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Increased platelet and microparticle activation in HIV infection: upregulation of Pselectin and tissue factor expression

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Abstract

OBJECTIVE—HIV-1 infected patients have an increased risk for atherothrombosis and cardiovascular disease, but the mechanism behind these risks is poorly understood. We have previously reported that expression of tissue factor (TF) on circulating monocytes is increased in persons with HIV infection and that TF expression is related to immune activation, to levels of HIV in plasma, and to indices of microbial translocation. In this study, we explore the activation state of platelets in HIV disease.

METHODS—Here, using flow cytometry-based assays, we measured platelet and platelet microparticle (PMP) activation in samples from HIV-1 infected donors and controls.

RESULTS—Platelets and PMPs from HIV-1 infected patients are activated (as reflected by expression of CD62 P-selectin) and also more frequently expressed the procoagulant tissue factor (TF) than did platelets and PMPs obtained from controls. Expression of these proteins was directly related to expression of TF on monocytes, to markers of T cell activation (CD38 and HLA-DR) and to plasma levels of soluble CD14, the coreceptor for bacterial lipopolysaccharide. Platelet and microparticle expression of TF was not related to plasma levels of HIV but expression of P-selectin was; neither TF nor P-selectin expression was related to CD4 T cell count.

CONCLUSIONS—Platelets and microparticles are activated in HIV infection and this activated phenotype may contribute to the increased risk for cardiovascular and thrombotic events in this population although a role for other confounding cardiovascular risks cannot be completely excluded.

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Keywords

tissue factor; platelets; HIV-1; immune activation

Introduction

Platelets, anuclear cell fragments, participate in the localization of coagulation reactionsin the intravascular compartment. Platelet reactivity, especially within ruptured atheromatous plaques, has been implicated in the pathogenesis of atherothrombosis and cardiovascular disease [1, 2]. Many agents aimed at preventing cardiovascular disease target platelet activation [2, 3]. Upon activation, platelets increase expression of the adhesion molecule, P-selectin, thereby promoting adhesion to cell surfaces and clot formation [2, 3]. Platelets also contain pre-mRNA for the procoagulant tissue factor. Tissue factor on platelets [4], and on other surfaces, forms a complex with factor VII to serve as a cofactor for factor VIIa to activate factor X and drive clot formation [5].

P-selectin, a glycoprotein found in the α-granules of platelets, binds to P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed by the endothelium and leukocytes. This interaction is integral to the recruitment of pro-inflammatory leukocytes to sites of injury and thrombosis, such as occurs in the settings of endotoxin-induced sepsis and atherosclerosis [3, 6–9]. P-selectin expressed by platelets captures microparticles, monocyte and platelet fragments, in both pathologic and non-pathologic thrombi to activate leukocytes and cause up-regulation of tissue factor expression by endothelial cells and monocytes [3, 10].

Patients with human immunodeficiency virus (HIV) infection have an increased risk of thrombotic cardiovascular disease [11, 12]. The SMART study, a clinical trial of continued versus interrupted antiretroviral treatment, found that the risk of death, including deaths due to cardiovascular events, was linked to higher plasma levels of the inflammatory cytokine interleukin-6 (IL-6), C-reactive protein, and D-dimers (markers of clot formation and lysis) [13]. We have earlier shown that HIV infection is associated with systemic translocation of microbial products such as bacterial lipopolysaccharide and bacterial DNAs from the intestinal lumen. These microbial products can bind to innate immune pattern recognition molecules such as Toll-like receptors (TLRs), resulting in activation of both innate [14] and adaptive immune cells [15]. Plasma levels of these products are correlated with indices of immune activation [16, 17].

We have also shown that freshly isolated monocytes from HIV-infected patients more frequently express cell surface tissue factor than do monocytes from uninfected controls. Monocyte TF expression correlates with markers of immune activation, with plasma levels of HIV RNA, with plasma levels of the LPS co-receptor CD14, and with levels of D-dimers [18].

Platelets, which have an important role in arterial thrombosis, interact with leukocytes [1, 2] and express functional Toll-like receptor 4 [19]. We hypothesized that platelets of HIV-infected patients might also be activated as a result of sustained exposure to microbial elements in circulation. In the present report we show that platelets and microparticles in HIV infection are activated to express both P-selectin and tissue factor and that expression of these markers correlate with indices of T cell activation and microbial translocation/ monocyte activation that are recognized predictors of morbidity in chronic HIV infection [20, 21].

Methods

All procedures were conducted in accordance with policies of the Case Western Reserve University/University Hospitals of Cleveland Institutional Review Board.

Peripheral blood was drawn, with consent, from HIV infected patients (n=46) and from healthy controls (n=18). All samples were conveniently collected from the populations available, in the morning, over a two month time span. Blood was collected in glass tubes for preparation of serum, and in EDTA-coated or citrate-anticoagulated plastic tubes for plasma. Anticoagulated (citrate) blood was centrifuged at 180x g for 10 minutes to separate platelet-rich plasma from cells. Peripheral blood mononuclear cells were isolated on a ficoll-hypaque cushion.

