Supplementary Material and Methods

Patients and healthy volunteers

Samples from patients and healthy controls were obtained under NIAID Institutional Review Board approved clinical research study protocols in the NIAID/CCMD intramural program. Both healthy controls and HIV infected patients supplied written informed consent for use of their blood. Samples from healthy controls were obtained from the NIH Blood Bank. Samples from patients with HIV infection were obtained from the NIH Clinical Center. Patients and healthy controls used in Figure 1, 2, 3 and 4 are described at Table S1. Treatment regime and associated complications of patients on Figure 2, 3, 4, are presented in Table S3 and S4.

Flow cytometry

Whole blood was collected and processed within 3 hours after blood draw. For whole blood staining, 400 µl blood was incubated for 10 minutes at room temperature with 10 µg/ml human IgG (Sigma-Aldrich, MO) to prevent potential Fc receptor binding. This was followed by incubation with a cocktail of mAbs: anti-CD3 FITC (BD Pharmingen, clone SK7); anti-CD4 Qdot605 (Invitrogen, clone S3.5) or anti-CD8 Qdot605 (Invitrogen, clone 3B5); anti-CD45RA Pacific Blue (Invitrogen, clone MEM-56); anti-CD27 APC-H7 (BD Pharmingen, clone M-T271); anti-CD42b PerCP (Biolegend, clone HIP1); anti-CD62P P-Selectin APC (BD Pharmingen, clone AK-4). After a 15 minute incubation at room temperature, red blood cells were lysed with BD FACS Lysing Buffer (BD Biosciences, CA). Samples were collected on a BD LSR II using FACSDiva software and the data was subsequently analyzed using FlowJo software (Tree Star).

Binding Recombinant CD62P-Fc: T cells were obtained from PBMCs from healthy controls (n=13) and HIV infected patients (n=18). cART treated HIV infected patients

had successfully suppressed viremia to <40 copies/ml (median months cART 65 (IQR: 44.8-135.3) and supplementary Table S1). T cells were isolated by negative selection (Pan T cell isolation kit, Miltenyi Biotec, CA). Purified T cells were rested overnight in X-Vivo Medium (Lonza, MD). Cells were washed twice with binding buffer (HBSS + Ca^{++} Mg^{++} + 1 mg/ml BSA) and incubated for 30 minutes at 4^0C with 10 μg/10^6 cells of recombinant human P-selectin/CD62P (Trp42-Ala771)-Fc chimera (CD62P-Fc) or recombinant human IgG Fc as control (R&D System, CA). CD62P-Fc binding was performed in absence or presence of 10 mM EDTA. After incubation, cells were washed twice and detection was done using a mouse anti-human IgG Fc APC labeled (Southern Biotech, clone H2). After washing, the cells were stained with surface markers for T cell subsets as above.

Thrombin stimulations: PBMCs were isolated by ficoll gradient centrifugation. PBMCs were stimulated with 50U/ml thrombin and analyze by flow cytometry or Amnis (EMD-Millipore, WA) as described in Supplementary material and methods.

Biomarkers

Serum levels of D-dimer and soluble CD62P were assessed in HIV infected patients (n=20) and healthy controls (n=23). The D-dimers were run using an ELFA (Enzyme-Linked Fluorescence Assay) on a VIDAS instrument (BioMerieux, Durham, North Carolina). Soluble CD62P serum levels were measured with a magnetic bead Milliplex on a Luminex xMAP (EMD Millipore, CA).

Thrombin stimulation: 3x10^6 total PBMCs were resuspended in stimulation buffer (HBSS + Ca^{++} Mg^{++} + 1 mg/ml BSA) and stimulated for 5 or 15 minutes at 37^0C with 50 U/ml of thrombin (Enzyme Research Laboratory, IN). After stimulation, cells were washed, stained and analyzed by flow cytometry.

