Coagulation biomarkers predict disease progression in SIV-infected nonhuman primates

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Abstract

HIV infection is associated with increased risk of cardiovascular complications, the underlying mechanism of which remains unclear. Plasma levels of the coagulation biomarker D-dimer (DD) have been correlated with increased mortality and cardiovascular events in HIV-infected patients. We compared the incidence of cardiovascular lesions and the levels of coagulation markers DD and thrombin anti-thrombin (TAT) in pathogenic SIV infections of rhesus (RMs) and pigtailed macaques (PTMs), and in nonpathogenic SIV infection of African green monkeys (AGMs) and sooty mangabeys (SMs). Hypercoagulability and cardiovascular pathology were only observed in pathogenic SIV infections. In SIVagm-infected PTMs, DD levels were highly indicative of AIDS progression and increased mortality and were associated with cardiovascular lesions, pointing to SIVagm-infected PTMs as an ideal animal model for the study of the mechanisms of HIV-associated cardiovascular disease. In pathogenic SIV infection, DD increased early after infection and was strongly correlated with markers of immune activation/inflammation and microbial translocation (MT) and only peripherally associated with VLs. Endotoxin administration to SIVagm-infected AGMs (which lack chronic SIV-induced MT and immune activation) resulted in significant increases of DD. Altogether, our data demonstrate that, in SIV infection, hypercoagulation and cardiovascular pathology are at least in part a consequence of excessive immune activation and MT.
Introduction

There is increasing data demonstrating the significant impact of non-AIDS defining comorbidities on the outcome of HIV infection. Cardiovascular disease (CVD) emerged as a major comorbidity during the era of highly active antiretroviral therapy (HAART)\textsuperscript{1-3}, especially in urban African-American and Hispanic communities, which are disproportionately affected by HIV and are also at high CVD risk. CVD may be intrinsically related to HIV infection\textsuperscript{2} or to long term HAART, which may increase CVD risk by adverse metabolic effects (lipid changes) or vascular toxic effects.\textsuperscript{3} To date, however, our understanding of the relationship between HIV infection, HAART and CVD risk is unclear and incomplete, which is a critical barrier to the field, preventing interventions that may reduce CVD mortality in these patients.

CVD complications in HIV-infected subjects include thrombotic micrangiopathy (TMA), arteriopathy, dilated cardiomyopathy, abnormal coronary artery pathology (including atherothrombotic disease) and myocarditis.\textsuperscript{4-12} Abnormal high levels of coagulation markers, endothelial activation markers, and platelet activation markers have also been documented in HIV-infected patients.\textsuperscript{13-15} The SMART study confirmed that a mild-to-moderate hypercoagulable state exists in HIV infection\textsuperscript{2}. This study showed that increased levels of the coagulation biomarker D-dimer (DD) are associated with increases in inflammation markers (i.e., IL-6), both being strongly linked to the loss of HIV/SIV control, disease progression and death,\textsuperscript{2} thus suggesting a causal relationship between immune activation/inflammation and CVD in HIV-infected patients.

It is widely accepted that immune activation levels more accurately predict HIV disease progression to AIDS and death than do levels of viral replication or CD4\textsuperscript{+} T cells.\textsuperscript{16-18} This paradigm is supported by data from our group and others showing that natural hosts of SIV (AGMs, mandrills and SMs) generally do not progress to AIDS despite having levels of viral replication similar to or higher than untreated HIV-1-
infected patients. This suggests that the ability of natural hosts to maintain low levels of immune activation in the face of SIV infection and high viral load (VL) may be a major determinant of their resistance to disease progression.

Immune activation is a multifactorial process. Although viral replication is a key factor driving immune activation during acute HIV/SIV infection, this association is not evident during chronic infection: (i) HIV-infected patients with similar VLs may have different levels of immune activation and different rates of disease progression; (ii) HIV patients that receive HAART and suppress viremia but continue to exhibit increased levels of immune activation have poor T cell restoration and prognosis; and (iii) natural hosts of SIV maintain chronically high levels of VLs that are independent of immune activation and disease progression. Therefore, factors in addition to viral replication were proposed to drive the excessive immune activation characteristic of HIV infection, most notably the translocation of gut microbial products into the bloodstream. This mechanism is supported by data from natural hosts of SIV in which normal levels of immune activation during chronic SIV infection are associated with lack of microbial translocation (MT).

A correlation between the levels of DD and sCD14, a product of bacterial LPS-activated monocytes, was recently established in HIV-infected patients. These cross-cutting correlations between cardiovascular biomarkers and inflammatory biomarkers on one hand and between inflammatory biomarkers and MT biomarkers on the other led us to hypothesize that the MT associated with HIV/SIV infection activates inflammatory mediators which in turn go on to play a role in the onset of CVD.