Flow cytometry

Platelets, from platelet-enriched citrated plasma, were identified in plasma by small size and low complexity on forward versus side scatter plots. These elements were positively identified as platelets, or platelet microparticles, by expression of the surface marker glycoprotein 1b (CD42b) using a phycoerythrin (PE) conjugated monoclonal antibody (BD Pharmingen, San Diego, CA). In selected experiments we confirmed that these labelled elements were neither monocyte derived-MPs nor monocyte PMP aggregates by the absence of CD14 expression (not shown). Surface expression of P-selectin (CD62P) and tissue factor was measured using conjugated monoclonal antibodies to CD62P (Allophycocyanin (APC)-conjugated - BD Pharmingen) and to tissue factor (fluorescein isocyanate (FITC)-conjugated (American Diagnostica, Stamford, CT).

Red blood cells within whole blood preparations were lysed by incubation in FACS Lyse buffer (BD Biosciences, San Diego, CA) for 15 minutes on ice. Monocytes were identified by light scatter characteristics and by reactivity with anti-CD14 Peridinin-chlorophyll-protein Complex (PerCP), and with anti-HLA-DR APC (BD Pharmingen). CD4+ and CD8+ T cells were identified by binding of anti-CD3 APC and anti-CD4 Pacific Blue, or anti-CD8 APC-Cy7 and levels of T cell activation were measured using anti-CD38 PE and anti-HLA-DR FITC (BD Pharmingen). Fluorochrome-conjugated monoclonal antibodies of appropriate isotype were used to establish gating. Cell preparations were stained for 30 minutes, then washed in phosphate buffered saline containing 1% fetal calf serum and 0.1% sodium azide, and resuspended in 1% paraformaldehyde. Platelet preparations were stained for 30 minutes on ice, then washed and resuspended in 1% paraformaldehyde before analysis. Cells, platelets and PMPs were acquired and analyzed using an LSR II flow cytometer (Becton-Dickinson, San Jose, CA) and FACSDiva software version 6.1.1 (BD Biosciences, San Diego, CA).

Measurement of D-dimers and Soluble CD14

Whole blood was collected into EDTA containing tubes and plasma was prepared by centrifugation for 10 minutes at 1610×g and was then frozen at −80°C until assay. Levels of D-dimers were measured using the Asserachrom D-DI immunoassay (Diagnostic Stago Asnieres, France) and soluble CD14 was measured by ELISA using the Quantikine soluble CD14 kit (R&D Systems Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical Methods

Differences between variables among patients and controls were tested with a Mann-Whitney's U test. We tested associations between pairs of continuous variables with Spearman's rank correlation. We processed the analyses using Graphpad Prism v. 5.02

(GraphPad Software, Inc., La Jolla, CA). All tests are two-sided, and a p-value of 0.05 was considered nominally significant.

Results

Platelets in HIV infected subjects have high surface expression of P-selectin and tissue factor

Previous studies have shown that HIV infected patients have an increased risk for thrombotic and cardiovascular events [11, 12, 22–24] and since platelets and microparticles play a major role in coagulation, we prepared these anuclear fragments from the plasma of 46 HIV infected donors and 18 healthy controls and measured surface expression of P-selectin and tissue factor by flow cytometry. All of our samples were collected from patients and controls in the University Hospital/ Case Western Reserve University population. Among the patients and controls for whom we have information, 67% of each group was male. The median ages for the patient and control populations were 42 years (range 26–65) and 33 years (range 20–61) respectively (p=0.015). Forty one percent of patients had plasma RNA levels >400copies/ml. The median plasma HIV RNA level for the entire patient population was 50 copies/ml (range = 50–590,000copies/ml) and the median CD4+ T cell count was 400 cells/ul (range = 6–1,063 cells/ul). Seventy three percent of the patients were being treated with antiretroviral therapy. A greater proportion of the patient population reported that they were current smokers (29%), compared to 17% of the controls While the HIV-infected group had a greater number of patients who were defined as obese (Body Mass Index, BMI> 30, N=7 for patients, N=0 for controls), the median BMI of each group was not statistically different (patients =25.8, controls 24.5, p=0.11). Among the HIV+ group, 6 patients reported daily treatment with aspirin, 5 patients reported using statins, 4 patients had a positive screen for Hepatitis B and 6 patients were positive for Hepatitis C. Among the healthy controls, 1 control reported taking aspirin daily, 2 were receiving statins, and none was diabetic or known to be infected with Hepatitis B or C. A greater proportion of the HIV infected population was African American (51%) compared to 6% among uninfected controls. Demographic information is summarized in Table 1.