For Amnis (EMD-Millipore, WA) analysis, $10x10^6$ PBMCs were cultured in media or 50 U/ml of thrombin for 15 minutes at 37^0C. Cells were washed and incubated for 10 minutes with 10 μg/ml human IgG (Sigma-Aldrich, MO) to prevent potential Fc receptor binding. The cells were then stained with anti-CD3 PE-Cy7 (BD, clone SP34.2); anti-CD4 PE (BD Pharmingen, clone SK3); anti-CD42b FITC (BD Pharmingen, clone HIP1); and anti-CD62P P-Selectin APC (BD Pharmingen, clone AK-4). After staining, the cells were fixed in paraformaldehyde 4% for 10 minutes. Samples were collected on the Amnis (EMD-Millipore, WA). IDEAS software (EMD-Millipore, WA) was used to generate the ImageStream gallery display. Image display was generated by setting the pixel range above threshold background noise. The display pixel upper range was set to 255 for all intensities above this background. The composite views facilitate image analysis between different channels for the same cell, i.e. platelet-T cell conjugates. For the overlay images, the contributing contribution between any 2 or more overlaid images can be varied, in particular to highlight fluorescence in any overlaid channels. The percent image contributions for each channel in these composite views (Figure 5A and Supplemental Figure S3C) were set to 100% for brightfield, 30% for CD42b and 35% for CD62P.

Scanning electron microscopy

Platelet isolation: Platelet rich plasma was obtained by centrifugation at 200g for 20 minutes and diluted in a 1:1 volume with HEP buffer (140 mM NaCl, 2.7 mM KCl, 3.8 mM Hepes, 5 mM EGTA, pH 7.4) followed by centrifugation at 100g for 20 minutes at room temperature. The supernatant was discarded and the platelet rich pellet was washed in buffer (10mM sodium citrate, 150 mM NaCl, 1 mM EDTA, 1% (w/v) dextrose, pH 7.35). The pellet was then resuspended in Tyrode's buffer (134 mM NaCl, 12 mM

NaHCO3, 2.9 mM KCl, 0.34 mM Na2HPO4, 1 mM MgCl2, 10 mM Hepes, 3 mg/ml BSA, 5 mM glucose, pH 7.4). Platelets were counted using a hemacytometer and adjusted to the desired concentration ($20x10^6$ cells/ml) before being added to T cells suspension as described below.

CD8 T cells and platelets stimulation: CD8 T cells were isolated by negative selection (Miltenyi Biotec, CA) and stained with anti-CD3 FITC (BD Pharmingen, clone SK7); anti-CD8 Qdot605 (Invitrogen, clone 3B5); anti-CD45RA Pacific Blue (Invitrogen, clone MEM-56); anti-CD27 APC-H7 (BD Pharmingen, clone M-T271); and the CD8 T cell memory subset ($CD3^+CD8^+CD45RA^-CD27^+$) was obtained by FACS sorting. The sorted CD8 memory T cells were resuspended (at $2x10^6$ cells/ml) in HBSS + Ca^{++} Mg^{++} + 1 mg/ml BSA and platelets were added at a 1:1 ratio. The cells were stimulated for 15 minutes at 37^0C with 50 U/ml of thrombin (Enzyme Research Laboratories, IN), spun down and washed with PBS. The samples were fixed in a cocktail of 4% formaldehyde and 2% glutaraldehyde in 0.1M cacodylate buffer and post fixed using a 1% osmium tetroxide solution. They were then dehydrated in a series of graded alcohols and air dried after a final dehydration course of tetramethylsilane. Subsequently, the samples were sputter coated with a thin layer of iridium and imaged utilizing a Hitachi S-4500 field emission scanning electron microscope (Tokyo, Japan).

Statistical analysis method

A nonparametric unpaired Mann-Whitney test was used to compare the data between the HIV infected patients and healthy controls. A nonparametric, paired Wilcoxon test was used to compare unstimulated and thrombin stimulated PBMCs. Statistically significant

differences were considered to be those whose p value was <0.05. Spearman's correlation coefficients were use to evaluate correlations.

