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

Supplemental Digital Content

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Supplemental Table 1. *Cohort clinical and epidemiological data.[a]*

	Seronegatives	HIV-1	HIV-2
Numbers (male/female)	16 (6/10)	22 (17/5)	28 (9/19)
Age (years)	44 ± 2 (27-57)	40 ± 2 (23-61)	49 ± 3 (19-78)
Ethnicity (Caucasian/other)	15/1	13/7	14/14
% CD3 cells	62.6 ± 1.9 (49-76.3)	67.4 ± 2.1 (48.6-82.8)	63.8 ± 2.0 (46.3-87.4)
CD3 cells/µl	1286 ± 93 (695-1929)	1621 ± 171 (538-3970)	1399 ± 112 (346-2930)
% CD4 T-cells	52.4 ± 2.5 (5.36-67.0)	24.6 ± 3.9 (1.7-70.1)***	31.6 ± 3.1 (6.8-62)***
CD4 T-cells/µl	906 ± 59 (518-1312)	569 ± 105 (18-1848)*	659 ± 81 (52-1511)*
% HLA-DR$^+$ in CD4	4.1 ± 0.4 (1.9-7.6)	17.1 ± 3.0 (1.7-54.5)***	11.4 ± 1.7 (1.8-36.3)**
% CD8 T-cells	24.0 ± 1.8 (11.7-35.2)	46.7 ± 2.8 (12.4-64.7)***	38.1 ± 2.3 (21-65.7)***#
CD8 T-cells/µl	503 ± 51 (212-802)	1107 ± 120 (138-2514)***	816 ± 75 (221-1817)**#
CD38 MFI in CD8	1237 ± 106 (838-2539)	5128 ± 848 (706-13553)***	2854 ± 537 (643-13659)* #
Viremia (RNA cp/ml)	NA	778459 ± 334956 (71-4.5x10^6)[b]	11266 ± 3,633 (1189-3.6x10^4)[c]

[a]Data are Mean±SEM with limits in brackets, unless indicated otherwise. NA, Not Applicable. Data referring to the early stage cohort are not included in this Table (see Supplemental Table 3). Statistical differences between a given HIV cohort and the seronegative controls: *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$. Statistical differences between HIV-1+ and HIV-2+ individuals: #, $p < 0.05$.

[b] HIV-1 viremia was < 40 RNA copies/ml (cutoff) in 3 of the 22 subjects studied.

[c] HIV-2 viremia was < 200 RNA copies/ml (cutoff) in 21 of the 28 subjects studied.

Supplemental Table 2. *Correlations between PD-1 or PD-L1 MFI on CD4 and CD8 T-cells and additional surrogate markers of HIV disease progression.*[a]

	PD-1 MFI					
	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
HLA-DR MFI in CD4	0.36;0.17	0.40;0.13	**0.70;0.0003**	0.36;0.10	**0.67;<0.0001**	**0.45;0.02**
%HLA-DR$^+$ in CD8	0.16;0.55	0.17;0.52	**0.47;0.02**	**0.43;0.04**	**0.63;0.0004**	**0.37;0.05**
HLA-DR MFI in CD8	0.23;0.39	0.21;0.43	0.35;0.12	**0.58;0.005**	**0.60;0.0008**	**0.47;0.01**
%CD38$^+$ in CD8	-0.11;0.68	-0.11;0.67	**0.55;0.009**	**0.50;0.02**	**0.46;0.01**	**0.44;0.02**
β2-microglobulin (mg/l)[b]	ND	ND	0.38; 0.18	0.10;0.72	**0.57;0.0075**	0.35;0.12
	PD-L1 MFI					
	Seronegatives		HIV-1		HIV-2	
	CD4	CD8	CD4	CD8	CD4	CD8
HLA-DR MFI in CD4	0.17;0.53	0.41;0.11	0.28;0.21	0.28;0.20	**0.53;0.004**	0.32;0.09
%HLA-DR$^+$ in CD8	0.16;0.54	-0.01;0.97	**0.56;0.01**	**0.52;0.01**	**0.51;0.005**	**0.39;0.04**
HLA-DR MFI in CD8	0.11;0.68	0.01;0.98	0.41;0.06	**0.44;0.04**	**0.49;0.008**	0.34;0.07
%CD38$^+$ in CD8	-0.31;0.25	-0.37;0.16	0.28;0.20	0.20;0.37	**0.53;0.004**	0.33;0.08
β2-microglobulin (mg/l)[b]	ND	ND	0.27;0.35	-0.01;0.98	**0.66;0.001**	**0.55;0.01**

[a] Relationships were tested for significance with Spearman correlations: r;*p* values are shown. Results with *p* values <0.05 are highlighted in bold. ND: Not Determined.

