COAGULATION ABNORMALITIES PLAY A CENTRAL ROLE IN HIV COMORBIDITIES AND SHOULD BE THERAPEUTICALLY TARGETED

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Despite the success of antiretroviral therapy (ART) that drastically decreased the deaths of acquired immunodeficiency syndrome (AIDS), human immunodeficiency virus (HIV)-infected patients remain at higher risk for non-AIDS comorbidities than the normal population, especially cardiovascular (CV) diseases. As this population ages with significantly prolonged lifespans by ART, these commonly age-related CV events are becoming more of a pressing issue. A growing body of evidence show that the increased CV risk in HIV patients is driven both by a hypercoagulable state and HIV-associated chronic immune activation and inflammation. However, the exact mechanism remains unclear.

In this dissertation, we examined the link between hypercoagulation and immune activation and inflammation, and identified tissue factor (TF) as a critical mediator between the two. We found that a subset of $CD14^{+}$ monocytes highly express TF during chronic HIV and pathogenic simian immunodeficiency virus (SIV) infection, and was not normalized even after ART. This subset of TF^{pos} monocytes not only triggers activation of Factor X, thus initiating the coagulation cascade, but also is capable of producing multiple inflammatory cytokines upon thrombin stimulation *via* protease activated receptor-1 (PAR-1) signaling. *In vivo* blockade of TF in acutely SIV-infected pigtail macaques (PTMs) by an innovative TF inhibitor, Ixolaris, resulted in reduction of coagulation marker D-dimer levels as well as markers of immune activation and inflammation. We also administered a direct thrombin inhibitor (dabigatran) and PAR-1 inhibitor (vorapaxar) in SIV-infected PTMs and monitored a large array of biomarkers related to

hypercoagulation and immune activation and inflammation in all three treatment groups. Dabigatran and vorapaxar also induced reduction of both hypercoagulation and immune activation and inflammation, although not to the extent of Ixolaris, and may have impacted different aspects of HIV/SIV pathogenesis. Altogether, these results point to a critical role of TF in bridging hypercoagulation and immune activation and inflammation to fuel HIV-associated comorbidities, and therapeutically targeting TF-related coagulation pathways is beneficial in reducing the risk of these comorbidities.

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PREFACE

These past six years towards a PhD degree has been one of the most eventful and amazing journeys in my life, and I am extremely fortunate to have many wonderful people by my side along the way, without whom this dissertation would not have been possible.

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A famous saying in China goes, "a journey of a thousand miles begins with a single step." I am extremely thankful that every step I took was with all of you. It was only because of you, here I am, towards the end of this journey, and I will carry all of your blessings for many more journeys ahead. Thank you all.

Nomenclature issue:

Human immunodeficiency virus (HIV) consists of two major subtypes: HIV-1 and HIV-2. HIV-1 is the most common and pathogenic strain of the virus, accounting for over 95% of all HIV infections worldwide. The vast majority of laboratorial and clinical research of HIV infection focuses on HIV-1. Therefore, throughout this dissertation, descriptions of HIV epidemiology, virologic characteristics and pathogenesis all refer to those of HIV-1, unless specified otherwise.

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LIST OF ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ADP	Adenosine diphosphate
AGM	African green monkey
AIDS	Acquired immunodeficiency syndrome
aPTT	Activated partial thromboplastin time
ART	Antiretroviral therapy
cART	Combinational antiretroviral therapy
CCR5	Chemokine receptor type 5
CD	Cluster of differentiation
CMV	Cytomegalovirus
CRP	C-reactive protein
CTL	Cytotoxic T lymphocyte
CV	Cardiovascular
CXCR4	C-X-C chemokine receptor type 4
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
FDA	Food and Drug Administration
GALT	Gut-associated lymphoid tissue
GLUT	Glucose transporter
HAND	HIV-associated neurocognitive disorders
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HLA-DR	Human Leukocyte Antigen – antigen D Related
hsCRP	High sensitivity C-reactive protein
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
IL	Interleukin
IL-1 RA	Interleukin-1 receptor antagonist
IP-10	Inducible protein 10
IRIS	Immune reconstitution inflammatory syndrome
LDL	Low-density lipoprotein
LE	Life expectancy

LPS	Lipopolysaccharide
MCP	Monocyte chemoattractant protein
MI	Myocardial infarction
MIP	Macrophage inflammatory protein
NF-κB	Nuclear factor kappa B
NHP	Nonhuman primate
NK	Natural killer
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PD-1	Programmed cell death protein 1
PF	Platelet factor
PrEP	Pre-exposure prophylaxis
PT	Prothrombin time
PTM	Pigtail macaque
RM	Rhesus macaque
RNA	Ribonucleic acid
SAA	Serum amyloid A
sICAM-1	Soluble intercellular adhesion molecule-1
SIV	Simian immunodeficiency virus
SM	Sooty mangabey
SMART	Strategies for Management of Antiretroviral Therapy
sVCAM-1	Soluble vascular cell adhesion molecule-1
TAFI	Thrombin activatable fibrinolysis inhibitor
TB	Tuberculosis
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF β	Transforming growth factor beta
TLR	Toll-like receptor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TRA 2°P-TIMI 50	Trial to Assess the Effects of Vorapaxar in Preventing Heart Attack and Stroke in Patients with Atherosclerosis-Thrombolysis in Myocardial Infarction
TRACER	Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome
Treg	T regulatory cell
uPA	Urokinase
VEGF	Vascular endothelial growth factor
vWF	Von Willebrand factor
α-NAPAP	N-alpha-(2-naphthylsulfonylglycyl)-4-amidinophenylalanine piperidide

1.0 INTRODUCTION

Ever since its emergence in the 1980s, human immunodeficiency virus (HIV) has been one of the most feared and widely spread viral pathogens in the modern human society. Despite three decades of continuous effort and remarkable achievement in combatting HIV infection and the deadly disease caused by HIV, acquired immunodeficiency syndrome (AIDS), about 35 million people have died of HIV and as of 2016, 36.7 million people are still living with HIV worldwide, with approximately 1.8 million new infections and 1.0 million AIDS-related deaths [1]. HIV/AIDS remains a global pandemic.

Over the years, from the development and scale-up of combination antiretroviral therapy (ART), wide acceptance of pre-exposure prophylaxis (PrEP), to blooming new approaches towards an HIV cure, treatment and prevention strategies for HIV have made enormous advancement; in the meantime, the field experienced ever-shifting paradigms as they coevolve with the pandemic to face constantly-emerging new challenges. Thus, the success of ART made the pathognomonic features of AIDS, i.e., opportunistic infections and cancers to be very rare events. As such, ART has dramatically improved the life expectancy of HIV-infected subjects, as such that it currently approaches that of uninfected population [2] As a result, the HIV-infected population is aging. In fact, an estimate of 3.6 million people living with HIV worldwide aged 50 years and older in 2013 [3]. The management of HIV infection is gradually transitioning into that of a chronic disease.

To date, there is no general cure for HIV infection except for the famous “Berlin” patient [4]. Aging HIV-infected individuals will be facing unique challenges of age-related diseases combined with chronic HIV-associated non-AIDS comorbidities, one of the leading ones being cardiovascular (CV) complications [5-7]. The special need of care for this new scenario calls for investigations of the underlying causes as well as innovative treatment strategies targeting the increased CV risk in HIV patients. In search of solutions to address this newly-evolved issue, the use of nonhuman primate (NHP) model infected with simian immunodeficiency virus (SIV) is essential in modeling the mechanisms of increased CV risk in the context of HIV infection, as well as assessing innovative therapies.

1.1 HIV INFECTION AS A CHRONIC DISEASE

1.1.1 Natural course of HIV disease progression

Untreated HIV infection generally undergoes three stages of disease progression: (i) acute or primary infection, (ii) chronic infection, (iii) AIDS. Each stage represents a distinct series of events including the behavior of the virus, response of the immune system, and pathological impact on organ systems (Figure 1).

The infection is initiated through the interaction between the envelope glycoprotein of HIV, gp120, its corresponding receptor CD4 molecule [8], following by a conformational change and bind with the chemokine coreceptors, Chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) [9], the former being most predominantly use during early stages of infection [10]. This gp120 reconfiguration also exposes gp41, which facilitates virion

fusion with the target cell membrane allowing the virus core to enter the target cell [11]. Soon after entry, heterodimeric reverse transcriptase p66 and p51 starts reverse transcribing viral single-stranded ribonucleic acid (RNA) genome into a double-stranded deoxyribonucleic acid (DNA) genome [12]. The newly transcribed viral DNA, along with capsid, integrase, and host cell nuclear import factors, forms the preintegration complex, which then enters the host cell nucleus, and integrates viral DNA with host DNA genome to form provirus [13].

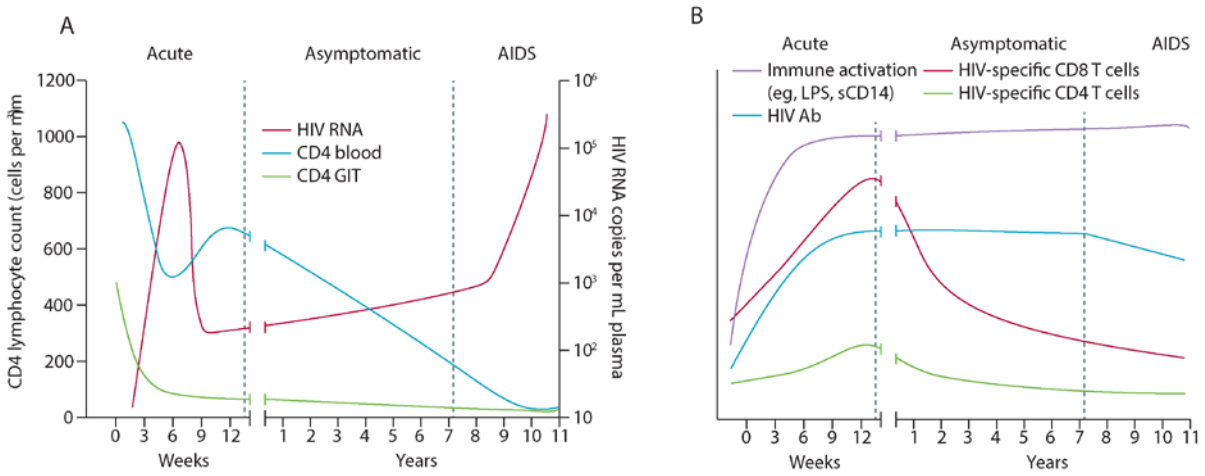

Figure 1. Natural course of untreated HIV infection
(A) In untreated HIV infection, viral load peaks during acute infection, accompanied with significant loss of $CD4^+$ T cells in blood and almost complete depletion of $CD4^+$ T cells in the gut. During asymptomatic chronic infection, viral load reaches set point plateau, and slightly recovered $CD4^+$ T cells in the blood gradually decrease again over time, with no recovery of $CD4^+$ T cells in the gut. Eventually, CD4 counts drop below 200 cells/mm^3 and viral load dramatically increases, marking AIDS progression. (B) Immune activation significantly increases during acute infection and remains high during chronic infection. HIV-specific CD8 and CD4 T cells decrease as the viral load decreases to set point. GIT – gastrointestinal tract. Figure reprinted with permission from Maartens et al. 2014 [14], ©2014 Elsevier Ltd.

Once integrated, the provirus utilizes host cell polymerase to produce genomic viral RNA and messenger RNA needed for new virion protein synthesis. These RNAs are then exported out of the nucleus under the protection of viral protein Rev [15]. The messenger RNA is translated into new viral proteins, which then selectively bind genomic viral RNA and localize to host cell membrane [16]. Viral structural proteins then multimerize at lipid rafts [17, 18], and eventually the new virion buds off utilizing the host endosomal sorting complexes required for transport

machinery [19]. Upon the successful completion of its replication life cycle, HIV infection enters its acute phase.

The acute phase of HIV infection is typically defined as the time from transmission of the virus to complete seroconversion, ranging from 12-14 weeks after virus entry [14, 20]. Immediately after viral transmission, usually established by one single founder virus [21], HIV infection quickly spreads among its primary target cells, $CCR5^+$ memory CD4 T cells, and causes cell death through either direct cell lytic consequence of the infection or virus-induced apoptosis [22]. This process consequently leads to massive CD4 T cell depletion at the mucosal sites, as this is where the majority of $CCR5^+$ memory CD4 T cells reside [22, 23]; the gut-associated lymphoid tissue (GALT) is also the major reservoir of lymphocytes in the human body [24]. Studies on both HIV-infected humans and SIV-infected rhesus macaques showed a rapid and almost complete loss of $CD4^+$ T cells in the intestinal lamina propria during acute infection [25-27]. Moreover, T cell subsets that are crucial for defense against bacteria, including T helper 17 (Th17) cells and mucosal-associated invariant T cells, are reported to be preferentially depleted [28-30]. This catastrophic and preferential depletion of gut $CD4^+$ T cells sets the basis for chronic disruption of mucosal barrier functionality, including consequences like enteropathy, microbial translocation, increased acquisition of opportunistic pathogens, etc. Meanwhile, HIV also infects other cells bearing CD4 or chemokine receptors, such as resting $CD4^+$ T cells [31], monocytes and macrophages [32], and dendritic cells [33], as part of the viral dissemination process and establishment of the latent HIV reservoir [34, 35], which is essentially the major roadblock to an HIV cure. In addition, CD4-independent HIV infection of cells also occurs, notably in astrocytes [36], hepatocytes [37] and renal epithelial cells [38], which later play important roles in the pathogenesis of HIV-associated neurocognitive disorder (HAND), hepatitis and nephropathy in chronic phase of the infection.

As viral load climbs to a peak viremia of 10^5 ~ 10^7 copies/mL of plasma, a "cytokine storm" is induced as part of the innate immune response to the viral infection, including the rapid increase of acute-phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) [39], as well as two major waves of cytokines and chemokines: the rapidly-induced interferon-alpha (IFN-α), interleukin-15 (IL-15), inducible protein 10 (IP-10), tumor necrosis factor alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1); followed by the delayed elevation of IFN-γ, IL-6, IL-8, IL-18 and IL-10 [40]. Meanwhile, both innate and adaptive immune cells are profoundly activated, including: a) dendritic cells activated by the interaction between toll-like receptor 7 (TLR7) and the endocytosed HIV [41]; b) natural killer (NK) cells, whose activation has an antiviral effect through CD107a-mediated cytolysis of HIV-infected cells, as well as producing antiviral cytokines and chemokines, such as IFN-γ [42]; c) $CD8^+$ T cells, especially HIV-1-specific $CD8^+$ T cells, are highly activated, undergo rapid expansion and exhibit strong cytolytic activity towards HIV-infected cells and IFN- γ production [43, 44]; d) SIV-specific $CD4^+$ T cells, can also be activated and exert virus-specific cytokine-producing protective effect, although they are less well-defined, because they are also target cells and are thus preferentially depleted [45, 46]; e) B cells, whose activation is responsible for effective HIV-specific antibody production, albeit non-neutralizing at the initial phase [47].

Up to 60%-90% HIV-infected patients experience non-specific symptoms similar to other viral infections such as influenza and infectious mononucleosis [48]. Yet, with the emergence of the rapid and robust initial responses to the virus infection, the vast majority of patients survive acute infection and step into the dreadfully long asymptomatic chronic phase, which may last ten years or longer.

Despite commonly being referred to as clinical latency, with almost no clinical manifestations, the chronic phase of HIV infection is a constant battle between the immune system and the viral infection, which without ART is more often than not a losing war for the host.

Over a period of 12-20 weeks after the peak of viremia, plasma viral load decreases to a stable level, known as the viral set point ("steady state replication"), mainly as a result of effective killing of productively infected cells by HIV-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) [49, 50], assisted by NK cells and production of broadly neutralizing antibody [51]. Since viral set point is the net result of effective immune response against robust replication and high turnover of the virus, it is highly predictive of the rate of progression to AIDS [52-54]. During the establishment of viral set point, virus diversification also occurs. The pressure of potent immune response selects for the emergence of immune escape mutants and extensive viral recombinations [55-57], which later become a major component of the latent viral reservoir, and present as yet another obstacle to an HIV cure as it indicates broad CTL responses are essential for reservoir eradication [58].

On the other hand, the general activation of the immune system (detailed in Section 1.2.1) also rapidly climbs up throughout acute infection. In contrast to viral load, the increase of immune activation is continuous, and by the time that HIV infection enters into chronic phase, immune activation also reaches a plateau, but of an unusually high level. Deeks *et al* brought forth the concept of immune activation "set point", describing a steady-state high level of immune activation established during early infection, although widely variable between individuals [59]. It is also shown that the immune activation set point is negatively correlated with the viral load set point. Multiple studies on the effect of ART administration on decreasing

the turnover and activation of T lymphocytes indicate that the level of viremia is one of the contributors to the T-cell activation set point [60-62].

Not coincidentally, despite a slight recovery of $CD4^+$ T cellcounts occurring simultaneously with the decrease of viral load to set point, during the chronic clinical latency, HIV-infected individuals experience a gradual loss of peripheral $CD4^+$ T cells. Traditionally, this decrease of $CD4^+$ T cells was thought to be due to the "tap-and-drain" model, where the rapid infection and direct killing of $CD4^+$ T cells by the virus outruns the rate of replenishment by homeostasis proliferation from the peripheral T cells pools. However, more and more evidence during the past two decades showed that virus has the ability to heighten the activation of bystander T cells that are not infected, turning them into activation phenotype which are then destined to a rapid death [63-65]. This hypothesis is supported by the observation that the number of productively infected peripheral T cells is very low (typically less than 0.01%) [66], and natural hosts of SIV infection who do not present immune activation during chronic infection recover their peripheral $CD4^+$ T cells to normal level despite high viral load [67]. In line with this theory, it is not surprising that immune activation is shown to have stronger prognostic significance than viral load towards disease progression [59, 68]. In rare cases of HIV infection, such as elite controllers who control viral load to undetectable levels, and long-term nonprogressors, who may have low viremia but do not progress to AIDS, CD4 counts are preserved, and these individuals present with lower immune activation [69, 70].

Eventually, during typical untreated HIV chronic infection, the continuous activation and turnover of T cells slowly exhaust the lymphocyte pool, and consequently, plasma viremia loses control and skyrockets to levels sometimes even higher than peak viral load. Once the $CD4^+$ T cell level hits the threshold of 200 cells/mm^3, AIDS develops as a result of profound destruction

of the immune system, marked by the frequent occurrence and severe outcome of opportunistic infections and malignancies.

Overall, although acute phase is critical for HIV infection and largely determines the magnitude and rate of progression in later stages, the fatal process of HIV pathogenesis mainly happens during chronic phase, which dictates the ultimate outcome of HIV infection.

1.1.2 The new landscape of HIV infection in post-ART era

Since the first effective therapy against HIV (zidovudine) was approved in 1987, a variety of antiretroviral drugs have emerged. ART, which is a cocktail of several types of antiretroviral regimens, was later introduced in 1996 to overcome the drug resistance and incomplete virus suppression issues of mono- and bi- therapy [71]. Current antiretroviral drugs are divided into several groups, each targeting a different stages of HIV life cycle: reverse transcriptase inhibitors, including nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs); protease inhibitors (PIs), integrase inhibitors (INSTIs); entry inhibitors; and fusion inhibitors (Table 1). The first-line ART for most adults is two NRTIs plus an NNRTI/INSTI [72, 73].

Table 1. Antiretroviral drugs approved by FDA

NRTIs	NNRTIs	PIs	INSTIs	Entry Inhibitors	Fusion Inhibitors
Lamivudine (3TC)	Rilpivirine (RPV)	Tipranavir (TPV)	Raltegravir (RGV)	Maraviroc (MRC)	Enfuvirtide (T-20)
Emtricitabine (FTC)	Etravirine (ETR)	Indinavir (IDV)	Dolutegravir (DTG)		
Zidovudine (AZT)	Delavirdine (DLV)	Saquinavir (SQV)	Elvitegravir (EVG)		
Zalcitabine (ddC)	Efavirenz (EFV)	Lopinavir (LPV)			
Abacavir (ABC)	Nevirapine (NVP)	Ritonavir (RTV)			
Tenofovir disoproxil fumarate (TDF)		Fosamprenavir calcium (FOS-APV)			
Didanosine (ddI)		Darunavir (DRV)			
Stavudine (d4T)		Atazanavir (ATV)			
		Nelfinavir (NFV)			

(Last Updated: 08/09/2016. https://www.fda.gov/forpatients/illness/hivaids/treatment/ucm118915.htm)

By inhibiting the most crucial steps of virus life cycle, ART effectively suppresses virus replication, often decreases viral load to concentrations below the lower limit of detection of common HIV-1 RNA load assays (50 copies/mL of plasma). However, a small proportion of patients show low level viremia (50 – 200 copies/mL) and may experience virological failure (>200 copies/mL, or two consecutive measurements, or >1000 copies/mL in resource-limited settings) which requires regimen switch [72, 73], due to drug intolerance/toxicity/resistance and/or suboptimal adherence.

Meanwhile, ART also increases $CD4^+$ T cell counts to an extent [74], although this recovery is highly variable between individuals and strongly depends on the severity of immunedeficiency at the time of treatment initiation [75, 76]. Autran *et al.* described successful CD4 increase by ART as three-phase [77]: (i) an early rise of memory $CD4^+$ cells, (ii) a reduction in T cell activation correlated to the decreasing retroviral activity together with an improved $CD4^+$ T cell reactivity to recall antigens, and (iii) a late rise of "naïve" $CD4^+$ T lymphocytes. However, even with the three-phase increase, these parameters are not completely normalized. It is not surprising that multiple factors may impact the outcome of this indirect effect of ART, summarized by Maartens *et al.* as host factors (e.g. older age, low CD4 nadir, high baseline HIV RNA), viral factors (e.g. CXCR4-tropic virus and coinfections), and immunological factors (e.g. low thymic output, senescence, increased programmed cell death protein 1 (PD-1) expression and apoptosis) [14]. While ART has a variable effect of improving $CD4^+$ T cell reconstitution in the peripheral blood, such effect is even less prominent in the gut. Studies have shown that gut $CD4^+$ T cells remain largely depleted even after five years of fully suppressive ART [78], potentially due to alterations in $CD4^+$ T cell homing [79]. Overall, poor

$CD4^+$ T cell recovery in patients with cART is associated with marked increases in mortality [80].

ART was also shown to reduce immune activation, especially T cell activation, as another indirect effect of successful suppression of viral replication [81]. However, the level of immune activation still remains elevated compared with uninfected control subjects [81-83], and the persistently elevated immune activation is associated with incomplete CD4+ T cell recovery and persistence of HIV on ART in blood or tissue [75, 81, 84-86]. The general impact of ART on the natural history of HIV infection is summarized in Figure 2.

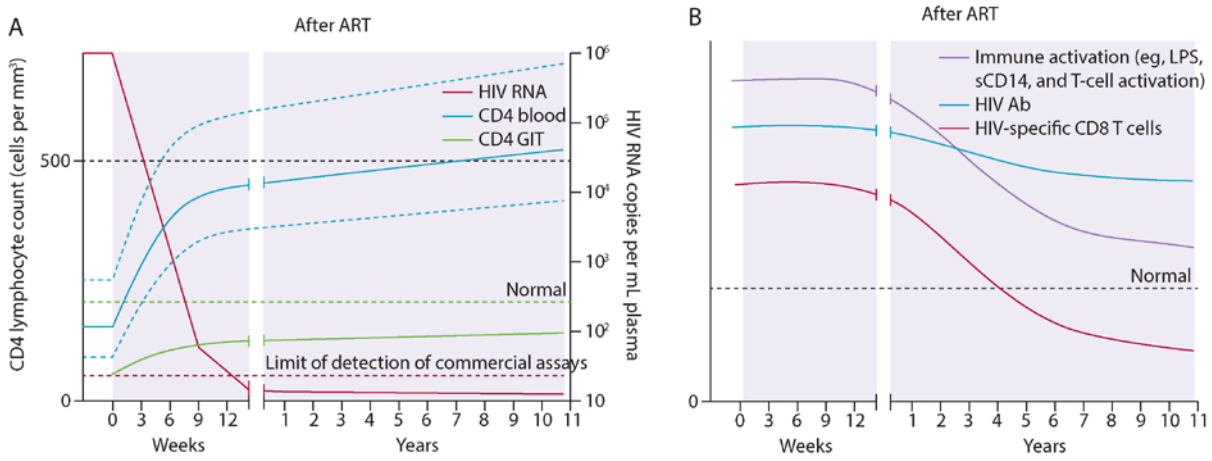

Figure 2. Altered course of HIV infection after antiretroviral therapy
(A) After ART initiation, HIV RNA significantly decreases followed by recovery of CD4 T cells, which varies between individuals (dotted blue line shows the interval between various response). By contrast, recovery of CD4 T cells in the gastrointestinal tract is reduced. (B) With reduction of HIV RNA and viral antigen, HIV-specific T cells decrease after antiretroviral therapy, whereas antibody persists in all patients. Immune activation decreases after ART but in most patients remains significantly increased compared with healthy controls. GIT – gastrointestinal tract. Figure reprinted with permission from Maartens et al. 2014 [14], ©2014 Elsevier Ltd.

The potent combination therapy has led to a 60% to 80% decline in the rate of AIDS, AIDS-related death and hospitalization [87], and is highly effective in reducing HIV transmission [88]. The death from AIDS-related causes declined from a peak of 1.9 million [1.7 million–2.2 million] in 2005 to 1.0 million [830,000–1.2 million] worldwide in 2016 [1]. Importantly, the advent of ART resulted in a substantial prolongation of the life expectancy of

HIV-infected individuals, albeit their life expectancy remains lower than that of the general population [2, 89]. A large cross-cohort study including 88,504 patients showed that the life expectancy of a 20-year-old patient starting ART during 2008–10, who had a $CD4^+$ T cell counts of more than 350 cells/mm^3 was 78.0 years (77.7–78.3) 1 year after starting ART. The study also showed that life expectancy increases with calendar period of initiation of ART, emphasizing the importance of early initiation of ART [2].