Supplementary Figures

Figure S1. PSGL1 expression on CD4 and CD8 T cell subsets and recombinant CD62P binding affinity on activated HLADR^{+}CD38^{+} T cells

(A) PSGL1 expression on CD4 and CD8 T cells. Flow cytometry gating strategy used to analyze PSGL1 expression on CD4 and CD8 T cell subsets: naïve (CD45RA+CD27+), memory (CD45RA-CD27+), memory/effector (CD45RA-CD27-), TEM (CD45RA+CD27-). **(B)** Overlay histogram of PSGL1 on the CD4 and CD8 T cells subsets: naïve (gray) memory (black), memory/effector (red), TEM (blue) and istotype control (dotted gray line). **(C)** PSGL1 MFI expression in the subsets in CD4 and CD8 T cells from healthy controls (black symbols, n=13) and HIV infected patients (red symbols, n=18). **(D)** Flow cytometry gating strategy used to analyze HLADR and CD38 expression on total CD4 and CD8 T cells. **(E)** Representative CD62P-Fc binding plots (gray) overlaid with the IgG control (blue) of a healthy control and HIV infected patient on HLADR^{+}CD38^{+} and HLADR^{-}CD38^{-} CD4 and CD8 T cell populations. **(F)** Percentage of rCD62P-Fc binding in CD8 and CD4 T cells in the HLADR^{+}CD38^{+} and HLADR^{-}CD38^{-} populations from healthy controls (black symbols, n=13) and HIV infected patients (red symbols, n=18). Paired Wilcoxon test was used for comparisons between binding of rCD62P-Fc in the HLADR^{+}CD38^{+} and HLADR^{-}CD38^{-} populations.

Figure S3. Increased activation of platelet and platelet-leukocyte conjugates HIV infected patients.

(A) Gating strategy of whole blood from HIV infected patients (n= 20) and healthy controls HC (n=23) from Figure 2 were analyzed for platelet bound to other leukocytes types such as CD3 negative lymphocytes and high FCS-SSC (monocytes) and

free/unbound platelets. **(B)** Represents the proportion of platelets bound to monocytes (left panel) and CD3 negative lymphocytes (middle panel). Right panel shows $CD62P^+$ expression on free/unbound platelets. Mann-Whitney test was used for comparisons between HIV infected patients and healthy controls. *p* value <0.05 was considered significant.

Figure S3. Association between circulating activated platelet and activated platelet-T cell conjugates in HIV infected patients.

(A) Association between the percentage of circulating (free/unbound) activated platelets ($CD42b^+CD62P^+$) and the proportion of activated platelets bound to CD4 and CD8 T cells in patients with HIV infection (n= 20). **(B)** Relationship between serum levels of D-dimer and circulating activated platelet-CD4 and –CD8 T cell conjugates in patients with HIV infection (n=20).

Figure S4. Increased activation of platelet and platelet-leukocyte conjugates HIV infected patients.

HIV infected patients (n= 20) and healthy controls HC1 (n=23) from Figure 2 and healthy controls (HC2, Supplementary Table S1 and S2) were analyzed for platelet T cell conjugates. Mann-Whitney test was used for comparisons between HIV infected patients and healthy controls. *p* value <0.05 was considered significant.

Figure S5. ImageStream gating Strategy.

Gating strategy of samples shown in Figure 5A. PBMCs from healthy controls (n= 10) and HIV infected patients (n= 5) were cultured in the presence or absence of thrombin

(50 U/ml) for 15 minutes. Cells were stained with CD3, CD4, CD42b and CD62P mAbs and fixed prior to acquisition in ImageStream (Amnis). **(A)** For analysis, acquired samples were pre-gated for those cells in focus followed by a singlets side-scatter gate and a $CD42b^+$ vs SSC gate was performed to eliminate free/unbound platelets (data not shown). The lymphocytes were gated using the equivalent to FSC vs SSC followed by $CD3^+CD4^+$ for (CD4 T cells, green) and $CD3^+CD4^-$ for (CD8 T cells, yellow). In addition, a gate based on $CD62P^+CD42b^+$ (pink) was performed to analyze the platelet-T cells conjugates formation in unstimulated and after thrombin stimulation. Example of analysis of 10 platelet-CD4 and –CD8 T cell conjugates cultured in media or after thrombin stimulation. **(B)** Overlay of the brightfield morphology, CD62P, and CD42b channels in10 representative free/unbound platelets in unstimulated and stimulated conditions (samples showed in Figure 5A).