[b] β2-microglobulin serum levels were assessed for 21 HIV-2 (3.0±0.4 mg/l) and 14 HIV-1 (3.0±0.4 mg/l) infected individuals, with no statistically significant differences having been observed between cohorts.

Supplemental Table 3. *Clinical and epidemiological data of the early stage cohorts.*[a]

	Seronegatives	HIV-1[a]	HIV-2[a]
Numbers (male/female)	8 (3/5)	12 (8/4)	7 (1/6)
Age (years)	42 ± 5 (26-57)	41 ± 3 (25-63)	50 ± 5 (33-66)
Ethnicity (Caucasian/other)	8/0	9/3	5/2
% CD3 cells	67.3 ± 3.8 (51.8-88.1)	69.7 ± 2.3 (56.6-86.7)	72.3 ± 2.1 (63.5-78.6)
CD3 cells/µl	1725 ± 307 (927-3135)	1730 ± 130 (1102-2756)	1547 ± 197 (1126-2517)
% CD4 T-cells	36.3 ± 3.6 (26.2-51.9)	26.9 ± 2.6 (19.4-50.1)*	31.6 ± 3.1 (19.5-45.7)
CD4 T-cells/µl	905 ± 118 (512-1385)	662 ± 74 (430-1361)	697 ± 143 (335-1464)
% HLA-DR$^+$ in CD4	5.8 ± 0.9 (3.8-10.3)	6.7 ± 1.2 (1.5-13.7)	5.9 ± 1.4 (3.1-11.6)
% CD8 T-cells	30.4 ± 5.4 (13.2-61.1)	42.1 ± 2.9 (26.3-67)*	40.4 ± 2.9 (28.1-47.7)
CD8 T-cells/µl	808 ± 289 (302-2175)	1054 ± 104 (493-1887)	834 ± 76 (526-1130)
CD38 MFI in CD8	2447 ± 298 (1394-3993)	4895 ± 626 (1865-9787)**	3098 ± 220 (2454-3665)
Viremia (RNA cp/ml)	NA	25,949 ± 8405 (295-73,110)	NA[b]

[a] Data are Mean±SEM with limits in brackets, unless indicated otherwise. NA, Not Applicable. Statistical differences between a given HIV cohort and the seronegative controls: *, $p < 0.05$; **, $p < 0.001$.

[b] HIV-2 viremia was < 200 RNA copies/ml (cutoff) in all of the subjects studied.