Here we used four animal models of SIV infection to: (i) identify the most relevant animal model to study SIV-related CVD, (ii) compare CVD-related biomarkers between progressive and nonprogressive SIV infections, (iii) dissect the timing and the pathways of CVD development during SIV infection, (iv) directly test the hypothesis that MT
associated with HIV/SIV infection plays a role in the development of CVD observed in HIV-1-infected patients.

We report that CVD pathology is present in pathogenic SIV infections and is associated with disease progression, while being absent in natural nonprogressive SIV infections. We identified the SIVagm-infected PTM as the most appropriate animal model for the study of SIV-related CVD pathology. In this model, hypercoagulability correlated with immune activation and MT. LPS administration to chronically SIVagm-infected AGMs (a species that lack MT during SIV infection\textsuperscript{22}) significantly impacted coagulability, strongly supporting MT as a major cause of CVD in progressive SIV infections.
Materials and Methods

Animals and infections

We used four NHP species representative of progressive SIV infections (RMs and PTMs) and of nonprogressive SIV infections (AGMs and SMs). The following study groups were included:

First group: 8 uninfected and 8 SIVmac239-infected RMs, 13 uninfected and 13 SIVagm-infected PTMs, 15 uninfected and 26 SIVagm-infected AGMs and 4 uninfected and 21 SIVsmm-infected sooty mangabeys. The goal was to compare and contrast the changes in the levels of coagulation biomarkers between progressive and nonprogressive SIV infections and to assess the differences in the levels of coagulation biomarkers between uninfected and chronically SIV-infected monkeys within the same species to identify an NHP model for the HIV-related hypercoagulability.

Second group: 9 PTMs (6 males and 3 females aged 6-13 years) and 8 RMs (males, aged 5-15 years). These monkeys were intravenously infected with 300 tissue culture infectious doses (TCID50) of SIVagmSab92018 and SIVmac239, respectively. The follow-up for these animals was until progression to AIDS [1-2 years postinoculation (p.i.) for PTMs and 4-18 months p.i. for RMs]. The goal was to determine the timing of biomarker changes and to establish correlations between coagulation biomarkers and other virologic/immunologic biomarkers at different stages of SIV infection, to unravel the pathways responsible for the coagulation abnormalities.

Third group: 6 adult AGM males aged 6-11 years experimentally inoculated with 300 TCID50 SIVagmSab92018. During chronic infection (starting from day 200 p.i.) these animals received lipopolysaccharide (LPS; endotoxin) intravenously every two days for a period of three weeks. Based on a preliminary study the initial LPS dose was of 18 international units (IU)/kg. To minimize tolerance to the LPS, dose was
increased by 5 IU every week. The goal was to directly assess the role of MT in the genesis of hypercoagulation in SIV-infected NHPs.

All RMs and SMs, 8 PTMs and 22 AGMs were housed and handled at the Tulane National Research Primate Center (TNPRC). Five PTMs and 4 AGMs were housed and handled at the Regional Industrial Development Corporation (RIDC) NHP facility of the University of Pittsburgh. Housing and handling of all animals were in accordance with American Association for Accreditation of Laboratory Animal Care, Guide for the Care and Use of Laboratory Animals (U.S. Public Health Service) and the Animal Welfare Act. All animal procedures were approved by the Institutional Animal Care and Usage Committees (IACUCs) of the Tulane University and University of Pittsburgh.

Tissue sampling

Blood and intestinal biopsies were collected as reported. Plasma and peripheral blood mononuclear cells (PBMCs) and mononuclear cells from the intestinal biopsies were isolated as described. At the necropsy, kidney, heart, aorta, coronary arteries, intestine, lung and brain were collected from SIVagm-infected PTMs and AGMs and fixed in 10% buffered formalin.

Viral quantification

Plasma VLs were quantified by specific real-time PCR for SIVagmSab92018 and SIVmac239, as described.

Antibodies and flow-cytometry

Whole blood and mononuclear cells isolated from intestinal biopsies were stained for flow cytometry as described to assess changes in the levels of major T cell
populations, as well as their immune activation status. Monoclonal antibodies used were: CD3-Pacific Blue, CD4-allophycocyanin (APC), CD8-Texas Red, HLA-DR-APC-Cy7, Ki-67-fluorescein isothiocyanate (FITC) (BD Biosciences Pharmingen San Diego, CA). All antibodies were validated and titrated using AGM, RM and PTM PBMCs. Samples were stained for Ki-67 using Ki-67-FITC conjugated mouse anti-human monoclonal antibody set (BD Pharmingen), as per manufacturer instructions. Stained cells were analyzed with a LSRII flow cytometer (BD Immunocytometry Systems) and FloJo software (Tree Star, Inc). CD4+ and CD8+ T-cell percentages were obtained by first gating on lymphocytes, then on CD3+ T cells. Activation markers were determined by gating on lymphocytes, then on CD3+ T cells and finally on CD4+CD3+ or CD8+CD3+ T cells.