Table S1. HIV infected Patients and Healthy controls

	HIV$^+$ Patients (n= 18) Fig 1	HIV$^+$ Patients (n= 20) Fig 2, 3, 4	HC1 (n=23) Fig 2,3,4	HC2 (n=24) Fig S2
HIV RNA (copies/ml)	40	40	N/A	N/A
CD4 counts (cells/μl)	691.5 (605-1068)	454 (309-582)	n/a	585.5 (410-812)
CD8 counts (cells/μl)	757.5 (534-920)	808 (504-1291)	n/a	424 (288-517)
% CD4 HLA-DR+ 38+	5.0 (3.8-7.0)	5.5 (4.3-10.5)	n/a	4.0 (2.3-4.8)
% CD8 HLA-DR+ 38+	15.5 (12.0-22.3)	17 (10.0-22.8)	n/a	8.5 (6.0-11.0)
cART (months)	65.0 (44.8-135.3)	85.5 (15.5-135)	N/A	N/A
Aspirin Warfarin (months)	27* (25-92)	34* (7-77.5) 115**	n/a	n/a
D-Dimer (ng/ml)	n/d	451 (259-884)	351 (262.3-665.5)	718 (355.3-947.7)
sCD62P (ng/ml)	n/d	291.3 (218-359)	280 (198-315)	160 (114.3-240)

Values are presented as medians (IQR: 25% and 75%)
N/A: not applicable, n/a: not available, n/d: not determined
Patients on *Aspirin (n=3), **warfarin (n=1)

Table S2. Comparisons of marker of activation and activated platelets

	%Free/unbound Platelets CD42b+CD62P+ R, *p* value	%(CD42b+CD62P+) Platelet-CD4 T cell conjugate R, *p* value	%(CD42b+CD62P+) Platelet-CD8 T cell conjugate R, *p* value
HIV (n=20) %HLADR+CD38+ CD4 T cells	-0.01, 0.990 (0.01, 0.986)*	0.05, 0.820 (0.29, 0.251)*	0.08, 0.720 (0.33, 0.187)*
HIV (n=20) %HLADR+CD38+ CD8 T cells	-0.08, 0.710 (0.02, 0.942)*	0.18, 0.440 (0.39, 0.110)*	0.21, 0.540 (0.40, 0.107)*
HIV (n=20) CD4 count cell/μl	-0.10, 0.656 (-0.12, 0.622)*	0.04, 0.855 (-0.16, 0.528)*	-0.10, 0.654 (-0.21, 0.416)*
HIV (n=20) CD8 count cell/μl	0.13, 0.571 (0.06, 0.807)*	-0.08, 0.709 (-0.20, 0.422)	-0.16, 0.485 (-0.24, 0.351)
HIV (n=20) D-dimer ng/ml	0.51, 0.026 (0.68, 0.007)*	0.56, 0.015 (0.68, 0.007)*	0.52, 0.023 (0.62, 0.013)*
HIV (n=20) sCD62P ng/ml	0.30, 0.229 (0.37, 0.157)*	0.39, 0.117 (0.40, 0.121)*	0.22, 0.389 (0.23, 0.386)*
HC1 (n=23) D-dimer ng/ml	-0.20, 0.473	-0.30, 0.280	-0.30, 0.287
HC1 (n=23) sCD62P ng/ml	0.01, 0.973	0.29, 0.354	0.26, 0.404
HC2 (n=24) %HLADR+CD38+ CD4 T cells	-0.01, 0.958	-0.07, 0.743	0.01, 0.965
HC2 (n=24) %HLADR+CD38+ CD8 T cells	0.31, 0.128	-0.08, 0.701	-0.01, 0.928
HC2 (n=24) CD4 count cell/μl	0.14, 0.512	0.07, 0.742	-0.03, 0.875
HC2 (n=24) CD8 count cell/μl	-0.01, 0.938	0.37, 0.069	0.37, 0.069
HC2 (n=24) D-dimer ng/ml	0.16, 0.430	-0.04, 0.837	-0.02, 0.900
HC2 (n=24) sCD62P ng/ml	-0.11, 0.582	0.36, 0.070	0.42, 0.070

*Correlation after removing 3 patients on Aspirin

Table S3. cART regime (months)