Platelets and microparticles were identified by low forward and side scatter characteristics, and by expression of CD42b (Figure 1A). Among healthy controls, these elements rarely expressed P-selectin (median 1.1% positive) or tissue factor (median 1.2% positive). The proportions of platelets and microparticles expressing P-selectin or tissue factor (17.3% and 4.7% respectively) in HIV-infected patient samples were 10 significantly greater ($p < 0.001$ for both) than among controls. Platelets and microparticles obtained from patients with detectable viremia (n =18) more frequently had detectible surface P-selectin than did platelets and PMPs obtained from patients with virologic control (HIV RNA<400 copies/ml) (n = 23, 23.3 versus 12.6%, p =0.022, Figure 1B). The proportions of TF+ platelets and PMPs were similar among patients with uncontrolled viremia and patients with virologic suppression (3.4% vs 5.0%, respectively, p=0.7, Figure 1C). The proportions of TF+ and CD62P+ platelets and microparticles were directly related when samples from all donors were analyzed (r=0.53 p<0.001, data not shown).

We previously described an increase in surface expression of TF on monocytes from HIV infected patients [18]. In this study, the proportions of monocytes and platelets/microparticles expressing tissue factor, from all donors, are directly related (r=0.4842, p=0.0078, Figure 1D), suggesting that the drivers of platelet/microparticle and monocyte expression of tissue factor may be related.

Platelet/microparticle activation is differentially related to plasma levels of the LPS coreceptor CD14 and to plasma HIV levels, but not to CD4 T cell counts

To obtain preliminary insight into the determinants of platelet activation in HIV-1 infection, we explored potential clinical and laboratory correlates of platelet activation. Unlike monocyte TF expression, there was no relationship between platelet/PMP expression of TF and the magnitude of plasma viremia or CD4+ T cell count (r=0.1066 p=0.51 and r= −0.12, p=0.46 respectively, not shown). There was a correlation between the proportion of P-selectin positive platelets/PMPs and levels of plasma viremia (r=0.45, p=0.003) but, there was no relationship between P-selectin expression and CD4+ T cell counts(r=−0.20, p=0.21, not shown). Thus, HIV replication or its consequences may drive platelet expression of P-selectin, but not of tissue factor. We speculate that monocytes may recognize and be activated by HIV-1 through surface receptors used for viral entry or by innate immune receptors expressed by monocytes that are likely absent on platelets and PMPs.

We have previously reported that the increased plasma levels of the LPS co-receptor (sCD14) in HIV infected patients correlated with the proportion of monocytes expressing TF [18]. In the present study, we find higher plasma levels of sCD14 in HIV infection than in healthy controls (2603ng/ml versus 1364ng/ml, p=0.004). In all donors, plasma levels of sCD14 were directly related to platelet/microparticle expression of P-selectin (r=0.71, p<0.001, Figure 2A) and tissue factor (R=0.39, p=0.026, Figure 2B). The relationship between platelet/microparticle expression of P-selectin and sCD14 remained significant in the HIV infected patients population (r=0.456, p=0.029, not shown).

Plasma levels of D-dimers, markers of coagulation and fibrinolysis, were predictive of all-cause mortality in the SMART study [13, 16]. Since we had earlier linked monocyte TF expression to D-dimer levels [18], we asked if this marker also was related to platelet/PMP activation. While plasma levels of D-dimers were increased in patients compared to levels in controls (436 FEU versus 256 FEU, respectively, p=0.029) there were only modest correlations between D-dimer levels and the proportion of platelets/microparticles expressing P-selectin (r=.40, p=0.041) or tissue factor (r=0.30, p=0.115) in samples from all donors.

Platelet/microparticle activation is directly related to CD8+ T cell activation

T cell activation indices have been linked to disease course in HIV infection [25, 26]. We next asked if T cell and platelet/PMP activation could also be related. The expression of CD38 and HLA-DR on CD8+ T cells was strongly correlated with platelet/microparticle surface expression of both P-selectin (r=0.63, p=0.0006) and tissue factor (r=0.45, p=0.0175) (Figure 3A and B) in all donors, and the relationship between CD38 and HLA-DR on CD8+ T cells and the proportion of platelets/PMPs that express TF remained when analyzing the patient population alone (r=0.530, p=0.02).

Discussion

Both in the pre-HAART and in the post HAART eras, HIV infected patients have experienced an increased risk of thrombosis and cardiovascular disease, [12, 27, 28] yet, the determinants of these risks are incompletely understood. In a recent study where continuous antiretroviral therapy was compared to intermittent therapy, all cause mortality, as well as morbidity due to cardiovascular disease, were linked to increased levels of D-dimers, interleukin-6, and C-reactive protein [13], implicating both inflammation and coagulation as potential mediators of these risks.