Supplemental Figure 1. *PD-1 expression according to cell-differentiation and viremia in HIV-2+ individuals.*
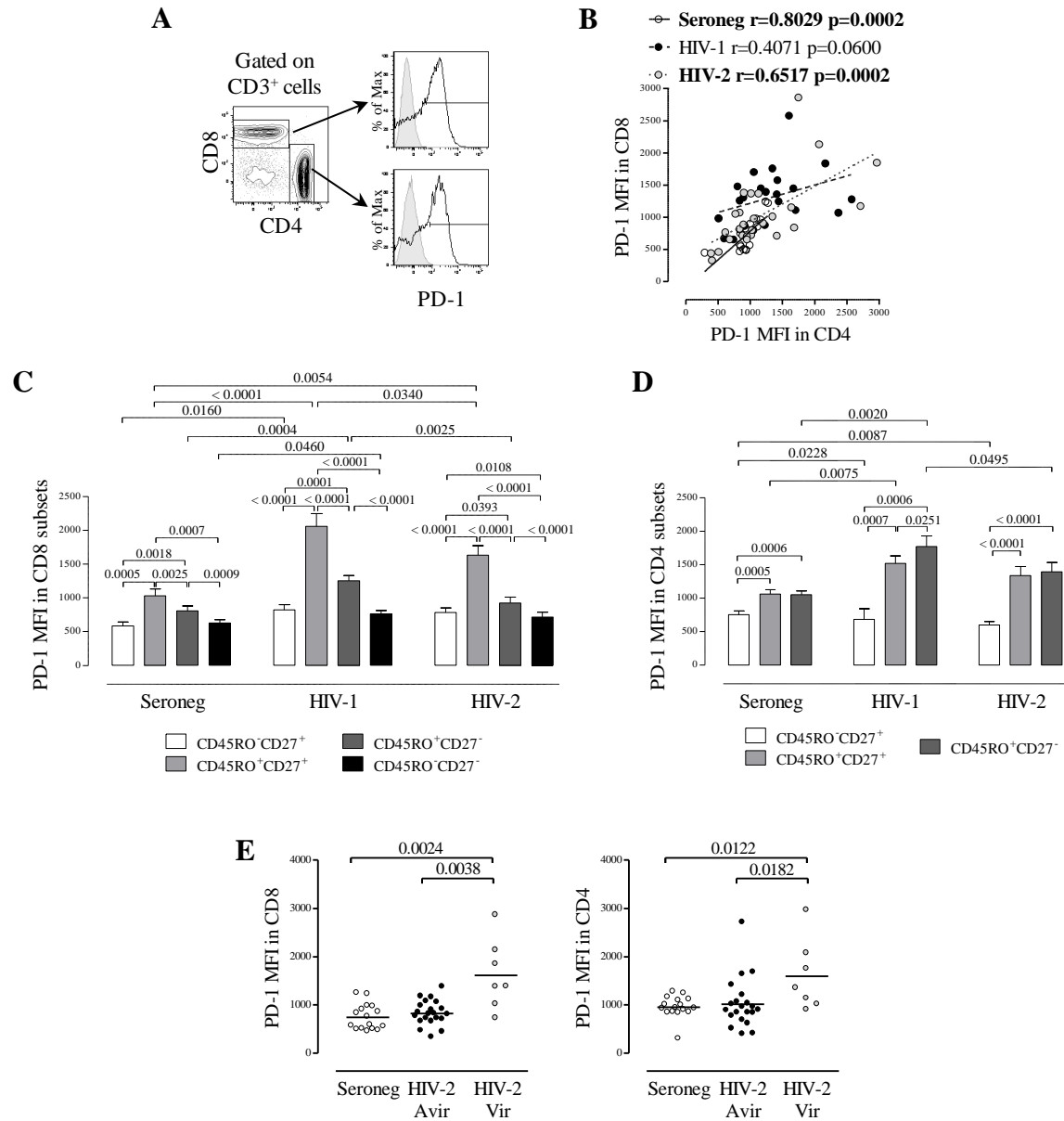

(A) Gating strategy used to assess PD-1 (clone: MIH-4) expression by flow cytometry: cells were first gated according to CD8 or CD4 expression (within a CD3+ gate) and PD-1 expression was then assessed using as reference a "fluorescence minus one" control (grey histogram). **(B)** Similarly to seronegative controls, HIV-2+ individuals exhibited a significant correlation between PD-1 expression on CD8 and on CD4 T-cell subsets. PD-1 expression was assessed within naïve and memory CD8 **(C)** and CD4 **(D)** T-cell subsets defined according to CD45RO and CD27 expression. Bars represent mean±SEM and statistically significant differences are shown. **(E)** PD-1 expression in HIV-2+ individuals stratified according to levels of circulating virus: undetectable (aviremic, avir) and viremic (vir). Each dot represents one individual.

Supplemental Figure 2. *Canonical correlation analysis.*

As none of the MLR models fully explained the variability in PD-1 expression seen among the different T-cell subsets ($r^2 < 0.6$ for all models), a canonical correlation analysis was performed in order to clarify the determinants of PD-1 expression within T-cells.