Patient Number	Months VL <40	ABC/ 3TC	ATV	DRV	EFV/ FTC/ TDF	FTC/ TDF	EVG/ COBI TDF/ FTC	ETR	3TC	MVC	NVP	RAL	RTV	TMP-SMX	TER
Figure 2 - Pt 1	282			42				212			54	171		130	212
Figure 2 - Pt 2	140	45								140					
Figure 2 - Pt 3	84				84										
Figure 2 - Pt 4	102		94			102						94			
Figure 2 - Pt 5	72		72			72							72		
Figure 2 - Pt 6	145		22			22							145		
Figure 2 - Pt 7	117					101					117				
Figure 2 - Pt 8	29				29										
Figure 2,3,4 - Pt 9	9						9								
Figure 2,3,4 - Pt 10	6				5										
Figure 2,3,4 - Pt 11	47					47						47			
Figure 2,3,4 - Pt 12	120	120				103							120		
Figure 2,3,4 - Pt 13	87			4		4							4		
Figure 2,3,4 - Pt 14	4														
Figure 2,3,4 - Pt 15	8				8										
Figure 2,3,4 - Pt 16	11			11		11							11		
Figure 2 - Pt 17	293					106		51				56		293	
Figure 2 - Pt 18	100			4		100									
Figure 2 - Pt 19	42					42						42			
Figure 2 - Pt 20	174			80		80				43		80	174		

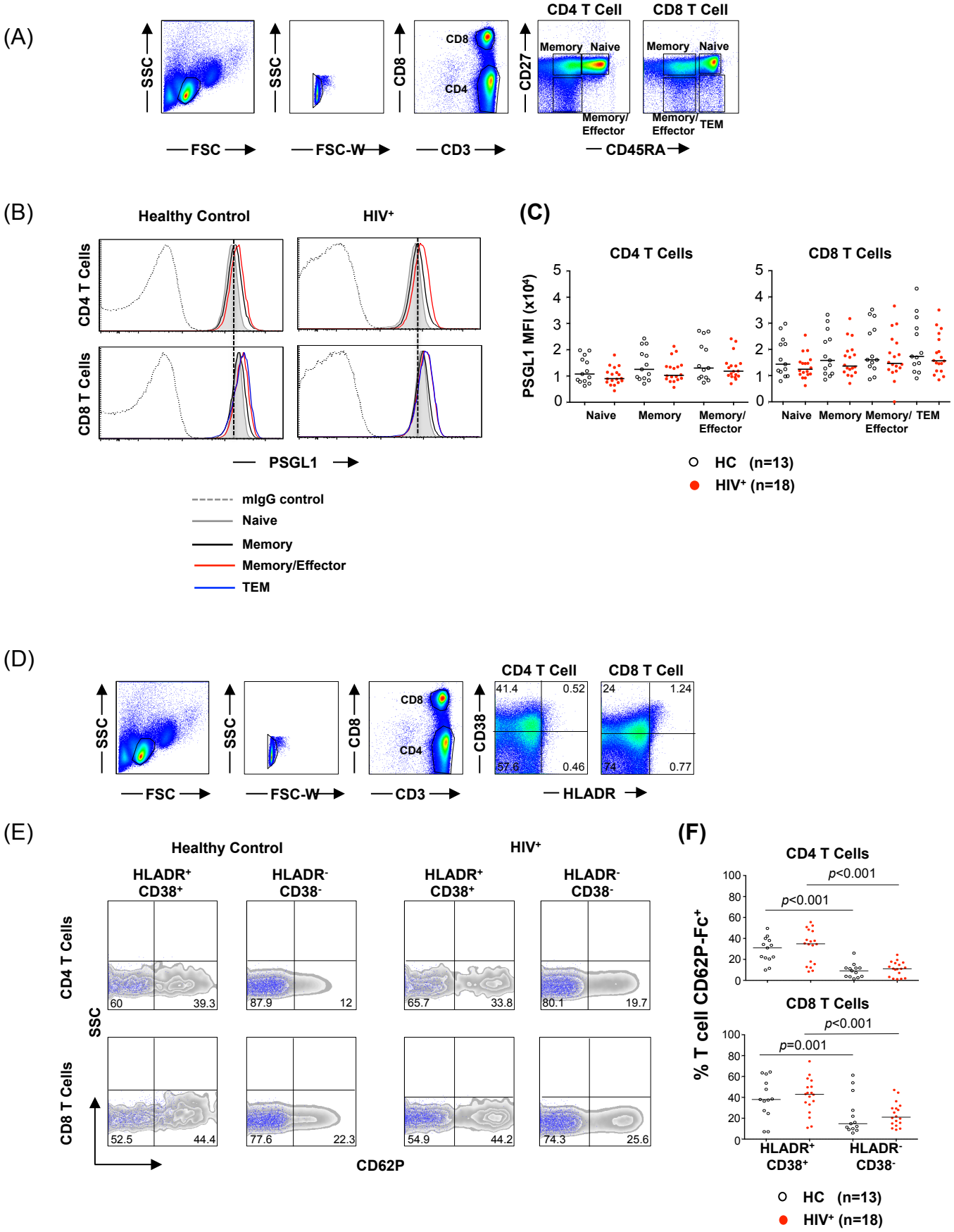
ABC: abacavir; **3TC:** lamivudine; **ATV:** atazanavir; **COBI:** Cobicistat; **EFV:** efavirenz; **EVG:** Elvitegravir; **FTC:** emtricitabine; **ETR:** etravirine; **DRV:** darunavir; **MVC:** maraviroc; **NVP**: nevirapine; **RAL**: raltegravir**; RTV:** ritonavir; **TDF:** tenofovir; **TER:** Terbinafine; **TMP-SMX:** Trimethoprim-sulfamethoxaz **ABC**: abacavir; **3TC:** lamivudine; **ATV:** atazanavir; **COBI:** Cobicistat; **EFV:** efavirenz; **EVG:** Elvitegravir; **FTC:** emtricitabine; **ETR:** etravirine; **DRV:** darunavir; **MVC:** maraviroc; **NVP:** nevirapine; **RAL:** raltegravir; **RTV:** ritonavir; **TDF:** tenofovir;; **TER:** Terbinafine; **TMP-SMX:** Trimethoprim-sulfamethoxaz