Dynamics of inflammatory cytokines and chemokines

Cytokine testing in plasma was done using the Human Cytokine 25-Plex system (Biosource International, Camarillo, CA), per manufacturer instruction. Results were read by the Bio-Plex reader (Bio-Rad Laboratories, Hercules, CA), which uses Luminex technology (Luminex Corporation, Austin, TX). The analysis was focused on interleukin 6 (IL-6), because plasma levels of this cytokine have a well-known association with CVD and may be produced as a result of bacterial exposure. In HIV-infected patients, increases in IL-6 and DD were strongly associated with disease progression and death.

Microbial translocation (MT)

Plasma soluble CD14 (sCD14) levels were measured by ELISA (Quantikine Human sCD14 Immunoassay, R&D Systems, Minneapolis, MN); analytical CV ranged between 7.19% and 10.9%.
sCD163

Plasma soluble CD163 (sCD163) levels were measured by ELISA (Trillium Diagnostics, LLC, Brewer, ME); analytical CV ranged from 4.6% to 13.6%.

Coagulation markers

Coagulation status was estimated by determining the plasma levels of DD and Thrombin-anti-thrombin complex (TAT).

DD is a terminal product of plasmin acting on a fibrin clot. It increases during the activation states of coagulation, disseminated intravascular coagulation and deep vein thrombosis. DD was measured using a STAR automated coagulation analyzer, (Diagnostica Stago) and an immuno-turbidometric assay (Liatest D-DI; Diagnostica Stago, Parsippany, NJ); analytical CV ranged from 5 to 14%.

TAT measures thrombin production and subsequent inhibition by antithrombin; antithrombin being in excess, TAT primarily reflects recent thrombin production. TAT was measured by ELISA (Enzygnost TAT micro, Siemens, Inc., Deerfield, IL); analytical CV ranged between 7.3-17.43%.

Histological assays

Heart, aorta, kidneys, brain, intestine and lung collected at the necropsy, fixed in 10% buffered formalin were embedded in paraffin. We routinely checked for pathology similar to that seen in HIV-infected patients including renal thrombotic microangiopathy (TMA), arteriopathy, myocardial hypertrophy and fibrosis, atherosclerosis (ATS), infarction and myocarditis. Four micron paraffin sections were stained with
haematoxylin-eosin (HE) for routine histopathology diagnosis and Masson trichrome for collagen detection.

Immunohistochemical (IHC) staining was performed on the formalin-fixed, paraffin-embedded tissues using an avidin-biotin complex horseradish peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) and a rabbit polyclonal anti-fibrinogen antibody (DAKO, Carpinteria, CA) as a primary antibody.

Statistical analysis

Paired t-test and Mann-Whitney U test were used to compare variables before and after infection, using GraphPad Prism. We used general estimating equations to test the relationship between D-dimer and the other variables (immune activation, inflammation, VLs) in pathogenic infection. We present the slope of these estimates and as a crude measure of fraction of variance explained by the model (analogous to a correlation coefficient, \( r^2 \)) we calculated \( f = 1 - \frac{s^2_{\text{model}}}{s^2_{\text{DD}}} \), where \( s_{\text{model}} \) is the scale parameter of the model and \( s^2_{\text{DD}} \) is the variance of the DD data, as suggested before.\(^{37}\) For these tests, it was assumed that the full covariate conditional mean assumption was verified.\(^{38}\) These statistical tests were performed in R (Comprehensive R-Archive Network, http://CRAN.R-project.org/). A significance level of 0.05 was used throughout.
Results

Chronic pathogenic SIV infections, but not nonpathogenic SIV infections, are characterized by a procoagulant state

A procoagulant state was reported to occur in HIV-1-infected patients.\textsuperscript{23,39} To determine if these findings are reproduced in the NHP models, we compared the levels of DD and TAT in a variety of uninfected and chronically SIV-infected NHPs. Two models of progressive SIV infection (SIVmac239-infected RMs and SIVagm-infected PTMs) and two models of natural, nonprogressive SIV infection (SIVagm-infected AGMs and SIVsm-infected sooty mangabeys) were compared.

Similar to HIV-infected patients, DD levels significantly increased during chronic pathogenic SIV infections in RMs and PTMs (Figure 1a). The average DD levels were 2.021±0.26 µg/ml in chronically infected RMs, significantly higher (p=0.0002) than in uninfected RMs (average: 0.38±0.058 µg/ml). Similar increases were observed in chronically SIVagm-infected PTMs (2.3±0.16 µg/ml vs. 0.25±0.05 µg/ml, p<0.0001). Conversely, no significant increase in the DD levels was observed in either chronically SIV infected AGMs or SMs (0.18±0.03 µg/ml vs. 0.23±0.05 µg/ml, p>0.05 in AGMs and 0.2±0.05 µg/ml vs. 0.26±0.05 µg/ml, p>0.05 in SMs) (Figure 1a).