This technique looks for correlations between linear combinations of a set of predictive variables and of outcome variables, commonly denominated variates or canonical variables. In our analysis, the predictive variate (variate 1) included CD4 T-cell counts, viral load and T-cell activation (HLA-DR expression on CD4 T-cells and HLA-DR and CD38 expression on CD8 T-cells, measured as MFI), and the outcome variate (variate 2) PD-1 expression (MFI) on CD4 and CD8 T-cells in all the individuals studied. The two most significant correlations found are shown.

A
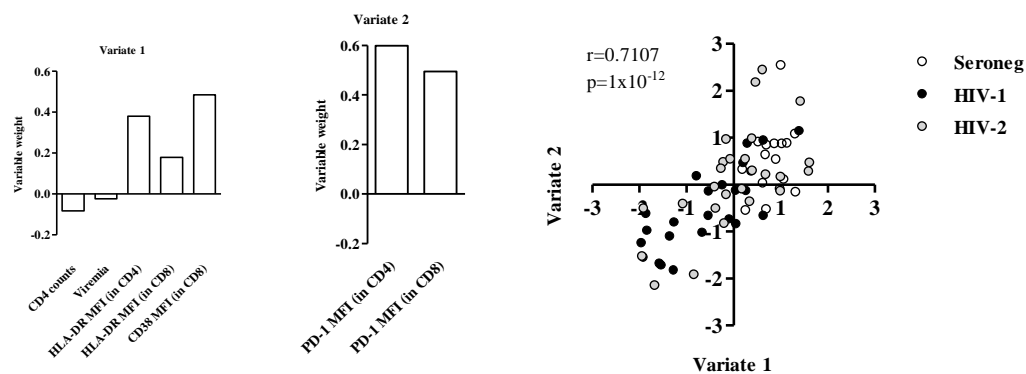
The first **(A)** shows a strong association between a predictive variate dominated by T-cell activation parameters and a outcome variate with high values for PD-1 expression on both total CD4 and CD8 T-cells, which implicates immune activation as the major determinant of PD-1 expression on both T-cell subsets.
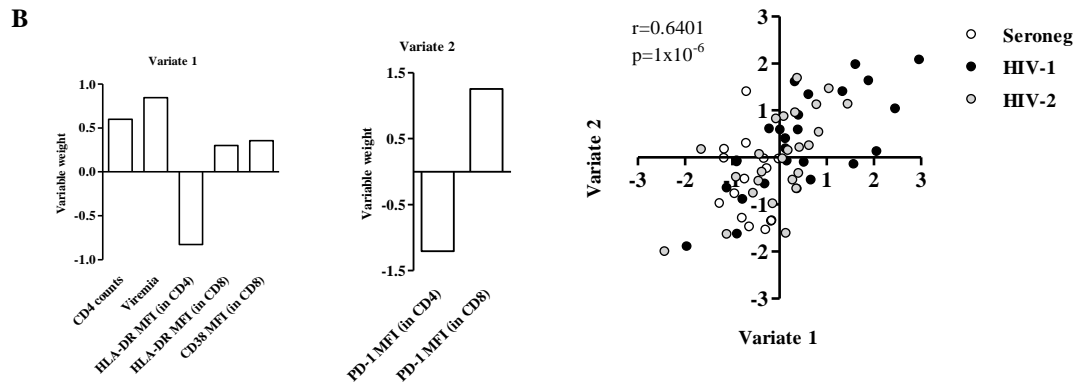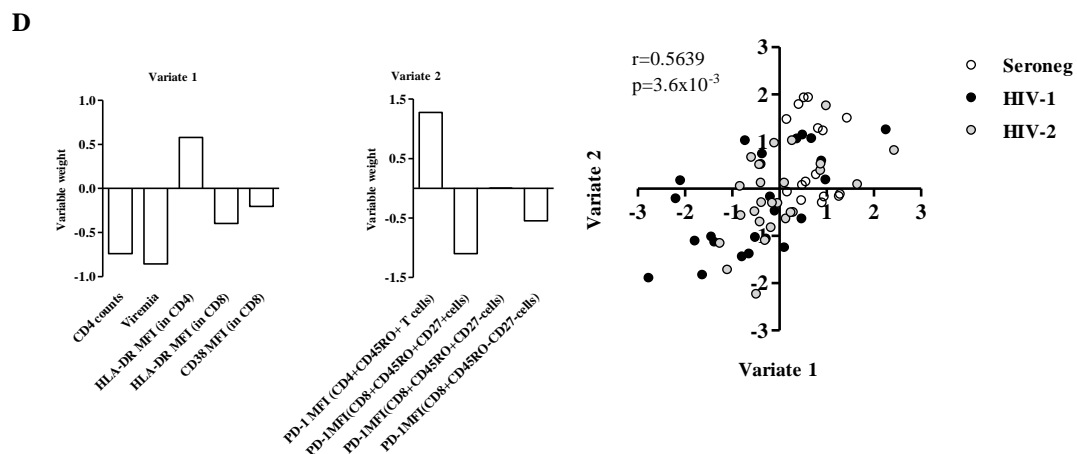
The second **(B)** represents an association between low HLA-DR expression on CD4 T-cells and high viremia with low PD-1 expression on CD4 T-cells and high PD-1 expression on CD8 T-cells, thus reinforcing the role of immune activation in driving PD-1 over-expression but also indicating a contribution of viremia in the up-regulation of PD-1 on CD8 T-cells.