Table S4. Associated complication

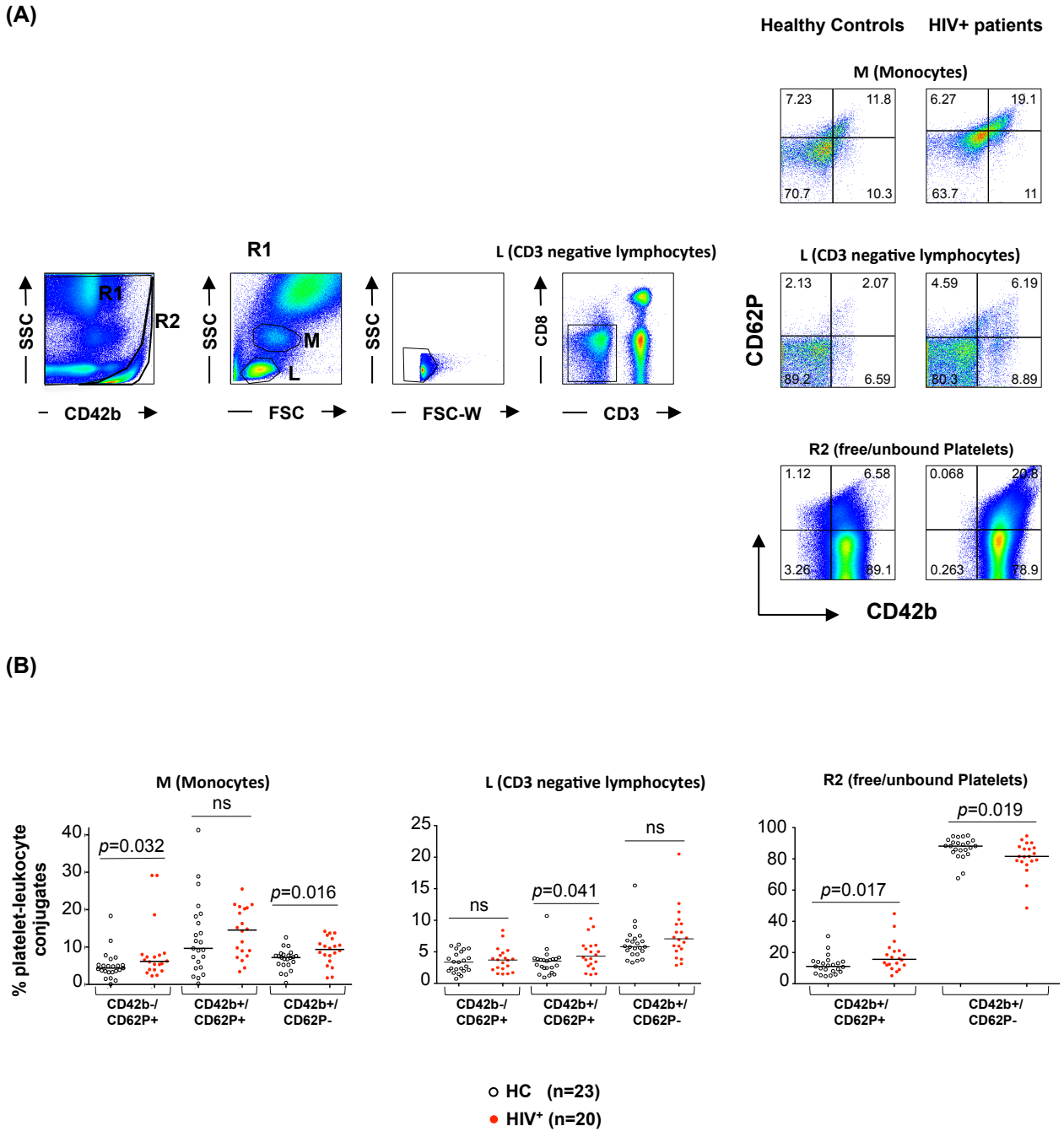
Patient Number	AD/CAD/PPx	DEPR	Dia	HSV	HLD/HTN/HYTG	HYT	Other
Figure 2 - Pt 1	**AD:** Warfarin (115)			Acyclovir (282)	**HLD:** Atorvastatin (172) **HTN:** Lisinopril (147)		
Figure 2 - Pt 2	**PPx**: Asprin (57)	Melatonin (92)					
Figure 2 - Pt 3			Metformin (128)		**HTN:** Lisinopril (90)		
Figure 2 - Pt 4		Clonazapam (150)					
Figure 2 - Pt 5							
Figure 2 - Pt 6							
Figure 2 - Pt 7		Citalopram (158);			**HLD:** Atorvastatin (140)	Levothyroxine (56)	**PD:** Carbidopa-levodopa-entacapone (46); Lidocaine transdermal patch (64); Ropinirole (67)
Figure 2 - Pt 8							
Figure 2,3,4 - Pt 9				Valacyclovir (26)			
Figure 2,3,4 - Pt 10							
Figure 2,3,4 - Pt 11	**PPx**: Asprin (84)			Valacyclovir (142)	**HLD:** Atorvastatin (132)		
Figure 2,3,4 - Pt 12		Bupropion (100)			**HLD:** Atorvastatin (39)	Levothyroxine (187)	**CS:** Diphenhydramine (44); Fexofenadine (55)
Figure 2,3,4 - Pt 13							
Figure 2,3,4 - Pt 14		Citalopram (16); Zolpidem (16)					**GERD:** Omeprazole (8)