Indeed, despite its encouraging effects, ART is not yet an ideal solution for HIV infection: (i) it does not "cure" or eradicate the virus, and has limited access to tissue-harboring viral reservoir due to inadequate drug penetration, (ii) it does not always achieve complete virus suppression, (iii) it frequently does not result in optimal immune reconstitution, particularly the mucosal $CD4^+$ T cell restoration, (iv) it does not fully normalize persistent chronic immune activation and inflammation, (v) it does not restore the life expectancy of HIV-positive population to that of the general population, (vi) drug toxicity often leads to side effects, excessive inflammation and increased risk for comorbidities, (vii) continuous care is needed and is highly resource-demanding, (viii) stigma persists for ART-treated patients as they are still seen as contracting "incurable" sexually-transmitted disease. Early initiation of ART has been shown to improve some of these drawbacks by preventing early virus dissemination and profound damage to the immune system, and was shown to result in lower T cell activation, improved CD4 recovery and smaller viral reservoir size [90-93]. Furthermore, in the VISCONTI ANRS cohort of HIV-infected adults living in France who were treated within the first 2 months of infection for over 3 years, identified promising results in 14 individuals for which post-treatment control was reporting consisting in control of viremia after ART interruption, for more than 5 years [94]. Unfortunately, early initiation of ART in an infant 30 hours after birth, famously

known as the “Mississippi baby”, which was discontinued after 18 months, eventually resulted in a viremic relapse after 27 months of viral suppression off ART [95]. A study of 16 patients from San Diego Primary Infection Cohort with similar early initiation of ART, maintained ART for a median of 2.04 years, and ART interruption after viral suppression as the VISCONTI cohort also did not replicate the successful viral control after treatment discontinuation [96], indicating further investigation of the underlying mechanism of post-treatment control is needed.

Nevertheless, the success of ART has turned the battle against HIV/AIDS from a progressive life-threatening condition to a chronic manageable disease setting, where more attention is now drawn to the pathological impact of chronic HIV infection despite viral suppression and patient’s quality of life after years of ART.

1.1.3 HIV-related non-AIDS comorbidities

As ART significantly reduces AIDS-related death in HIV-infected subjects, the proportion of death due to non-AIDS-defining illnesses becomes more prominent, reported to have risen from 13.1% in 1996 to 42.5% in 2004 [97]. Particularly revealed by the Strategies for Management of Antiretroviral Therapy (SMART) trial [98], and corroborated by many other studies, HIV-infected subjects face higher risks of non-AIDS comorbidities than general population, including but not limited to CV disease, cancer, kidney disease, liver disease, osteopenia/osteoporosis and neurocognitive disease [99-104].

Importantly, many of these non-AIDS comorbidities are also age-related non-communicable diseases. Aged HIV-infected individuals over 50 years of age not only have higher risk for each individual comorbidity, but also show a higher proportion with three or more age-associated noncommunicable comorbidities simultaneously [104]. This is particularly

concerning as the prolonged life expectancy of HIV-infected population by ART also means the proportion of aged HIV-infected individuals will be increasing. Currently, an estimate of 47% Americans living with diagnosed HIV are aged 50 and over [105]. A study conducted in the Netherlands based on data from 10,278 patients from the Dutch ATHENA cohort predicts that the median age of patients receiving treatment for HIV will increase from 43.9 years in 2010 to 56.6 years in 2030, the proportion of patients older than 50 years will increase to 73%, and as a result, the number of HIV-infected subjects with at least one noncommunicable disease is projected to increase from 29% in 2010 to 84% in 2030 [106]. The aging of HIV-positive population poses unique challenges in the clinical management for these patients, and calls for more investigation on the care for non-AIDS comorbidities [107].

Severe events of non-AIDS comorbidities that are life-threatening, cause prolonged hospitalization and persistent incapacity or are associated with significant morbidity are categorized as serious non-AIDS events (SNAEs), among which CV event is one of the most fatal and leading cause of death [97, 108, 109]. HIV-infected individuals have significantly higher rates of hospital admission for coronary artery disease and risk for myocardial infarction (MI) [110-112]. In the US Veterans Aging Cohort Study, incidence rate for MI is almost 2-fold higher in HIV-positive individuals compared with uninfected controls, especially in those aged 50 years or above [103]. A larger cohort of patients from the California Medicaid population corroborated this increase risk of acute MI in younger patient population, and also showed significant increased risk for other acute and subacute ischemic heart disease [111]. This is in line with the increased subclinical marker levels of atherosclerosis, such as the carotid, femoral, or iliac intima-media thickness, and earlier progression of atherosclerosis in HIV-positive population [113, 114]. Other studies also showed that HIV-positive subjects are at increased risk

of other chronic CV diseases such as pulmonary hypertension, myocardial fibrosis, congestive heart failure, ischemic stroke, peripheral artery disease and venous thromboembolism [115-122].

Importantly, high risk of CV disease is not normalized by ART. In fact, exposure to antiretroviral drugs is shown to be an independent risk factor for premature coronary artery diseases observed in HIV-infected subjects [123, 124]. ART that modifies lipid metabolism in particular, may have a greater impact on CV health, such as d4T-associated lipoatrophy is associated with high triglycerides and low high-density lipoprotein (HDL) [125]. Besides, protease inhibitor is shown by many studies to accelerate atherosclerosis and subsequent risk of MI due to its effect of inducing dyslipidemia [100, 126, 127]. As such, CV risk is an ongoing concern for both ART-naïve and treated HIV-infected subjects, rendering the desperate need to investigate the mechanism of increased CV risk in HIV-positive individuals and strategies to prevent CV event in this population.

HIV infection may alter the risk of SNAEs through a combination of over-presentation of traditional risk factors (e.g., smoking) in the HIV-infected population, exacerbation of traditional risk factors (e.g. ART-induced dyslipidemia), and HIV-specific pathogenic processes [112]. The underlying pathophysiological cause of HIV-associated SNAEs apart from traditional risk factors is not yet completely elucidated. Multiple factors are thought to contribute to the pathogenesis of SNAEs, including ART toxicity, direct effect of HIV and associated immunodeficiency, persistent viremia, coinfections, microbial translocation, immune activation with associated inflammation and coagulopathy [108]. The use of ART is generally considered to increase the risk for SNAEs, while certain integrase inhibitor-based ART is shown to reduce monocyte activation and vascular inflammation [128, 129].

Table 2. Potential strategies to reduce SNAEs

Potential strategies to reduce SNAEs	Interventions investigated or under evaluation	References
Preventing immunodeficiency	Initiate ART prior to advanced immunodeficiency	[88, 130-136]
Increasing CD4+ T cell recovery		
Cytokine therapy	Subcutaneous IL-2	[137-139]
	Subcutaneous IL-7	[140, 141]
	Subcutaneous IL-21	[142]
Modulating lymphoid tissue fibrosis	Pirfenidone	[143]
	Angiotensin receptor antagonist	[144]
	ACE inhibitor	[144]
Managing comorbidities	Smoking cessation	[145, 146]
	Optimize blood pressure, lipids and diabetic control	[147, 148]
	ART switch	[149-157]
Reducing chronic antigen stimulation		
Residual viremia	Raltegravir intensification	[129, 158-166]
	Maraviroc intensification	[167-170]
HBV and HCV co-infection	Hepatitis B and C treatment	[171-173]
CMV co-infection	Valganciclovir	[174]
HSV co-infection	Valacyclovir	[175]
Reducing inflammation	Statins	[176-181]
	COX-2 inhibitors	[182, 183]
	Aspirin	[184]
	Hydroxychloroquine & Chloroquine	[185-187]
	Leflunomide	[188]
	Prednisone	[189-192]
Reducing microbial translocation		
Balancing microbiota	Prebiotic, probiotic and synbiotic	[193-196]
Reducing bacterial/endotoxin load	Rifaximin	[197]
	Bovine colostrum	[164]
	Sevelamer	[198]
Improving mucosal integrity	Lubiprostone	NCT01839734 (ongoing clinical trial)
Reducing inflammation in the gut	Mesalamine	[199-201]

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Over the years, various strategies were investigated to reduce SNAEs (Table 2). However, the majority of these studies are small in size, variable with regards to ART status of the patients and their levels of virus suppression and conflicting findings with moderate improvement of SNAEs at best. Specific for CV risk, studies have investigated the use of statins in HIV patients has been investigated, with observational promising outcomes in small HIV patient cohorts [202,

203]. A large-scale clinical trial of pitavastatin with an estimate of 6,500 enrollment is being conducted and anticipating results in 2020 (NCT02344290). Aspirin use has also been investigated in HIV-infected population [204, 205]. However, these studies are mostly observational, and these interventions are not directly targeting the coagulation system itself. Complete understanding of the effect of more direct CV event-targeting therapies (i.e. anticoagulant therapies) is still lacking.

1.2 CHRONIC HIV/SIV PATHOGENESIS

The advent of ART dramatically increased the life expectancy of HIV-infected individuals. However, their life expectancy is still lower than that of uninfected individuals in the same age group, largely due to high risks for SNAEs and non-AIDS mortality. In the post-ART era of managing HIV as a chronic disease with frequent end-organ manifestations, understanding the mechanism of chronic HIV pathogenesis, especially aspects that are not completely normalized by ART, is crucial for designing and applying interventions specifically targeting these aspects to further improve the life expectancy of HIV patients and reduce the incidence of comorbidities.

1.2.1 Immune activation and inflammation associated with chronic HIV infection

Well before the widespread of ART, studies dedicated to identifying prognostic markers for HIV disease progression have shown that coexpression of HLA-DR (Human Leukocyte Antigen – antigen D Related) and CD38 on $CD8^+$ T cells is an independent indicator for CD4+ T-cell loss and progression to AIDS [206]. Around the same time, innate immune activation markers such

as TNF receptor II, soluble IL-2 receptor, and neopterin were also shown to have important prognostic values for HIV progression [207, 208]. Later on, a large array of evidence has shown that immune activation and inflammation is a hallmark of chronic HIV infection: (i) T lymphocyte activation is more closely related to shorter survival in advanced HIV infection and faster progression to AIDS than viral load or coreceptor use [59, 209], (ii) $CD4^+$ T-cell depletion is only indirectly linked to viral load, but directly linked to immune activation, as defined by activation markers (HLA-DR, CD38, CD69, Fas molecules), frequency of cycling CD4+ T cells (Ki67+) and proliferative responses to CD3 stimulation [210], (iii) the extent of the excessive immune activation in ART-treated subjects is associated with increased morbidity and mortality [211].

The most important evidence of the connection between chronic immune activation and inflammation with HIV disease progression was in fact revealed in the investigation of natural hosts of SIV. Multiple species of African NHPs naturally harbor species-specific SIV (e.g., SIVagm-infected African green monkeys (AGMs), SIVsmm-infected sooty mangabeys (SMs), and SIVmnd-infected mandrills), but do not typically manifest AIDS. However, Asian macaques infected with the same or similar viruses, for example, SIVagm-infected pigtail macaques (PTM) and SIVmac-infected rhesus macaques (RMs) (SIVmac is originated from SIVsmm), show pathogenic features and progression to AIDS without treatment similar to those observed in HIV-infected humans [67, 212, 213]. The most striking contrast between the natural hosts and pathogenic/progressive NHP infected with SIV is that, while both models harbor highly replicating SIV and experience massive gut $CD4^+$ T-cell depletion during acute infection, the natural hosts lack immune activation and inflammation and preserve both peripheral and mucosal $CD4^+$ T cells in chronic infection [198, 212-216]. Moreover, experimentally inducing immune

activation and inflammation in natural hosts results in increased viral replication and CD4+T cell depletion [217-219] The findings in NHPs are also corroborated by low immune activation observed in HIV long-term nonprogressors despite high viral loads [220]. These observations provide direct evidence for the immune activation and inflammation correlation with HIV/SIV progression.

Chronic HIV/SIV-associated immune activation and inflammation involves the general activation of both adaptive and innate immune system. The levels of a series of biomarkers representing distinct arms of immune activation and inflammation are shown to be aberrantly high in HIV subjects, although to different extent in different subpopulations of patients (e.g. acute infection *vs*. advanced infection, treatment-naïve *vs*. treated, young *vs*. old, etc.). As such, these biomarkers are utilized as valuable tools to predict disease progression, or to assess the effect of interventions to reduce immune activation and inflammation (Table 3).

Table 3. Common biomarkers for HIV/SIV-associated immune activation and inflammation

Adaptive immune activation	Innate immune activation	Inflammation
CD38 and HLA-DR	CD80	CRP
Ki-67 (also proliferation marker)	CD86	IL-6
CD69	CD69	IL-1β
CD25	Glut-1	IL-1RA
CD95 (also apoptosis marker)	Neopterin	IL-8
Glut-1	Soluble CD14	IL-15
Soluble CD27	IP-10	TNF-α
	Soluble CD163	IFN-γ
	Soluble TNF Receptor II	MCP-1
	Soluble IL-2 Receptor	MIP-1α and -1β
		MCP-1

*Glut-1 – Glucose transporter-1. MIP – macrophage inflammatory protein. IL-1RA – interleukin-1 receptor antagonist

The underlying cause of this generalized immune activation and inflammation during chronic HIV and progressive SIV infection is thought to be complex and multifactorial [221, 222]: (i) direct effect of virus production and replication, including low grade viremia under ART, mediated by TLRs and CCR5 [41, 223, 224], (ii) coinfections, such as cytomegalovirus

(CMV), Epstein-Barr virus (EBV) and hepatitis C virus [171, 174, 225], (iii) ART toxicity, lipodystrophy, and other traditional risk factors (e.g., smoking) [226], (iv) loss of mucosal integrity of the gastrointestinal tract and subsequent microbial translocation, where microbial products in the intestinal lumen such as lipopolysaccharide (LPS), flagellin, and peptidoglycan translocate into systemic circulation and other organs, such as liver and lymph nodes, and induce innate immune activation and production of proinflammatory cytokines through TLR stimulation [197, 198, 227-230], (v) imbalance of $CD4^+$ T cell subsets, such as Th17/Treg (T regulatory cells) ratio and effector to central memory $CD4^+$ T cell ratio [231-233].

Driven by the multiple mechanisms of HIV/SIV pathogenesis, immune activation and inflammation then exert deleterious consequences on the disease progression and outcome: (i) T cell activation induces increased expression of CCR5 and CXCR4, generating more target cells for the virus [234, 235], and at the same time, the activated intracellular nuclear factor kappa B (NF-κB) enhances transcription of the integrated virus [236], thus initiating a vicious cycle of promoting virus spreading and persistence, (ii) the heightened activation and turnover of lymphocytes drives homeostatic proliferation and results in the drainage of the lymphocyte progenitor pool and early onset immune senescence (marked by the high proportion of $CD28^-$ /$CD57^+$ $CD8^+$ T cells) [44, 237], (iii) T cell activation accelerates the exhaustion of their function (marked by elevated PD-1 expression) [85, 238], (iv) fibrosis of lymphatic tissues through collagen deposition mediated by TGFβ (Transforming growth factor beta) expression by Tregs, dampening the architecture of lymphatic tissues and preventing T cell homeostasis [239, 240], (v) activation of immune cells, especially monocytes/macrophages, along with prolonged inflammatory response can induce damage to vasculature and lipid abnormalities, causing CV

diseases [241, 242], as well as facilitating proliferation of premalignant and malignant cells, and inducing non-AIDS associated malignancy [243].

To tackle these adverse effects, various strategies aiming at reducing immune activation and inflammation targeting the different underlying causes have been investigated, many of which overlap the strategies listed in Table 2 that ultimately sought to reduce HIV comorbidities. However, as mentioned, most of these studies are small in size and their benefice is at best moderate [244]. By far, the best strategy to decrease immune activation and inflammation in HIV remains early initiation of ART [245]. Notably, due to the complexity of the causes of immune activation and inflammation, some are virus-related related while others are not, thus it is likely that not all root drivers of immune activation and inflammation can be equally attenuated by early initiation of ART [246]. A better understanding of which immunologic pathways and how they are driving specific disease manifestations is needed to help prioritize interventional targets and identify the populations at highest risk.

1.2.2 Hypercoagulation associated with chronic HIV infection

1.2.2.1 Common coagulation pathways

Common coagulation involves both a cellular (platelet) and a protein (coagulation) component (Figure 3). Upon endothelial damage, the von Willebrand factor(vWf)-Factor VIII complex bind and activates platelets, linking platelets with extracellular matrix, resulting in platelet aggregation and forming a platelet plug, which is usually the initiation of primary hemostasis [247].

Endothelial or tissue damage can also initiate coagulation through the contact activation (intrinsic) pathway and the tissue factor (extrinsic) pathway. It was traditionally thought that the

two pathways are parallel and of equal importance in initiating fibrin clot production. However, it was later shown that hemostasis is primarily initiated by extrinsic pathway, and only augmented by intrinsic pathway [248].

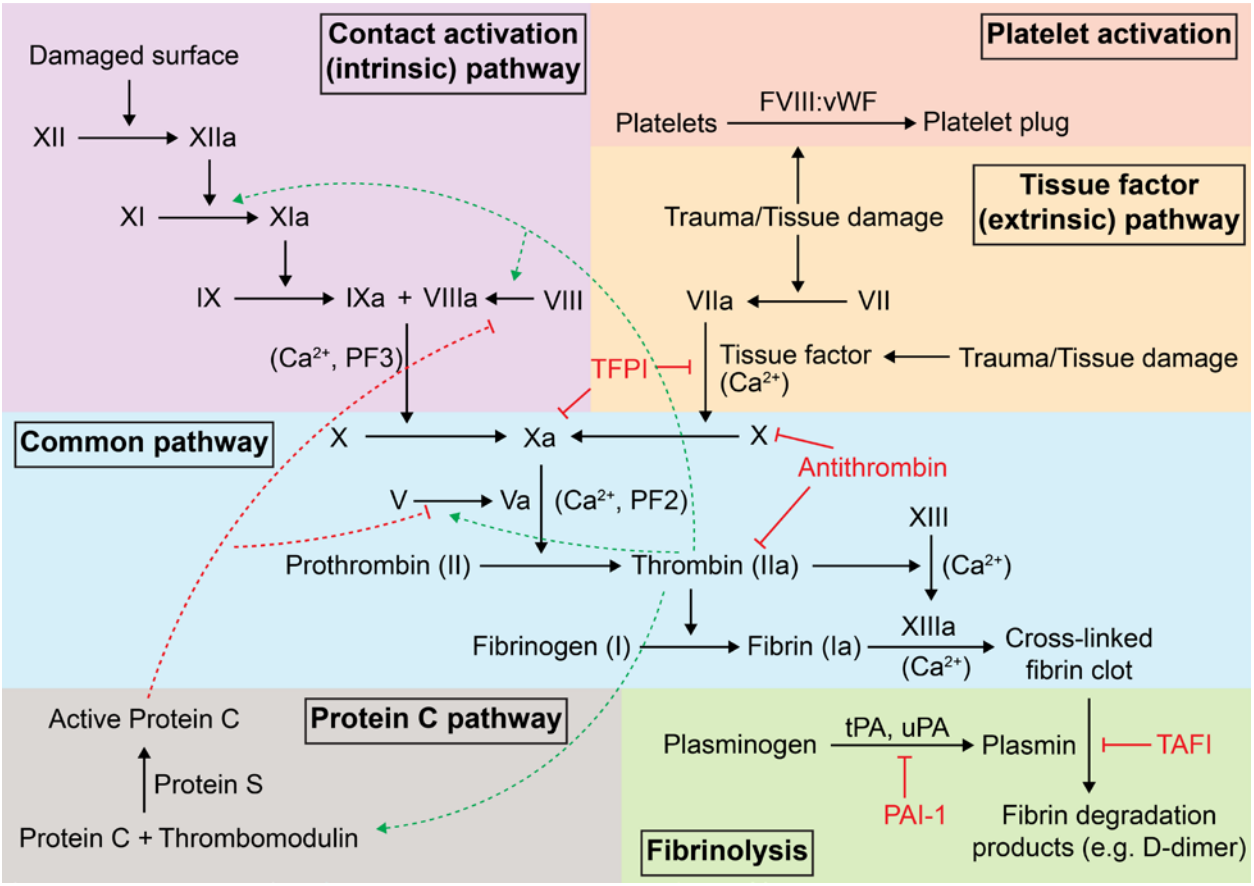


Figure 3. Common coagulation pathways
An illustration of common coagulation cascade (intrinsic pathway, extrinsic pathway, common pathway), with platelet activation, Protein C pathway and fibrinolysis. Black and green arrows indicate activation or facilitating activation, and red bar-headed lines indicate inhibition. PF – platelet factor; vWF – von Willebrand factor; TFPI – tissue factor pathway inhibitor; tPA – tissue plasminogen activator; uPA – urokinase; PAI-1 – plasminogen activator inhibitor-1; TAFI – thrombin activatable fibrinolysis inhibitor.

The extrinsic pathway is activated by tissue factor (TF), which binds with Factor VIIa and calcium, to promote Factor X conversion to Xa. The intrinsic pathway begins with the formation of the complex of Factor XII, high-molecular-weight kininogen and prekallekerin, with activates Factor XII to XIIa. FXIIa converts FXI into FXIa. Factor XIa activates FIX, which, with its cofactor FVIIIa, forms the tenase complex, which on a phospholipid surface

activates FX to FXa. After the activation of FX, the extrinsic and intrinsic pathway converge to the common pathway, where FXa along with its cofactor (Factor V), phospholipid surface and calcium, forms the prothrombinase complex which converts prothrombin to thrombin. Thrombin then cleaves fibrinogen to insoluble fibrin and activates Factor XIII, which covalently crosslinks fibrin polymers incorporated in the platelet plug, stabilizes the clot and forms a definitive secondary hemostatic plug [249, 250]. Notably, thrombin also plays an important role in the coagulation amplification loop by activating platelets, FXI, FVIII, and FV. On the other hand, thrombin can also promote activation of Protein C with cofactor thrombomodulin, which has an inhibitory role on coagulation.

Coagulation cascade is naturally regulated by intrinsic anticoagulants in the body, such as Protein C, which, when activated by cofactors thrombomodulin and Protein S, inhibits FV and FVIII activation [251]. Antithrombin is a serine protease inhibitor, which binds and inactivates thrombin, factor IXa, Xa, XIa and XIIa [252]. Tissue factor pathway inhibitor (TFPI) limits the action of TF, and also inhibits excessive TF-mediated activation of FVII and FX [253].

Lastly, fibrinolysis is activated along with the activation of coagulation cascade and serves to limit the clot size. Catalyzed by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA), plasminogen is cleaved into plasmin which dissolves the fibrin clot into fibrin degradation products (FDPs). FDPs, such as D-dimer, are specific indicators of fibrinolysis used in the assessment and diagnosis of pulmonary embolism, DIC or deep vein thrombosis [254].

1.2.2.2 Hypercoagulation observed in HIV patients

Even before the advent of ART, HIV has been recognized as a prethrombotic condition with hematological abnormalities [255, 256]. With the HIV-infected population having longer life

span and aging, coagulopathy is becoming a growing concern due to its role in inducing CV comorbidities. Increasing evidence in recent years has shown that HIV-related coagulopathy affects multiple coagulation pathways and involves general activation of the vasculature.

In the pre-ART era, the hypercoagulability in HIV-infected patients that led to high incident rate of thromboembolic events was thought to be due to: (i) decreased activities of natural anticoagulants (i.e. Protein C and Protein S deficiency) [257-259]; note that one South African study showed that protein S deficiency did not differ significantly between patients with stroke who are HIV-positive and patients with stroke but without HIV infection [260]; (ii) the presence of antiphospholipid-anticardiolipin antibodies, but the results of related studies are also inconsistent, with reports of either no association [261, 262] and of positive association [263, 264] with venous thromboembolism in HIV-infected subjects; (iii) increased platelet activation [265, 266], as well as the accumulation of platelet-activating factor [267] and the detection of platelet-monocyte complexes in circulation [268].

In the post-ART era, with more patients suffering from catastrophic thrombotic events rather than AIDS-defining illnesses, more research focus has been directed to the investigation of hypercoagulation, and as a result, many other CV biomarkers revealed to be at aberrant levels in HIV-infected individuals, are associated with CV comorbidities and are not completely normalized by ART, despite viral suppression. The SMART study generated wide interest in hypercoagulation by demonstrating that increased plasma levels of CRP, IL-6 and D-dimer were predictive of all-cause mortality and CV events in HIV-infected subject [241, 269]. CRP and IL-6, despite being inflammation markers, are recognized also for their close relationship with CV risk, being strong predictors of CV dysfunctions [270, 271]. The buildup of D-dimer indicates excessive fibrin production, therefore indirectly reflects elevated thrombotic risk. When

compared with Coronary Artery Development in Young Adults study participants for age group 33-44, the levels of hsCRP (high sensitivity CRP) and IL-6 levels were 55% (P<.001) and 62% (P<.001) higher in HIV-positive subjects from SMART study; when compared with Multi-Ethnic Study of Atherosclerosis (MESA) study participants for age group 45-76, hsCRP, IL-6, and D-dimer levels were 50%, 152%, and 94% higher in HIV-positive subjects from SMART study [83]. Even in patients with suppressed viremia (HIV RNA ≤ 400 copies/mL) aged 45-76 yrs., D-dimer levels were 49.1% higher than general population [83]. Multiple studies on other HIV cohorts later corroborated the elevation of these markers in both untreated and ART-treated HIV-infected subjects and their predictive values of morbidity and mortality [226, 272-275].