Similarly, TAT levels were significantly increased (p=0.0002 and 0.0063) in chronic pathogenic SIV infections in PTMs and RMs, respectively, but not in chronically-infected AGMs and SMs (p>0.05) (Figure 1b).

Thus, cross-sectional testing of DD and TAT revealed for the first time that DD is a reliable marker for measuring coagulation status in SIV infection and that hypercoagulation is characteristic of progressive SIV infections in macaques (similar to HIV-1 infection in humans), while SIV-infected nonprogressive hosts maintain normal coagulation markers.
Coagulation biomarkers DD and TAT increase early after SIV infection and are excellent predictors of SIV disease progression

To assess the timing of the SIV-related coagulation biomarker disturbances and their ability to serve as predictive markers of HIV/SIV disease progression, we prospectively assessed changes in DD and TAT levels at key time points in the two models of pathogenic SIV infection (SIVagm/PTMs and SIVmac/RMs) (Figure 2). DD and TAT levels were measured throughout the acute and chronic stages of experimental infection, including endstage AIDS. This comparative study showed that DD levels were significantly elevated in both species during acute SIV infection (0.24±0.21 µg/ml vs. 2.02±0.58, p<0.0001 in PTMs and 0.38±0.16 µg/ml vs. 1.2±0.7 µg/ml, p=0.0281 in RMs) (Figure 2), with PTMs exhibiting the greatest increase (Figure 2a). With the transition to chronic infection, differences between the two models were uncovered: in SIVagm-infected PTMs, the levels of DD remained significantly increased throughout infection (Figure 2a). Furthermore, DD levels significantly increased in PTMs with the progression to AIDS (p=0.0227) (Figure 2a). Conversely, in SIVmac239-infected RMs, DD levels transiently decreased around the set-point and during the early chronic infection (Figure 2b), albeit not returning to baseline levels. With the progression to AIDS, DD levels rebounded in RMs, but, differently from PTMs, the increase was not statistically different from chronic infection (p=0.072) (Figure 2b).

TAT had similar dynamics to DD during the course of acute and chronic SIV infection in both RM and PTMs, thus confirming the existence, the timing and the predictive value for disease progression of the SIV-related coagulation disorder (Figure 2).

The dynamics of coagulation markers in progressive SIV infections thus showed that hypercoagulability occurs very early post-SIV infection and that, similar to HIV-
infected patients, increases in DD arising in SIVagm-infected PTMs are strongly correlated with AIDS progression and death. Overall, these results suggest that the SIVagm-infected PTM is a better model for investigating HIV-related CVD hypercoagulability than the SIVmac-infected RM.

**Histological analyses reveal a high incidence of cardiovascular lesions in pathogenic SIVagm/PTM compared to nonpathogenic SIVagm/AGM infections**

To confirm that the SIVagm/PTM model reliably reproduces the broad spectrum of cardiovascular abnormalities reported in HIV patients, we investigated numerous tissues collected at necropsy from 9 chronically SIVagm-infected PTMs. Cardiovascular pathology has already been reported in persistent progressive SIVmac/HIV-2 infected RMs and PTMs. However, the spectrum of cardiovascular lesions in SIVagm-infected PTMs is currently unknown. We also compared tissues from SIVagm-infected PTMs with SIVagm-infected AGMs to assess if differences in the incidence of cardiovascular lesions exist between progressive and nonprogressive hosts infected with the same virus strain.

We report that SIVagm-infected PTMs develop pathological evidence of CVD. Similar to the renal TMA described in HIV-infected patients, numerous thrombi were present in the glomerular capillary loops and small arteries in the kidneys (Figure 3a) in seven out of nine SIVagm-infected PTMs. Presence of thrombi in the kidney was confirmed by IHC for fibrinogen (brown) (Figure 3b). TMA was also detected in the small blood vessels in the intestine (data not shown), lung (Figure 3c) and brain (Figure 3d) in SIVagm/PTMs (consistent with the frequent neurological disease observed in this species). TMA appear to be specifically associated with SIV infection, as it was reported to be absent in uninfected PTMs.
Five out of nine SIVagm-infected PTMs also had histological lesions similar to those described in HIV associated nephropathy, i.e., focal and segmental glomerulosclerosis and collapsing glomerulopathy (Figures 3a and e) and HIV-arteriopathy (in 2 out of 9), i.e., thickened-wall arteries and vessel occlusion (Figure 3f). Numerous myocardial lesions, similar to those identified in HIV patients, were also identified in SIVagm-infected PTMs, such as: myocardial hypertrophy (in 7 out of 9 PTMs) (enlarged myocytes with irregular nuclei) (Figure 3g), fibrosis (in 3 out of 9 PTMs), with increased collagen deposition replacing either small groups of drop-out myocytes (in 1 out of 9 PTMs) (Figure 3h) or larger areas of infarction (in 2 out of 9) (Figure 3i) and myocarditis (in one out of 9 PTMs), with mononuclear cell infiltration (Figure 3j) and myocytolysis (Figure 3k). Finally, although ATS lesions are very rare in young adult SIV-negative macaques, fatty streaks (the first visible lesions in the development of ATS) composed of foamy macrophages (Insert in Figure 3l) were found to accumulate in the tunica intima and under the aortic endothelium in two out of nine SIVagm-infected PTMs (Figure 3l).

