

PD-1 and its ligand PD-L1 are progressively up-regulated on CD4 and CD8 T-cells in HIV-2 infection irrespective of the presence of viremia

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Objective: Hyper-immune activation is a main determinant of HIV disease progression, potentially counter-acted by T-cell inhibitory pathways. Here we investigated, for the first time, inhibitory molecules in HIV-2 infection, a naturally occurring attenuated form of HIV disease, associated with reduced viremia and very slow rates of CD4 T-cell decline.

Design: Programmed death (PD)-1/PD-L1, an important pathway in limiting immunopathology, and its possible relationship with T-cell immunoglobulin and mucin-domain containing molecule-3 (TIM-3), a recently identified inhibitory molecule, were studied in untreated HIV-2 and HIV-1 cohorts, matched for degree of CD4 T-cell depletion, and noninfected individuals.

Methods: Flow cytometric analysis of T-cell expression of PD-1, PD-L1 and TIM-3, combined with markers of cell differentiation, activation, cycling and survival. Statistical analysis was performed using ANOVA, Mann–Whitney/Wilcoxon tests, Spearman's correlations, multiple linear regressions and canonical correlation analysis.

Results: T-cell expression of PD-1 and PD-L1 was tightly associated and directly correlated with CD4 T-cell depletion and immune activation in HIV-2 infection. No such correlation was found for PD-L1 expression in HIV-1-positive patients. Central memory and intermediate memory cells, rather than terminally differentiated T-cells, expressed the highest levels of both PD-1 and PD-L1 molecules. Conversely, TIM-3 expression was independent of T-cell differentiation and dissociated from cell cycling, suggesting distinct induction mechanisms. Importantly, in contrast with HIV-1, no significant increases in TIM-3 expression were found in the HIV-2 cohort.

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Conclusions: Our data suggest that PD-1/PD-L1 molecules, rather than markers of T-cell exhaustion, may act as modulators of T-cell immune activation, contributing to the slower course of HIV-2 infection. These data have implications for the design of antiretroviral therapy-complementary immune-based strategies.

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Introduction

The hallmark of HIV immunopathogenesis is a hyper-immune activated state that directly correlates with progression to AIDS [1,2]. Since the process of T-cell activation is tightly regulated, resulting from a balance between stimulatory and inhibitory signaling pathways [3], it is plausible that inhibitory molecules play a major role during HIV infection. The programmed death-1 (PD-1) receptor/ligand network is considered one of the main pathways capable of limiting the deleterious effects of inappropriate/exacerbated immune activation [4]. In the context of HIV-1 infection, PD-1 was first described as a cellular exhaustion marker for HIV-specific T-cells, being associated with reduced proliferation and cytokine secretion capacities, and its blockade leading to partial recovery of these functional deficits [5–7]. Recently, however, several groups have suggested that increased PD-1 levels may instead limit tissue damage resulting from chronic immune activation [8,9] and/or persistent antigenemia associated with ongoing viral replication [10–12]. Furthermore, an increasing body of evidence indicates that PD-1 expression alone, may not always be associated with, nor sufficient to characterize an exhausted phenotype, with the co-expression of other inhibitory molecules, like the immunoglobulin superfamily member T-cell immunoglobulin and mucin-domain containing molecule-3 (TIM-3), being essential to define T-cell exhaustion [13–18].

Here we sought to investigate the contribution of the PD-1/PD-L1 pathway and TIM-3 to HIV/AIDS immunopathogenesis through the study of HIV-2 infection, a naturally occurring form of attenuated HIV disease [19]. HIV-2-positive individuals typically feature lower rates of CD4 depletion, reduced amounts of circulating virus and slower disease progression as compared to HIV-1-positive patients [20]. Nevertheless, when matched for the degree of CD4 depletion, both infections feature the same levels of immune activation [2,21]. Data regarding the expression of PD-1, PD-L1 and TIM-3 in HIV-2-positive patients are extremely limited [11]. Investigating the contribution of inhibitory pathways to the slower progression to AIDS observed in HIV-2 infection may provide a rationale for novel therapeutic interventions in HIV/AIDS.

Methods

Study populations

The study included 28 HIV-2-positive and 22 HIV-1-positive antiretroviral therapy (ART)-naïve, asymptomatic individuals and 16 seronegative controls (Supplemental Digital Content, Table 1, <http://links.lww.com/QAD/A215> for clinical and epidemiological data). Written consent for blood sampling and processing was provided, and the study was approved by the Ethical Board of the Faculty of Medicine of the University of Lisbon.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood and surface and/or intracellularly stained, as previously described [22,23]. At least 100 000 events were acquired with a FACSCanto. Analysis was performed using a ‘fluorescence minus one’ strategy with FlowJo software.