Another major procoagulant factor that was shown to be elevated in HIV-infected subjects is tissue factor (TF), which is the initiator of the extrinsic pathway of the coagulation cascade (Figure 3). The important role of TF in thrombosis is well-documented [276]. Inappropriate clot formation mediated by TF can lead to blood vessel occlusion, and depending on the location and the size of the clot, can lead to deep vein thrombosis, pulmonary embolism, stoke, or MI. Funderburg *et al* and others showed that the levels of soluble TF and TF expression on monocytes are significantly increased in HIV-infected subjects, and are correlated with the D-dimer levels [226, 277-279], supporting a direct relationship between HIV-associated hypercoagulation and increased CV comorbidities in HIV-infected subjects.

Other CV biomarkers elevated in HIV-infected individuals and also predictive of mortality include: (i) fibrinogen, which is associated with platelet aggregation and atherosclerosis [280], is as much as 2.6 fold higher in HIV-infected subjects than in uninfected controls [281, 282], (ii) vWf, a marker of endothelial cell damage [283, 284], (iii) p-selectin, which can induce generation of prothrombotic microparticles from leukocytes and upregulation

of TF expression on monocytes [273, 285, 286], (iv) endothelial dysfunction markers, such as soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) [287, 288], (v) cardiac function marker, such as N-terminal prohormone of brain natriuretic peptide (NT-proBNP) [289, 290], (vi) lipids, such as low-density lipoproteins (LDL) and HDL [291, 292]. A recent study comprehensively measuring the composition of extrinsic pathway coagulation factors, and based on which estimating thrombin generation through computational modeling, showed that untreated HIV infection led to declines in all major anticoagulants (e.g., antithrombin, protein C and protein S) and that the net effect of HIV replication was to increase coagulation potential [279].

Hypercoagulation marked by increased CV biomarkers not only provides prognostic values for thrombotic events, but also is predicative of anemia, which is another common hematological abnormality in HIV disease, also associated with mortality [293-295]. Therefore, investigating strategies to reduce hypercoagulation in HIV-infected patients are needed to mitigate the risk of hematological and CV abnormalities and prevent CV comorbidities.

1.2.3 Linkage between hypercoagulation and immune activation and inflammation

The elevation of both hypercoagulation and immune activation and inflammation associated with chronic HIV infection, and both being strongly predictive of non-AIDS comorbidities and mortality, is hardly a coincidence.

Traditionally, inflammation is thought to activate coagulation and that this is only a one-way process. For example, in atherosclerosis plaque, macrophages primed by IL-6, platelet-derived growth factor and MCP-1 highly express TF, which upon plaque rupture, initiates the extrinsic pathway of coagulation cascade and results in thrombin and subsequent fibrin formation

[296, 297]. Platelets can also be activated by proinflammatory mediators, such as platelet-activating factor [298]. Mediated by p-selectin, platelets binding to leukocytes, endothelial cells, and monocytes, enhances the expression of TF on monocytes through NF-κB activation [299]. Moreover, inflammation is shown to downregulate physiological anticoagulant pathways through decreasing antithrombin and Protein C synthesis and accelerating degradation by neutrophil elastase [300, 301], as well as upregulating plasminogen activator inhibitor type-1 (PAI-1) through TNF-α and IL-1β signaling [302, 303], which indirectly inhibits fibrin removal.

More recently, it was shown that these two systems interact way more closely, and that this relationship is bidirectional [304]. Serine proteases yielded in coagulation cascade activation, such as thrombin, Factor Xa, and Factor VIIa, can impact inflammation and immune function through binding to protease activated receptors (PARs) and trigger these G-protein-coupled receptors for downstream signaling [305]. PAR-1 can be receptor for thrombin, tissue factor-factor VIIa complex, and Factor Xa; PAR-2 can be activated by the tissue factor-factor VIIa complex, factor Xa, and trypsin; PARs 3 and 4 are thrombin receptors [305]. PAR-1 activation by tissue factor or thrombin was shown to enhance activation of p38 mitogen-activated protein kinase and induction of IFN-β and CXCL10 expression [306], as well as enhance effector function of $CD8^+$ T cells [307]. Binding of TF-Factor VIIa to PAR-2 results in upregulation of inflammatory responses in macrophages and was shown to affect neutrophil infiltration and proinflammatory cytokine (TNF-α, IL-1β, IL-6, IL-8) expression [308]. Increases in TFPI levels were shown to reduce systemic inflammation [309, 310], further supporting the modulatory effect of serine proteases on inflammation.

Moreover, activated platelets can release various proinflammatory cytokines (such as CD40 ligand and IL-1β) and chemokines (such as RANTES and PF-4), which may result in

activation of monocyte integrins and increase the probability of monocyte recruitment to form atherosclerotic plaques [311]. Fibrinogen and fibrin can directly stimulate expression of proinflammatory cytokines (such as TNF-α and IL-1β) on mononuclear cells mediated by TLR-4, which is also the receptor of endotoxin [312]. Fibrin can also induce chemokine production (including IL-8 and MCP-1) by endothelial cells and fibroblasts [312]. On the other hand, activated Protein C has been found to inhibit endotoxin-induced production of TNF-α, IL-1β, IL-6, and IL-8 by cultured monocytes/macrophages [313], and abrogates endotoxin-induced cytokine release and leukocyte activation in rats *in vivo* [314]. Therefore, the deficiency of Protein C observed in HIV-infected subjects indicates potential reduction of the inhibitory effect of Protein C on inflammation.

In the specific context of HIV infection, microbial products (such as LPS) translocated from the intestinal lumen into the systemic circulation due to mucosal damage, not only induce endothelial activation directly through TLR-4 [315], but also drive immune activation [316] and significantly increases TF expression on monocytes, which subsequently induce hypercoagulation [277]. Hypercoagulation in turn can act back on immune activation and inflammation through PAR signaling potentially by TF, FVIIa, thrombin or activated platelets. PAR-1 activation not only enhances effector function of $CD8^+$ T cells [307], but also directs $CX3CR1^+$ $CD8^+$ T cells homing to endothelium, directly interact with clot-forming elements, and enhance T-cell receptor-mediated IFNγ production [317]. Moreover, recent studies showed ample evidence that coagulation plays a crucial role in orchestrating inflammatory and fibroproliferative responses following tissue injury, thus promoting tissue fibrosis [318, 319], which underscores an overlooked role of hypercoagulation in the high prevalence of lymphatic,

liver and myocardial fibrosis in HIV-infected individuals,. This was thought to be mostly due to persistent chronic immune activation and inflammation [117, 320, 321].

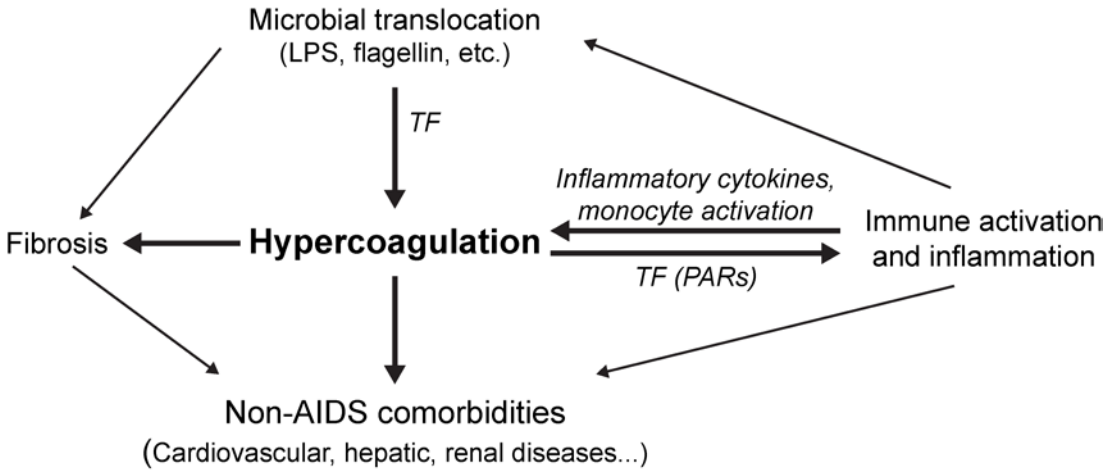


Figure 4. Hypercoagulation plays a central role in HIV-related comorbidities
A schematic of the central role that hypercoagulation plays in directly causing CV comorbidities in HIV-infected patients, and indirectly exacerbating HIV-related non-AIDS comorbidities by forming a vicious cycle with immune activation and inflammation, as well as inducing tissue fibrosis in various organ systems. The schematic also highlights the crucial role of tissue factor in bridging hypercoagulation with other aspects of chronic HIV pathogenesis. TF – tissue factor; PAR – protease-activated receptor; LPS – lipopolysaccharide.

With abundant evidence of a close cross-talk between hypercoagulation and immune activation and inflammation, we propose that hypercoagulation plays an essential part of the chronic HIV pathogenesis and forms a vicious cycle with immune activation and inflammation to increase the risk of comorbidities and end-organ diseases, especially CV events, being potentially the missing piece in solving the complex immune activation and inflammation puzzle (Figure 4). Therapeutically targeting hypercoagulation can put a break on the vicious cycle, alleviate the deleterious effects of chronic HIV pathogenesis and eventually reduce the risk of non-AIDS comorbidities and improve clinical outcome of HIV infection.

1.2.4 Using a model of pathogenic SIV infection to study hypercoagulation and CV comorbidity

As both HIV subtypes originated from cross-species transmission of SIV, HIV-1 from SIVcpz in chimpanzees/gorillas, and HIV-2 from SIVsmm in sooty mangabeys, NHP models are widely used in HIV research to study HIV transmission, prevention, pathogenesis and cure research, and provided important insights in all of these fields [322-324].

One of the paradigm-shifting advancement on the understanding of HIV pathogenesis is gained through the comparative studies of the nonprogressive SIV infection in African-origin natural hosts with the progressive SIV infection in Asian macaques. For example, SIVsab (the SIV substrain naturally carried by *Chlorocebus sabaeus*)-infected AGMs and SIVsab-infected PTMs, despite being infected with the same strain of virus and having similarly high viral replication *in vivo*, the two models present opposite disease outcomes. Remarkably, while SIVsab-infected AGMs generally lack any clinical pathogenic manifestation, SIVsab-infected PTMs recapitulate the most important pathogenic features of HIV infection, including similar $CD4^+$ T cell depletion pattern, mucosal damage, microbial translocation, persistent immune activation and inflammation during chronic infection, and the eventual AIDS-like disease progression including opportunistic infection, malignancy and neuropathology-neuro-AIDS [324, 325].

Previously, our lab showed that SIVsab-infected PTMs and SIVmac239-infected RMs also present hypercoagulation during chronic infection, marked by significantly increased D-dimer and thrombin-antithrombin complex (TAT) levels, similar to that of HIV-infected humans. Especially in SIVsab-infected PTMs, the rising of these biomarkers occurs very early after SIV infection, persists throughout the course of chronic infection, and strongly correlates with AIDS

progression and death [218]. Furthermore, a broad spectrum of CV abnormalities was presented histologically in SIVsab-infected PTMs, such as thrombotic microangiopathy in kidney, lung, and brain, myocardial hypertrophy and fibrosis, myocarditis, as well as areas of MI [218]. These evidences suggest that SIVsab-infected PTM is a good model for investigating HIV-related hypercoagulation and CV comorbidities. Using this model, we demonstrated that experimental interventions aimed at controlling microbial translocation and persistent inflammation also lowered the biomarkers of hypercoagulation [197, 198], further supporting the close relationship of hypercoagulation with immune activation and inflammation.

Studying HIV-related CV comorbidities can be challenging due to multiple limitations: (i) the long duration of HIV infection limits the feasibility of large-scale long-term observatory studies; (ii) interference of numerous confounding factors, including ART toxicity itself [100], and traditional risk factors for CV disease such as smoking, alcohol, obesity etc. (iii) ethical considerations that limit invasive tissue sampling, to assess tissue pathology and directly investigate specific interventions in human subjects, for example, experimentally introduce anticoagulation therapies in HIV-infected subjects to assess the effect of anticoagulation drugs on HIV-related CV is unethical, as anticoagulants are known to pose significantly increased bleeding risk. Therefore, the use of progressive SIVsab-infected PTM model is a valid approach for studying HIV-related hypercoagulation and CV comorbidity, addressing the limitations of human subjects for studies of this nature, while well-recapitulating the characteristics of hypercoagulation associated with HIV infection.

1.3 ANTICOAGULANT THERAPY

Although the important role of hypercoagulation in chronic HIV infection is increasingly recognized, the effort to dissect the exact mechanism and identify specific interventions is only at starting stage, calling for more research to better understand the issue. Currently, there are very few studies investigating the administration of anticoagulants, and the focus is mostly on drug interactions with ART, rather than assessing the effect of the anticoagulants on reducing CV risk or even elucidating the involvement of coagulation in chronic HIV infection and comorbidities [326-329]. Utilizing the model of pathogenic SIV infection in PTMs, we will be able to experimentally administer different anticoagulant and antiplatelet therapies without the confounding coadministration with ART, so as to not only better elucidate the mechanism of how coagulation pathways play into the chronic HIV pathogenesis, but also directly assess the effect of different anticoagulants and antiplatelet agents on preventing CV complications and potentially other non-AIDS comorbidities.

As coagulation pathways are composed of many coagulant and anticoagulant factors, multiple classes of anticoagulant and antiplatelet agents exist, targeting specific component of the coagulation pathways (Table 4).

Recently, Francischetti *et al* also isolated a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, Ixolaris, which specifically targets FVIIa/TF complex on the scaffold of FX [330]. To investigate the specific mechanism of hypercoagulation in association of HIV/SIV pathogenesis, as well as to elucidate the effect of therapeutically targeting hypercoagulation in the context of HIV/SIV infection, we propose to experimentally administer specific anticoagulant and antiplatelet drugs in progressive SIV-infected PTMs: Ixolaris, which specifically inhibits TF activity, as we hypothesize that TF may be the bridge

between hypercoagulation and immune activation and inflammation; Dabigatran, which directly inhibits thrombin, due to the broad role of thrombin in activating and consequently amplifying multiple coagulation pathways; Vorapaxar, which is PAR-1 antagonist, as we hypothesize that TF may exert its effect in fueling the vicious cycle of hypercoagulation and immune activation and inflammation through PAR-1 signaling.

Table 4. Common FDA-approved anticoagulant and antiplatelet drugs

Drug Generic Name	Brand Name	Route of Administration	Mechanism of Action
Warfarin	Coumadin, Jantoven	Oral	Vitamin K antagonist
Dabigatran	Pradaxa	Oral	Direct thrombin inhibitor
Lepirudin	Refludan	Intravenous injection	Direct thrombin inhibitor
Bivalirudin	Angiomax	Intravenous injection	Direct thrombin inhibitor
Argatroban	Acova	Intravenous injection	Direct thrombin inhibitor
Desirudin	Iprivask	Subcutaneous injection	Direct thrombin inhibitor
Rivaroxaban	Xarelto	Oral	Direct factor Xa inhibitor
Apixaban	Eliquis	Oral	Direct factor Xa inhibitor
Edoxaban	Savaysa, Lixiana	Oral	Direct factor Xa inhibitor
Heparin	Hemochron, Hep-Lock, Hep-Lock U/P	Subcutaneous/Intravenous injection	Activates antithrombin
Low molecular weight heparin	-	Subcutaneous/Intravenous injection	Activates antithrombin
Aspirin	-	Oral	Cyclooxygenase inhibitor
Clopidogrel	Plavix	Oral	ADP receptor inhibitor
Ticagrelor	Brilinta, Brilique, Possia	Oral	ADP receptor inhibitor
Vorapaxar	Zontivity	Oral	PAR-1 antagonist
Abciximab	Reopro	Intravenous injection	Glycoprotein IIb/IIIa inhibitor
Eptifibatide	Integrilin	Intravenous injection	Glycoprotein IIb/IIIa inhibitor
Tirofiban	Aggrastat	Intravenous injection	Glycoprotein IIb/IIIa inhibitor
Dipyridamole	Persantine	Oral	Adenosine reuptake inhibitors

*ADP – Adenosine diphosphate. PAR-1 – Protease activated receptor-1.

1.3.1 Ixolaris – specific TF inhibitor

Ixolaris is a specific TF pathway inhibitor isolated from tick *Ixodes scapularis* saliva, containing 140 amino acids (15.7 kd) including 10 cysteines, and a pI of 4.56. Ixolaris is similar to other members of the Kunitz family of protease inhibitor proteins including human TFPI precursor, but structurally and functionally distinct. Structurally, Ixolaris lacks two cysteines of the second

Kunitz domain compared with human TFPI, with less amino acids separating the first and second Kunitz domains and 4 additional amino acids between the fourth and fifth cysteine residues of Kunitz-type domain 2, as well as a short and basic carboxy terminus compared with human TFPI. Functionally, Ixolaris is much faster binding to FXa, in contrast to the slow binding TFPI, and potently inhibits FVIIa/TF-induced FX activation with an IC_{50} in the picomolar range. Importantly, FX zymogen is an efficient scaffold for Ixolaris to form tight FVIIa/TF/Ixolaris/FX complexes. This inhibitory strategy is particualrly effective because Ixolaris/FX may inhibit FVIIa/TF *in vivo* before and independently of FXa production [330].

Ex vivo experiments in rats showed that Ixolaris (up to 100 µg/kg) did not affect the activated partial thromboplastin time (aPTT), which evaluates the intrinsic coagulation pathway, while the prothrombin time (PT), which evaluates the extrinsic coagulation pathway, was increased by approximately 0.4-fold at the highest Ixolaris concentration. Subcutaneous or intravenous administration of Ixolaris in rats *in vivo* caused a dose-dependent reduction in thrombus formation, with complete inhibition attained at 20 µg/kg and 10 µg/kg, respectively, and was not associated with bleeding, demonstrating the effectiveness and safety of Ixolaris as an antithrombotic agent [331].

Interestingly, besides antithrombotic effect, Ixolaris was shown to inhibit tumorigenic potential and block angiogenesis of U87-MG human glioblastoma cells, with downregulation of vascular endothelial growth factor (VEGF) and reduced tumor vascularization [332]. Another two recent studies also confirmed the effect of Ixolaris to reduce primary tumor growth as well as metastatic potential in murine model of melanoma B16F10 cells and human MDA-MB-231mfp or murine PyMT breast cancer cells [333, 334]. The study by *Carneiro-Lobo et al* on a breast cancer model demonstrated that the anti-tumor effects of Ixolaris are through inhibition of

direct TF-FVIIa-PAR2 signaling [333], supporting other potential pathophysiological effects of TF signaling besides initiating coagulation which can be targeted by Ixolaris. Currently, the investigation of the effects of Ixolaris still remains in preclinical phase, and more studies are warranted to fully establish the efficacy and safety profile of Ixolaris in humans.

1.3.2 Dabigatran – direct thrombin inhibitor

Dabigatran is the first broadly approved novel oral anticoagulant as an alternative to the parenteral indirect thrombin inhibitors (e.g., heparins, low-molecular-weight-heparins) and vitamin K antagonists (e.g. warfarin). For over 50 years, warfarin was the only available oral long-term anticoagulant therapy. Although it has been proven effective in inhibiting thrombosis, clinical administration can be problematic due to slow onset-offset of activity, and drug-drug and drug-food interactions which require frequent monitoring and dose adjustment to maintain appropriate anticoagulation [335]. Alternatives that have better efficacy, safety and tolerability profile, as well as easier management, are thus called for.

Dabigatran was identified among a panel of chemicals with similar structure to benzamidine-based thrombin inhibitor α-NAPAP (N-alpha-(2-naphthylsulfonylglycyl)-4-amidinophenylalanine piperidide), and stood out because of its favorable selectivity profile and strong *in vitro* and *ex vivo* anticoagulant activity in rats and RMs [336]. It was soon selected for clinical development and approved by U.S. Food and Drug Administration (FDA) in 2010.

Orally taken dabigatran reaches peak plasma concentration after 2-3 hours, with a half-life of 12-14 hours [337]. Dabigatran is predominately metabolized by esterase-mediated hydrolysis and cytochrome P450 plays minimal role [338], therefore reducing the potential interactions with drugs metabolized by cytochrome P450. Dabigatran inhibits both clot-bound

and free thrombin, as well as thrombin-induced platelet aggregation, but has no inhibitory effect on platelet aggregation induced by other stimuli (e.g. arachidonic acid, collagen, ADP) [339]. Dabigatran is effective in preventing venous thromboembolism, comparable to warfarin with no increased rates of major bleeding [340]. Additionally, in prevention of stroke in patients with atrial fibrillation, dabigatran was more effective than well controlled warfarin with significantly decreased risk of intracranial hemorrhage [341]. However, there is a significant higher risk of gastrointestinal bleeding with dabigatran 150 mg bid compared with warfarin in atrial fibrillation patients [341]. In 2015, the first specific reversal agent for dabigatran, idarucizumab, was developed to address emergency major bleeding events associated with dabigatran use and was approved by FDA in the same year [342]. Moreover, a meta-analysis of 14 randomized controlled trials on dabigatran showed a significantly increased risk of myocardial infarction associated with dabigatran [343], pointing to more strict monitoring while using this medication than advertised on market.

In the context of HIV infection, recent few case reports of HIV patients on atazanavir/ritonavir or lopinavir/ritonavir that were put on dabigatran for asymptomatic atrial fibrillation or multiple bypass surgeries showed that there is minimal drug-drug interaction and no bleeding complications [329, 344].

Besides antihemostatic effects, based on the recently revealed role of thrombin-PAR signaling in mediating inflammation [305], dabigatran use in other inflammation-related disease settings was also investigated and showed promising beneficial effects preclinically. Studies utilizing respective mice models showed that dabigatran reduces atherosclerotic lesion size along with enhanced plaque stability [345], inhibits primary tumor growth and metastasis of breast cancer [346], and reduces the extent of fibrosis and collagen release in bleomycin-induced

pulmonary injury [347]. However, whether these effects can be translated clinically remains unknown.

1.3.3 Vorapaxar – PAR-1 antagonist

Vorapaxar is an orthosteric inhibitor of PAR-1 that binds reversibly to the ligand-binding pocket on the extracellular surface of PAR-1, which differs distinctly from the mechanisms of older generation antiplatelet agents, such as aspirin (TxA2 biosynthesis inhibitor by cyclooxygenase-1 inhibition) and clopidogrel (ADP receptor antagonist) [348]. Although these older generation antiplatelet agents were effective in reducing ischemic CV events when used concomitantly, the bleeding risk is largely increased, and the rate of recurrent ischemic events remains high [349]. Therefore, additional targets for platelet inhibition to further decrease CV risk in addition to standard antiplatelet therapies are sought for, with the hope of not increasing bleeding liability.

PAR-1 is of particular interest, as it is the primary mediator of thrombin-stimulated platelet activation and aggregation [350]. Theoretically, specifically blocking PAR-1 may exert antiplatelet effect without affecting the ability of thrombin to generate fibrin or platelet activation by other stimuli such as collagen and ADP. Vorapaxar was discovered in light of this interest based on a lead generated from the natural product himbacine [348].

Orally administered vorapaxar is rapidly absorbed and reaches peak plasma concentrations within 60-90 minutes, with a half-life of three to four days and a terminal elimination half-life of eight days [351]. Although vorapaxar binds to PAR-1 reversibly, the long half-life essentially makes the antagonism effectively irreversible. Vorapaxar is metabolized by CYP3A4 and CYP2J2, thus potential drug interactions with ART metabolized by CYP3A4 (such as indinavir, nelfinavir, ritonavir, saquinavir) may exist.

Two major Phase III trials assessed the efficacy and safety of vorapaxar, TRACER (Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome) [352] and TRA 2°P-TIMI 50 (Trial to Assess the Effects of Vorapaxar in Preventing Heart Attack and Stroke in Patients with Atherosclerosis-Thrombolysis in MI) trials [353]. The TRACER trial did not demonstrate a significant reduction in a broad primary composite endpoint of CV causes, MI, stroke, recurrent ischemia with rehospitalization, or urgent coronary revascularization, and was terminated early in patients with prior history of stroke or transient ischemic attack due to significantly increased risk of intracranial hemorrhage [352]. The TRA 2°P-TIMI 50 with more traditional composite endpoint showed significantly reduced the occurrence of CV death, MI, or stroke, with the greatest benefit in patients with prior MI [353, 354]. Based on these results, FDA approved vorapaxar in May 2014 for reduction of thrombotic CV events among patients with a history of MI or peripheral arterial disease and no history of stroke or transient ischemic attack [355].

Notably, both trials investigated vorapaxar as an addition to standard antiplatelet therapy, and currently there is limited clinical investigations of vorapaxar therapy by itself [356]. Although the new perspectives on PAR signaling and its involvement in inflammation spurred studies investigating the effect of anticoagulants on inflammation-related diseases, due to the significant bleeding risk, vorapaxar has hardly been studied in other disease settings. Nevertheless, in the context of HIV infection, a phase I/II clinical trial is underway to assess the safety and efficacy of vorapaxar in reducing D-dimer expression and markers of cellular immune activation in ART-treated HIV patients, and the results are expected soon (NCT02394730).

2.0 HYPOTHESIS AND SPECIFIC AIMS

Effective ART has led to a significant increase in the life expectancy of patients infected with HIV [357]. However, mortality rates of HIV-patients are still higher than the general population, due to high risk of comorbidities, such as CV disease, liver disease and renal disease [358, 359]. A hypercoagulable status has been observed in patients with HIV infection and has been associated with CV disease risk and mortality [83, 241].

We reported that a similar hypercoagulable status can be observed in an NHP model of progressive HIV infection developed in our laboratory (SIVsab-infected PTMs) [212], in which elevated levels of D-dimer and thrombin-antithrombin complex (TAT) are associated with CV lesions and are highly indicative of progression to AIDS and increased mortality [218]. Notably, hypercoagulability only develops in SIVsab-infected PTMs progressors that display excessive immune activation and inflammation, but not in the SIVsab-infected AGMs, which do not progress to AIDS and control chronic immune activation and inflammation after SIVsab infection [218]. A connection between coagulation abnormalities and immune activation and inflammation has also been established in both HIV-infected patients and SIV-infected NHPs [241, 272].