Analysis of tissues collected from 4 PTMs sacrificed during acute SIVagmSab infection (data not shown) did not reveal the cardiovascular lesions observed in chronically infected animals, in agreement with previous studies that reported that dilative cardiomyopathy, fibrosis and arteriopathy are present in chronically but not in acutely-infected macaques. These findings, in conjunction with the lack of TMA in uninfected PTMs demonstrates that microangiopathy and cardiovascular lesions observed are clearly the result of the infection and not simply due to a background increase in cardiovascular lesions in PTMs.

Finally, histological examination of the kidneys (Figure 4a), brain (Figure 4b) lung (Figure 4c), intestine (Figure 4d), aorta (Figure 4e) and heart (Figure 4f), collected at necropsy from 9 chronically-infected AGMs failed to identify any of the lesions described
in SIVagm-infected PTMs.

Histological analyses confirmed that the SIVagm/PTM has a high incidence of tissue cardiovascular lesions in addition to hypercoagulability. Furthermore, the nonprogressive natural hosts do not develop cardiovascular lesions during chronic SIV infection.

*sCD163 increases in SIVagm-infected PTMs and is correlated with DD levels*

CD163 was reported to be a biomarker of coronary ATS based on its significant increases in both HIV uninfected patients with coronary artery disease \(^{46}\) and in HIV patients with noncalcified ATS plaques. \(^{46}\) We therefore measured sCD163 in SIVagm-infected PTMs and detected significant increases of this monocyte/macrophage activation marker during all stages of SIV infection (Figure 5). sCD163 was increased in all PTMs, the highest levels being observed in the two animals with incipient ATS lesions. We found a strong correlation between the levels of sCD163 and DD levels (p=0.0016). Our results thus support a possible role of monocyte/macrophage activation in the development of CVD.

Altogether, our data suggest that the SIVagm infected PTM is a relevant NHP model for the study of HIV-related CVD mechanisms and for developing therapeutic interventions to reduce the risk of HIV-related CVD, since it has a high incidence of cardiovascular lesions, hypercoagulability during SIV infection and increased markers of monocyte/macrophage activation.

*Procoagulant state correlates with MT and immune activation biomarkers*

In Figure 6, we show the SIV associated changes in: (i) viremia (Figure 6a), (ii) CD4+ T cell depletion (Figure 6b), (iii) T cell immune activation (measured as the
fraction of CD4+ and CD8+ T cells expressing Ki-67 (Figures 6c and d) and HLA-DR (data not shown)] (iv) systemic inflammation (measured by plasma levels of IL-6) (Figure 6e), and (v) MT (estimated by plasma levels of sCD14) (Figure 6f).

To identify candidate mechanisms for HIV/SIV related procoagulant state in the SIVagm-infected PTM model of CVD, we correlated DD and those disease parameters. DD was strongly correlated with levels of immune activation (for Ki-67+ CD8+ T cells p=0.01, slope=0.062, f=0.73 and for Ki-67+ CD4+ T cells p<0.00001, slope=0.102, f=0.9) and inflammation (p=0.0002, slope=0.504, f=0.76 for IL-6) as the PTMs progressed from uninfected to chronic infection and then AIDS.

A strong correlation was also observed between DD and sCD14 (p=0.00002, slope=2.23, f=0.75). This correlation was reinforced by two cases of peritonitis that showed high levels of sCD14, immune activation and DD, in spite of an exquisite control of viral replication (Figure 6).

We could not formally test the association between DD and VL because there is no preinfection viral replication. However, cross-sectional correlations between DD and viremia showed that the two parameters were not correlated during chronic SIV infection (p=0.261, r²=0.18). The lack of correlation between DD and viremia was reinforced by the animals that strongly controlled chronic virus replication but maintained increased DD levels. These findings are in agreement with the data from nonprogressive models in which viral replication is very high while DD and TAT remain at preinfection levels during chronic infection (Figure 1).