Colinearity analysis failed to reveal any significant association between PD-1 expression on the different T-cell subpopulations, which suggests that PD-1 expression is regulated in a subset-independent manner. To address the regulation of PD-1 expression within the different T-cell subpopulations, we performed an additional canonical correlation analysis in which disease progression markers (variate 1) were correlated to PD-1 expression on memory-effector CD4 and CD8 T-cell subsets (variate 2 – among memory CD4 T-cells, CD45RO$^+$ cells were considered as a whole as no difference in PD-1 expression was observed between CD27$^+$ and CD27^{neg} counterparts). The two most significant correlations found are shown.

C
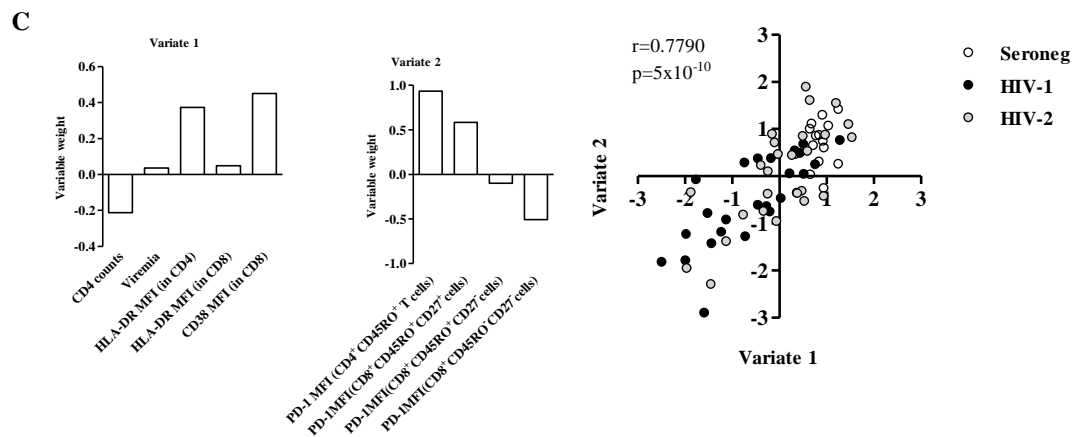
The first **(C)** demonstrates a relationship between a predictive variate combining mostly T-cell activation parameters and a outcome variate combining PD-1 expression within CD4$^+$CD45RO$^+$ and CD8$^+$CD45RO$^+$CD27$^+$ T-cells.

D

The second **(D)** represents direct associations between CD4 T cell counts, viremia and PD-1 expression on CD8$^+$CD45RO$^+$CD27$^+$ T-cells and CD4 T cell activation (HLA-DR expression) and PD-1 expression on memory CD4 T-cells. Overall, this analysis supports a major role for T-cell activation in PD-1 up-regulation on both CD4 and CD8 memory T-cells and a contribution of CD4 T-cell depletion and viremia to PD-1 overexpression on CD8$^+$CD45RO$^+$CD27$^+$ T-cells.