Immune activation and inflammation generally upregulates thrombotic events [360], while conversely, thrombin generated by the activation of coagulation pathways can enhance both immune responses via PAR-1 signaling [306, 307, 361]. To date, interventions aimed only

at targeting immune activation and inflammation have not been very successful in patients receiving ART, potentially due to this coagulation-inflammation vicious cycle. Based on these observations, and on the finding that D-dimer is a strong independent predictor of mortality in both HIV-infected patients [241] and progressive NHP model [218], **we hypothesize that coagulation abnormalities play a central role in HIV pathogenesis and should be therapeutically targeted**. To test this hypothesis, anticoagulants specifically targeting the extrinsic, intrinsic or common coagulation pathways will be administered to SIVsab-infected PTMs. These interventions will enable us to investigate the mechanisms of HIV/SIV-related hypercoagulation and establish the impact of the procoagulant status on HIV/SIV pathogenesis, HIV/SIV infection-associated immune activation and inflammation and response to ART. Such *in vivo* mechanistic experiments cannot be performed in HIV-infected patients, due to the risk of hemorrhage and death. To test our hypothesis, we propose the following Specific Aims (SAs):

SA1. To test the hypothesis that TF is the major activator of coagulation in progressive HIV/SIV infections. Rationale: Previous studies [277, 279, 362] and our preliminary data indicate that TF is a major determinant of the prothrombotic status in both HIV-infected patients and SIVsab-infected PTMs. Approach: We will assess the TF expression on lymphocytes and monocytes and investigate its relationship with chronic HIV/SIV pathogenesis, particularly immune activation and inflammation, microbial translocation and hypercoagulation. We will also experimentally administer a new TF inhibitor (Ixolaris [330]) *in vivo* to SIV-infected PTMs, and assess its effect in improving coagulation status, as well as the other aspects of SIV pathogenesis. Significance: This interventional approach will allow us to directly gain insight into the mechanisms of SIV-related hypercoagulability independent of factors that usually confound human studies (i.e., ART, smoking, alcohol, diet).

SA2. To assess the effect of different anticoagulant and antiplatelet therapy on HIV/SIV pathogenesis, reducing HIV/SIV-associated comorbidities and improving clinical outcome of HIV/SIV infection. Rationale: Relieving hypercoagulation may reak the coagulation-inflammation vicious cycle, thus may be important in reducing immune activation and inflammation, and improving the overall clinical outcome of HIV/SIV infection. Approach: We will administer Ixolaris (specific TF inhibitor), dabigatran (direct thrombin inhibitor) and vorapaxar (PAR-1 antagonist) *in vivo* in SIVsab-infected PTMs, and compare immune activation and inflammation markers, $CD4^+$ T cell levels, incidence of SIV-related comorbidities, and survival between SIVsab-infected PTMs receiving the therapeutic interventions. Significance: This approach will shed light on the potential of anticoagulant therapy as an adjuvant to interventions targeting immune activation and inflammation and improving the clinical outcome.

3.0 TISSUE FACTOR IS THE KEY PLAYER IN THE CROSSTALK OF HYPERCOAGULATION AND CHRONIC IMMUNE ACTIVATION AND INFLAMMATION

This work, essentially as presented here, was published in Science Translational Medicine: Schechter ME, Andrade BB, He T, *et al.* Sci Transl Med. 2017 Aug 30;9(405). Results from this work were partially presented as poster discussion titled "Tissue factor pathway inhibitor Ixolaris improves disease outcome in progressive SIVsab-infected pigtail macaques" at the 9th IAS Conference on HIV Science (IAS 2017) in Paris, France. My contribution includes conducting the *in vitro* and *in vivo* experiments of Ixolaris on NHPs, isolation of NHP samples for assessing TF expression and activity on monocytes, data analysis, figure construction and manuscript preparation of the NHP portions. To provide the proper context for my contributions, the paper is presented in its entirety. The supplementary materials are included in Appendix A.

3.1 INTRODUCTION

Monocytes are key mediators of innate immunity and have been closely associated with pathogenesis of chronic viral infections, including HIV [363, 364]. Heightened circulating levels of monocyte activation markers, such as soluble (s) TF, sCD14 and sCD163 have been associated with increased risk for death [365], non-infectious complications [366, 367],

subclinical atherosclerosis [368], and immune reconstitution inflammatory syndrome (IRIS) in HIV-infected individuals [369]. Moreover, differential activation of monocyte subsets has recently been described as a predictor of tuberculosis (TB)-associated IRIS in patients with HIV-TB co-infection [369]. One important feature of monocytes in HIV pathogenesis is their capacity to produce TF [370-372]. TF is expressed in response to inflammatory stimuli such as toll-like receptors (TLR) [373-375] and cytokine-driven signals [304, 376] and initiates the extrinsic coagulation cascade by cleaving coagulation factors leading to formation of Factor Xa, thrombin and fibrin, which when degraded forms the coagulation biomarker D-dimer [377, 378]. For these reasons, augmented TF expression is associated with increased levels of D-dimer [277] and thus may be associated with an increased risk for cardiovascular complications in HIV-infected individuals [379]. These findings support a direct role of activated monocytes in the persistent inflammatory milieu observed in chronic HIV infection.

The need to investigate the link between coagulation and inflammation in chronic viral infections is pressing. Inflammatory and coagulation markers are both independent predictors of morbidity and mortality in treated HIV individuals [241, 269, 274, 380] and are clearly associated with non-infectious complications of HIV such as cardiovascular and thromboembolic disease [379] which are rising due to the aging of treated HIV-infected persons [381]. In an experimental model of nonhuman primates (NHP) infected with SIVsab, we previously demonstrated that increases in D-dimer as well as monocyte activation markers (sCD14) predict disease progression [382]. These findings highlighted monocyte activation as a key event driving persistent coagulation in SIV/HIV chronic infection, suggesting a need to delineate the role of monocyte-derived TF in SIV/HIV-driven systemic inflammation and coagulopathy.

In the present study, we evaluated the role of TF-expressing monocytes in HIV and SIV pathogenesis and related coagulopathy. We examined the links between inflammation and coagulation with the aim to identify potential targets for therapeutic interventions in HIV-infected persons.

3.2 MATERIALS AND METHODS

3.2.1 Study design

Ethics statements are detailed in the Supplementary Materials and Methods. Participants were prospectively enrolled in a clinical observational study to evaluate impact of ART in ART-naïve HIV-infected persons. The study objectives included the evaluation of phenotype and function of monocytes at different stages of HIV infection. Participants were selected retrospectively from a completed trial for evaluation of the monocyte function based on PBMC availability, compliance with ART and lack of aberrant immune responses or clinical course that would entail administration of medications that may affect coagulation such as corticosteroids or chemotherapy or other immune therapies.

For the *in vivo* NHP studies, 8 pigtailed macaques were infected with SIVsab. In PTMs, Ixolaris was administered starting from the day of the infection to assess its impact on key parameters of SIV infection (survival, viral replication, $CD4^+$ T cell counts and activation status, systemic inflammation and coagulation status). The study design involved assessment of the impact of Ixolaris on key variables of acute SIV infection, as this stage of infection is when differences in these parameters can reach statistical significance even in small animal groups.

Also, the study design permitted not only comparisons between the animals in the Ixolaris-treated group and controls, but also in the same animals before and after SIVsab infection and Ixolaris treatment. Multiple samples collected at well-defined key time points of SIVsab infection enabled these comparisons. Primary data are located in table S6.

3.2.2 Description of HIV-infected patients

Cross-sectional analysis was performed in HIV-infected individuals enrolled in protocols at the National Institutes of Health. Cryopreserved PBMC from 10 HIV^+ ART-naïve donors and 10 HIV^+ virally suppressed patients (median: 128 weeks on ART, IQR: 112-128 weeks) were used. The characteristics of the participants from the cross-sectional analysis are shown in Table S2. Prospective analysis was performed in HIV infected ART-naïve patients over the age of 18 with $CD4^+$ T-cell counts <100 cells/μL prior to therapy initiation. Cryopreserved PBMC samples were used from 15 patients pre and post ART initiation after HIV suppression (median: 160 weeks on ART, IQR: 96-192 weeks). The characteristics of the participants from the prospective analysis are shown in Table S3. Blood from age- and gender-matched healthy donors was collected from the NIH blood bank.

3.2.3 Description of the Nonhuman Primates samples used for *in vitro* and *in vivo* studies

Cryopreserved PBMCs from six pigtailed macaques (PTMs) and six age-matched African green monkeys (AGMs) were studied from pre-SIVsab infection (SIV^-, naïve) and 72 days post-SIVsab infection (SIV^+). The characteristics of the animals before and after SIVsab infection are shown in Table S1. For the pre-clinical study in vivo, eight PTMs were intravenously infected

with plasma equivalent to 300 tissue culture infectious doses (TCID50) of SIVsab strain BH66 [212]. Ixolaris therapy (20 µg/kg, subcutaneously, daily) was initiated in five PTMs at the time of infection and was maintained for 80 days. Blood was collected from all animals twice prior to infection, twice a week for the first two weeks postinfection (p.i.), weekly during early chronic infection (up to ten weeks p.i.) and then monthly. The Ixolaris-treated group was closely monitored for signs of bleeding. Plasma viral RNA loads were quantified using quantitative real-time PCR specifically developed for SIVsab, as described previously [383, 384].

3.2.4 LPS stimulation

Cryopreserved PBMCs were thawed and resuspended in RPMI-1640 media supplemented with 10% human AB serum at 10^6 cells/well in 96-well plates. Cells were washed and resuspended in complete media with 5µg/mL brefeldin-A (Sigma-Aldrich, St. Louis, MO) and stimulated with indicated doses of ultrapure LPS (Sigma-Aldrich) in the presence or absence of purified Ixolaris (10nM) for 6 hours at 37ºC in 5% CO_2. Cells were then stained following the flow cytometry assay described above.

3.2.5 *In vitro* experiments with HIV^+ and HCV^+ serum

Column-purified $CD14^+$ monocytes were obtained from healthy blood donors and resuspended in RPMI-1640 media supplemented with 10% of indicated serum (commercialized human AB serum or pooled 0.22µm filtered sera from 12 healthy controls, 20 ART-naïve HIV^+ patients and at week 12 and 48 of ART, and 16 HCV^+ persons) at 10^6 cells/well in 96-well plates. Cells were left overnight (18h) at 37ºC in 5% CO_2. Cells were then washed and lysed for measurement of

TF protein expression in cell lysates. In additional experiments, cells were incubated with 10μg/mL of anti-IL1R1 (polyclonal, R&D Systems), anti-IL6Ra (clone 17506, R&D Systems), anti-IFNγR (clone 92101, R&D Systems), anti-IFNAR (polyclonal, ab10719, Abcam) and anti-TNFR1 (clone 16803R, R&D Systems) for 1h before incubation with HIV^+ serum and persisted in cultures for additional 18h. HIV^+ serum was also treated with Polymyxin (0.5μg/μL, Sigma-Aldrich) for 1h and filtered before incubating for 18h.

3.2.6 Testing the efficacy of Ixolaris in inhibiting coagulation in NHP *in vitro*

The *in vitro* efficacy of Ixolaris was assessed by measuring its impact on clotting time on citrate plasma from SIV-naïve healthy pigtail macaques (PTMs). Results were compared to the in vitro testing of Ixolaris-treated plasma from healthy human subjects. Plasma samples from PTMs and humans were incubated with Ixolaris (0.5, 1, 1.5, 2, or 2.5 μg/ml) for two minutes at 37˚C. PT/INR and aPTT were then immediately measured. Testing was performed by ITxM Diagnotics.

3.2.7 Statistical Analysis

Median and interquartile ranges were used as measures of central tendency. All statistical comparisons were pre-specified and two-tailed. Differences with P-values < 0.05 were considered statistically significant. Data from continuous variables comparing two groups were analyzed using Wilcoxon matched-pairs, Mann-Whitney and Spearman rank correlation tests. Comparisons between more than two groups were performed using the Kruskal-Wallis test with Dunn's multiple comparisons *ad hoc* test. Nominal variables and the expression profile of

cytokine production by monocytes were compared using the Fisher's exact test (two groups) or Chi-square test (more than 2 groups). Hierarchical cluster analysis (Ward's method with bootstrap 100x) was used to test if TF^{pos} and TF^{neg} monocytes could be clustered separately based on expression profile of selected proinflammatory genes measured by qPCR (after data was z-score normalized). A model of principal component analysis with an associated vector analysis were employed to visualize the distinction between TF^{pos} and TF^{neg} cells and the direction in which each marker influences mathematically the distribution of the data. These data analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc.) and JMP 11.0 (SAS) softwares. Additional description of statistical analyses is in Supplementary Materials and Methods (Appendix A).

3.3 RESULTS

3.3.1 A specific monocyte subset is the major source of TF amongst peripheral blood mononuclear cells

To determine the potential of monocytes to express TF, peripheral blood mononuclear cells (PBMCs) from healthy blood donors were stimulated with LPS in vitro and TF expression was assessed by flow cytometry (monocyte gating strategy shown in Figure 16, Appendix A). The frequency of monocytes expressing TF (TF^{pos}) in unstimulated cultures was very low (median and interquartile range (IQR); median, 0.49% of total monocytes, IQR: 0.24-0.69). Upon LPS stimulation, the expression of TF by monocytes was significantly increased ($P<0.0001$, Figure 5A).

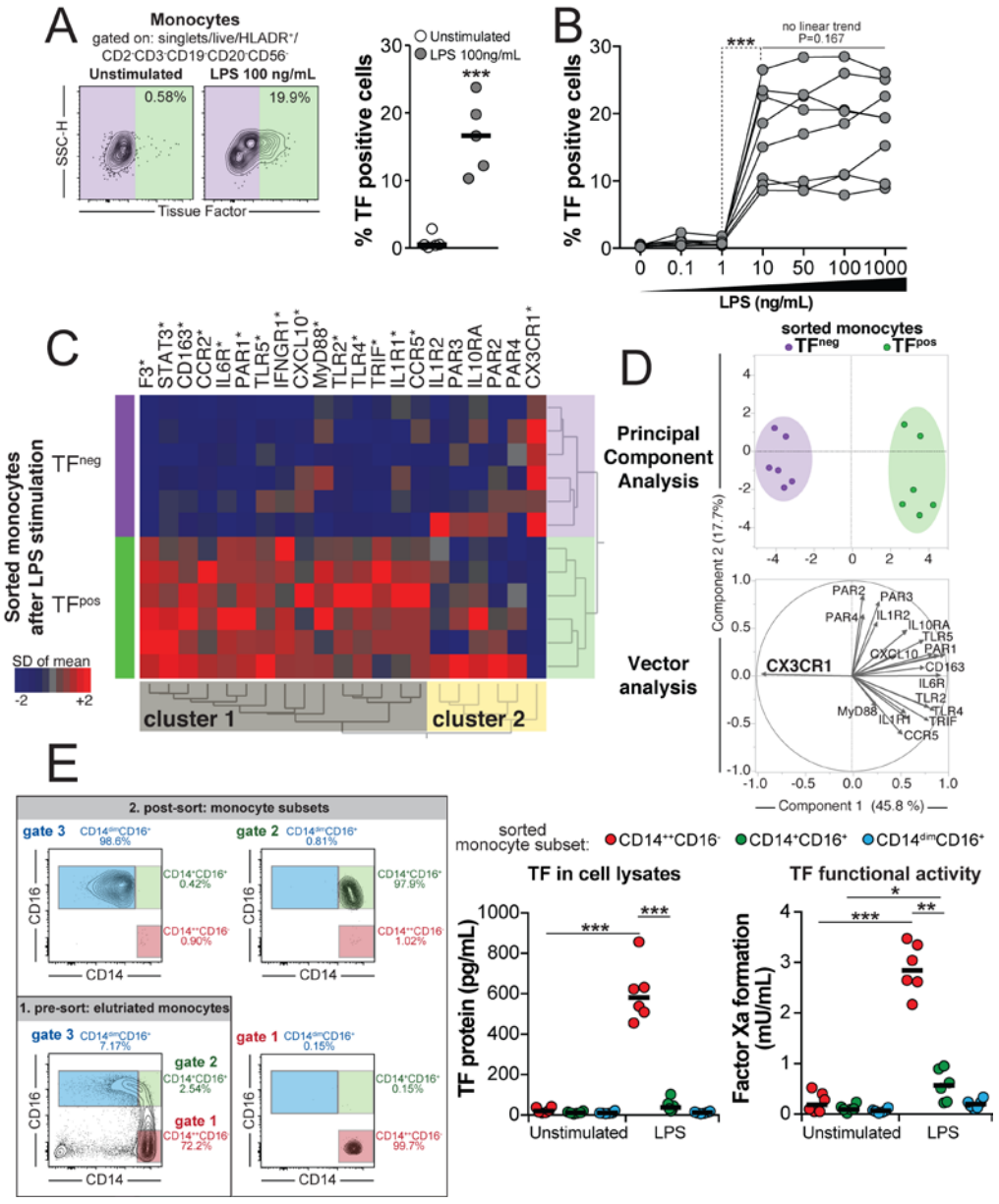


Figure 5. LPS drives TF expression on human monocyte subsets

(A) Left panel shows representative FACS plots of tissue factor (TF) expression on human monocytes from healthy controls upon LPS stimulation in vitro. Right panel shows summary data (n=5) of frequency of TF positive monocytes. Lines represent median values. Data were analyzed using Mann-Whitney test. (B) Frequency of TF-expressing monocytes on PBMCs from healthy controls stimulated with increasing doses of LPS in vitro (n=8). Data were analyzed using Kruskal-Wallis test with Dunn's multiple comparisons and linear trend ad hoc test. (C) Hierarchical cluster analysis of the expression profile (z-score normalized) of indicated genes assessed by qPCR in monocytes (n=6 healthy donors) sorted after 6h of LPS stimulation (100ng/mL) as described in Methods. (D) Principal Component Analysis of the expression level of indicated genes was performed. (E) Different monocyte subsets were sorted based on surface expression of CD14 and CD16 (n=6 healthy donors). Representative plots show monocytes pre and post sorting (left panel). TF protein expression in cell lysates and TF functional activity measured by formation of Factor Xa in vitro were compared between the different monocyte subsets in vitro using Kruskal-Wallis test with Dunn's multiple comparisons post test. Lines represent median values * P<0.05, ** P<0.01, *** P<0.001.

We next tested whether TF expression reflects a state of cellular activation by quantifying the frequency of TF^{pos} monocytes following stimulation with increasing doses of LPS. A robust expression of TF by monocytes was observed only when cells were stimulated with ≥10 ng/mL of LPS whereas lower doses triggered a minor induction of the enzyme (Figure 5B). The frequency of TF^{pos} monocytes did not increase further with escalated doses of LPS (Figure 5B), suggesting that only a subset of cells is capable of producing the coagulation factor in vitro. We next performed a phenotypic analysis to better delineate the subpopulation of mononuclear cells from PBMC that produce TF upon LPS stimulation. We observed that TF expression is restricted to $HLADR^{+}$, $Dump^{-}$ ($CD2^{-}CD3^{-}CD19^{-}CD20^{-}CD56^{-}$) cells, which were further characterized as $CD14^{+}$, $CD16^{-}$ and $CCR2^{+}$ monocytes (Figure 17, Appendix A). A more detailed analysis revealed that TF^{pos} cells exhibit differential expression of activation markers and co-stimulatory molecules, such as CD36, CD40, CD86, CD62L, CD163, TLR4 and IL-6R (Figure 17, Appendix A). To further investigate these populations, we sorted TF^{pos} and TF^{neg} monocytes from six healthy donors after LPS stimulation and examined the expression profile of a customized assortment of genes associated with monocyte activation and coagulation using a multiplex qPCR assay. Hierarchical clustering analysis of the overall gene expression profile of monocytes revealed that TF^{pos} cells exhibit a unique signature highlighted by increased expression of TLRs, proinflammatory cytokine receptors, and signaling molecules such as MyD88 and TRIF (Figure 5C-D) whereas TF^{neg} cells exhibited increased relative expression of CX3CR1 (Figure 5C-D). Principal Component Analysis (PCA) with vector analysis of the expression profile of all genes confirmed the observation that TF^{pos} and TF^{neg} monocytes have distinct gene expression profiles associated with inflammation and coagulation (Figure 5D). These findings indicate that rather

than representing a general state of cell activation, TF expression by circulating mononuclear cells may be restricted to a specific monocyte subset.

It is known that expression of surface markers on monocytes is dynamic and can change depending on the stimulation and microenvironment conditions [385]. To address whether this dynamic expression of surface markers was confounding our interpretation of which monocyte subset expresses TF in response to LPS, we sorted the three major monocyte subsets based on the dichotomous surface expression of CD14 and CD16 ($CD14^{++}CD16^{-}$, $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{+}$), stimulated each subset with LPS and then examined TF expression in cell lysates and measured TF functional activity by a colorimetric assay which quantifies the formation of the coagulation factor Xa [386] (Figure 5E). In unstimulated conditions, TF protein expression and functional activity by each monocyte subset was uniformly low in cultures (Figure 5E). LPS stimulation induced an increase in TF protein expression and activity in $CD14^{++}CD16^{-}$ monocytes, a marginal increase of TF functional activity (by means of factor Xa formation) in $CD14^{+}CD16^{+}$ cells and no effect on $CD14^{dim}CD16^{+}$ monocytes (Figure 5E). These data demonstrate an inherent capacity for the $CD14^{++}CD16^{-}$ monocyte subset to express TF after LPS-driven activation.

3.3.2 TF-expressing monocytes are expanded in chronic HIV infection independent of antiretroviral treatment

It has been previously observed that monocytes from HIV^{+} patients display higher levels of TF expression compared to healthy controls [277]. In the present study, we extended these observations by comparing TF expression in HIV^{+} individuals before and after ART initiation (after virological suppression was achieved) and age and gender-matched healthy controls. In a

cross-sectional comparison of ex vivo TF expression, measured by flow cytometry, we observed that the frequency of TF^{pos} monocytes was significantly higher in both ART-naïve and ART-treated HIV^+ patients compared to healthy controls (P<0.001 and P<0.01 respectively, Figure 6A), albeit with considerable variability in expression levels. The median frequency of TF^{pos} cells was not statistically different between treatment naïve HIV^+ patients and those with ART-induced suppression of HIV viremia (Figure 6A). This finding suggests that TF protein expression in HIV^+ patients may not be substantially affected by ART.

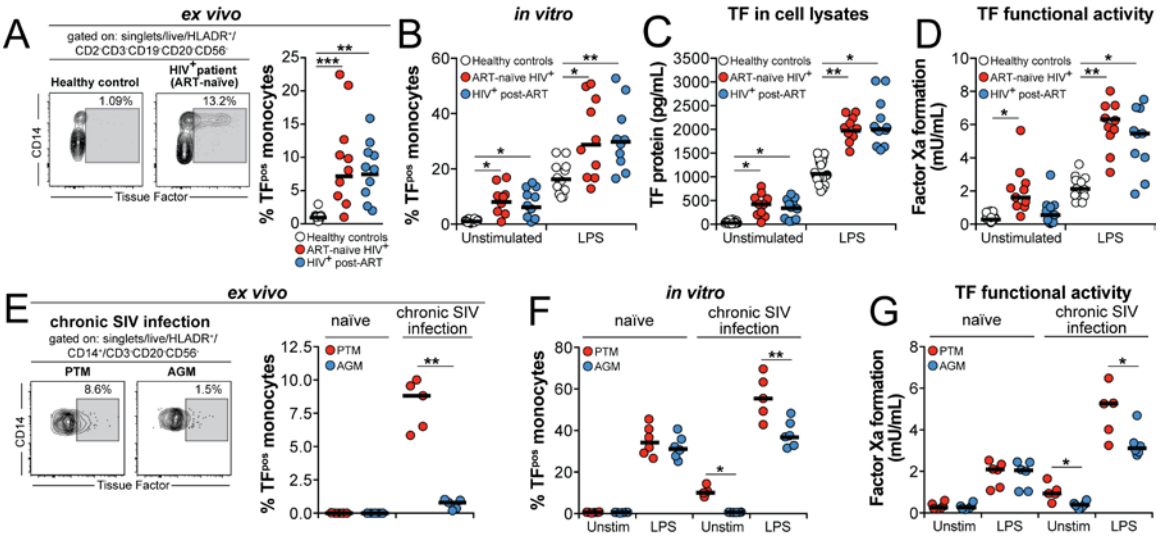


Figure 6. Frequency of TF-expressing monocytes is increased in chronically HIV infected individuals and in SIV infected non-human primates despite virological suppression status
(A) Left panel shows representative plots of TF positive (TFpos) monocytes ex vivo in healthy controls and HIV+ patients. Right panel shows summary data of frequency of TFpos monocytes from a cross-sectional analysis including healthy controls (n=12), ART-naïve HIV+ patients (n=10) and HIV+ individuals post-ART induced virological suppression (HIV+ post-ART; n=10). PBMCs were stimulated with LPS in vitro and frequency of TFpos monocytes (B) as well as TF protein expression in cell lysates (C) and TF functional activity (D) were compared between the cross-sectional study groups using Kruskal-Wallis test with Dunn's multiple comparisons post-test. (E) Left panel shows representative plots of TF expression on monocytes ex vivo in chronically SIV-infected PTMs (n=6) and AGMs (n=6). Right panel shows summary data of frequency of TFpos monocytes ex vivo from both naïve and chronically SIV-infected animals. PMBCs were stimulated with LPS in vitro and frequency of TFpos monocytes (F) and TF functional activity (G) were compared between naïve or SIV-infected PTMs and AGMs. Lines represent median values. Data were analyzed using Mann-Whitney test. * P<0.05, ** P<0.01, *** P<0.001.

