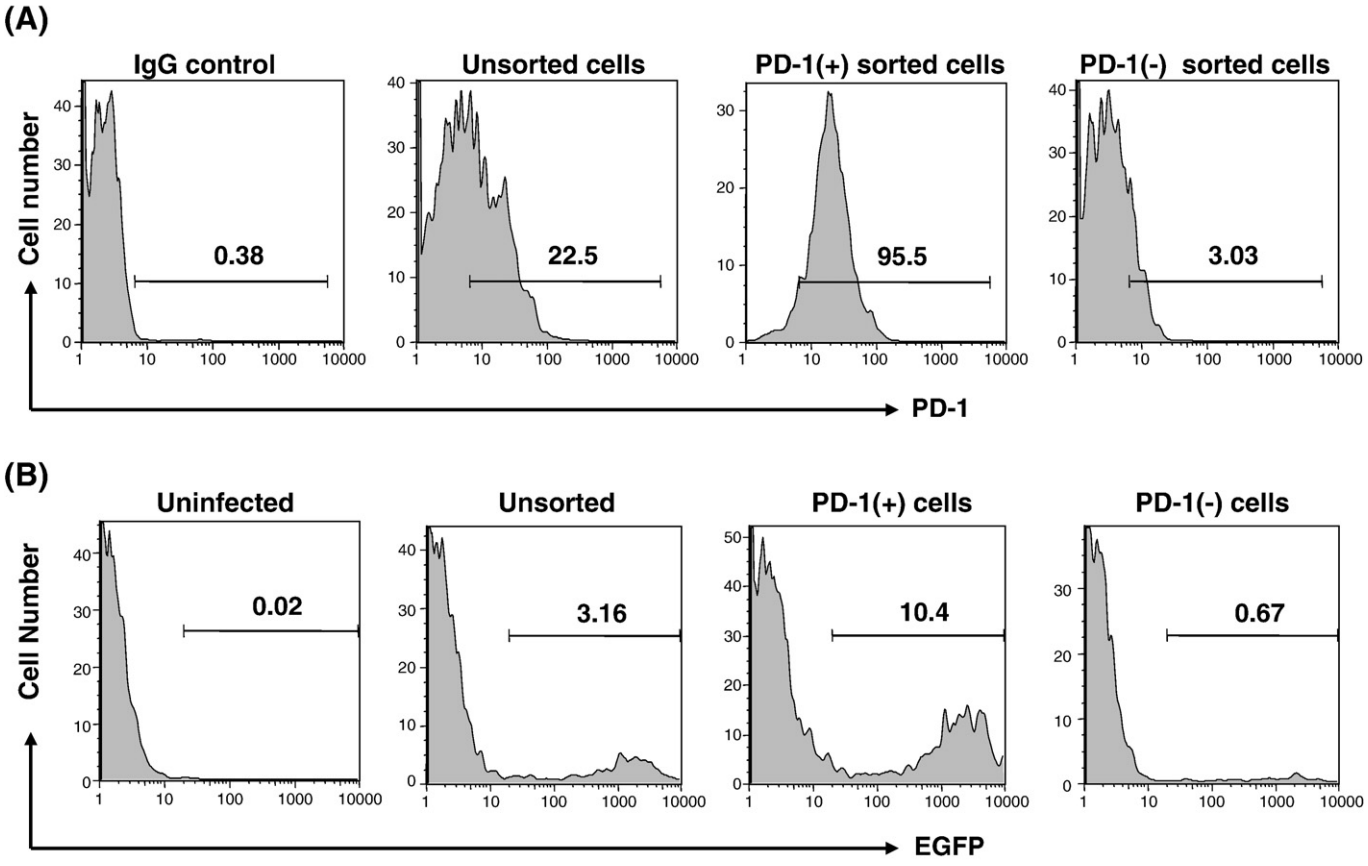

Fig. 4. PD-1 positive lymphocytes are more susceptible to HIV-1 infection. (A) Normal donor PBMC were activated for 3 days, stained for surface expression of PD-1. PD-1(+) and PD-1(−) cells were sorted and purity of sorted cells was confirmed. (B) Unsorted, PD-1(+) and PD-1(−) sorted cells were infected with HIV-1 EGFP reporter virus as mentioned in Materials and methods. Percentage of productively infected cells were evaluated in these groups are presented. Figure represents one of the multiple donors (n=6) evaluated. (C) Results