Figure 2,3,4 - Pt 15							**PSO:** Hydrocortisone (12)
Figure 2,3,4 - Pt 16				Valacyclovir (89)			**HYP**: Potassium phosphate (47)
Figure 2 - Pt 17							
Figure 2 - Pt 18			Metformin (12)		**HYTG:** Atorvastatin (17)		**OP**: Calcium carbonate (125); Cholecalciferol (71)
Figure 2 - Pt 19					**HTN:** Lisinopril (264)		**C.Tox:** Clinadmycin (376); Dapsone (748); leucovorin (44); Pyrimethamine (34) **SzD:** Levetiracetam (44); Phenytoin (44)
Figure 2 - Pt 20	**CAD:** Brilinta (2) **PPx**: Asprin (66)		Metformin (153)		**HYTG:** Pravastatin (101)		

Months of treatment is indicated in parenthesis. Abreviations: **AD:** Atrial Dysrythmia; **CAD:** Coronary Artery Disease; **PPx:** Prophylaxis; **C.Tox.:** Cerebral Toxoplasmosis; **CS:** Chronic Sinusitis; **DEPR:** Depression; **Dia:** Diabetes; **GERD:** Gastroesophageal reflux disease; **HSV:** Herpes Simplex Virus; **HYP:** Hypophosphatemia; **HLD:** hyperlipidemia; **HTN:** Hypertension; **HYT:** Hypothyroidism**; HYTG:** Hypertriglyceridemia; **PD**: Parkinson's Disease; **PSO:** Psoriasis; **OP:** Osteoporosis; **SzD:** Seizure Disorder.