We next compared the potential of monocytes to produce TF in response to LPS between the different study groups. We found that the frequencies of TF^{pos} monocytes were significantly higher in HIV^+ patients, before and after ART, compared to healthy individuals in unstimulated

cultures as well as upon LPS stimulation (Figure 6B). There was no observed difference in response to LPS between treatment-naïve HIV^+ patients and those on ART with virological suppression (Figure 6B). These results mirrored the findings obtained by quantification of TF protein expression in PBMC lysates (Figure 6C). We further demonstrated that TF expressed on the cell surface of monocytes from HIV^+ individuals was able to trigger Factor Xa formation, demonstrating that TF was functionally active in vitro (Figure 6D). Again, no difference was detected in TF functional activity between the groups of HIV^+ patients (Figure 6C-D). The differences in TF expression on monocytes were independent of the total monocyte counts in PBMC amongst the HIV-infected groups, which were not significantly different (median 4451 cells/μL, IQR: 2383-6829 in ART-naïve vs. 4507 cells/μL, IQR: 3308-7771 in virologically suppressed individuals, P=0.34).

These primary observations indicated an increased in the capacity of monocytes to produce TF and promote factor Xa formation in vitro upon cellular activation in HIV infection which persists after ART-induced HIV suppression. To further test this hypothesis, we prospectively assessed ex vivo TF expression as well as plasma levels of D-dimer and C-reactive protein (CRP) in an additional set of HIV^+ patients examined longitudinally with paired samples available from pre-ART and post-ART (at virological suppression) timepoints. This longitudinal paired analysis confirmed that the frequency of TF^{pos} monocytes was not significantly altered by ART (Figure 18, Appendix A). Out of 12 HIV^+ patients prospectively evaluated, 6 (50%) exhibited increases whereas 4 (33.3%) displayed reduction in TF expression upon ART-induced HIV suppression. Mean fluorescent intensities (MFI) values for TF expression did not differ between the timepoints (MFI at pre-ART: 575 arbitrary units [AU] ± 49.5 vs. MFI at virologic suppression timepoint: 553 AU ± 89.6, P=0.785). However, D-dimer levels decreased whereas

CRP values remained unchanged after ART initiation (Figure 18, Appendix A). These findings show sustained elevation of TF expression on circulating monocytes regardless of the reduction in D-dimer levels, which strongly argues that HIV-associated coagulopathy may persist even after ART-induced HIV suppression, as has been previously suggested [387]. In ART-naïve patients, the ex vivo frequency of TF^{pos} monocytes was positively correlated with the levels of D-dimer ($r=0.69$, $P=0.015$; Figure 18, Appendix A) and did not associate with concentrations of CRP ($r=0.04$, $P=0.908$; Figure 18, Appendix A).

To further assess the role of TF on monocytes in HIV pathogenesis and coagulopathy, we next examined the expression and activity of this coagulation factor in PBMC from NHP before and after SIVsab infection in vivo. In this biological system, AGMs are the natural hosts of SIVsab and undergo infection but do not progress to SIV disease and coagulopathy despite active chronic viral replication reflected by lifelong high plasma SIV viremia [388, 389]. Contrastingly, SIVsab infection causes progressive disease in PTMs [388]. We have previously shown that PTMs infected with SIVsab experience increased systemic inflammation and coagulopathy compared to naïve animals [382]. Prior to SIVsab infection, both PTMs and AGMs exhibited similarly low frequency of TF^{pos} monocytes (Figure 6E). The frequency of monocytes expressing TF ex vivo was substantially increased in chronically infected PTMs compared to naïve animals ($P<0.001$), whereas there was no marked effect of SIV infection in AGMs (Figure 6E). Importantly, monocytes, but not myeloid dendritic cells, were the major source of TF ex vivo and after LPS stimulation in vitro (Figure 19, Appendix A). Notably, monocytes isolated from naïve PTMs and AGMs displayed indistinguishable TF expression in response to LPS stimulation in vitro (Figure 6F). During chronic SIVsab infection, the frequency of TF^{pos} monocytes was higher in PTMs compared to AGMs in unstimulated cells and this difference was

further increased upon LPS stimulation (Figure 6F). Comparable results were obtained by quantification of TF functional activity (Figure 6G). In addition, we found in our cohort of NHP that concentrations of D-dimer and CRP in plasma were significantly higher in accordance with the augmented TF monocyte expression in chronically infected PTMs compared to naïve animals (Table 5, Appendix A). Neither TF expression nor levels of these plasma markers of inflammation and coagulation were significantly altered upon SIVsab infection in AGMs (Table 5, Appendix A). To further investigate the role of TF in SIV pathogenesis, we examined TF expression prior to infection and at different time points post-infection in the mucosal tissues sampled from PTMs. We observed continuous increase of TF expression in the gut with disease progression (Figure 20, Appendix A). Furthermore, few TF positive cells were present in the lamina propria prior to infection, but their numbers increased after infection, first at the tip of the villi and then more profoundly in the lamina propria. The same dynamics were also observed in Peyer's patches (Figure 20, Appendix A). The significant increase in TF expression of both mucosal tissues and peripheral monocytes of chronically SIV-infected PTM suggest that TF expression may contribute to the pathogenesis of SIV-driven persistent coagulation and inflammation.

3.3.3 Proinflammatory cytokines increase TF expression during chronic HIV infection

A potential explanation for the activation of circulating monocytes and subsequent persistent TF expression in HIV-infected persons could be a soluble factor. To test this hypothesis, we column-purified $CD14^+$ monocytes from healthy blood donors and incubated them overnight with medium supplemented with either heat inactivated serum from human AB plasma (HAB serum), filtered pooled sera from healthy controls, or sera from individuals chronically infected with HIV

or hepatitis C virus (HCV). TF protein expression was then quantified in cell lysates. Samples incubated with HIV^+ or HCV^+ sera displayed augmented production of TF (Figure 7A). TF expression was indistinguishable between cultures of monocytes incubated with sera from ART-naïve HIV^+ patients and at different time points upon treatment initiation (Figure 7B). This observation indicates that the factors driving TF expression by monocytes persist after ART implementation. Moreover, we performed a series of blocking experiments trying to delineate the molecules that are potentially driving monocyte activation and TF production from the sera of HIV infected individuals.

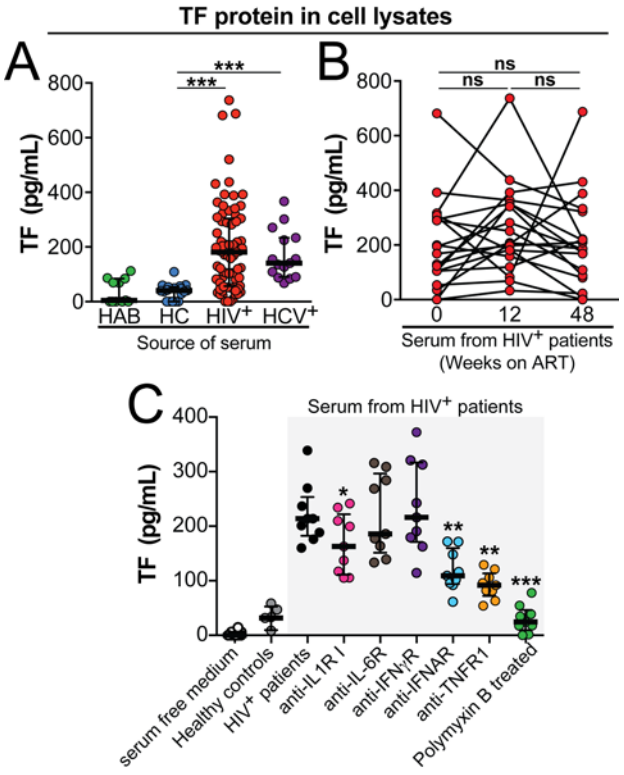


Figure 7. Inflammatory mediators and microbial products may drive TF expression by circulating monocytes in the context of HIV infection

(A) Column-purified CD14+ monocytes from 10 healthy controls were cultured for 18h in the presence of RPMI medium supplemented with 10% manufactured human AB serum, or serum isolated from healthy controls, ART-naïve HIV+ patients or HCV+ individuals as described in Methods. (B) Monocytes were also cultured in the presence of serum from ART-naïve HIV-infected patients at different time points after ART initiation. (C) Monocytes were cultured for 18h in the presence of indicated blocking antibodies (10µg/mL) or with serum previously treated with polymyxin B (0.5µg/µL). Cells were washed and lysed for assessment of TF protein expression using ELISA. Unmatched data were analyzed using Mann Whitney U test whereas matched pairs were compared using Wilcoxon matched pairs test. * P<0.05, ** P<0.01, *** P<0.001. ns, nonsignificant.

We found that IL-1RI blocking resulted in a slight but consistent reduction of TF production in monocytes cultured in the presence of HIV^+ serum (Figure 7C). Neutralizing IL-6R and interferon-gamma receptor (IFNγR) did not alter TF expression in this experimental setting (Figure 7C). Notably, TF production exhibited a substantial drop in monocyte cultures incubated with blocking antibodies against type I interferon receptor (IFNAR) or tumor necrosis factor receptor 1 (TNFR1) (P=0.003 and P=0.005, respectively, Figure 7C). These experiments confirmed that inflammatory signals present in serum from HIV-infected persons are capable of inducing TF production in monocytes. The specific drivers of these inflammatory signals could be coming from inflamed tissues or the blood itself. Microbial translocation in the gut has been described as an important trigger of persistent systemic inflammation detected in HIV^+ persons [390]. To test the possibility that monocyte activation in blood could be resulting from a residual leakage of microbial products from mucosal interfaces, we incubated HIV^+ serum with polymyxin B before culturing the monocytes. Serum treated with this bactericidal compound resulted in the lowest TF expression among all the experimental conditions tested (Figure 7C). These findings demonstrate that circulating microbial products are indeed relevant drivers of TF expression by activated monocytes.

3.3.4 TF-expressing monocytes produce multiple proinflammatory cytokines

Chronic HIV infection has been associated with persistent immune activation and elevated markers of coagulation [83, 269]. We hypothesized that TF-expressing monocytes, aside from promoting coagulation, could contribute to systemic inflammation. Indeed, in non-HIV-infected healthy individuals, TF^{pos} monocytes more frequently produced multiple proinflammatory cytokines, such as TNF-α, IL-1β and IL-6, simultaneously upon LPS stimulation (Figure 8A-C).

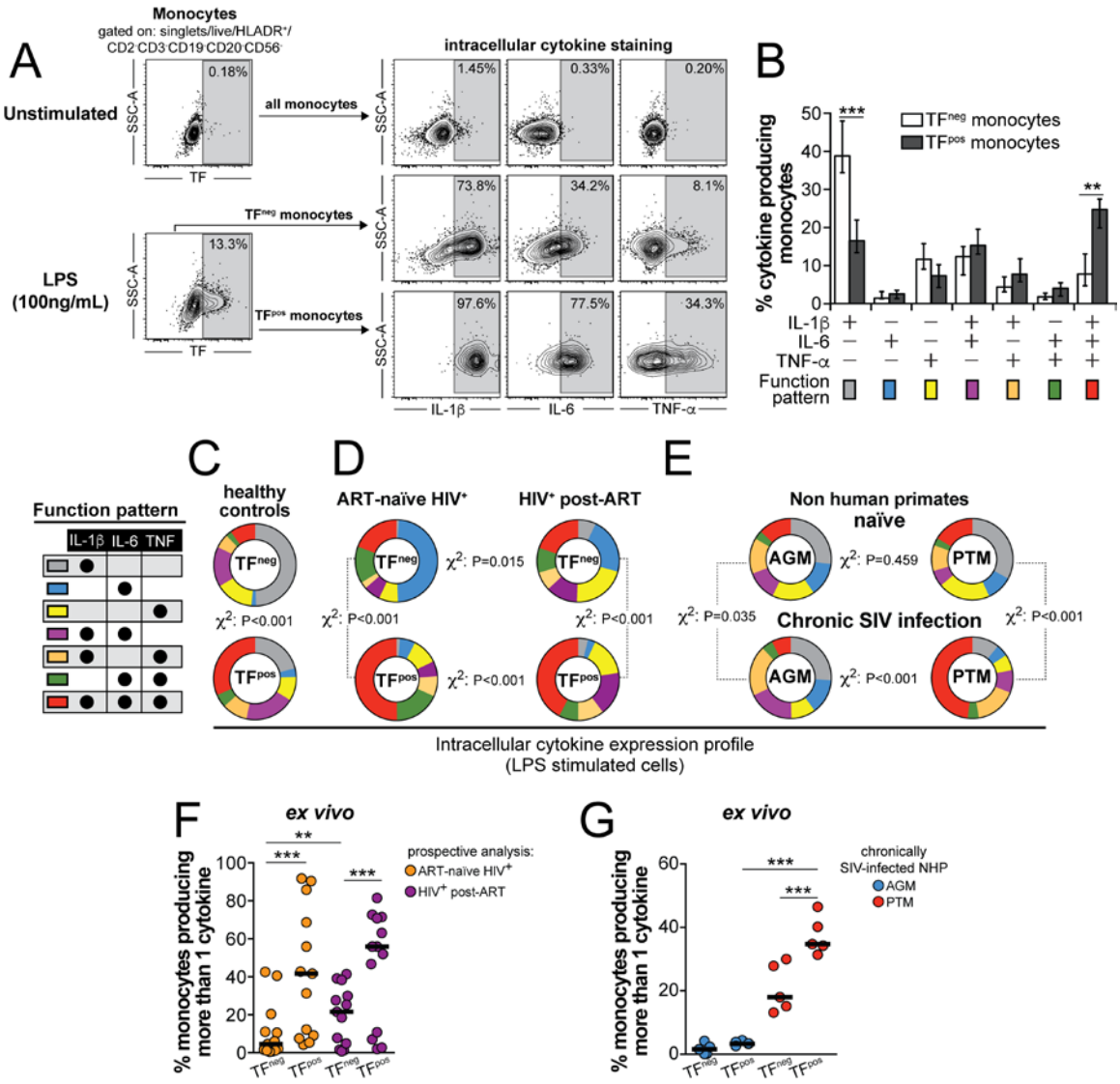


Figure 8. TF-expressing monocytes produce multiple pro-inflammatory cytokines
(A) Representative plots show intracellular cytokine staining for IL-1β, IL-6 and TNF-α in monocytes from healthy donors (n=12) upon LPS stimulation in vitro. (B) Polyfunctional analysis of TFneg and TFpos monocytes upon LPS stimulation. (C-E) The cytokine expression profiles in TFneg and TFpos monocytes were compared using Chi-square tests. (F) Frequency of monocytes producing more than one cytokine ex vivo was compared between TFneg and TFpos monocytes in a prospective cohort of ART-naïve HIV+ patients (n=15) before therapy initiation and after ART-induced virological suppression (HIV+ post-ART). (G) Frequency of monocytes producing more than one cytokine ex vivo was compared between TFneg and TFpos monocytes in chronically SIV-infected PTMs (n=6) and AGMs (n=6). Unmatched data were compared using the Mann-Whitney test whereas matched comparisons were performed using the Wilcoxon matched pairs test. ** P<0.01, *** P<0.001.

We further investigated the profile of intracellular cytokine expression in monocytes ex vivo from our longitudinal cohort of HIV$^+$ patients before ART initiation and after virological suppression. HIV$^+$ individuals, regardless of treatment status, exhibited high frequencies of

monocytes spontaneously producing pro-inflammatory cytokines (Figure 21A, Appendix A). Furthermore, by testing the monocyte response to LPS stimulation in our prospective cohort of HIV^+ patients, we detected markedly different intracellular cytokine expression profiles between TF^{neg} and TF^{pos} monocytes in ART-naïve HIV^+ patients (chi-square $P<0.001$). In contrast to monocytes from healthy controls (Figure 4C), following LPS stimulation, the vast majority of TF^{neg} monocytes from HIV infected individuals produced IL-6 alone (median 48.7%, IQR: 33.5-52.8% of all the cytokine producing TF^{neg} cells) whereas TF^{pos} monocytes more frequently produced TNF-α, IL-1β and IL-6 simultaneously (53.1% of all the cytokine producing TF^{pos} cells, IQR: 49.5-56.7%) (Figure 8D). The intracellular cytokine expression profile of LPS-stimulated monocytes significantly changed after ART initiation (chi square $P=0.015$ for the expression profile in TF^{neg} cells and $P<0.001$ for TF^{pos} monocytes from pre vs. post-ART initiation; Figure 8D). In virologically suppressed HIV^+ individuals, TF^{neg} cells exhibited a mixed cytokine expression profile with monocytes producing one or various combinations of two cytokines whereas the majority of TF^{pos} monocytes remained polyfunctional, producing a combination of three cytokines (TNF-α, IL-1β and IL-6) simultaneously (chi-square $P<0.001$ profile of TF^{neg} vs. TF^{pos} cells; Figure 8D). In addition, the frequency of TF-expressing monocytes strongly correlated with the frequency of polyfunctional monocytes before ART initiation ($r=0.89$, $P<0.001$) and in patients with virological suppression ($r=0.91$, $P<0.001$).

We next examined the monocyte response to LPS in our NHP cohort by assessing the intracellular expression of TNF-α, IL-1β and IL-6 *in vitro* and found that monocytes isolated from both naïve and chronically SIVsab infected PTMs and AGMs were capable of producing these cytokines upon LPS stimulation (Figure 21B, Appendix A). The cytokine expression profile of stimulated monocytes was not different between uninfected PTMs and AGMs, with a

majority of the cytokine-producing cells expressing IL-1β alone in both species (chi-square P=0.459; Figure 8E and Figure 21B, Appendix A). The overall frequency of monocytes simultaneously expressing multiple pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) ex vivo was significantly higher in chronically infected PTMs than in AGMs (Figure 8F). Upon activation in vitro, frequency of cytokine producing monocytes was higher in PTMs than in AGMs (Figure 21B, Appendix A). SIVsab infection caused changes in the cytokine expression profile of stimulated monocytes from both NHP species (chi-square P=0.035 for AGM naïve vs. chronically infected, P<0.001 for PTM naïve vs. chronically infected; Figure 8E). During chronic SIVsab infection, the majority of the activated monocytes from PTMs produced TNF-α, IL-1β and IL-6 simultaneously (median 43.5% of all the cytokine producing monocytes, IQR: 35.2-55.8%) whereas the activated monocytes from AGMs had a mixed expression profile and a low frequency of triple cytokine producers (median 12.5% of all the cytokine producing monocytes, IQR: 8.5-18.9%, P<0.001 vs. frequency of triple producers from PTMs) (Figure 8E).

We next quantitatively compared the dynamics of monocyte polyfunctionality in HIV-infected patients as well as in SIVab-infected NHP. In HIV^+ patients, the polyfunctionality of TF^{pos} monocytes remained high after ART initiation and was significantly increased in TF^{neg} cells post-ART compared to TF^{neg} cells before ART initiation (P<0.001, Figure 8F). The polyfunctionality of TF^{pos} monocytes from chronically $SIVsab^+$ PTMs was significantly higher when compared to TF^{neg} monocytes from the same animals and also when compared to TF^{pos} monocytes isolated from chronically $SIVsab^+$ AGMs (P<0.001 Figure 8G). These findings from both HIV-infected patients and SIV-infected NHPs support the hypothesis that in monocytes, increased TF expression is associated with an enhanced potential to produce multiple proinflammatory cytokines.

3.3.5 Thrombin triggers TF expression on CD14high monocytes and induces production of multiple proinflammatory cytokines via PAR-1 signaling

Our results so far demonstrated that $CD14^{high}$ monocytes have increased capacity both to promote activation of coagulation factors and to produce multiple proinflammatory cytokines linked to HIV and SIV pathogenesis. Activation of the coagulation cascade by TF in vivo ultimately results in formation of thrombin, an essential protein leading to thrombus development [391]. Thrombin has been shown to activate NF-κB via cleavage of the cytoplasmic tail of protease-activated receptors (PAR) [392]. Amongst the various types of PARs, PAR-1 and PAR-3 are known to be expressed on monocytes [393]. Interestingly, a recent study has demonstrated that thrombin triggers TF expression on human monocytes via activation of PAR-1 [394, 395]. We postulated that activated $CD14^{high}TF^{pos}$ monocytes might also be able to sense thrombin generated from coagulation. To test this hypothesis, we performed a series of experiments and first confirmed that thrombin triggers TF expression on monocytes from healthy donors (Figure 22A, Appendix A). Subsequently we found that this effect was associated with reduced expression of PAR-1 on the surface of these cells upon thrombin stimulation (Figure 22B, Appendix A). Importantly, we observed that TF expression triggered by thrombin was restricted to $CD14^{high}$ monocytes (Figure 22C, Appendix A) and we further established that $CD14^{++}CD16^{-}$ and $CD14^{+}CD16^{+}$ cells exhibited similarly high expression of PAR-1 (Figure 22C, Appendix A). Additional assays in HIV^{+} patients suggested that PAR-1 is preferentially expressed in $CD14^{high}$ cells and that it is shed in response to thrombin in monocytes, a phenomenon that was associated with induction of TF expression by these cells (Figure 22D, Appendix A). These results indicate that the subset of monocytes that produces TF upon LPS-

driven activation is the same that can respond to thrombin stimulation in vitro possibly via PAR1.

These results led us to speculate that monocytes activated by thrombin also produce multiple pro-inflammatory cytokines. We tested this idea by examining cytokine production by PBMC directly stimulated with thrombin in vitro. We found that thrombin induced robust production of intracellular cytokines (TNF-α, IL-1β and IL-6) by $CD14^{high}$ monocytes from healthy individuals (Figure 22E, Appendix A). Pharmacologic inhibition of PAR-1 signaling by SCH79797 completely neutralized the effects of thrombin on TF expression and cytokine production (Figure 22E, Appendix A). These results delineate how thrombin triggers an inflammatory response by monocytes in a PAR-1 dependent manner.

3.3.6 Ixolaris blocks TF activity without suppressing monocyte immune function *in vitro*

The results at this point support the hypothesis that monocytes are highly responsive to thrombin and act as a critical link between TLR-driven persistent inflammation and coagulation in chronic viral infections. Interventional therapies focusing on TF inhibition and/or blockade could therefore be key to breaking this vicious cycle of coagulation and inflammation in HIV and SIV pathogenesis. Ixolaris is a small molecule isolated from the saliva of the tick *Ixodes scapularis* and acts as a potent TF pathway inhibitor (TFPI), by blocking Factor VIIa/TF-induced coagulation [330, 333]. Although Ixolaris has been tested in thrombosis models [331], it has not been evaluated in the setting of HIV-driven coagulopathy.

In our in vitro model, Ixolaris inhibited TF functional activity in monocytes from healthy donors stimulated with LPS in a dose-dependent fashion (Figure 9A). Importantly, even at doses 10 times higher than the maximum inhibitory concentration (10 ng/mL), Ixolaris did not exhibit

substantial cytotoxicity (Figure 23, Appendix A). We next examined if Ixolaris blocks TF activity in pathological settings such as HIV and SIV infection. Indeed, Ixolaris completely inhibited formation of factor Xa in unstimulated conditions and after LPS stimulation in vitro in PBMCs from both ART-naïve and ART-treated, virologically suppressed HIV$^+$ persons (Figure 9B). Ixolaris also significantly inhibited TF activity in PBMCs from chronically SIVsab-infected PTMs and AGMs (Figure 9C). Ixolaris did not affect TF protein expression or cytokine production triggered by LPS stimulation in vitro (Figure 9D). These results demonstrate that, at low doses, Ixolaris can potently inhibit TF functional activity in cells from HIV$^+$ patients and from SIVsab-infected NHP and suggest that inhibition of TF activity could be used to suppress monocyte-driven activation of coagulation in these settings without directly affecting the capacity of these cells to respond to TLR stimulation.

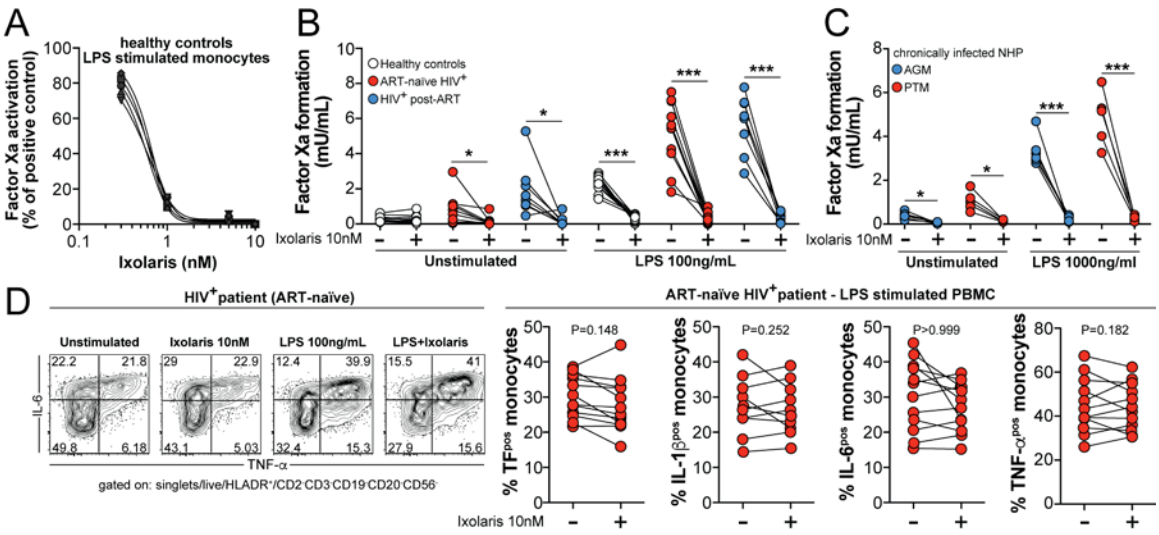


Figure 9. Ixolaris potently blocks TF activity but not protein expression in activated monocytes from HIV$^+$ patients and chronically SIV-infected NHPs

(A) Tissue factor functional activity in vitro measured by Factor Xa formation in elutriated monocyte cultures from healthy donors stimulated with LPS and treated with indicated doses of Ixolaris (n=5). Data represent percentage of the positive control (LPS 1μg/ml without Ixolaris). TF activity upon treatment with Ixolaris was compared between (B) healthy controls (n=12) and unmatched ART-naïve HIV+ (n=10) patients and those who achieved ART-induced virological suppression (post-ART; n=10) as well as between (C) chronically SIV-infected PTMs (n=6) and AGMs (n=6). (D) Representative plots of intracellular IL-6 and TNF-α production in monocytes from ART-naïve HIV+ individuals stimulated in the presence or absence of Ixolaris (left panel). Summary data showing frequency of TFpos monocytes as well as percent of cytokine producing monocytes in stimulated cultures treated or non-treated with Ixolaris (right panel). Data were analyzed using the Wilcoxon matched pairs test. * P<0.05, *** P<0.001.