Plasma viral load assessment

Viremia was quantified by reverse transcriptase PCR (RT-PCR). Detection thresholds were: 40 RNA copies/ml for HIV-1 (Roche, Basel, Switzerland), and 200 RNA copies/ml for HIV-2, using a previously described in-house assay [24].

Statistical analysis

Statistical analysis was performed using GraphPad Prism v5.00, SPSS v15.0 and MATLAB v7.3. Data were compared using one-way or repeated-measures ANOVA (with the Dunns post-test), followed by Mann–Whitney or Wilcoxon tests, as appropriate; associations were assessed by Spearman’s correlation coefficient, multiple linear regressions and canonical correlation analysis. *P* values below 0.05 were considered significant.

Results

PD-1 expression on T-cells

We compared ART-naïve HIV-2 and HIV-1 cohorts matched for CD4 T-cell depletion, with similar levels of T-cell activation, but distinct viremia (Supplemental

Digital Content, Table 1). PD-1 expression tended to be higher on both CD4 and CD8 T-cells in HIV cohorts as compared to seronegatives, though HIV-2 featured significantly lower levels within CD8 T-cells than HIV-1 (Fig. 1a). Of note, similar to seronegatives, HIV-2-positive individuals exhibited a significant correlation between PD-1 expression on CD8 and CD4 T-cells, which was not found in the HIV-1 cohort (Supplemental Digital Content, Fig. 1, <http://links.lww.com/QAD/A215>).

Next, we investigated whether increased PD-1 expression was related to HIV-associated T-cell imbalances [25–27]. PD-1 expression varied with CD8 T-cell differentiation in HIV-2-positive in a similar way to that previously described for HIV-1-positive and seronegative individuals [28,29]. PD-1 levels were lowest on naïve cells, and peaked at the CD45RO⁺CD27⁺ stage (Supplemental Digital Content, Fig. 1, <http://links.lww.com/QAD/A215>), supporting the view that PD-1 up-regulation, rather than being related to terminal T-cell differentiation and exhaustion, may indicate T-cell activation upon antigen encounter. Whilst PD-1 expression was significantly increased on all CD8 T-cell subsets in HIV-1-positive relative to seronegative individuals, this was only observed for CD45RO⁺CD27⁺ cells in the HIV-2 cohort. PD-1 expression on CD4 T-cells was similarly linked to cell differentiation, with expression on naïve cells being significantly lower in HIV-1-positive and HIV-2-positive as compared to seronegative individuals; conversely, PD-1 expression on memory subsets was increased in both HIV cohorts, although only reaching statistical significance for HIV-1 (Supplemental Digital Content, Fig. 1, <http://links.lww.com/QAD/A215>).

Programmed death-1 expression on CD4 T-cells was strongly associated with the degree of CD4 depletion and T-cell activation in both HIV cohorts (Table 1 and Supplemental Digital Content, Table 2, <http://links.lww.com/QAD/A215>). Additionally, significantly increased levels of PD-1 on CD4 and CD8 T-cells were found in viremic as compared to both aviremic HIV-2-positive and seronegative individuals (Supplemental Digital Content, Fig. 2, <http://links.lww.com/QAD/A215>).

Importantly, no statistically significant correlations were found between PD-1 expression levels on CD8 T-cells and the aforementioned parameters in either HIV cohort, except for CD38 mean fluorescence intensity (MFI) within CD8 T-cells (Table 1).

To investigate which of these factors most strongly influenced PD-1 expression on T-cells, we conducted multiple linear regression (MLR) analyses using combined data from all cohorts. Colinearity analysis revealed associations between CD4 cell counts and T-cell activation levels (data not shown), in agreement with variable inter-dependency [21]. MLR analysis revealed a

statistically significant association between PD-1 and human leucocyte antigen DR expressions on CD4 T-cells ($\beta = 0.444$, $P = 0.030$). Moreover, PD-1 expression levels within CD45RO⁺CD27⁺ CD8 T-cells were significantly associated with CD38 expression on CD8 T-cells ($\beta = 0.542$, $P = 0.025$) and nearly significantly associated with viremia ($\beta = 0.286$, $P = 0.055$). Moreover, a canonical analysis further supported a role for immune activation in driving increased PD-1 expression on CD4 and CD8 T-cells, as well as an additional contribution of viremia to PD-1 up-regulation on CD8 T-cells (Supplemental Digital Content, Fig. 2, <http://links.lww.com/QAD/A215>).