In plasma samples from persons with chronic HIV infection, we and others have found increased levels of microbial products; evidence of microbial translocation from the

damaged gut [16, 17, 29]. These levels were linked both to indices of immune activation and also to T cell homeostasis as reflected by a strong relationship to the magnitude of CD4+ T cell restoration after application of combination antiretroviral therapies [16, 17]. Moreover, we have demonstrated that a broad variety of microbial TLR ligands can induce an activation phenotype on both CD4+ and CD8+ T cells in vitro that is similar to the activation phenotype seen *in vivo* in chronic HIV infection [15]. More recently, we have shown that selected TLR ligands can induce tissue factor expression on blood monocytes and that monocytes from HIV infected donors commonly express high levels of cell surface TF [18]. Soluble TF levels were also increased in HIV infection and levels of monocyte TF were related to plasma levels of the LPS coreceptor sCD14 and to plasma levels of D-dimers, potentially linking this procoagulant to microbial translocation/monocyte activation and to ongoing clot formation and fibrinolysis. Recently, Hashimoto et al have also found that *in vitro* exposure to LPS could induce platelets to express P-selectin [30]. Thus, it is reasonable to propose that monocyte and platelet activation and increased procoagulant expression in chronic HIV infection may be linked at least in part to in vivo exposure to microbial products.

We identified platelets/microparticles by their size and light scatter characteristics and confirmed that these small anuclear fragments are of platelet origin by the concordant expression of CD42b, a glycoprotein that is uniquely expressed by platelets [31]. The absence of CD14 expression assured that these elements were not microparticles derived from macrophages/monocytes, nor had they bound their membrane fragments. Based on our gating strategy, we could not discriminate between platelets and PMPs. These subcellular platelet fragments are mainly produced by activated platelets and express the same cell surface antigens as do activated platelets, including CD42b, tissue factor, and P-selectin [32–34]. While we cannot distinguish activated platelets from platelet derived microparticles, these two populations are functionally and phenotypically nearly identical and the main finding of this study is that the proportion of small, CD42b+ anuclear cell fragments that express TF or P-selectin are increased in the circulation of HIV-1 infected patients, compared to these proportions in the circulation of healthy uninfected controls. High levels of P-selectin have been previously reported in plasma [35] and on the surfaces of platelets in HIV infected patients [36], in consumptive coagulopathies, [37] and in settings of direct endothelial injury and platelet activation [38]. Unlike the finding reported by Holme et al [36], we did not see a relationship between platelet/microparticle expression of P-selectin and CD4+ T cell counts among our HIV infected donors [36].

Platelet/microparticle expression of TF was not directly related to clinical indices of HIV disease (plasma viremia and CD4+ T cell count), but both TF and P-selectin expression on platelets/microparticles were related to T cell activation. This work suggests that the drivers of chronic immune activation and inflammation that are independent of the magnitude of viral replication may be important determinants of morbidity in both treated and untreated HIV infection.

While our findingsrelate the chronic immune activation of HIV-1 infection to platelet activation and coagulation in HIV disease, the results of this cross sectional study may be limited by the small sample size and by significant differences in our patient and control populations. Patients tended to be older (mean age 42 years) than our controls (mean age 33 years, p=0.015) but, age has not been associated with differences in P-selectin expression in other studies [39–42] nor did we show a significant relationship between age and platelet expression of Pselectin (r=0.159, p=0.334) or TF (r=0.035, p=0.832) in our patient population. Although there were proportionally more smokers among patients (29%) than among controls (17%), in our patient population, smokers did not have higher platelet tissue factor expression (mean= 5.7 %TF+) or platelet activation (16.29% CD62P+) than did non-

smokers (5.5 %TF+, 20.77%CD62P+, p=0.39 and p=0.34, respectively), suggesting smoking does not influence the increased levels of platelet activation in these patients. While we also had more obese patients among the HIV infected population (N=7) than among the controls (N=0), expression of CD62P or TF on platelets from obese patients and non-obese patients within the HIV+ population was not significantly different (p= 0.43 and p=0.42, respectively). While the proportion of African Americans was much greater in our patient population (51%) than in controls (6%), we did not see a significant difference in markers of platelet activation between African Americans and Caucasians within the HIV-infected population. The proportions of CD62P+ or TF+ platelets within the African American population were 19.4+/−3% and 6.15+/−2%, and among the Caucasian population, the proportions of CD62P+ and TF+ platelets were 18.7+/−3% and 5.1+/−3%, (p=0.75 and p=0.87, respectively.) These results suggest that race/ethnicity was not a major determinant of the increased platelet activation seen here in persons with HIV infection. Several of these indices (smoking, BMI, ethnicity) contribute to an increased risk of cardiovascular disease and platelet activation. Due to the small overall sample size in this study and the differences between our HIV+ and HIV− subjects as noted above, we cannot entirely rule out some contribution of these confounders on the differences in platelet activation reported herein. Future studies, where a larger and or more comparable groups of participants are examined, will be needed to confirm our findings that platelets and microparticles are activated in HIV infection. Conceivably this activation contributes to the apparent increased cardiovascular risk experienced by persons with HIV infection that is linked both to HIV-1 replication and to other indices of immune activation.