3.3.7 *In vivo* administration of Ixolaris is safe and decreases coagulation and immune activation in SIVsab infection in PTMs

To assess the efficacy of Ixolaris, we first measured its anticoagulant effect in vitro (Figure 10). Ixolaris resulted in dose-dependent prolongation of prothrombin time (PT) /international normalized ratio (INR) when added to human plasma collected from healthy individuals (Figure 10A). Changes in PT/INR were similar when Ixolaris was added to plasma from uninfected healthy PTMs (Figure 10B). Activated partial thromboplastin time (aPTT), an intrinsic pathway coagulation marker, was not affected by Ixolaris in either monkey or human plasma, indicating that Ixolaris has a specific inhibitory effect on the extrinsic pathway. Our results thus confirmed the high efficacy and specificity of Ixolaris in NHP in vitro.

Chronic progressive HIV/SIV infection is characterized by high levels of immune activation, inflammation and hypercoagulation, which are robust independent prognostic factors of progression to AIDS and comorbidities [241, 396, 397]. To investigate the effect of the anticoagulant therapy on the outcome of a highly pathogenic SIVsab infection, we administered Ixolaris to five PTMs upon SIVsab infection (at day of infection) and compared the natural history of SIVsab infection in Ixolaris-treated PTMs and untreated controls. Therefore, we first assessed the effects of the anticoagulant treatment by comparing the levels of immune activation and inflammation markers in PTMs receiving Ixolaris and in controls. The animals treated with Ixolaris showed significantly reduced levels of the proinflammatory cytokine IL-17 during early chronic infection ($P=0.03$, Figure 10C). The anticoagulant treatment also impacted T cell immune activation, as demonstrated by a lower frequency of $CD4^+$ T cells expressing HLA-DR^+ and $CD38^+$ (Figure 10D), and by significantly lower frequency of $CD8^+$ T cells expressing HLA-DR^+ and $CD38^+$ during early chronic infection ($P<0.001$, Figure 10E).

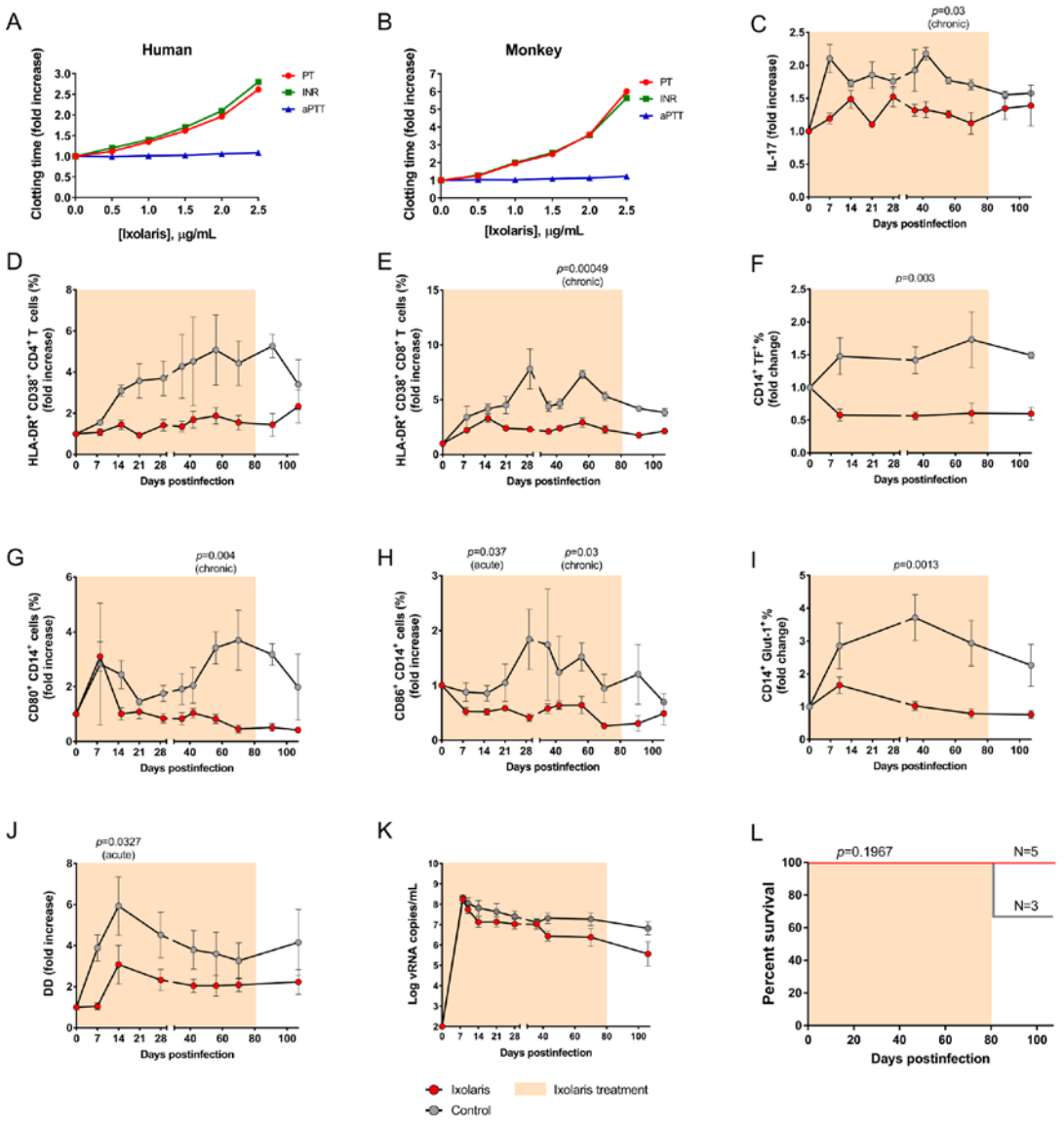


Figure 10. Anticoagulant treatment positively impacts the immune activation and systemic inflammation of highly pathogenic SIVsab infection in PTMs

(A) Dose-dependent dynamics of PT/INR and aPTT after Ixolaris addition to plasma isolated from uninfected human subjects. (B) Dose-dependent dynamics of PT/INR and aPTT after Ixolaris addition to plasma isolated from uninfected PTMs. (C) Dynamics of the pro-inflammatory cytokine IL-17 in untreated vs Ixolaris-treated SIVsab-infected PTMs assessed by Luminex. Dynamics of activated double positive HLA-DR+/CD38+ CD4+ (D) and CD8+ T cells (E) in untreated vs Ixolaris-treated SIVsab-infected PTMs assessed by flow cytometry. Dynamics of TF expression (F) on CD14+ monocytes in untreated vs Ixolaris-treated SIVsab-infected PTMs assessed by flow cytometry, as well as CD80 (G), CD86 (H) and Glut-1 (I) expression on CD14+ monocytes in untreated vs Ixolaris-treated SIVsab-infected PTMs assessed by flow cytometry. (J) Dynamics of D-Dimer in untreated vs Ixolaris-treated SIVsab-infected PTMs assessed by an immunoturbidimetric assay. (K) Dynamics of plasma SIVsab viremia in untreated vs Ixolaris-treated SIVsab-infected PTMs assessed by a real-time PCR assay. (L) Survival in untreated vs Ixolaris-treated SIVsab-infected PTMs. Statistical analyses were performed with grouping of acute vs chronic infection time points as described in Methods, except for TF and Glut-1 for which the statistical analyses were done on whole dynamics, due to limited availability of samples for flow staining. Survival analysis was performed using the Mantel-Cox test.

Following treatment, TF expression on circulating $CD14^+$ monocytes decreased compared with untreated controls (Figure 10F). In addition, Ixolaris treatment significantly lowered both CD80 expression in chronically infected PTMs (P=0.004, Figure 10G) and CD86 expression in both acutely and chronically infected PTMs (P=0.03, Figure 10H), compared with the untreated controls. To further validate the reduction of monocyte activation induced by Ixolaris treatment, we measured Glut-1 expression on $CD14^+$ monocytes, an important monocyte activation marker [398-400]. Ixolaris-treated animals showed a significantly reduced Glut-1 expression following SIV infection, compared to untreated controls (P=0.001, Figure 10I). Furthermore, we examined associations between expression of monocyte markers and lymphocyte activation using generalizing estimating equations, due to the nature of the data distribution and small sample size of the experimental groups. Using this approach, we found that the monocyte activation markers strongly associated with lymphocyte activation. CD80 and CD86 expression on $CD14^+$ monocytes were directly associated with HLA-DR^+ $CD38^+$ expression on $CD4^+$ T cells ($p<0.001$ and $p<0.001$, respectively), while Glut-1 expression on $CD14^+$ monocytes was strongly associated with HLA-DR^+ $CD38^+$ expression on both $CD4^+$ and $CD8^+$ T cells ($P<0.001$ and $P<0.001$, respectively). Altogether, these results suggest that Ixolaris treatment had a beneficial effect in reducing immune activation and inflammation associated with SIV infection.

The hypercoagulable status exhibited in SIVsab-infected progressive NHPs and HIV-infected patients is marked by elevated D-dimer, which is associated with increased incidence of cardiovascular comorbidities and mortality [274, 324, 382]. Therefore, we next compared the D-dimer levels in Ixolaris-treated PTMs and controls. Ixolaris administration significantly reduced plasma D-dimer levels in acute SIVsab-infection of PTMs (P=0.033) (Figure 10J), and resulted

in lower D-dimer levels during early chronic infection, indicating a clear effect of Ixolaris in improving the coagulation status in the SIV-infected animals. To monitor the infection in SIVsab-infected PTMs, we measured plasma SIV viremia in all infected animals [67]. Interestingly, the Ixolaris-treated group showed overall lower viral loads compared to controls (Figure 10K).

Most importantly, Ixolaris-treated animals did not develop disease during the first 100 days postinfection (Figure 10L). In accordance with our previous studies in which two out of five SIVsab-infected PTMs were rapid progressors [197, 212], one animal out of three progressed to AIDS in the first 100 days postinfection in the untreated group, however, no rapid progression was registered in the Ixolaris group. Statistical significance was not reached in the present study, possibly due to the small sample size. This result is of potential translational interest, as it suggests that Ixolaris treatment may abrogate rapid progression in treated animals, likely due to the combination effect of reduced immune activation and inflammation, reduced hypercoagulable status and small reduction in plasma viremia.

3.4 DISCUSSION

Chronic HIV infection has been associated with elevated circulating levels of biomarkers of coagulation, in particular D-dimer, and systemic inflammation, such as IL-6, sCD14 and CRP [241, 269, 401] which have been independently linked to a higher risk of non-AIDS related death and mortality even in persons treated with ART [402]. Although ART induced suppression of HIV viremia is shown to significantly reduce plasma levels of D-dimer, plasma levels remain higher than in non-HIV infected populations [83].

Monocytes have been previously described as immune cells involved in cardiovascular disease in both HIV^- and HIV^+ populations [366, 402-404] and are an important source of TF [371]. In the current study we performed a detailed immune profiling of monocyte subsets and delineated molecular signatures that characterize TF-expressing cells in the context of TLR activation. Our results demonstrated that TF positive monocytes exhibit elevated expression of several genes associated with innate immune activation. These findings argue that the subpopulation of monocytes able to upregulate TF expression upon TLR4 activation is the classical subset defined as $CD14^+CD16^-$. Activated monocytes not expressing TF in the same circumstances exhibit markers of patrolling monocytes ($CD14^{dim}CD16^+$) such as upregulation of CX3CR1 gene expression. In the setting of coagulopathy, it is possible that patrolling monocytes, expressing high levels of CX3CR1, a vascular homing receptor, might interact with thrombin clots. Our results indicate that the patrolling monocytes express the lowest level of PAR-1 and that PAR-1 expression is decreased by thrombin stimulation. Therefore, in vivo, it is possible that the interaction between CX3CR1-expressing patrolling monocytes and thrombin clots accounts for the lower expression of PAR-1. Furthermore, we demonstrated that the frequency of monocytes expressing TF upon activation could not be further increased with augmenting doses of TLR activation, suggesting an inherent capacity of these circulating monocytes to express this coagulation factor. Importantly, we confirmed that non-monocytic cells from peripheral blood are unable to express TF upon LPS stimulation. Flow cytometry-based phenotypic analysis revealed that compared to TF^{neg} monocytes, TF^{pos} cells displayed increased expression of HLA-DR and co-stimulatory molecules such as CD40 and CD86 as well as IL-6R. These results indicate a unique monocyte phenotype capable of producing TF. Previous flow-cytometry based studies have indicated that $CD16^+$ monocytes are able to produce

TF [278]. Our results however indicate that the molecular signature associated with TF expression in monocytes is restricted to the classical proinflammatory subset that lacks expression of CX3CR1. It is possible that different experimental settings and/or gating strategies have resulted in discrepancies between the studies, and for this reason we performed TF expression assessment in sorted monocyte subsets to identify the main TF producers. In addition, molecular gene analysis was performed in healthy individuals and gene expression might differ in HIV^+ individuals due to the trained immunity phenomenon [405]. Our findings clearly indicate that classical proinflammatory monocytes are the major subset capable of robustly expressing TF protein and also upregulating TF functional activity upon LPS stimulation.

Previous studies have indicated that TF expression on monocytes is increased in HIV-infected individuals compared to healthy controls and that frequency of TF^{pos} cells is associated with HIV disease progression [277]. Here we found that the frequency of TF^{pos} monocytes as well as TF functional activity was similar between ART-naïve HIV^+ individuals and those who achieved HIV suppression after ART initiation. These findings strongly indicate that TF expression by monocytes remains high in HIV^+ individuals regardless of ART-induced virological suppression, which could be associated with increased potential to activate the coagulation cascade and cause cardiovascular disease. Consistent with this concept, previous work has demonstrated a positive correlation between frequency of TF^{pos} monocytes and D-dimer levels in HIV^+ patients [277]. We validated these findings in our analysis of ART-naive HIV^+ patients. Intriguingly, while D-dimer plasma levels were significantly reduced after ART-induced suppression of HIV viremia, the frequency of TF^{pos} monocytes remained elevated. It is plausible that some degree of coagulopathy persists despite the decreases in D-dimer and supports a more complex relationship between pro- and anti-coagulant factors in untreated HIV

due to poor synthetic liver function [380]. This also suggests that assessing TF expression on monocytes would increase sensitivity for the detection of coagulopathy. Our findings further reveal TF as a potential therapeutic target in ART-treated patients with evidence of coagulopathy.

The pathogenic role of coagulation during progressive SIVsab infection in PTMs has been established previously [382], and NHPs serve as an important HIV model that can be controlled for key variables, such as time of infection. In the present study, we observed that compared to AGMs, which experience active viral replication but no disease progression, PTMs have significantly increased frequency of TF-expressing monocytes as well as TF functional activity upon chronic SIVsab infection. Importantly, no differences were observed in TF expression and activity in vitro between PTMs and AGMs prior to SIV infection, suggesting that monocyte subsets from these two NHP species diverge drastically in response to chronic viral infection. These results also link the occurrence of SIV progression and systemic coagulopathy with increased capacity of monocytes to produce TF upon activation.

Aside from their role in coagulation, monocytes are also important in inflammatory processes due to their production of cytokines. There is a growing body of evidence indicating that persistent inflammation is associated with increased mortality in HIV [241, 406]. Our experiments assessing polyfunctionality of monocytes by means of production of IL-1β, IL-6 and TNF-α upon TLR activation in vitro clearly demonstrate that TF^{pos} cells are more frequently triple cytokine producers when compared to TF^{neg} monocytes in healthy individuals and in HIV^{+} patients, as well as in chronically SIV-infected PTMs. These results suggest that the same monocyte subset that expresses TF upon activation may also be implicated in persistent inflammation by producing multiple cytokines. This idea was reinforced by our findings

demonstrating an increased frequency of polyfunctional monocytes in PTMs compared to AGMs after SIVsab infection, but not in naïve animals. Thus, it is reasonable to propose that TF^{pos} cells may be critically implicated in promotion of systemic inflammation and coagulation associated with disease progression in chronic HIV and SIVsab infection (Figure 24, Appendix A).

Having identified a role of TF-expressing monocytes at the intersection of inflammation and coagulation, we hypothesized that interfering with TF could serve as a therapeutic approach to target hypercoagulation. Administration of Ixolaris in vivo decreased T cell activation as well as plasma IL-17 and D-dimer levels. Ixolaris produced no evidence of toxicity and was not associated with significant $CD4^+$ or SIVsab viremia changes. The results of the in vivo study are significant for two reasons. First, they point to a causal relationship between coagulation and immune activation and inflammation. Thus, not only did the Ixolaris treatment reduce inflammation, treatment also lowered monocyte and lymphocyte activation in the treated animals. The reduction of lymphocyte activation may be the direct result of reduced expression of the costimulatory markers CD80 and CD86 (two potent T cell activators) on monocytes. Alternatively, Ixolaris treatment could have impacted the levels of immune activation by directly reducing T cell expression of protease activator receptor 1 (PAR-1). Such a scenario is supported by our finding that Ixolaris treatment appears to have a stronger impact on the activation of $CD8^+$ T cells activation, as these cells express higher levels of PAR-1. In vitro assays showed that Ixolaris treatment did not diminish TF expression on monocytes in response to LPS, however these assays did not have the capacity to generate thrombin. Importantly, the in vivo data show reduction of monocyte activation after Ixolaris treatment, highlighting the importance of TF-generated thrombin on monocyte activation.

Our study limitations included the small sample size of our longitudinal cohort and the cross-sectional nature of many experiments. In addition, the in vivo Ixolaris administration study included only a small number of ART-naïve acutely infected animals, prohibiting a more detailed evaluation of possible toxicity, drug interaction, complications and potential survival benefit in untreated or treated animals.

The impact of anticoagulant therapy on immune activation and inflammation in SIVsab-infected PTMs demonstrates that hypercoagulation is a significant source of persistent immune activation and inflammation in this model and probably in HIV-infected patients as well. Anticoagulant therapy by itself reduced important measurements of immune activation and inflammation and therefore has potential to improve the clinical management of HIV-infected patients. Second, we show that anticoagulant treatment improved the natural history of highly pathogenic SIVsab infection even in the absence of any other intervention aimed at either controlling viral replication (ART) or improving the health of the gut. Therefore, our study suggests that targeting the coagulation pathway in HIV-infected patients may be effective in reducing the immune activation and inflammation that are linked to cardiovascular comorbidities in HIV infection. These findings indicate that targeting TF may be employed as a host-directed therapy in chronic HIV infection as well as other inflammatory diseases with similar immune pathology.

4.0 ANTICOAGULANT THERAPIES ALLEVIATE SIV-ASSOCIATED HYPERCOAGULATION, IMMUNE ACTIVATION AND INFLAMMATION

4.1 INTRODUCTION

With the advent of antiretroviral therapy (ART), clinical management of HIV-infected subjects is shifting towards that of a chronic disease. The threat of AIDS is gradually replaced by increased risk of multiple serious non-AIDS events (SNAEs), among which cardiovascular disease (CVD) is one of the most fatal comorbidities. HIV-positive individuals face almost two-fold higher risk of myocardial infarction (MI) than HIV-negative individuals [103], earlier progression of atherosclerosis [114], along with higher rates of pulmonary hypertension, myocardial fibrosis, and venous thromboembolism [117, 118, 120]. Since CVD is known to be age-related, and with half of the HIV-positive population in the US getting aged over 50 years [105], the need for better care specifically targeting this issue is pressing.

The exact mechanism for the heightened CV risk in HIV-infected individuals is not fully elucidated. It has been reported that HIV-infected subjects have a hypercoagulable status characterized by elevated levels of D-dimer, high sensitivity C reactive protein (hsCRP), and IL-6. All of these markers are predictors of high CV risk and mortality [83, 241]. Soluble tissue factor (sTF), the initiator of the extrinsic coagulation pathway, was also shown to be associated with coagulation activation in HIV-infected subjects [279]. Additionally, HIV/SIV-associated

hypercoagulation is accompanied by endothelial dysfunction, as well as platelet activation [287, 362], as suggested by the elevated levels of sP-selectin, sICAM-1 and von Willebrand factor (vWF) occurring in HIV-positive individuals [121, 284, 362]. The heightened endothelial and platelet activation may further increase the CV risk. The association between the hypercoagulable status and disease progression and development of CV lesions was best recapitulated in the progressive SIVsab infection of the pigtail macaques (PTMs), a model of HIV infection previously developed in our lab [212, 218]. Furthermore, another common feature shared by chronically HIV-infected individuals and SIVsab-infected PTMs is the persistent immune activation and inflammation (IA/INFL) [197, 198, 212, 245, 407, 408], which are thought to be the source of multiple SNAEs [409] and are not fully normalized even under ART [211].

Growing evidence suggests a bidirectional relationship between hypercoagulation and chronic IA/INFL. Thus, inflammation is a traditional trigger for platelet and endothelial activation, recruitment of activated macrophages, and formation of atherosclerosis plaque [297]; activated monocytes expressing TF, which are highly increased in HIV-infected persons [277], also directly initiate the extrinsic coagulation cascade and promote thrombin production [248]. Meanwhile, serine proteases produced and activated through the coagulation cascade, such as thrombin and Factor Xa, can in turn promote inflammation *via* protease activated receptor (PAR) signaling [305].

We have recently demonstrated that TF-expressing monocytes are the epicenter of the crosstalk between HIV-associated hypercoagulation and inflammation [410]. TF is preferentially expressed on activated $CD14^{++}CD16^{neg}$ monocytes and induce increased thrombin production. Upon thrombin stimulation, the same monocyte subpopulation can produce a plethora of

inflammatory cytokines through PAR-1 signaling, creating a vicious cycle of ongoing hypercoagulation and inflammation. In time, these processes that potentiate each other, not only compromise CV health, but also lead to an increased risk for other IA/INFL-related comorbidities, such as liver disease, kidney disease and neurocognitive disorders [411-413]. *In vivo* blockade of TF using a TF-specific inhibitor (Ixolaris), which essentially breaks this vicious cycle, showed promising results in improving survival of SIVsab-infected PTMs [410].

To further delineate the interaction between hypercoagulation and HIV/SIV-associated IA/INFL, as well as to investigate innovative therapies to mitigate CV comorbidities associated with HIV/SIV infection, we separately administered to SIVsab-infected PTMs two FDA-approved anticoagulants: a direct thrombin inhibitor (dabigatran), and a PAR-1 inhibitor (vorapaxar). By monitoring their effects on coagulation and IA/INFL biomarkers, together with key biomarkers of SIV pathogenesis, and contrasting these effects with those observed after the Ixolaris treatment, we assessed the utility of these new therapies on controlling the hypercoagulant state in HIV-infected subjects.

4.2 MATERIALS AND METHODS

4.2.1 Ethics statement

Animals used in this study were housed at the Plum Borough NHP facility of the University of Pittsburgh, in accordance with the recommendations of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and with the recommendations included in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. The study was approved by the IACUC of the University of Pittsburgh (protocol #15127345 and #17040178). Efforts were made to minimize NHP suffering, in agreement with the recommendations of the Weatherall report, "*The use of nonhuman primates in research*" and the regulations set forth by the *Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act [414]. The NHP facility is air-conditioned, with an ambient temperature of 21-25°C, a relative humidity of 40-60% and a 12 h light/dark cycle. Animals were socially housed in suspended stainless-steel wire-bottomed cages. A variety of environmental enrichment strategies were employed including providing toys to manipulate and playing entertainment videos in the animal rooms. In addition, the NHPs were observed twice daily and any signs of disease or discomfort were reported to the veterinary staff for evaluation. At the end of the study, the NHPs were euthanized following procedures approved in the IACUC protocol.

4.2.2 Infection, treatment, and sample collection

Twenty PTMs were intravenously infected with plasma equivalent to 300 tissue culture infectious doses (TCID50) of SIVsab strains SIVsabBH66 [212]. At the time of infection, five animals started dabigatran treatment (150 mg/animal, oral, BID), five animals started vorapaxar treatment (1/2 tablet [1.04 mg]/animal, oral, SID), five animals started Ixolaris treatment (20 µg/kg, subcutaneously, SID), and five animals received no treatment and were used as controls. During treatment administration, animals were closely monitored for any signs of major bleeding events and none was observed. For Ixolaris treatment, as it has not yet been FDA-approved, PT/INR was routinely measured twice a week for extra monitoring. The treatment was administered throughout the acute and postacute stages of SIVsab infection, and was

discontinued after 80 days (Ixolaris); 112 days (Vorapaxar) and until the euthanasia due to disease progression (70-316 days) (Dabigatran).

Blood was collected prior to infection, during acute infection (10 days postinfection [dpi]), during set point stage (35 or 42 dpi), and every month during chronic infection. Plasma were separated after centrifugation of whole blood at 2200 rpm at 25˚C for 20 min, and PBMCs were collected through Ficoll layers, as described [212, 415]. Intestinal tissues were digested with EDTA for 1 hour followed by collagenase for 1 hour, and then separated with Percoll layer, as described [212, 384]. LN cells were separated by mincing the tissue and filtering with 70μm cell strainers, as described [212, 416]. Tissues not used for cell separation were fixed in 10% buffered formalin, embedded in paraffin, and cut into four-micron sections for further staining.