Therefore, our data point to MT and immune activation as potential factors responsible for increased HIV/SIV-related cardiovascular risk and suggest that viral replication has a limited impact on HIV-associated hypercoagulation.
LPS administration induces immune activation and a procoagulant state in chronically SIV-infected AGMs

To directly test the hypothesis that HIV/SIV-associated MT is responsible for increased immune activation and the procoagulant status of SIV-infected NHPs, 6 chronically SIVagm-infected AGMs were treated with LPS for three weeks. Choice of SIVagm-infected AGMs for this study is justified by their lack of MT and their normal levels of immune activation during chronic SIVagm infection despite high levels of virus replication.22 Due to the remarkable stability of the VLs in this system (high levels of chronic viremia maintained for decades) and to the exquisite control of immune activation and MT, the SIVagm/AGM system allows accurate detection of discrete alterations in immune activation and coagulation biomarkers after therapeutic interventions.

LPS administration induced increased sCD14 levels (Figure 7a), demonstrating increased activation of macrophages due to bacterial products and validating sCD14 as a surrogate marker to accurately assess the magnitude of MT during HIV infection or other clinical conditions.

The levels of DD also increased significantly post LPS administration (Figure 7c). Finally, VLs showed moderate but sustained increases after LPS administration (Figure 7b) that were statistically significant (p=0.0313). Our study therefore established a direct causal relationship between MT and hypercoagulation in SIVagm-infected AGMs, providing proof of concept data for the mechanism of the procoagulant status in HIV-infected patients.
Discussion

There is a critical need to identify a relevant animal model to study the mechanisms underlying HIV-associated CVD and design therapies to improve clinical outcomes. CVD pathology is a major non-AIDS defining comorbidity that significantly impacts the outcome of HIV infection. However, a systematic approach to comprehensively define the mechanisms of HIV-related CVD is difficult in HIV-infected patients for several reasons: (i) the long duration of HIV infection in humans; (ii) late HIV diagnosis, which often focuses investigation on the chronic infection only; (iii) interference of multiple confounding factors: smoking, diet and diverse ART regimens which may impact the development of the cardiovascular lesions; and (iv) ethical limitations that prevent invasive tissue sampling for the assessment of cardiovascular lesions, or interventions aimed at reducing the frequency of these lesions. As a result, most of the published HIV-associated CVD studies have been cross-sectional and limited to the chronic phase of infection, while being correlative in nature.

Use of animal models that reproduce HIV-associated CVD permit circumventing these limitations, exploring the mechanisms of CVD occurring in HIV-1 infection and design and testing of therapies aimed at controlling CVD and improving the clinical outcome of chronic HIV infection.

By comparing and contrasting changes in coagulation biomarkers and CVD tissue lesions in NHP models representing pathogenic and nonpathogenic SIV infections, we demonstrated that CVD occurs with high frequency and is specifically associated with pathogenic SIV infection. The SIVagm-infected PTM appears to be the model of choice for the study of SIV-associated CVD based on its similarities to HIV-1 infection. These similarities include significant increases in immune activation and coagulation biomarkers, specifically those predictive of disease progression and mortality in humans, and a high incidence of cardiovascular lesions and significant
increases in sCD163 (a marker of activated monocytes/macrophages associated with coronary disease in HIV patients\textsuperscript{4,11,46}).

We identified DD as the best predictive marker of SIV disease progression in SIVagm-infected PTMs: during chronic infection, progression to AIDS was associated with significant increases in DD, while neither VLs nor CD4$^+$ T cell counts significantly changed (Figure 6). The mechanistic basis for this observation is that, differently from HIV infection, the highly pathogenic SIV infection of PTMs is characterized by a very poor control of viral replication and virtually no CD4$^+$ T cell restoration during early chronic infection. Therefore, during transition to AIDS, neither the VL nor CD4$^+$ T cell loss significantly increases,\textsuperscript{48} and this is a major limitation of the studies in NHP models. Our observation that coagulation biomarkers predict disease progression and death in SIVagm-infected PTMs is therefore a major achievement to the field and may significantly improve the follow-up of SIV-infected macaques.

DD can be nonspecific as a biomarker of SIV-related hypercoagulability, as it may be elevated in the presence of infectious disease processes other than SIV, which may account for false-positive results of DD testing. To validate the DD testing, we measured TAT as a second marker associated with the hypercoagulable status. Our study strongly suggests that DD level increases are directly related to the stage of SIV infection, as both DD and TAT gave similar results in both cross-sectional and prospective studies. Furthermore, increases in these two markers were associated with the presence of TMA in several tissues, which confirmed the usefulness of DD to assess HIV/SIV-associated coagulopathy.