The proposed linkage between platelet activation and microbial translocation is also limited by the fact that we did not measure LPS directly. While sCD14 levels have been correlated directly with plasma levels of LPS in some studies[16, 43] other studies were unable to find such a relationship[21, 44]. Activation of monocytes by microbial products, such as LPS, or inflammatory cytokines, such as IL-6 [44], can induce shedding of CD14. Thus, increased levels of plasma sCD14 may also reflect monocyte activation through a variety of stimuli. We have previously demonstrated a relationship between monocyte expression of TF and sCD14 [18] and in this current work, we report a relationship between platelet expression of TF and Pselectin to plasma levels of sCD14, suggesting that there may be a common “driver,” such as microbial translocation or inflammatory cytokines, that may be helping to drive monocyte and platelet activation in HIV disease.

In summary, we show here that platelets/microparticles of HIV infected persons are activated *in vivo* to express P selectin and tissue factor, and that expression of these markers of adhesion and coagulation are related to soluble levels of the LPS receptor CD14. The proportions of TF+ monocytes and TF+ platelets/microparticles appear to be directly related, and our data suggest that they may be driven, at least in part, by sustained exposure to microbial elements or represent another consequence of monocyte activation. Both platelet/microparticle and monocyte TF expression were strongly related to levels of the LPS coreceptor sCD14 while monocyte TF, [18] but not platelet/microparticle TF, was strongly correlated to plasma levels of HIV. We have recently found that monocytes can be directly activated by HIV in vitro to express tissue factor (unpublished); this is not likely the case for platelets. Additional study is needed to clarify the mechanisms of TF upregulation and platelet activation in HIV infection and to ascertain the relationship among these indices and in vivo thrombosis and cardiovascular morbidity. We suspect that the recognized increases in cardiovascular morbidities that are complicating the course of HIV infection may be at least in part, a result of the sustained exposure of these patients to the proinflammatory environment that persists even among HIV infected persons in whom viremia is well controlled by antiretroviral therapies [17].

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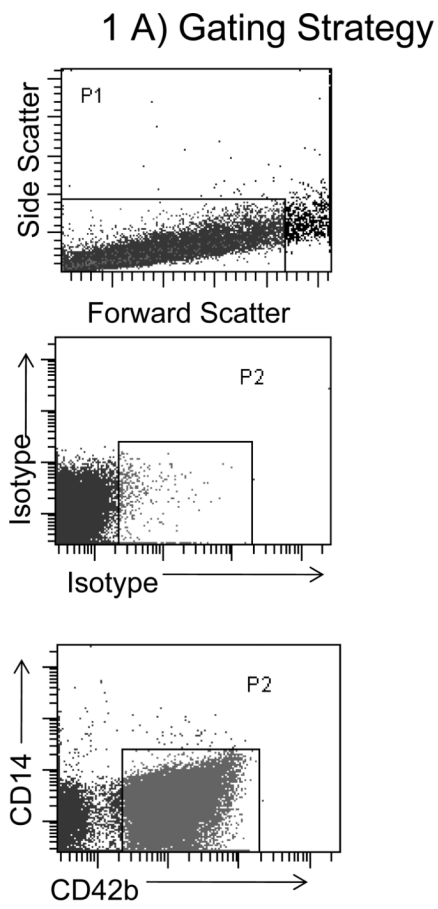
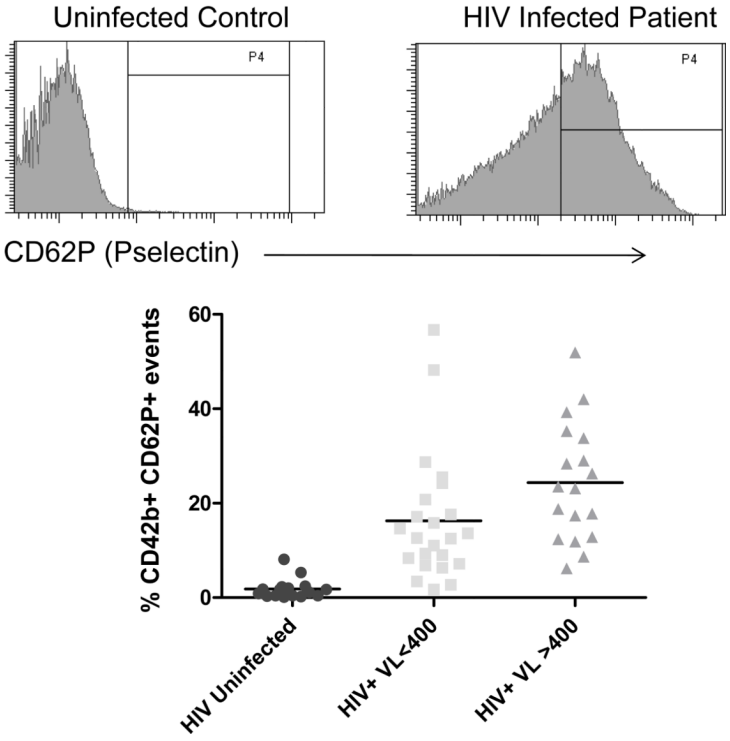