from multiple donors indicate the increased susceptibility (fold difference considering unsorted as 1) of PD-1 positive cells for productive infection with HIV-1. (D) Activated PBMC were stained for activation markers CD25 and HLA-DR using florescence tagged monoclonal antibodies and the expression of activation markers in PD-1(+) cells and PD-1(−) cells was evaluated by flow cytometry. (E) Expression of HIV-1 co-receptors CXCR4 and CCR5 was evaluated in PD-1(+) and PD-1(−) cells by flow cytometry.

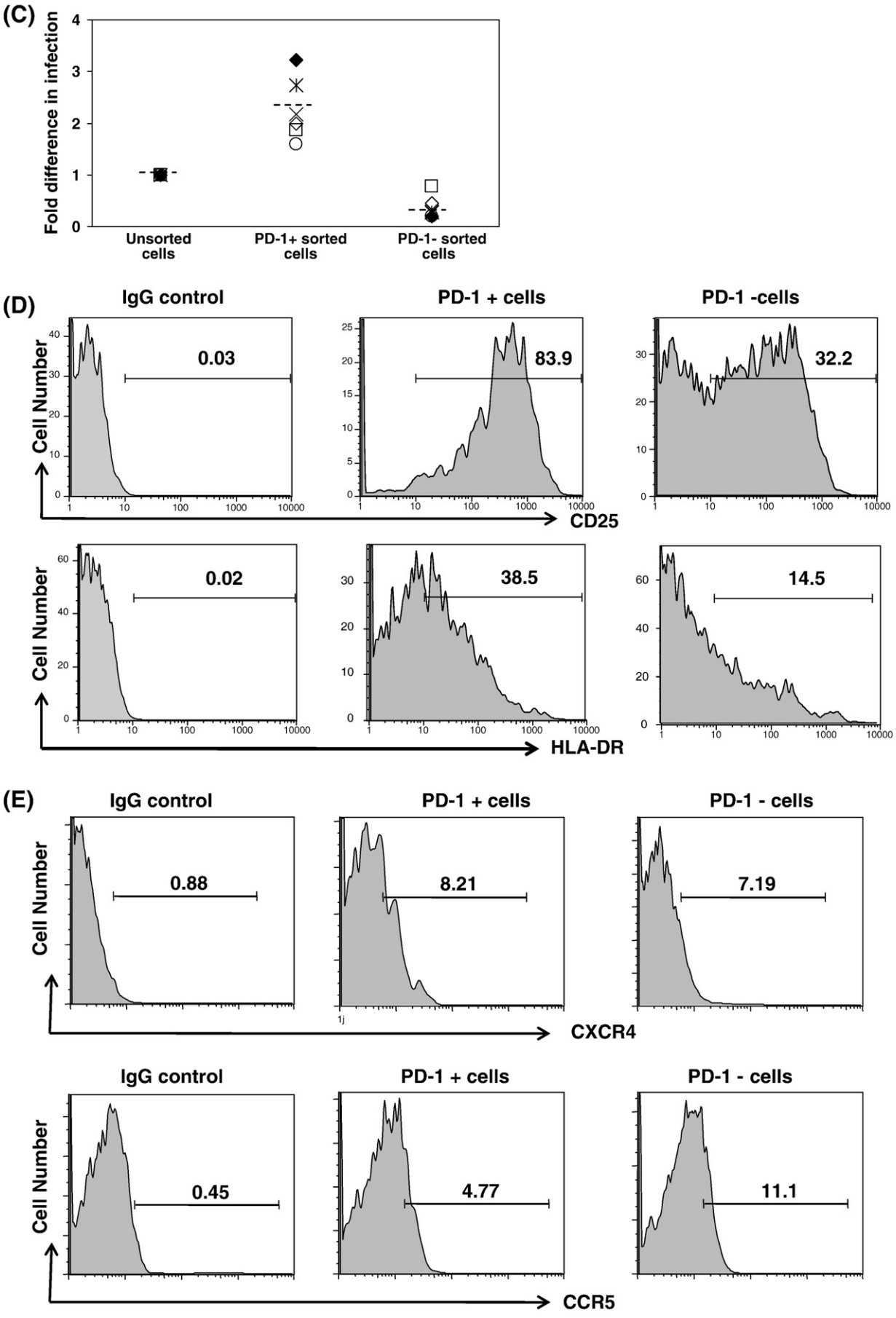

Fig. 4 (*continued*).