Overall, our results suggest that immune activation is the main factor contributing to the increased PD-1 levels during HIV infection. Additionally, they also indicate a role for viremia in PD-1 up-regulation on CD8 T-cells, particularly on the CD45RO⁺CD27⁺ subpopulation.

Programmed death-L1 expression on T-cells

Programmed death-L1 can also be up-regulated on T-cells upon activation [30]. Strong direct correlations were observed in HIV-2-positive individuals between PD-L1 expression on CD4 and CD8 T-cells and both the degree of CD4 depletion and T-cell activation (Table 1 and Supplemental Digital Content, Table 2, <http://links.lww.com/QAD/A215>). Such correlations were largely absent in the HIV-1 cohort and nonexistent in seronegative controls (Table 1), suggesting that PD-L1 expression on T-cells increases specifically during HIV-2 disease progression. This was observed despite the lack of a significant increase in PD-L1 expression levels on CD8 and CD4 T-cells in HIV-2 infection and of a similar differentiation-associated pattern of PD-L1 and PD-1 expression in all cohorts (Supplemental Digital Content, Fig. 3, <http://links.lww.com/QAD/A215>). Moreover, the expression of PD-L1 and PD-1 expression within CD4 and CD8 T-cells was apparently highly related (Fig. 1b). Overall, our data revealed that HIV-2-positive individuals showed strong correlations between PD-L1 and CD4 depletion and T-cell activation, suggesting that the strict control of PD-L1 expression may contribute to the slow HIV-2 disease progression.

TIM-3 expression on T-cells

In order to investigate TIM-3 expression in HIV-2 infection and its relationship with PD-1/PD-L1, we studied additional age-matched cohorts of ART-naïve HIV-1-positive and HIV-2-positive individuals with more than 300 CD4 T-cells/ μ l, and seronegative controls (Supplemental Digital Content, Table 3, <http://links.lww.com/QAD/A215> for clinical and epidemiological data).

The mechanisms regulating TIM-3 expression remain poorly understood [31–34]. Regarding the CD8 T-cell subset, the frequency of TIM3⁺PD1⁺ cells was very low