Figure 1 B)



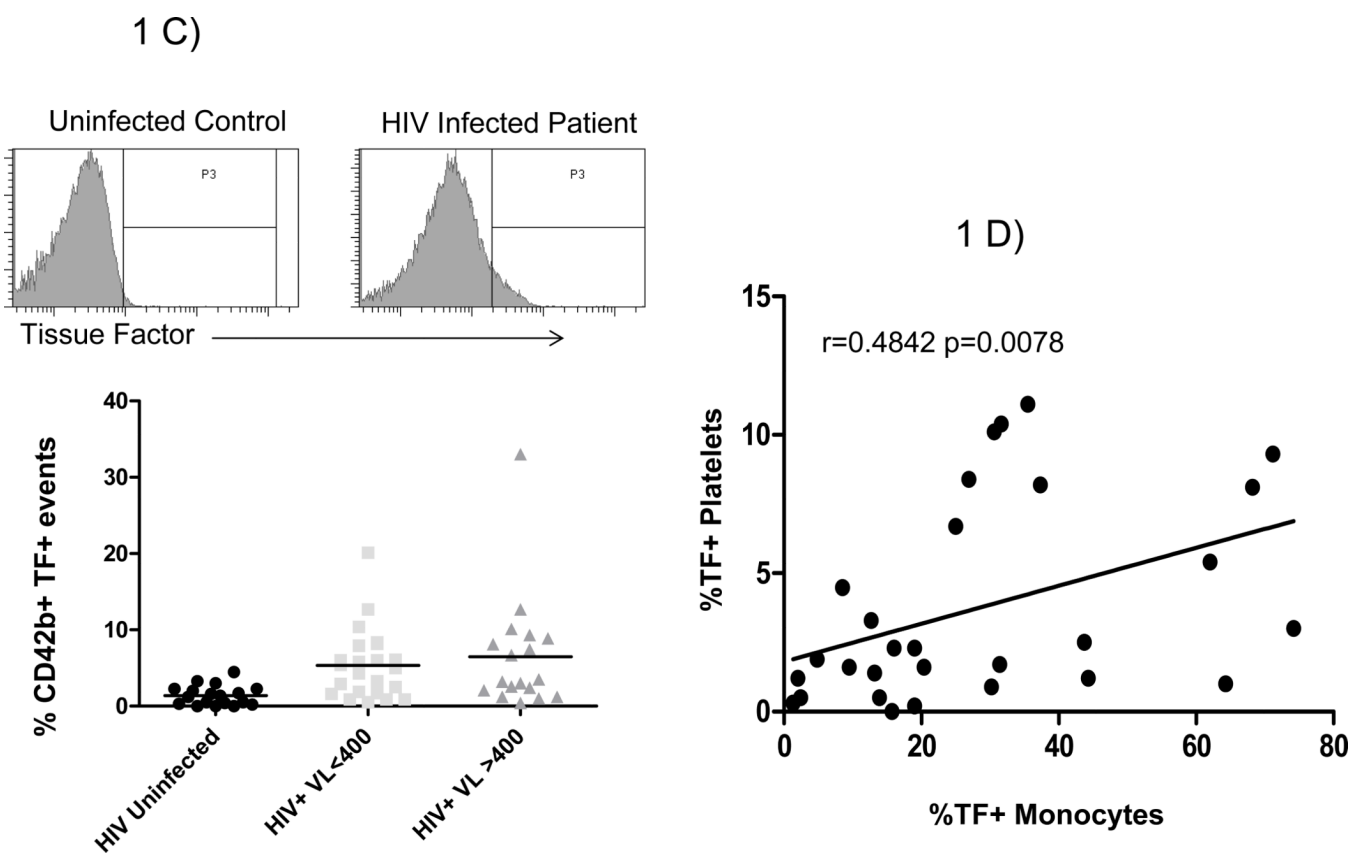


Figure 1. Platelets/microparticles from HIV infected patients have an activated phenotype

A) Platelets/microparticles are defined by their low complexity and small size in plasma and by expression of CD42b. Representative histograms and summary data among platelets/microparticles from healthy controls (N=18), patients with controlled viremia (HIV RNA<400 copies/mL, N=23), and uncontrolled viremia (HIV RNA>400 copies/mL, N=18) for surface expression of B) CD62P and C) tissue factor. The proportions of platelet/PMP that expressed CD62P in each of our patient groups were significantly different from the proportion in controls ($p<0.001$ for both). Likewise, the proportions of TF+ platelets/PMPs in each of our patient groups were significantly different from the proportion of TF+ platelets/PMPs in controls ($p>0.001$ for both) D) The proportions of TF+ platelets/microparticles and TF+ monocytes are directly related ($r=0.4842$ $p=0.0078$).