The study of the SIVagm/PTM model has identified several important features of cardiovascular disease in SIV-infected monkeys. First, changes in coagulation biomarker levels occurred in the absence of HAART, suggesting that, similar to SIV-infected NHPs, the hypercoagulability observed in HIV-infected patients is directly
related to viral infection and not only to HAART. Furthermore, CVD occurred in SIV-infected PTMs in the absence of risk factors specific for human subjects, such as high cholesterol diet and smoking, pointing to a direct role of lentiviral infection in inducing cardiovascular comorbidity. A direct comparison between PTMs and AGMs infected with the same viral strain (SIVagmSab92018) demonstrated that the SIV-associated CVD is host-dependent, and not virus-related. Furthermore, our prospective studies showed that significant changes of coagulation biomarkers occur early in SIV infection. Finally, we identified a very diverse pattern of CVD presentation in SIVagm-infected PTMs. Since this plethora of cardiovascular lesions was observed in a relatively small number of monkeys, we concluded that the incidence of CVD is very high in SIVagm/PTMs. This may also indicate that the incidence of CVD is underestimated in HIV-infected patients due to limited access to tissues.

To identify mechanisms underlying the hypercoagulable state in SIV-infected NHPs, we compared increases in coagulation biomarkers to other major parameters of pathogenic SIV infection. We report that during chronic infection, increases in coagulation markers are specifically associated with immune activation and MT and not with VLs. Thus both PTMs and AGMs have high VLs during chronic infection, but experience divergent levels of immune activation, MT and different incidences of coagulopathy. Two PTMs had low VLs during chronic infection, but died with peritonitis and associated high levels of MT and INFL. These two animals had high levels of DD despite their ability to control viral replication. Similarly, occasional progression of HIV-infection occurring in elite controllers associates high immune activation levels and abnormally high atherosclerosis in spite of viral control.49,50

Conversely, during acute infection massive increases in coagulation biomarkers were associated with both increased immune activation and MT and high VLs. To reconcile these findings, we propose that during chronic infection MT drive the levels of
immune activation and cardiovascular pathology. Conversely, during acute SIV infection, additional factors such as viral replication impact immune activation and subsequent coagulopathy.

Such dichotomy was observed for other pathogenic parameters of HIV infection, leading Hunt and Deeks to propose that acute and chronic HIV infection are two different diseases. In this context, during acute infection immune activation responses and the consequent changes in the coagulation markers limit HIV/SIV replication and establish a steady-state infection which limits perturbation of CD4+ T cell homeostasis and maintains the optimal function of the immune system. During chronic infection, immune activation results from persistent damage of the mucosal barrier leading to persistent bacteremia, which may give rise to altered cardiovascular function and subsequent CVD.

We have tested the hypothesis that MT may be the main determinant of the CVD observed in SIV infected NHPs and HIV-infected patients in two sets of experiments. First, in the cross-sectional study, we showed that both AGMs and SMs, which maintain immune system homeostasis during chronic SIV infection, also maintain a healthy coagulation status during chronic SIV infection. Moreover, no cardiovascular lesions could be identified in chronically SIVagm-infected AGMs. In natural hosts the mucosal barrier is preserved, therefore the levels of MT and immune activation during chronic infection are similar to the levels in uninfected AGMs and SMs. Altogether, these results strongly suggest that CVD may be due to the endotoxemia related to immune dysfunction observed in pathogenic HIV/SIV infection.

Note, however, that similar to studies performed in humans, such observations made in both cross-sectional and/or longitudinal analyses are intrinsically correlative.

Therefore, to identify the mechanisms of CVD in HIV infection and to confirm our hypothesis that MT associated with pathogenic HIV/SIV infection is at the origin of CVD,
we administered LPS to chronically SIVagm-infected AGMs. The rationale of performing this study in AGMs is that in this animal model high VLs, mucosal barrier integrity and control of MT and immune activation are maintained at stable levels for decades, thus permitting easy identification of any minor perturbation of the system.

LPS administration impacted the virus/host equilibrium in chronic SIVagm-infected AGMs by significantly activating macrophages (measured by the levels of sCD14) and by elevating levels of viral replication. This experiment demonstrated that the maintenance of sCD14 at baseline levels in chronically-infected AGMs is not due to an intrinsic tolerance to the LPS or LPS neutralization. More importantly, LPS administration resulted in a significant increase in the levels of coagulation biomarkers, confirming our hypothesis that MT observed in chronically HIV-infected patients and SIV-infected macaques is at the origin of hypercoagulation. The significance of this experiment relies on the fact that it was performed in a stable system. The administration of LPS to chronically-infected macaques would likely not have a similar predictive value, as it would be performed in the context of a damaged mucosal barrier, persistent MT and preexisting coagulopathy and CVD. Therefore, our study provides for the first time proof of concept data that experimental modeling of MT observed in chronically HIV-infected patients results in an altered coagulation status.