4.2.3 D-dimer and clotting time testing

D-dimer was measured using a STAR automated coagulation analyzer (Diagnostica Stago) and an immunoturbidimetric assay (Liatest D-DI; Diagnostica Stago) at the Laboratory for Clinical Biochemistry Research at the University of Vermont. The analytical coeficient of variation ranged from 5%-14%. Activated partial thromboplastin time (aPTT) and thrombin time were measured in the clinical coagulation laboratory at ITxM Diagnostics.

4.2.4 ELISAs

Inflammatory and anti-inflammatory cytokines and chemokines were measured in plasma using the Cytokine Monkey Magnetic 29-Plex Panel (Invitrogen), as per the manufacturer's instruction. Results were read by a Bio-Plex reader (Bio-Rad Laboratories, Hercules, CA), using Luminex

technology (Luminex Corporation). C reactive protein (CRP) was tested using a monkey CRP ELISA Kit (Life Diagnostics, PA). sTF levels were measured with IMUBIND® Tissue Factor ELISA (Sekisui Diagnostics, LLC, Lexington, MA). Soluble p-selectin was measured by monkey sP-selectin Platinum ELISA (eBioscience, Inc., San Diego, CA). Soluble ICAM-1 levels were measured by ICAM-1 (Soluble) Monkey Instant ELISA™ Kit (Thermo Fisher Scientific, Waltham, MA). vWF levels were measured by Human von Willebrand Factor ELISA Kit (Abcam, Cambridge, MA). Platelet factor 4 (PF4) was measured by Human CXCL4/PF4 DuoSet ELISA Kit (R&D Systems, Inc., Minneapolis, MN). Soluble CD40 Ligand (sCD40L) levels were measured by Human CD40 Ligand/TNFSF5 DuoSet ELISA (R&D Systems, Inc., Minneapolis, MN). Microbial translocation was monitored by measuring the plasma LPS levels with Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD), according to manufacturer's protocol. Hepatic fibrosis and cirrhosis biomarker, hyaluronic acid (HA) levels were measured with Monkey Hyaluronic Acid ELISA Kit (MyBioSource, Inc., San Diego, CA).

4.2.5 Flow cytometry

Whole blood or mononuclear cells isolated from blood, intestinal and lymph node samples were stained for flow cytometry, as described previously [212, 218]. The mAbs used were: CD3 (Clone SP34-2), CD4 (Clone L200), CD8 (Clone RPA-T8), CD38 (Clone HB7), HLA-DR (Clone L243), Ki-67 (Clone B56), CD80 (Clone L307.4), CD86 (Clone 2331[FUN-1]), TF(Clone HTF-1) from BD Biosciences, and CD14 (Clone M5E2) from BioLegend. All antibodies were validated and titrated using PBMCs from PTMs. Samples were stained for Ki-67 using the Ki-67/FITC–conjugated mouse anti–human mAb set (BD Pharmingen) as per the manufacturer's instructions. Stained cells were analyzed with an LSRII flow cytometer (BD

Biosciences) and FlowJo Version 7.6 software (TreeStar). $CD4^+$ and $CD8^+$ T cell percentages were obtained by first gating on lymphocytes, then on $CD3^+$ T cells. The relative proportion of $CD4^+$ T cells in intestinal samples was calculated as the "index" of baseline total $CD4^+$ T cells at these sites (considering baseline as 100%) to more clearly represent the depletion of $CD4^+$ T cells. T cell activation (HLA-DR^+ $CD38^+$ or Ki-67^+) were determined by gating on lymphocytes, then on $CD3^+$ T cells, and finally on $CD4^+$ $CD3^+$ or $CD8^+$ $CD3^+$ T cells. CD14 was used to identify circulating monocyte, by gating first on the $CD3^-$ HLA-DR^+ immune cell population. Monocyte/ activation was determined based on the expression of CD80 and CD86 on monocytes/macrophages. The absolute CD3 T cell counts in peripheral blood were obtained with BD Trucount™ Tubes (BD Biosciences). The absolute counts of circulating $CD4^+$ and $CD8^+$ T cells was further calculated through gating on the lymphocytes and $CD3^+$ T cells.

4.2.6 Viral quantification

Plasma viral RNA loads were quantified using quantitative real-time PCR specifically developed for SIVsab, as described previously [212, 417].

4.2.7 Statistics

All statistical analyses were performed using Prism 7 software (GraphPad Software, San Diego, CA), except for the multiple correlation between variables, which was performed using RStudio v1.1.383 (RStudio Inc., Boston, MA). Data are represented as means ± standard errors of the means (SEM) unless otherwise specified. Due to the relatively small size of the groups and individual variations between different animals, for most of the measured parameters, we used

baseline levels (average of three different time points collected before SIV infection) to normalize the results as fold change from the baseline. To compare the difference between treatment groups at critical stages of SIV infection, results from acute infection (10 dpi), set point (42 dpi), and chronic infection (1 week before treatment termination) were compared with Mann-Whitney U test. CD4+ T cell levels in circulation were presented as the index of preinfection levels (i.e. the absolute CD4+ T cell counts prior to infection in each animal was considered 100%, and the absolute CD4+ T cell counts after infection were calculated as the percentage compared with the preinfection counts) to better reflect the CD4+ T cell depletion while excluding individual variation. The survival of the PTMs were estimated with Kaplan-Meier analyses, and were compared between groups with Mantel-Cox method. Correlation between variables were performed with Spearman rank-order test using Hmisc package in R, with Spearman's correlation coefficient (rho) displayed using the corrplot function. P values less than 0.05 were considered to be significant.

4.3 RESULTS

4.3.1 Anticoagulant therapies reduce SIV-associated hypercoagulation

To assess the effects of the anticoagulant therapies on reducing SIV-associated hypercoagulation, we measured multiple biomarkers of elevated risk for CV comorbidities that were reported to be elevated in HIV-infected subjects. A the set-point and during chronic infection, plasma D-dimer level, which is a strong predictor of serious non-AIDS events, vascular disfunction and microbial translocation in HIV infection [241, 274, 418], was significantly lower in Ixolaris-treated PTMs

compared to controls (Figure 11A), but not in the PTMs treated with dabigatran or vorapaxar, in which the D-dimer levels were not significantly changed. Ixolaris also significantly reduced sTF levels at the set point and during the chronic infection in SIV-infected PTMs, while in the PTMs receiving dabigatran and vorapaxar the levels of sTF were significantly reduced only during the chronic infection (Figure 11B).

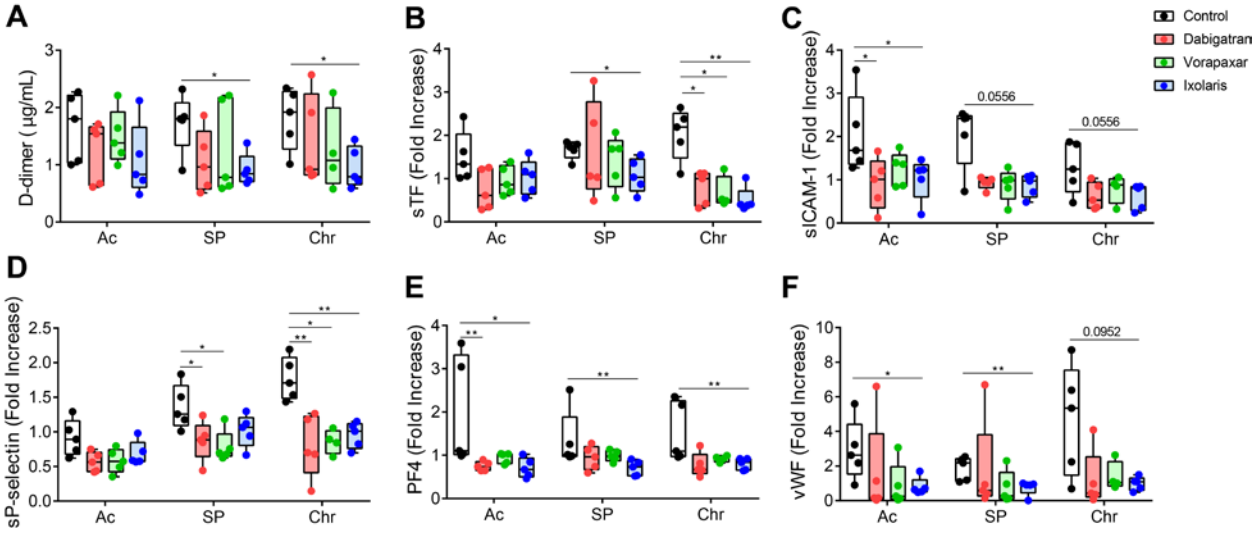


Figure 11. Anticoagulant treatment positively impacts the immune activation and systemic inflammation of highly pathogenic SIVsab infection in PTMs
D-dimer levels (A), and fold increase of sTF (B), sICAM-1 (C), sP-selectin (D), PF4 (E), and vWF (F) levels in control PTMs, dabigatran-, vorapaxar- and Ixolaris-treated PTMs during acute (Ac), set point (SP), and chronic (Chr) infection were compared using Mann Whitney U test. Data are presented as min to max, with the line representing the median. P values are presented as: * P<0.05, ** P<0.01, or otherwise the exact number.

The levels of the endothelial activation marker sICAM-1 were significantly lower in dabigatran- and Ixolaris-treated PTMs during acute infection, and showed trends towards reduction in the Ixolaris group at the set point and during chronic infection (Figure 11C). Endothelial and platelet activation marker sP-selectin levels were significantly reduced by dabigatran and vorapaxar treatment at the set point, and in all three treatment groups during chronic infection (Figure 11D). When assessing specifically platelet activation, all three anticoagulant therapies induced different extent of decrease of the plasma levels of platelet factor 4 (PF4), with only Ixolaris group reaching statistical significance throughout all stages of SIV

infection (Figure 11E). Ixolaris-treated PTMs also showed significantly lower vWF levels during acute infection and at the set point, and a trend towards decrease during chronic infection, while dabigatran and vorapaxar did not significantly change the vWF levels (Figure 11F).

In previous studies performed on ART-naive chronically SIV-infected PTMs, we found significant decreases in activated partial thromboplastin time (aPTT), significant increases in thrombin time and unchanged levels of the prothrombin time (PT) (Figures B25A, B and C, Appendix B). Therefore, we also measured aPTT and thrombin time during chronic infection in the PTMs receiving anticoagulants. In all three treatment groups, aPTT was not significantly different from preinfection level, indicating mild improvement compared to controls (Figure B25D, Appendix B). Thrombin time, however, was not improved by any of the anticoagulant treatments (Figure B25E, Appendix B), with the dabigatran-treated PTMs exhibiting the most prolonged thrombin time, due to the direct inhibitory effect of the drug on thrombin.

4.3.2 Anticoagulant therapies reduce inflammation in SIV-infected PTMs

Prompted by the close interactions between hypercoagulation and inflammation, we further assessed the impact of the different anticoagulant therapies on inflammation by measuring the plasma levels of multiple cytokines and chemokines. Vorapaxar treatment significantly reduced the levels of the proinflammatory cytokine IL-1β in PTMs during acute SIVsab infection, and showed a trend towards reduction during chronic infection (Figure 12A). The levels of IL-1β were also lower in the PTMs receiving Ixolaris during both the acute infection and at the set point, also showing a trend towards reduction during chronic infection (Figure 12A). Dabigatran treatment did not result in significant changes of IL-1β level. Another proinflammatory cytokine,

IL-17, was significantly reduced in all three treatment groups during acute SIVsab infection (Figure 12B).

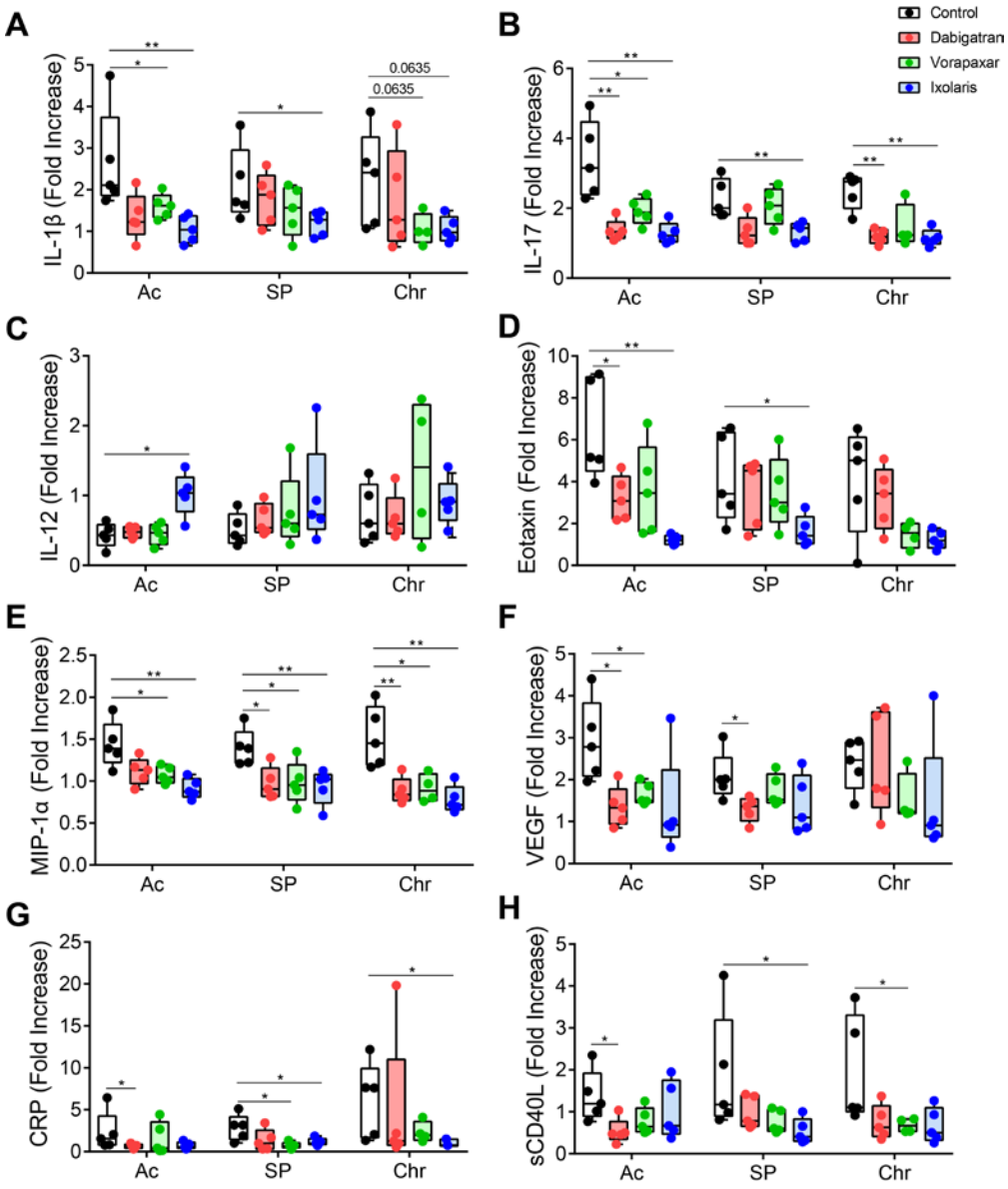


Figure 12. Anticoagulant treatment positively impacts the immune activation and systemic inflammation of highly pathogenic SIVsab infection in PTMs

Fold increase of IL-1β (A), IL-17 (B), IL-12 (C), Eotaxin (D), MIP-1α (E), VEGF (F), CRP (G), and sCD40L (H) levels in control PTMs, dabigatran-, vorapaxar- and Ixolaris-treated PTMs during acute (Ac), set point (SP), and chronic (Chr) infection were compared using Mann Whitney U test. Data are presented as min to max, with the line representing the median. P values are presented as: * P<0.05, ** P<0.01, or otherwise the exact number.

However, during the chronic SIV infection, the significance of the IL-17 reduction was only maintained in the PTMs treated with Dabigatran and Ixolaris, and was lost in the Vorapaxar

group because of a moderate reduction of the IL-17 levels in controls as they transitioned into the chronic infection (Figure 12B). Interestingly, the levels of IL-12 [the administration of which prolongs survival in SIV-infected macaques [419]], were significantly elevated in acutely SIVsab-infected PTMs receiving Ixolaris (Figure 12C).

The eosinophil chemoattractant eotaxin was significantly lower in Ixolaris-treated PTMs during acute SIV infection and at the set point, and in the dabigatran-treated PTMs during the acute SIV infection (Figure 12D). Macrophage chemoattractant MIP-1α was significantly reduced in the acutely SIV-infected PTMs receiving Vorapaxar and Ixolaris. Starting from the set-point and throughout the chronic SIV infection, MIP-1α was significantly reduced in all three treatment groups (Figure 12E). Vascular endothelial growth factor (VEGF), which has both angiogenic and proinflammatory properties, was significantly lower in Dabigatran-treated PTMs during the acute SIV infection and at the set point. In the animals receiving Vorapaxar, VEGF was lower only during the acute SIVsab infection, while in the PTMs receiving Ixolaris, VEGF was not significantly modified compared to controls (Figure 12F).

At the junction of hypercoagulation and inflammation, CRP is not only an acute-phase inflammatory protein, but also highly predictive of coronary heart disease [420]. Dabigatran treatment resulted in significant reduction of CRP in acutely SIV-infected PTMs, while Vorapaxar reduced CRP only at the set point stage. Ixolaris-treated PTMs showed lower levels of CRP at the set point and throughout the chronic SIVsab infection (Figure 12G). Another biomarker that indicates both hypercoagulation and inflammation is soluble CD40 ligand (sCD40L). sCD40L is a risk factor for acute coronary syndrome [421], which has also been involved in the inflammatory responses by platelet-monocyte complexes, as well as T cell activation [422]. Dabigatran treatment significantly reduced sCD40L during the acute SIV

infection. sCD40L levels also showed a trend towards reduction during chronic SIV infection of the Dabigatran-treated PTMs. Conversely, in the PTMs treated with Vorapaxar sCD40L was significantly reduced only during chronic SIV infection. In the PTMs receiving Ixolaris, the levels of sCD40L were significantly reducted at the set point, and showed a trend towards reduction during the chronic infection (Figure 12H).

4.3.3 Anticoagulant therapies reduce immune activation in SIV-infected PTMs

During HIV/SIV infection, systemic inflammation always accompanies persistent immune activation of both adaptive lymphocytes and innate monocytes [221]. We therefore flow-cytometrycally monitored the activation status of $CD4^+$ and $CD8^+$ T cells (i.e. HLA-DR^+ $CD38^+$ coexpression, and Ki-67) as well as $CD14^+$ monocytes (i.e. CD80 and CD86). All three anticoagulant therapies significantly reduced HLA-DR^+ $CD38^+$ coexpression on $CD4^+$ T cells during acute and chronic infection (Figure 13A). Ixolaris-treated PTMs presented significantly lower $CD8^+$ HLA-DR^+ $CD38^+$ T cells throughout all stages of SIV infection, whereas the Dabigatran administration did not result in significant changes of the HLA-DR^+ $CD38^+$ expression on the $CD8^+$ T cells. Vorapaxar induced significant reduction the HLA-DR^+ $CD38^+$ expression on the $CD8^+$ T cells only during the acute SIV infection (Figure 13B). In terms of Ki-67 expression, Ixolaris significantly reduced $CD4^+$ Ki-67^+ expression on the T cells during acute and chronic infection, while vorapaxar induced significant reduction only during acute infection (Figure 13C). All three treatment groups showed significantly lower Ki-67^+ expression on the $CD8^+$ T cells during chronic infection, whereas only Ixolaris-treated PTMs had significantly lower levels of the Ki-67^+ $CD8^+$ T cells during acute infection (Figure 13D).

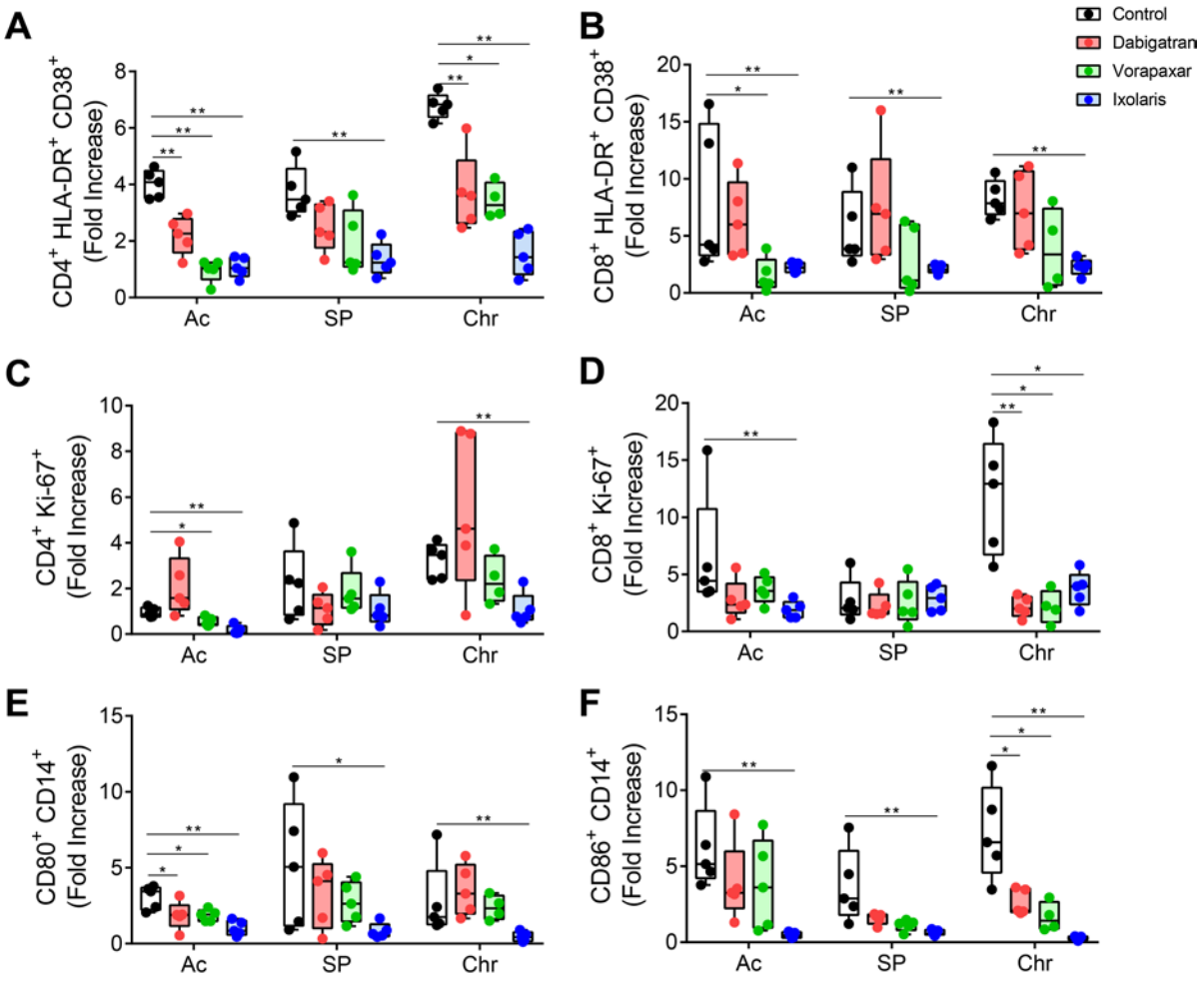


Figure 13. Anticoagulant therapies reduced T cell and monocyte activation
Fold increase of HLA-DR+ CD38+ coexpression on CD4+ (A) and CD8+ (B) T cells, Ki-67+ expression on CD4+ (C) and CD8+ (D) T cells, and CD80+ expression (E) as well as CD86+ expression (F) on CD14+ monocytes in control PTMs, dabigatran-, vorapaxar- and Ixolaris-treated PTMs during acute (Ac), set point (SP), and chronic (Chr) infection were compared using Mann Whitney U test. Data are presented as min to max, with the line representing the median. P values are presented as: * $P<0.05$, ** $P<0.01$.

On circulating $CD14^+$ monocytes, CD80 expression was significantly lower in all three treatment groups during acute infection, but was only significantly lower in Ixolaris-treated PTMs at the set point and during the chronic SIV infection (Figure 13E). Only Ixolaris-treated PTMs showed significant lower levels of CD86 expression on $CD14^+$ monocytes during the acute SIV infection. During the chronic infection, however, all three anticoagulant therapies significantly reduced the expression of $CD86^+$ on $CD14^+$ monocytes of the SIV-infected PTMs (Figure 13F).

4.3.4 Anticoagulant therapies reduced microbial translocation and liver fibrosis markers, but did not alter the natural history of SIV infection

To assess whether the anticoagulant therapies impacted other key aspects of SIV pathogenesis, we measured plasma levels of surrogate biomarkers for microbial translocation (i.e. lipopolysaccharide [LPS]) and liver fibrosis (i.e. hyaluronic acid). Dabigatran and Ixolaris treatment resulted in significantly lower LPS levels during both acute and chronic infection (Figure 14A). No significant changes were observed in vorapaxar-treated group. Vorapaxar and Ixolaris administration significantly reduced the plasma levels of hyaluronic acid only during the acute SIV infection, with no effect on the hyaluronic acid levels being observed at the set point and during the chronic infection in any of the treatment groups (Figure 14B).