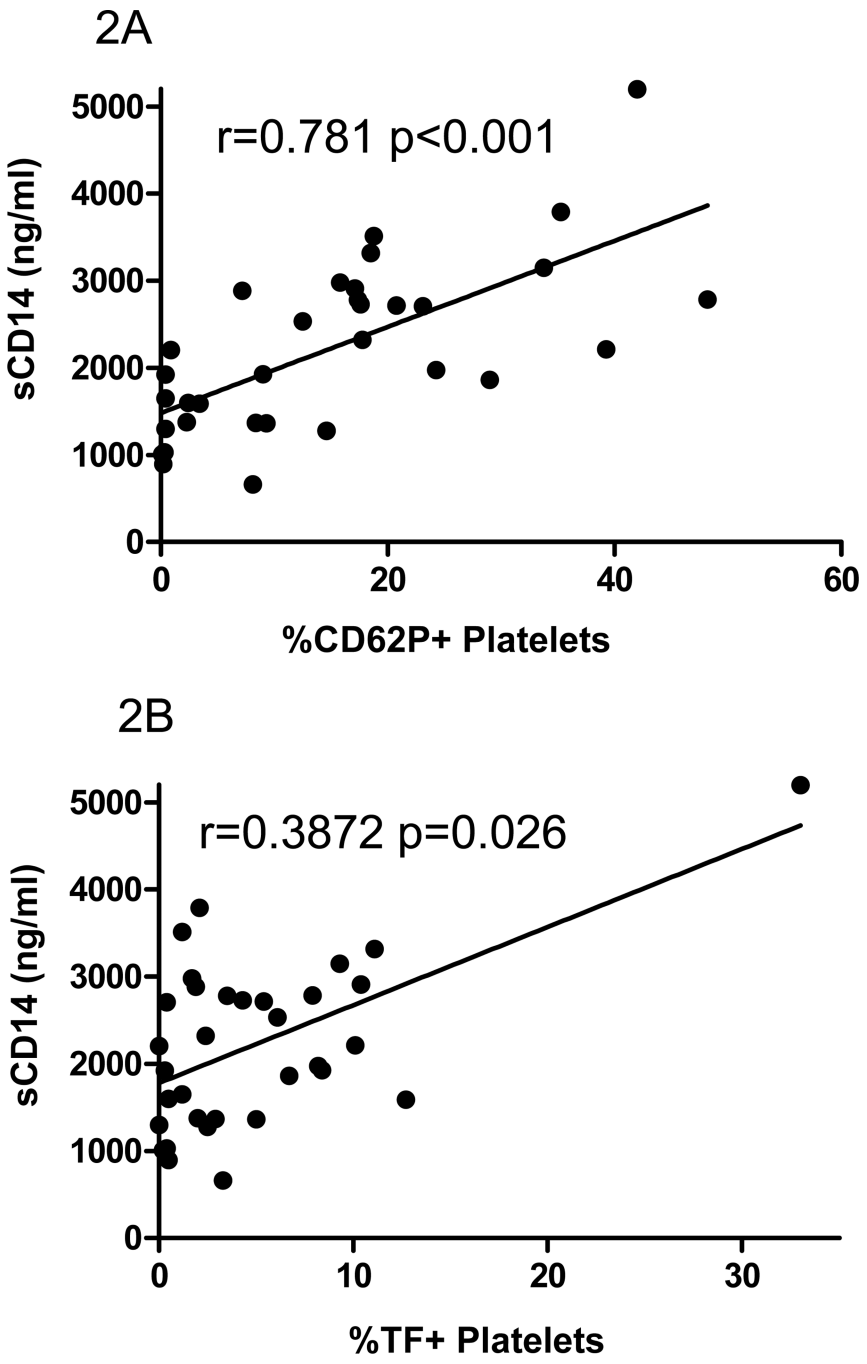


Figure 2. The proportions of platelets/microparticles expressing P-selectin or tissue factor are related to plasma levels of sCD14

Plasma samples were thawed in batch and levels of sCD14 were measured. In samples from all donors A) the proportion of P-selectin positive platelets/microparticles is directly related to plasma levels of sCD14, r=0.781 p<0.001; and B) the proportion of tissue factor positive platelets/microparticles is directly related to plasma levels of sCD14, r=0.3872 p=0.026.