Supplemental Figure 3. *PD-L1 expression on T-cells.*
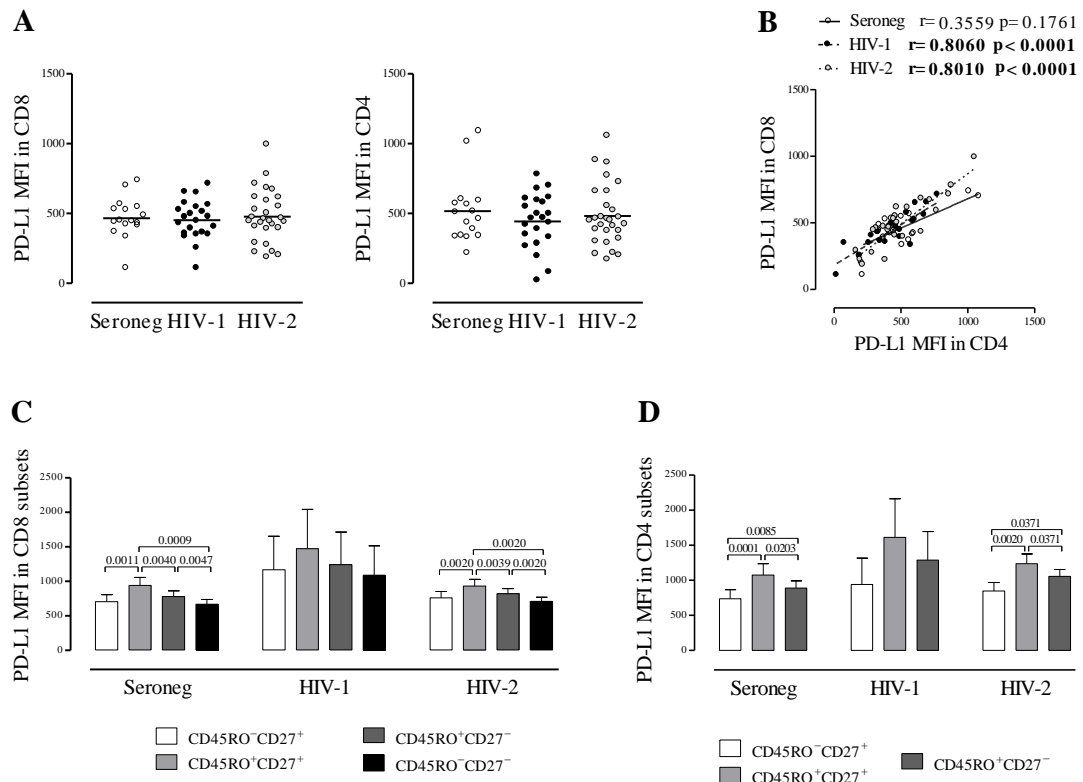
(**A**) PD-L1 MFI (clone MIH-1) within total CD8 and CD4 T-cells in seronegative, HIV-1 and HIV-2 cohorts. Bars represent mean and each dot represents one individual. (**B**) Direct correlations were found in both infected cohorts between PD-L1 expression levels within CD4 and CD8 T-cells. PD-L1 expression was further addressed on naïve and memory CD8 (**C**) and CD4 (**D**) T-cell subsets in a group consisting of 14 seronegative, 5 HIV-1+ and 10 HIV-2+ individuals. Naïve and memory T-cell subsets were defined according to CD45RO and CD27 expression and PD-L1 Mean Fluorescence Intensity (MFI) was then assessed within each cell population. Bars represent mean±SEM.