Supplementary Figure S1

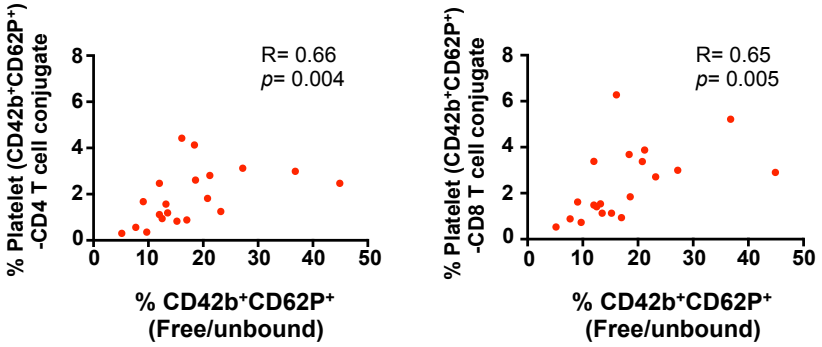


Supplementary Figure S2



Supplementary Figure S3

(A)



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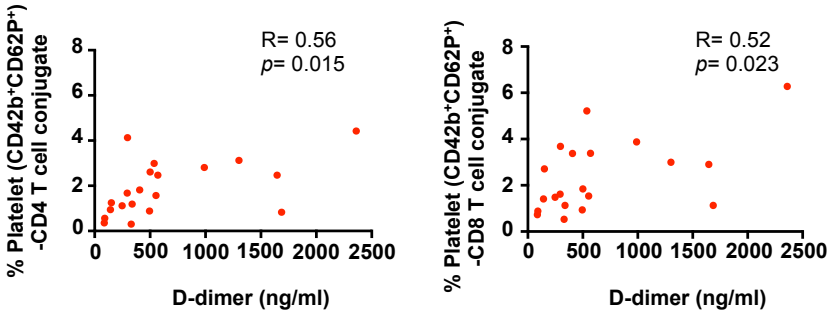
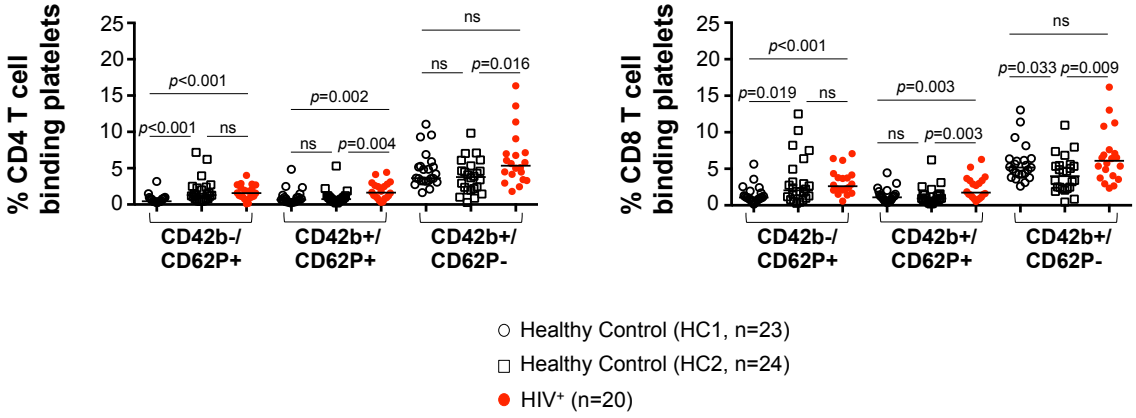


Supplementary Figure S4



Supplementary Figure S5