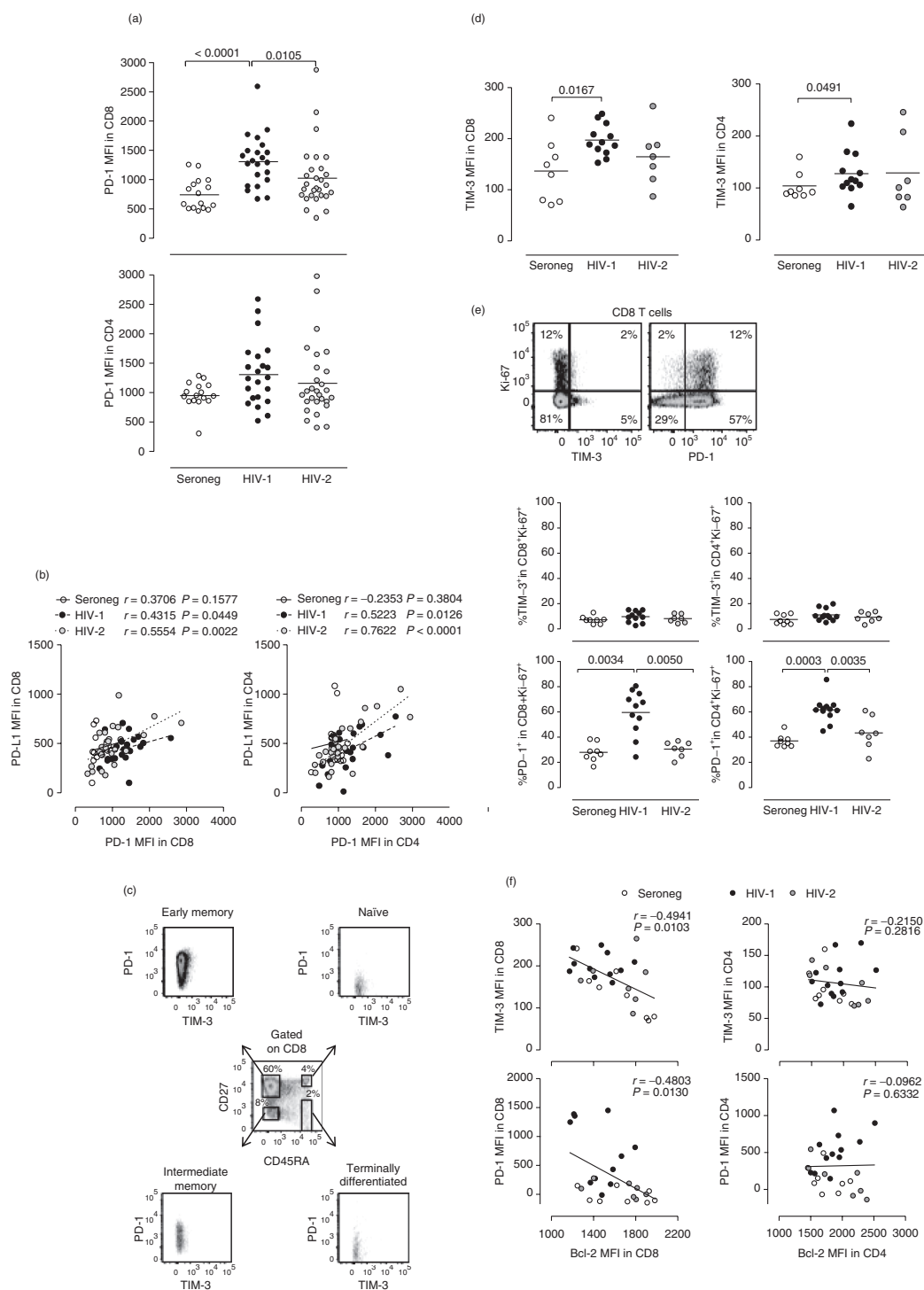


Fig. 1. T-cell expression of PD-1, PD-L1 and TIM-3 in HIV-2 infection. (a) PD-1 mean fluorescence intensity (MFI) assessed within total CD8 and CD4 T-cells in seronegative, HIV-1 and HIV-2 cohorts. (b) Correlations between PD-1 and PD-L1 MFI within total CD8 and CD4 T-cells in the three cohorts. (c) Illustrative dot-plots of PD-1 and TIM-3 expression within naïve and memory CD8 T-cell subsets, defined according to CD45RA and CD27, in an HIV-1-positive patient with 650 CD4 T-cells/ μ l and 21 731 RNA copies/ml. (d) TIM-3 MFI within total CD8 and CD4 T-cells in seronegative, HIV-1 and HIV-2 cohorts in early-stage disease as described in Supplementary Digital Content, Table 3. (e) Dot-plots illustrate the analysis of TIM-3 and PD-1 in relation to Ki-67 within total CD8 T-cells in the same patients as in (c). The relative contribution of TIM-3⁺ and PD-1⁺ cells to the pool of cycling CD8 and CD4 T-cells (Ki-67⁺) are shown in the graphs. (f) Correlations between the expression of the pro-survival factor Bcl-2 and TIM-3 or PD-1 expression within CD8 and CD4 T-cells in the three cohorts. Spearman correlation coefficients are shown. Each dot represents one individual. Bars represent mean.

Table 1. Correlations between PD-1 or PD-L1 expression (MFI) on CD4 and CD8 T-cells and surrogate markers of HIV disease progression.^a

	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
PD-1 MFI and:						
% CD4 T-cells	0.33;0.21	0.01;0.96	−0.83;<0.0001	−0.20;0.37	−0.62;0.0004	−0.20;0.30
CD4 T-cells/ μ l	0.36;0.18	0.13;0.63	−0.85;<0.0001	−0.39;0.07	−0.50;0.007	−0.27;0.17
%HLA-DR ⁺ in CD4	0.19;0.49	0.32;0.23	0.64;0.001	0.19;0.39	0.68;<0.0001	0.30;0.11
CD38 MFI in CD8	−0.006;0.98	−0.10;0.72	0.54;0.009	0.59;0.004	0.58;0.001	0.59;0.001
Viremia (RNA cp/ml) ^a	NA	NA	0.43;0.04	0.41;0.05	NA	NA
PD-L1 MFI and:						
% CD4 T-cells	0.04;0.87	0.19;0.47	−0.42;0.05	−0.43;0.04	−0.44;0.02	−0.43;0.02
CD4 T-cells/ μ l	0.01;0.98	−0.17;0.52	−0.31;0.16	−0.39;0.07	−0.58;0.001	−0.46;0.01
%HLA-DR ⁺ in CD4	0.16;0.55	0.28;0.30	0.21;0.35	0.25;0.27	0.54;0.003	0.35;0.07
CD38 MFI in CD8	−0.15;0.57	−0.22;0.41	0.29;0.18	0.26;0.25	0.58;0.001	0.40;0.03
Viremia (RNA cp/ml) ^b	NA	NA	0.18;0.43	0.11;0.64	NA	NA

^aRelationships were tested for significance with Spearman correlations: *r*; *P* values are shown. Results with *P* values less than 0.05 are highlighted in bold. NA, not applicable.