Supplemental Figure 4. *TIM-3 expression on CD8 T-cell subsets.*
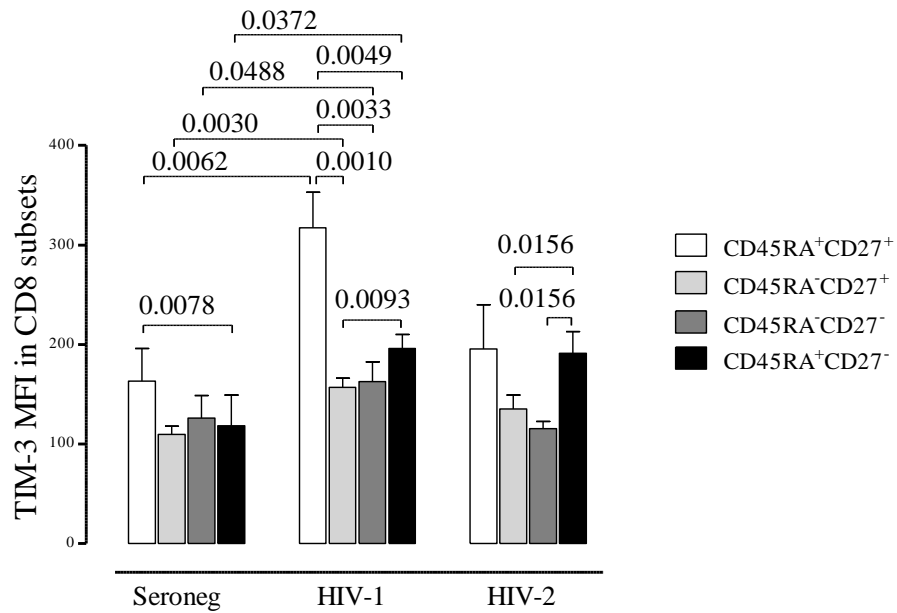
Graph shows TIM-3 MFI (clone: 344823) within each CD8 T-cell sub-population in all cohorts. Bars represent mean±SEM.

Supplemental Figure 5. *PD-1, PD-L1 and TIM-3 expression on regulatory, memory Foxp3^{neg}, and naïve CD4 T-cells.*
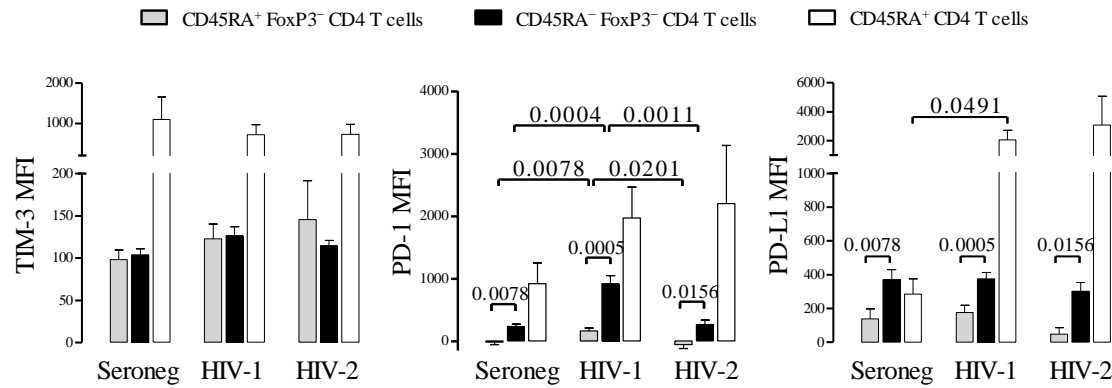
TIM-3 (left), along with PD-1 (middle) and PD-L1 (right) MFI was assessed within CD45RA$^+$FoxP3$^-$ and CD45RA$^-$FoxP3$^-$ populations and FoxP3$^+$ CD4 T-cells in seronegative, HIV-1+ and HIV-2+ individuals. Bars represent mean±SEM. The results obtained with naïve and memory FoxP3$^-$ subsets were broadly similar to those observed within total CD4 T-cells. Regarding the Treg population, both HIV-2 and HIV-1 cohorts featured significantly higher levels of PD-1 expression than the seronegative individuals, but TIM-3 levels were similar in all cohorts. Also, a preliminary analysis revealed a strong correlation between PD-L1 MFI on CD4 T-cells and the frequency of FoxP3-expressing CD4 T-cells in the HIV-2 (*p*=0.007, r=0.881, n=8) but not in the HIV-1 cohort (*p*=0.808, r=-0.143, n=6).