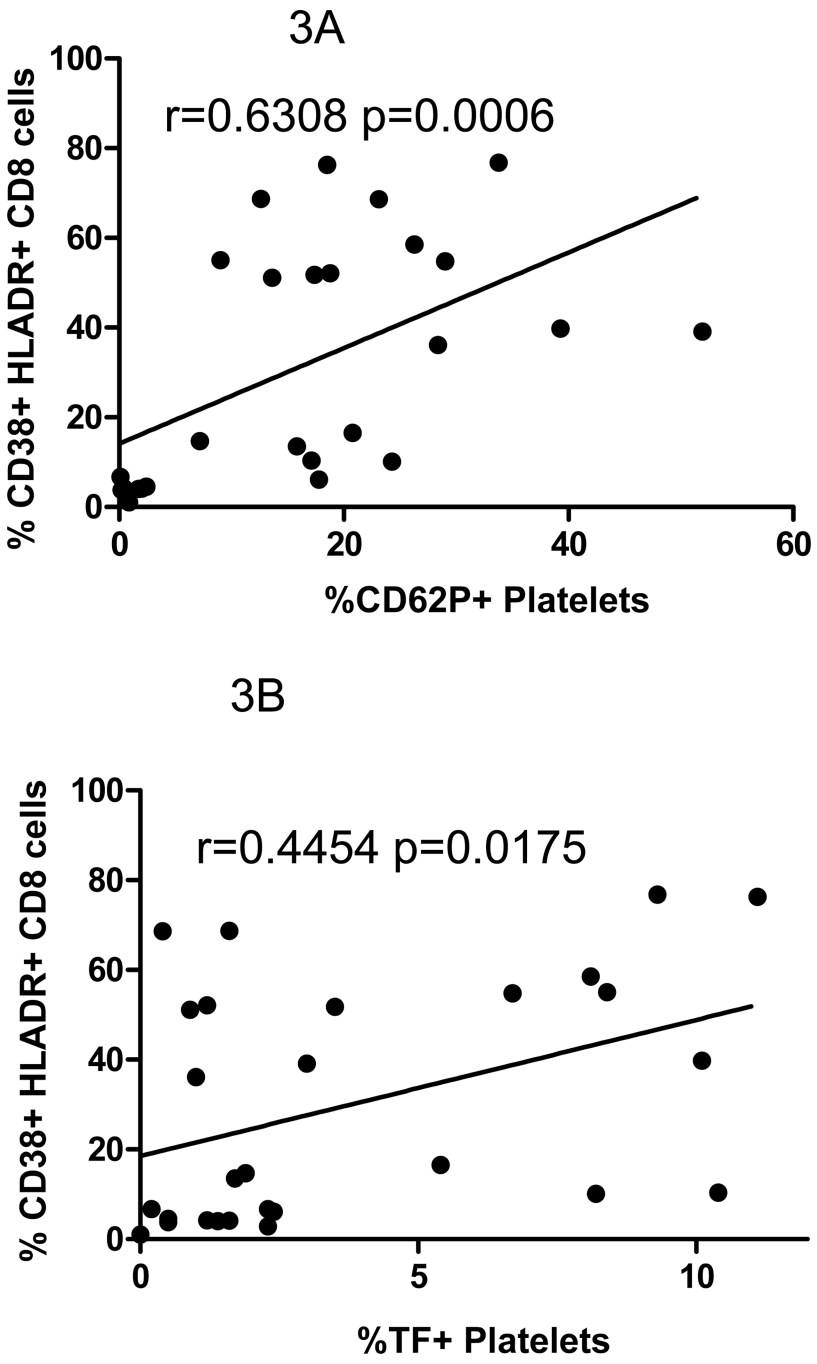


Figure 3. The proportions of platelets/microparticles expressing P-selectin or tissue factor are related to markers of T cell activation

CD8+ T lymphocytes were identified by size, complexity, and by expression of CD3 and CD8, and markers of activation (CD38 and HLA-DR) were measured on phenotypically gated cells. In samples from all donors A) the proportion of P-selectin positive platelets/microparticles is directly related to CD8+ T cell activation, r=0.6308 p=0.0006; and B) the proportion of TF positive platelets/microparticles is directly related to CD8+ T cell activation, r=0.4454 p=0.0175.

Table 1

Demographic information of the study cohort

	Gender	Age (Median yrs)	Tobacco use	Antiretroviral usage	Ethnicity (# individuals, self reported information)	BMI (median)
Controls	7 females 11 males	33 range [20–61]	Non-smokers 15 Smokers 3	Not applicable	1- African American 12- Caucasian 2 -Hispanic/Latin 2-Asian 1 –African	24.5 range [16.7–28.3]
Patients	14 females 28 males	42 range [26–65]	Non-smokers 33 Smokers 13	55 on HAART	19 -African American 18- Caucasian	25.8 range [18.9–42.1]