At this stage we cannot conclude that hypercoagulability is the only pathophysiologic mechanism driving CVD in HIV-infected patients. Interventions aimed at normalizing coagulation in SIV/HIV-infected patients and macaques should be performed to address this question. Alternative mechanistic pathways driven by inflammation/immune activation which are increased in SIV/HIV infection are also equally possible. It is plausible that CVD results from the interaction of multiple factors triggered by MT.

In conclusion, we identified an animal model which, due to its high incidence of
CV lesions, hypercoagulability and increased markers of monocyte/macrophage activation during SIV infection, is appropriate for the study of CVD complications of HIV/SIV infection. This model permits establishing the pathogenic determinants of the HIV/SIV-related CVD which may significantly impact the management of HIV-infected patients. This model also allows validation of biomarkers that can be used to identify those HIV-infected patients that, in spite of similar presentations (i.e., similar VLs in the absence or the presence of therapy), may have a variety of underlying pathobiologies requiring individualized therapeutic interventions, such as anti-inflammatory therapy and/or anticoagulant or anti-ATS drugs. Use of NHP models allowed us to directly test the hypothesis that MT is the root cause of CVD comorbidities in HIV-infected patients. Therefore, our results indicate that interventions aimed at reducing gut inflammation and MT have the potential to reduce CVD risk in HIV-infected patients. These findings can be readily translated to clinical practice to improve the management of HIV-infected patients. Preventing, diagnosing and treating HIV-related cardiovascular complications that represent a major cause of morbidity and mortality in HIV-infected patients may significantly impact the outcome of HIV infection, as HAART did 15 years ago.
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Author Contribution

IP, CW, CA, AL and RT designed the study; IP, EC, DM, JK, CX, CA performed the experiments; GSHR and AT, were in charge of animal studies; IP, RMR and CA analyzed data; IP, AL, CW, CA and RT wrote the paper.
References


Figure legends

Figure 1. Differences in coagulation status between natural, nonprogressive SIV infections of African green monkeys and sooty mangabeys and pathogenic SIV infections of rhesus macaques and pigtailed macaques. (a) Levels of 2DD are unchanged between uninfected and SIV-infected African non-human primates, while significantly increased in chronically SIV-infected rhesus macaques and pigtailed macaques; (b) similar changes are observed by testing a second coagulation marker, TAT. P values were calculated by the Mann-Whitney U-test.

Figure 2. Changes in the levels of 2DD and TAT, assessed at critical time points of SIV infection. (a) SIVmac239-infected rhesus macaques; (b) SIVagm-infected pigtailed macaques. SP-setpoint. P values were calculated by paired t-test.

Figure 3. Diverse spectrum of cardiovascular lesions in pathogenic SIVagm infection of PTMs. Kidney (HE) – TMA (a), kidney (IHC for fibrinogen) - TMA (b), lung (HE) - TMA (c), brain (HE) - TMA, (d), kidney (HE) – glomerulopathy (e), Kidney, small artery (HE) – arteriopathy (f), heart (HE) – myocardial hypertrophy (g), heart (collagen staining) – diffuse fibrosis (h), heart (collagen staining) – old infarctus (i), heart (collagen staining) – myocarditis, mononuclear infiltration (j), heart (collagen staining) – myocarditis, myocytolysis (k), aorta (HE) – incipient ATS lesion, foamy macrophages (insert) (l).

Figure 4. Lack of cardiovascular pathology in SIVagm-infected African green monkeys. Histological investigations of kidney (a), brain (b), lung (c), intestine (d), aorta (e) and heart (f) in chronically SIVagm-infected African green monkeys failed to identify cardiovascular lesions.

Figure 5. Significant increase of sCD163 levels during acute and chronic SIVagm infection in PTMs.

Figure 6. Dynamics of virologic and immunologic parameters of pathogenic
**SIVagm infection in pigtailed macaques.** (a) viral replication; (b) mucosal CD4⁺ T cells; (c) levels of immune activation (Ki-67) of CD4⁺ T cells; (d) levels of immune activation (Ki-67) of CD8⁺ T cells; (e) dynamics of inflammation, as assessed by changes in the levels of IL-6; (f) prospective assessment of microbial translocation based on testing of the surrogate marker sCD14. Two pigtailed macaques (□) died with peritonitis due to surgery complications. In these monkeys, the coagulation markers paralleled the levels of sepsis and inflammation and not viral replication, which was controlled. Index of CD4⁺ T cells is defined by % depletion from the baseline.

**Figure 7. LPS administration to chronically SIvagm infected AGMs.** (a) macrophage activation (sCD14) (b) DD and (b) viral loads.
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Coagulation biomarkers predict disease progression in SIV-infected nonhuman primates

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