^bFor statistical analyses, half of the cut-off value was considered in the cases in which viremia was below the detection limits.

(Fig. 1c) and, in contrast to PD-1, TIM-3 expression seemed to be independent of T-cell differentiation in all cohorts (Supplemental Digital Content, Fig. 4, <http://links.lww.com/QAD/A215>).

TIM-3 expression was higher in HIV-1-positive patients as compared to both HIV-2-positive and seronegative individuals on CD8 and CD4 T-cells, although only reaching significance in comparison with the latter (Fig. 1d).

Of note, the contribution of TIM-3⁺ cells to cycling T-cells was negligible in all cohorts in marked contrast to PD-1⁺ cells (Fig. 1e), which represented a significant proportion of cycling T-cells, particularly in the HIV-1 cohort, further supporting the requirement of T-cell receptor stimulation for PD-1 up-regulation.

Conversely, both PD-1 and TIM-3 expression seemed to impact on CD8 T-cell survival, as previously described [35,36], as indicated by the inverse correlation found with Bcl-2 expression levels within CD8 T-cells (Fig. 1f). No such correlations were found on CD4 T-cells (Fig. 1f).

We also confirmed that the above reported imbalances in the expression of inhibitory molecules were unrelated to regulatory CD4 T-cells (Treg), defined by FoxP3 expression (Supplemental Digital Content, Fig. 5, <http://links.lww.com/QAD/A215>) that have been reported to express high levels of PD-1 and TIM-3 [37,38].

Overall, our data showed that over-representation of a TIM-3⁺ population in T-cells, particularly within the CD8 subset, was much more marked in HIV-1 than in HIV-2 infection. Moreover, TIM-3 expression was apparently independent of T-cell differentiation and disassociated from cell cycling.

Discussion

We report here, for the first time, that HIV-2 infection was associated with increased PD-1/PD-L1 expression on both CD4 and CD8 T-cells, in direct correlation with the degree of CD4 depletion and T-cell activation. PD-1 up-regulation occurred in the majority of untreated HIV-2-positive individuals despite their low to undetectable viremia. We also found strong correlations between the expression of its main ligand, PD-L1, within both CD4 and CD8 T-cells, and the degree of CD4 depletion and immune activation that were exclusive to the HIV-2 cohort, in agreement with our previous data on plasmacytoid dendritic cells [23]. The expression of these inhibitory markers seemed to be mainly driven by immune activation, the major determinant of the rate of CD4 T-cell loss and AIDS progression, both in HIV-1 and HIV-2 infections [5,8,39,40]. Our data suggest that PD-1/PD-L1 up-regulation may reflect an attempt to limit immunopathology, hence contributing to a more benign course of HIV infection.

We showed that HIV-2-positive individuals did not feature a significant up-regulation of TIM-3, in contrast to the HIV-1 cohort which exhibited an over-expression of TIM-3 within CD4 and CD8 T-cells, as previously reported [15,16], even in early-stage disease. TIM-3 expression was dissociated from PD-1 expression, and markers of cell differentiation and cell cycling, but inversely correlated with the levels of the antiapoptotic molecule Bcl-2 on CD8 T-cells. Thus, PD-1 and TIM-3 may be differentially regulated, resulting in their expression by functionally distinct T-cell populations. Overall, we found no significant changes in TIM-3 expression in HIV-2-positive individuals, in contrast to its up-regulation throughout HIV-1 infection, suggesting a role of TIM-3 in the protected HIV-2 disease course.

In conclusion, we showed that, during the relatively benign course of HIV-2 infection, there was a progressive increase in the expression of PD-1 and PD-L1 within CD4 and CD8 T-cells but not of TIM-3. Our data suggest that the PD-1/PD-L1 pathway may counter-act the hyper-immune activation associated with AIDS progression with implications for the design of ART-complementary immune-based strategies.

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Author contributions: R.T., R.B.F. and A.P.B. designed and performed experiments, analysed data and wrote the paper; F.P. performed statistical analysis; R.S.S., R.C., and P.G. performed experiments; E.V. and R.M.M.V. contributed to the design of the study and data interpretation; A.E.S. designed the research and wrote the paper.

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Conflicts of interest

The authors have no conflicting financial interests.

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