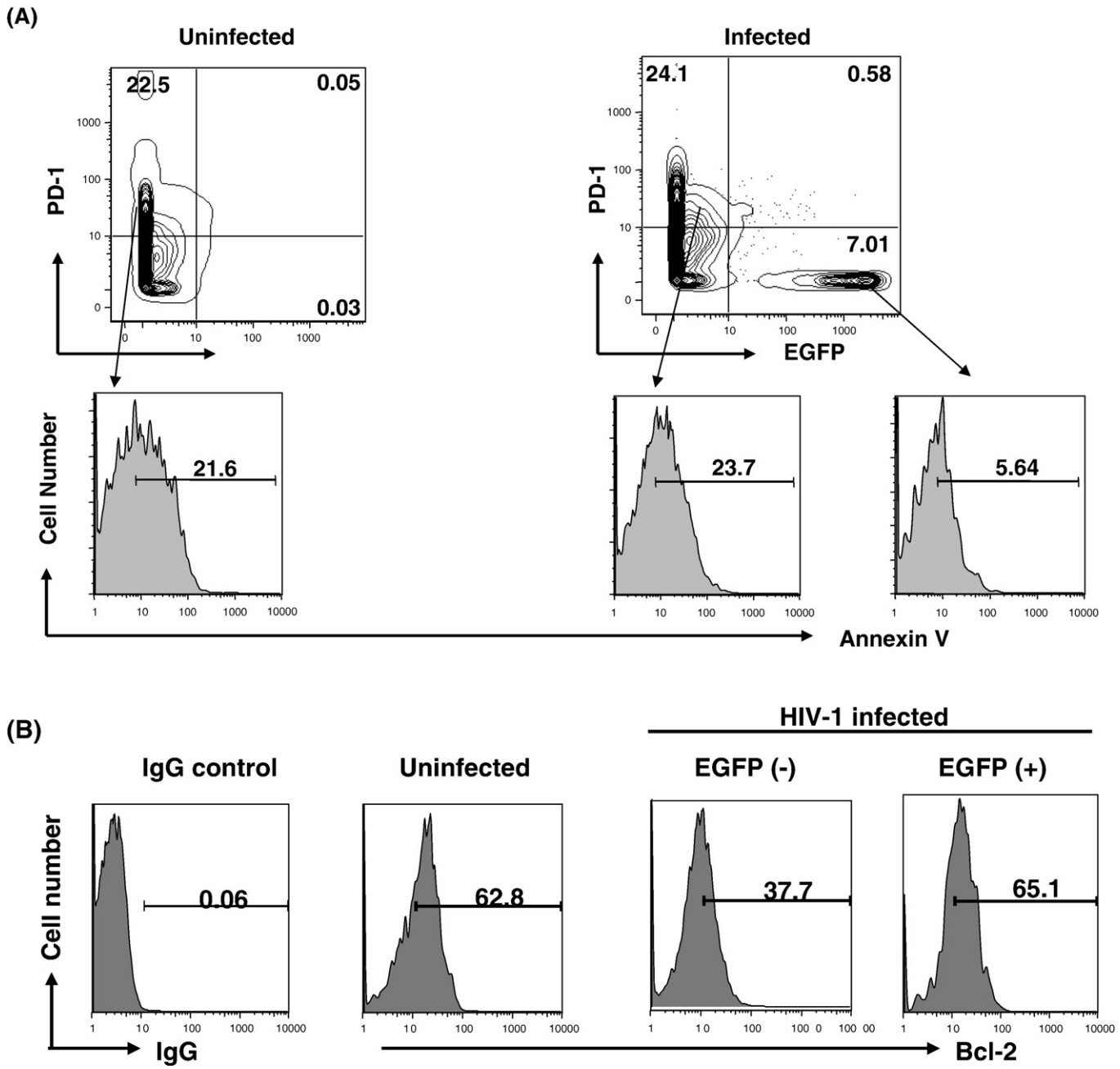

Fig. 5. Loss of PD-1 in infected cells reduces the susceptibility of cells to apoptosis: (A) PBMC were infected with HIV-1 EGFP reporter virus as described in Materials and methods. Two days postinfection the cells were stained for CD3, PD-1 and Annexin V, and analyzed by flow cytometry. Viable lymphocytes were gated based on side scatter and forward scatter dot plot. The CD3 expressing cells were then gated, and the infected (EGFP+) and bystander (EGFP−) cells were identified based on EGFP expression. The percentage of cells positive for Annexin V staining in PD-1(+) and PD-1(−) cells was analyzed in both the infected and uninfected control group. (B) Bcl-2 expression in infected and bystander cells. Infected, bystander and their counterpart uninfected cells were assessed for Bcl-2. Results represent one of six independent experiments.

quantitate RPLPO, PD-1 and HIV-1 gag–pol transcripts by real-time PCR. Briefly, a two-step RT-PCR was performed as follows: RNA was reverse transcribed using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA); Real-time PCR was carried out in triplicate using commercially available primer/probe sets specific for PD-1, HIV-1 Gag and ribosomal large protein (RPLPO, Applied Biosystems). The comparative C_T method was used to determine the relative level of PD-1 transcript by normalizing to the RPLPO control transcript.

Study population

The study population consisted of 11 HIV-1-infected participants from the Pittsburgh portion of the Multicenter AIDS Cohort Study with

Fig. 6. Loss of PD-1 on infected cells in HIV infected patients: (A) PBMC obtained from HIV infected patients, were evaluated for PD-1 expression by flow cytometry. Intracellular staining for p24 was done using the p24-FITC to detect infected cells. Expression of PD-1 on infected cells was evaluated on fresh and activated cells. The top panel (Fresh) represents infected cells that are detected at day 0, and the lower panel (Stimulated) represents lymphocytes used on day 4 upon activation with PHA-P. (B) Bcl-2 expression was assessed in the infected cells (p24+) obtained from HIV infected donor PBMC. Uninfected donor was used as a control for intracellular staining. Results represent one of three independent donors.

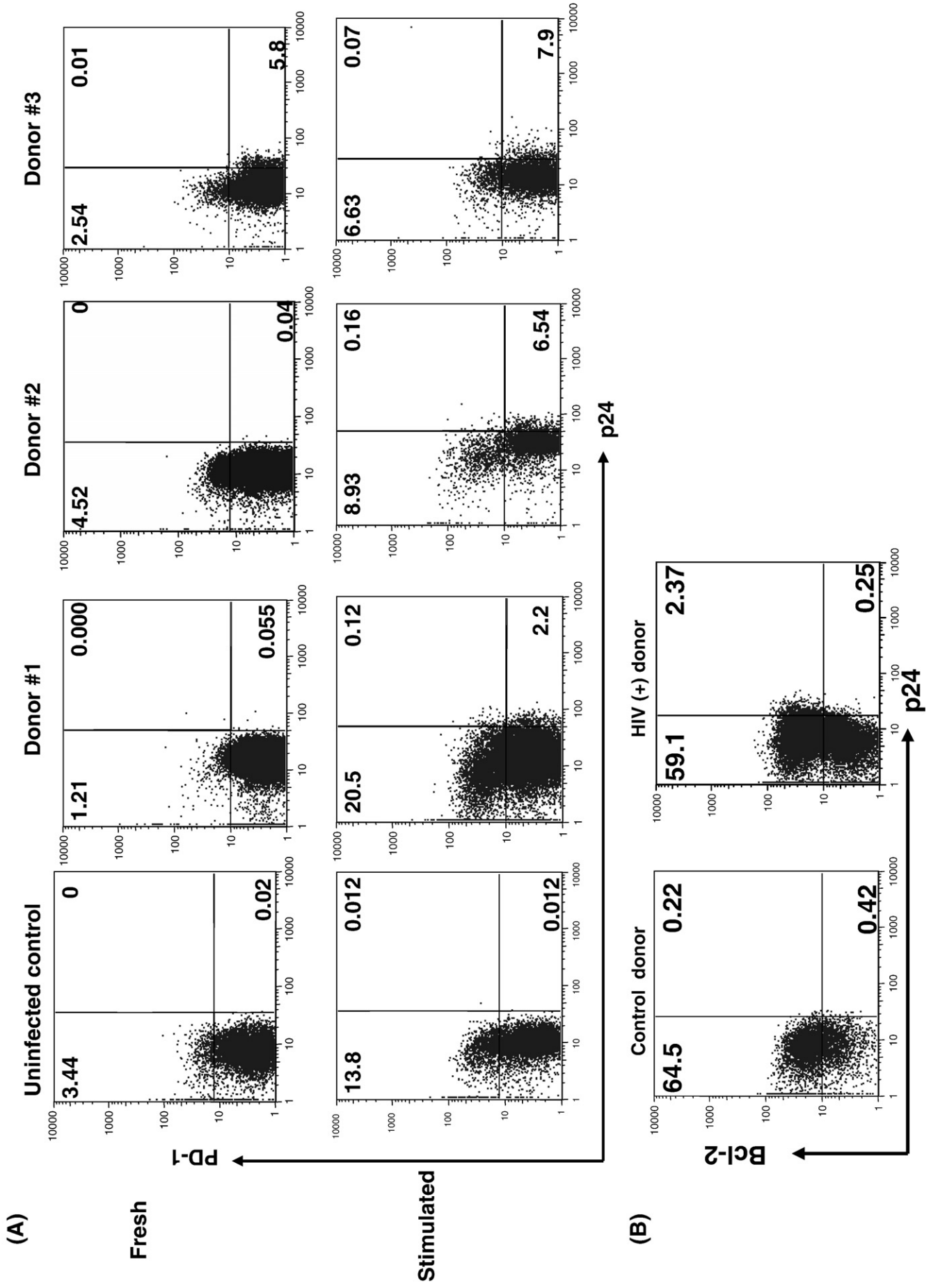
(A)
Fresh
Stimulated
Uninfected control
Donor #1
Donor #2
Donor #3
PD-1
p24
3.44
0
0.02
1.21
0.000
0.055
4.52
0
0.04
2.54
0.01
5.8
13.8
0.012
0.012
20.5
0.12
2.2
8.93
0.16
6.54
6.63
0.07
7.9
(B)
Control donor
HIV (+) donor
Bcl-2
p24
64.5
0.22
0.42
59.1
2.37
0.25

Table 1
Profile of HIV-1 positive patients used in this study

Sample	Years post Serocon-version	HAART status	Virus titer	p24 (%) positive cells	PD-1 positive cells (%)	
					Bystander	Infected
71a	4 months	Naive	60488	6.7	9.5	2.38
90a	1.5 years	Yes	<50	–	16.6	N/A
83	2.5 years	Yes	319	0.4	6.9	1.46
11	7.5 years	Yes	50	2.3	21.0	5.21
53	5.5 years	Naive	26724	1.5	10.6	0.06
93	1 year	Naive	41355	2.9	9.4	1.3
71b	20 years	Yes	50707	2.5	10.9	1.77
90b	>20 years	Naive	55910	7.5	3.8	0.22
19	4 months	Naive	36978	6.0	7.0	1.16
13	6.5 years	Naive	16191	3.0	12.8	2.74

viral loads (median, 31,851; range, <50 to 60,488) and their therapy status (Table 1). The HIV-1 plasma viral load was measured by a quantitative reverse transcription-PCR assay (Amplicor; Roche Diagnostics, Alameda, CA). Blood from HIV-1 positive patients who are enrolled in the MACS/Pitt's Men Study was collected using an informed consent form. All IRB protocols were reviewed and approved by the University of Pittsburgh. PBMC isolated from HIV-1 positive donors were stained for HIV-1 p24 gag and PD-1 on day 0 (fresh) as described above and on day 4 following activation with PHA-P (4 μg/ml) and rIL-2 as described (Arlen et al., 2006) to initiate virus production.

Statistical analysis

The data were analyzed using the Student *t* test for paired samples. A *p* value of <0.05 was considered significant.

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