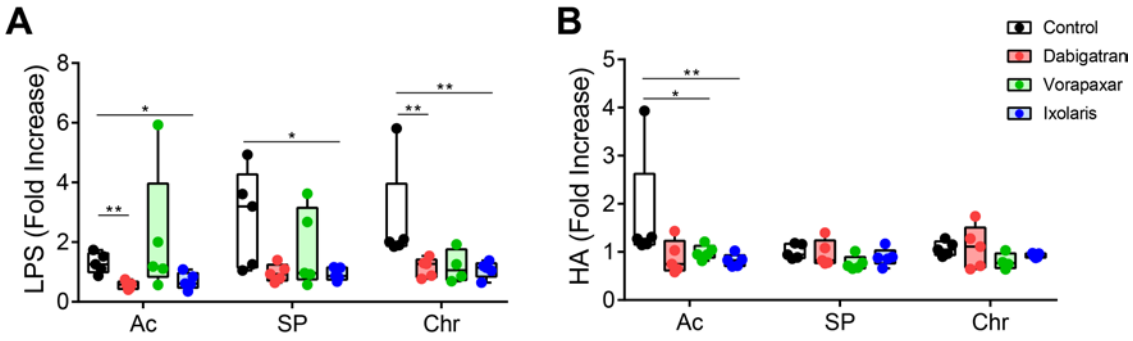


Figure 14. Anticoagulant therapies reduced surrogate biomarkers for microbial translocation and liver fibrosis
Fold increase of microbial translocation surrogate marker LPS (A) and liver fibrosis surrogate marker hyaluronic acid (HA) (B) levels in control PTMs, dabigatran-, vorapaxar- and Ixolaris-treated PTMs during acute (Ac), set point (SP), and chronic (Chr) infection were compared using Mann Whitney U test. Data are presented as min to max, with the line representing the median. P values are presented as: * $P<0.05$, ** $P<0.01$.

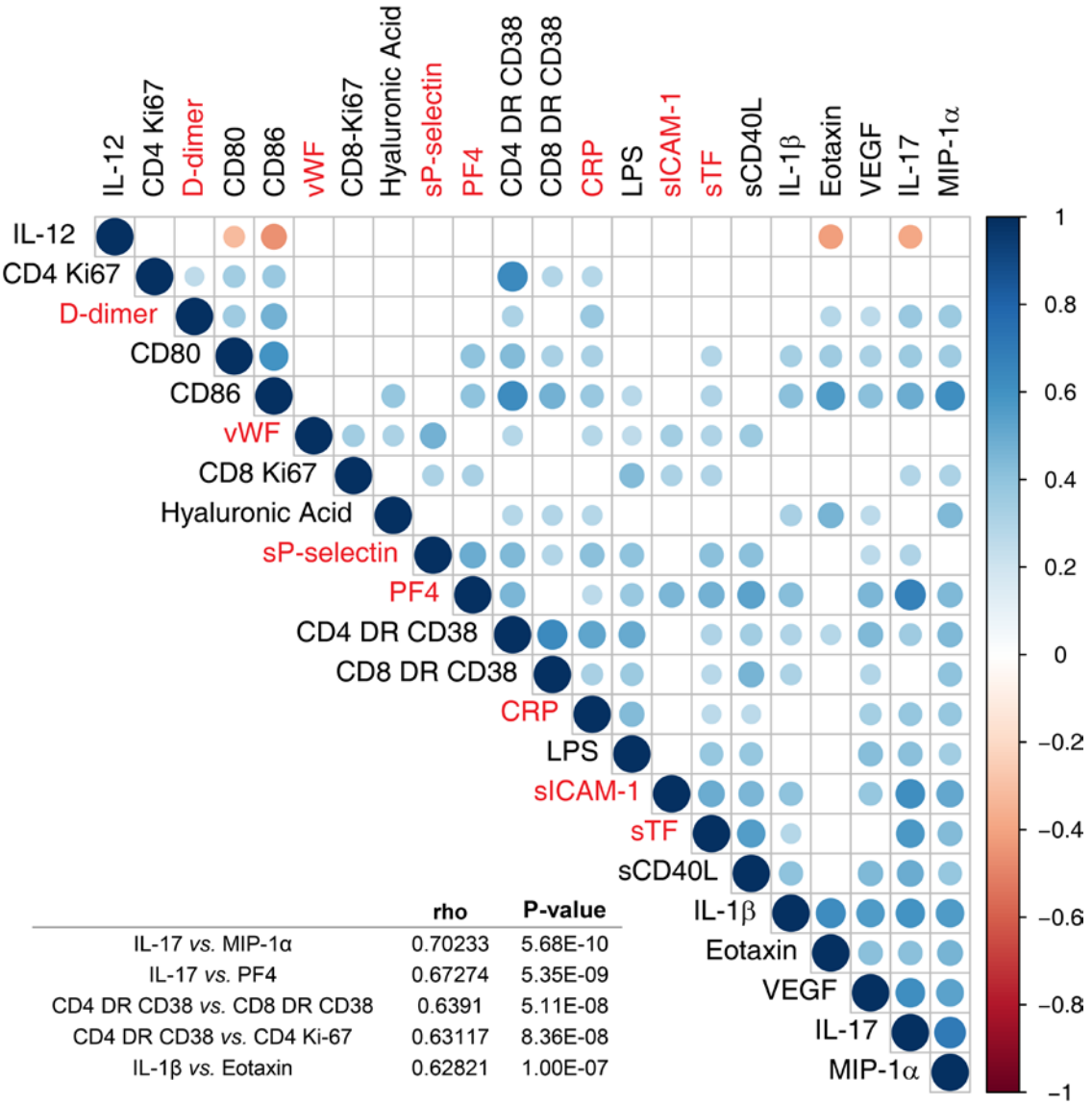
Despite the various extent of modifications of biomarkers of SIV pathogenesis, the anticoagulant therapies did not significantly reduce viral load or $CD4^+$ T cell depletion following SIV infection and did not significantly improve the survival of SIV-infected PTMs (Figure 26, Appendix B).

4.3.5 Hypercoagulation biomarkers modified by anticoagulant therapy closely correlate with IA/INFL markers

To further validate the relationship between the modifications of hypercoagulation and changes in IA/INFL observed after administration of the different anticoagulant therapies, we performed multiple Spearman's correlations between all the biomarkers measured in all the animals during acute, set point and chronic infection. A correlogram was then constructed based on the Spearman's correlation coefficients (rho), with only significant correlations ($P<0.05$) shown (Figure 15). For example, IL-12 level was significantly correlated negatively with CD80, CD86, Eotaxin and IL-17 levels. Although the highest rho values lie in the correlations between those within the IA/INFL markers (Figure 15, lower left panel), the hypercoagulation-associated biomarkers which were modified by the anticoagulant therapies (i.e. D-dimer, vWF, sP-selectin, PF4, CRP, sICAM-1, and sTF) all significantly correlated with multiple markers of IA/INFL (Figure 15). CRP showed significant correlation with the most biomarkers (16 out of the 22 variables), followed by sTF (15 variables), PF4 (14 variables), sP-selectin (11 variables), D-dimer (9 variables), vWF (9 variables), and sICAM-1 (9 variables). In particular, the correlation between PF4 and IL-17 has the second highest rho value and P-value among all correlations.

Interestingly, while all the hypercoagulation-related biomarkers significantly correlated with T cell activation (HLA-DR CD38 coexpression or Ki-67 expression on $CD4^+$/$CD8^+$ T cells), D-dimer levels mostly correlated with $CD4^+$ T cell activation (marked by HLA-DR CD38 coexpression and Ki-67) and monocyte activation (marked by CD80 and CD86). Meanwhile, sP-selectin, PF4 and sICAM-1 levels were more prominently correlated with inflammatory cytokines (i.e. VEGF, IL-17, MIP-1α) than D-dimer and vWF. Among all CV biomarkers, only CRP and vWF levels significantly correlated with the marker of liver fibrosis, hyaluronic acid.

These observations indicate that the biomarkers of hypercoagulation may be linked with IA/INFL through different pathways during SIV infection. Altogether, these results strongly suggest a close interaction between hypercoagulation and IA/INFL during SIV infection, which can be impacted by various anticoagulant therapies.



	rho	P-value
IL-17 *vs.* MIP-1α	0.70233	5.68E-10
IL-17 *vs.* PF4	0.67274	5.35E-09
CD4 DR CD38 *vs.* CD8 DR CD38	0.6391	5.11E-08
CD4 DR CD38 *vs.* CD4 Ki-67	0.63117	8.36E-08
IL-1β *vs.* Eotaxin	0.62821	1.00E-07

Figure 15. Correlation between biomarkers for hypercoagulation, immune activation and inflammation
The correlations between biomarkers were visualized in correlogram based on the Spearman's correlation coefficients (rho). Only significant correlations (P<0.05) were presented in correlogram as dots. The color shade and size of the dots were proportional to their rho values, corresponding to the color key on the right. Hypercoagulation-related biomarkers were highlighted in red. The correlations with the highest rho values were listed in the bottom left panel, with their respective P values listed.

4.4 DISCUSSION

Despite successful viral suppression with ART, HIV-infected subjects still face high risks of non-AIDS comorbidities, among which CVD remains one of the leading cause of death [97]. Emerging evidence have shown that the increased CVD risk in HIV-positive population may be driven in concert by hypercoagulation and HIV-associated persistent IA/INFL. In recent years, studies have emerged to investigate the use of statins and aspirin in HIV-infected subjects to reduce inflammation and as prevention for CVD and atherosclerosis [202, 204]. However, there is yet no knowledge on how anticoagulant therapies specifically targeting coagulation pathway or platelet activation may impact HIV-associated hypercoagulation and inflammation.

We previously established a key role of TF-expressing monocytes in bridging hypercoagulation and inflammation in HIV/SIV infection [410]. Here, we performed several in vivo studies in which we administered, for the first time, two FDA-approved anticoagulant therapies whose mechanisms revolve around TF signaling pathway, dabigatran (direct thrombin inhibitor) and vorapaxar (PAR-1 inhibitor) to SIVsab-infected NHPs. We monitored the effects of these two anticoagulants on hypercoagulation, IA/INFL and other markers of disease progression in SIV-infected PTMs. These effects were also compared with those of the TF inhibitor Ixolaris, to assess which anticoagulant therapy may exert the most beneficial effect on the natural history of SIV infection, particularly SIV-associated hypercoagulation and IA/INFL.

Overall, all three anticoagulant therapies exerted a certain reduction of hypercoagulation and IA/INFL. However, each anticoagulant therapy has a distinct profile of biomarker changes. Thus, with regards to the hypercoagulation profiles, all three anticoagulants reduced coagulation biomarkers at different stages of SIV infection, albeit only Ixolaris administration successfully reached statistical significance in reducing most of the markers.

With regard to the inflammatory cytokines and chemokines, a noticeable pattern of the Dabigatran treatment is that it successfully reduced the levels of IL-1β and Eotaxin in acutely SIV-infected PTMs, but this effect wane during the chronic infection (Figure 12A and 12D). The tandem changes of these two markers are not surprising, as Eotaxin can be directly induced by IL-1β secreted during the activation of the innate immune system [423], particularly through monocyte activation [424]. It is therefore conceivable that thrombin may play an important role in the inflammatory monocyte response during acute SIV infection, but not be directly involved in monocyte activation during chronic infection. This inference is corroborated by the CD80 expression on $CD14^+$ monocytes, which was significantly lowered during acute infection in the Dabigatran-treated PTMs, but returned to levels comparable to those observed in the untreated controls during chronic infection (Figure 13E). Another observation is that Vorapaxar had a much more limited effect on reducing IL-17 compared to Dabigatran and Ixolaris (Figure 12B), suggesting that hypercoagulation may be linked to Th17 activation through signaling pathways other than PAR-1.

In terms of immune activation, Dabigatran has the least effect on T cell activation, as illustrated by the levels of HLA-DR^+ $CD38^+$ expression on the $CD8^+$ T cells and the Ki-67^+ expression on the $CD4^+$ T cells (Figure 13B and 13C). Nevertheless, all three therapies reduced monocyte activation marked by CD80 and CD86 expression, albeit the timing was rather different. The reduction of CD80 was more significant during acute infection (Figure 13E), and the reduction of CD86 was more significant during chronic infection (Figure 13F), suggesting that CD80- and CD86-expressing monocytes may respond differently to hypercoagulation at different stages of the infection. Altogether, these observations suggest that the interaction

between hypercoagulation and HIV/SIV-associated IA/INFL is rather intricate and complex, and further investigation is needed to elucidate the exact mechanism.

Among all three anticoagulant therapies, Ixolaris exerted the most significant effect in reducing the levels of the biomarkers for hypercoagulation, inflammation and immune activation during SIV infection. Furthermore, Ixolaris administration also lowered the levels of the microbial translocation biomarker (LPS), and of the liver fibrosis surrogate marker (hyaluronic acid) during acute infection. This is not surprising as TF acts upstream of thrombin production and PAR-1 signaling. It is conceivable that once the vicious cycle of hypercoagulation with IA/INFL bridged by TF is established, targeting the downstream factors may not fully abrogate the deleterious effects. Additionally, TF may also impact SIV pathogenesis through other mechanisms than thrombin and PAR-1 signaling, such as Factor Xa (FXa). There is evidence showing that FXa (activated by TF:FVII complex) can induce proinflammatory cytokine production in macrophages *via* PAR-2 signaling [425]. TF may also promote tissue fibrotic response either through its own cytoplasmic signaling domain binding with PAR-2 [426], or through FXa-induced TGF-β activation [427]. Moreover, PAR-2 activation was also shown to induce intestinal inflammation and result in increased gut permeability [428]. These studies corroborated our findings suggesting that directly targeting of the TF represents a more effective strategy to break the hypercoagulation-IA/INFL vicious cycle and may achieve better results in reducing various HIV/SIV-related pathogenesis and comorbidities.

Here, we also correlated biomarkers measured for hypercoagulation with markers for IA/INFL, and identified significant correlations between multiple variables. These results not only strongly confirm the interaction between hypercoagulation and IA/INFL, but also provide insight for future studies involving the use of anticoagulation therapies in the context of HIV/SIV

infection. Due to the anticoagulation nature of these interventions and the accompanied bleeding risk, tissue sampling poses a big challenge and thus making the assessment of the effect of these therapies on tissue level rather difficult. It is therefore crucial to identify surrogate biomarkers to both accurately measure the anticoagulation effect, and in the meantime, readily and fully assess the effect of these therapies on HIV/SIV pathogenesis.

One of the main limitations of this study is the anticoagulant therapies were administered in a highly pathogenic SIV infection model in the absence of ART. Although this model would have best recapitulate the pathogenic evens during HIV progression, and thus best reflect any improvement on hypercoagulation and IA/INFL by anticoagulant therapies, the benefits of these therapies could have been offset by the highly pathogenic viral infection. The anticoagulant treatments did not reduce viral load or $CD4^+$ T cell depletion, nor were they able to significantly halt disease progression of the treated PTMs. Note, however, that none of the Ixolaris-treated PTMs progressed to AIDS within 100 days of infection, which is rare based on our experience working with SIV-infected PTMs [29, 212, 218, 324]. Frequently, two out of five animals will progress within 100 dpi. Unfortunately, this improvement on survival was not carried through as Ixolaris treatment was stopped after 80 dpi due to unavailability of the drug.

In conclusion, our NHP trials clearly demonstrate that anticoagulant therapies have beneficial impact on SIV pathogenesis by alleviating hypercoagulation and IA/INFL. Future studies to administer anticoagulant therapies along with ART are warranted to better assess their effects on comorbidities in a viral-suppressed and more clinic-mimicking setting.

5.0 GENERAL DISCUSSION

5.1 SUMMARY OF FINDINGS

HIV-infected patients, even on ART, are subject to increased risk of multiple non-AIDS comorbidities, especially CV diseases. Besides persistent and systemic immune activation and inflammation, they also present with a hypercoagulable state which is directly linked with increased chances of thrombotic events. Evidence in recent years have shown that these factors may function independently to drive the onset of CV events [277, 317, 387]. However, they can also potentiate each other and the exact mechanism of how they are linked is not yet defined. Driven by the fact that during HIV infection, activated monocytes highly express TF [365], that is also the major activator of the coagulation cascade [377], which eventually leads to thrombin and fibrin clot formation, we reasoned that TF plays a key role in bridging hypercoagulation with immune activation and inflammation.

In Chapter 3, we described a subset of CD14-expressing monocytes which during chronic HIV and pathogenic SIV infection in PTMs, highly express TF, while such increase is absent in the nonpathogenic SIV infection in the natural host, AGMs. These TF-positive monocytes in PTMs during chronic infection also respond to LPS stimulation more dramatically than those in AGMs, which suggest a potential connection between microbial translocation, one of the contributors to immune activation and inflammation in chronic HIV/SIV infection, and

hypercoagulation. Indeed, we observed a continuous increase of TF expression in the gut that paralleled the increased levels of local microbial translocation during chronic SIV infection. Besides triggering Factor Xa formation to initiate coagulation, these TF-positive monocytes are also capable of producing multiple inflammatory cytokines, such as IP-10, IL-6, and TNF-α. Interestingly, we found that the production of these cytokines can be induced by thrombin via PAR-1 signaling, and thrombin itself is one of the downstream products of TF-initiated coagulation cascade. These findings thus closed the circle of hypercoagulation and immune activation and inflammation bridged by TF. When we blocked TF *in vivo* in SIV-infected PTMs using the innovative TF inhibitor anticoagulant Ixolaris, there was not only a decrease in the hypercoagulation marker, D-dimer, but also an increase of protective cytokine IL-12 (the administration of which prolonged survival of SIV-infected macaques [419]), a decrease in proinflammatory cytokine IL-17 and IL-10, and a decrease of multiple markers of lymphocyte and monocyte activation. Most importantly, the survival of these progressively SIV-infected PTMs was improved during the time of Ixolaris administration.

Altogether, these results provide strong support of TF playing a critical role in linking hypercoagulation and immune activation and inflammation, potentially with the involvement of thrombin and PAR-1 signaling, and therefore, may be a new therapeutic target in HIV patients to reduce the risk of CV comorbidity. To translate these findings into clinical therapeutic settings, we then identified two FDA-approved anticoagulant therapies, dabigatran (direct thrombin inhibitors) and vorapaxar (PAR-1 inhibitors), and administered them in progressively SIV-infected PTMs. We compared their effects on hypercoagulation as well as immune activation and inflammation with those of Ixolaris, to determine whether they have similar effects and to identify the best therapeutic strategy for alleviating HIV-associated CV risk. We reasoned that if

the effects of dabigatran and vorapaxar are comparable to those of Ixolaris, these beneficial therapies may then be readily translated clinically to HIV patients, as they are already FDA-approved.

In Chapter 4, we reported 22 biomarkers ranging from those that are indicative of CV risk, endothelial and platelet activation, to those of inflammation and activation of lymphocytes and monocytes, measured throughout key stages of the infection in SIV-infected PTMs treated with different anticoagulant therapies and compared with control PTMs with no treatment. Overall, all three anticoagulant therapies reduced hypercoagulation-related biomarkers, but the reduction from dabigatran and vorapaxar were not to the extent of Ixolaris potency, potentially due to the fact that TF plays more upstream than thrombin and PAR-1 in the coagulation pathways. While Ixolaris resulted in a general reduction of immune activation and inflammation, dabigatran and vorapaxar seemed to differ in the way that they might impact the immune system. For example, dabigatran and Ixolaris both reduced IL-17 levels in the plasma after SIV infection, while vorapaxar did not have a significant impact, indicating that TF and thrombin may promote IL-17 production by Th17 cells through pathways other than PAR-1 signaling. On the other hand, T cell activation marker HLA-DR$^+$ CD38$^+$ coexpression and Ki-67 expression was modified less by dabigatran than by vorapaxar and Ixolaris, suggesting that hypercoagulation may promote general T cell activation through TF and PAR-1 signaling, and not directly or only involving thrombin, but through other component of the coagulation cascade, such as via Factor Xa [429]. Moreover, dabigatran and Ixolaris resulted in a reduction in plasma LPS levels, while vorapaxar did not have such an effect, suggesting that TF and thrombin may also have a role in exacerbating gut inflammation and consequent microbial translocation, but this effect is not induced through PAR-1 signaling. Interestingly, it was shown that PAR-2 activation can cause

intestinal inflammation and increase gut permeability [428], which may partly explain our finding.

These results suggest that the interaction between hypercoagulation and immune activation and inflammation may be more very intricate and may involve multiple pathways, in which case targeting the upstream player TF directly may represent the most effective strategy.

Nevertheless, different types of anticoagulant therapies all showed alleviation of hypercoagulation and immune activation and inflammation to different extent, pointing to not only a preventative role for CV comorbidities, but also beneficial effects on general HIV pathogenesis for HIV patients to start anticoagulant therapies. These findings, combined with the clinical presentation of increased CV risk in HIV patients, calls for potential reevaluation of the criteria to initiate anticoagulation therapies and/or innovative therapies that target coagulation pathways specifically in the setting of HIV infection.

Furthermore, by correlating the 22 biomarkers we measured in this study, we found strong correlations between the biomarkers modified by anticoagulant therapies with those related to immune activation and inflammation. This not only confirmed the close interaction between hypercoagulation and immune activation and inflammation, but also provided valuable insights for biomarker selection in future studies monitoring the effect of anticoagulants in the context of HIV infection, where invasive sampling may be difficult due to the bleeding risk associated with these therapies.

5.2 LIMITATIONS AND FUTURE DIRECTIONS

One limitation of our study is that the therapies are administered in PTMs infected with SIVsab and with no ART treatment. Although the highly pathogenic and progressive nature of this infection best recapitulates the pathogenesis feature of untreated HIV infection in humans, including hypercoagulation and immune activation and inflammation, the fast and rather uncontrolled disease progression renders any therapy not targeting the viral replication itself not able to significantly alter the natural history of the infection. In our study, the anticoagulant therapies did not significantly rescue the massive CD4 depletion associated with high viral loads or significantly prolong survival, except for Ixolaris, which was able to eliminate rapid progressors who progress to AIDS within 100 days of infection. The reason that this improvement did not reach statistical significance may also be due to the relatively limited sample size and the limited availability of the drug, calling for larger groups of animals and longer duration of treatment for future studies.

Thus, an obvious future direction is to combine the most effective anticoagulant therapy, Ixolaris, with ART, to assess its effect under viral-suppressed condition. This will not only allow us to further assess the effects of the therapy on hypercoagulation and inflammation, and eventually CV comorbidity, without the confounding factors of the pathogenicity of the virus, but also more closely mimic the clinical scenario of chronically infected HIV patients. In addition, it will also allow the possibility to fully assess the potential drug interactions between anticoagulant therapies and ART, and any complications those may entail.

In conclusion, our study directly confirmed that there is an intimate interaction between hypercoagulation and HIV/SIV-associated immune activation and inflammation, and TF is a critical player in bridging the two. Although future studies to clearly delineate the pathways

involved in the complex interaction are still in demand, our results showed that this vicious cycle can and should be therapeutically targeted, to slow down HIV pathogenesis and reduce the risks of HIV-associated CV comorbidities.

APPENDIX A

SUPPLEMENTARY MATERIALS FOR CHAPTER 3.0

This appendix includes the supplementary materials for manuscript “Inflammatory monocytes expressing tissue factor drive SIV and HIV coagulopathy”, Published 30 August 2017, Sci. Transl. Med. 9, eaam5441 (2017) DOI: 10.1126/scitranslmed.aam5441.

A.1 MATERIAL AND METHODS

A.1.1 Ethics statement

All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all study participants. The human clinical study was approved by the NIAID Ethics committee, and the studies are registered in Clinicaltrials.gov (NCT00286767 and NCT00101374). Serum samples from patients with chronic hepatitis C virus infection were originally from another clinical study (Long-term study of liver disease in people with hepatitis B and/or hepatitis C with or without HIV infection, NCT01350648). In addition, 14 male PTMs and 6 male AGMs were included in

this study. All animals were housed and maintained at the RIDC Park or Plum Boro animal facility of the University of Pittsburgh according to the standards of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC) (IACUC protocol: #12121250, approved in 2012, IACUC protocol: #12080831, approved in 2012, and IACUC #14043645). The animals were fed and housed according to regulations set forth by the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act. All animals included in this study were socially housed (paired) indoors in stainless steel cages, had 12/12 light cycle, were fed twice daily, and water was provided ad libitum. A variety of environmental enrichment strategies were employed including housing of animals in pairs, providing toys to manipulate and playing entertainment videos in the animal rooms. In addition, the animals were observed twice daily, and any signs of disease or discomfort were reported to the veterinary staff for evaluation. For sample collection, animals were anesthetized with 10 mg/kg ketamine HCl (Park-Davis, Morris Plains, NJ, USA) or 0.02-0.04 mg/kg tiletamine HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA) injected intramuscularly. When the endpoints of the study were reached, the animals were sacrificed by intravenous administration of barbiturates.

A.1.2 *Ex vivo* flow cytometry assays

Cryopreserved PBMCs from humans and NHPs were thawed and resuspended in RPMI- 1640 media supplemented with 10% human AB serum (106 cells/well in 96-well plates). Cells were washed and stained with Live/Dead fixable blue dead cell stain (Life Technologies) for 20 minutes at room temperature (RT) then washed with 1% PBS/BSA and stained with antibodies

for extracellular surface markers for 1 h at RT. Cells were then fixed and permeabilized (Foxp3/ Transcription Factor Staining Buffer Set, eBioscience) overnight at 4oC. After permeabilization, cells were stained for intracellular markers for 1hr at RT. All panels and antibodies used in both human and NHP samples are listed in Table S4. To measure the effect of the anticoagulant treatment on immune activation associated with SIV infection in vivo, whole blood was stained for flow cytometry, as described previously (25, 49). Data were acquired on a BD LSR II flow cytometer (BD biosciences). All compensation and gating analyses were performed using Flowjo 9.5.3 (TreeStar).

A.1.3 Cell sorting

To determine the population of cells producing TF, elutriated monocytes from healthy donors were stained using CD14 (clone TüK4), CD16 (clone 3G8) and HLA-DR (clone L243) then sorted on a FACSAria III (BD Biosciences) into the following subsets: $CD14^{++}CD16^{-}$, $CD14^{+}CD16^{+}$, and $CD14^{-}CD16^{+}$. Post-sort analysis demonstrated a purity of 99% or greater. Monocytes were considered to be SSC^{lo}, $HLA\text{-}DR^{+}$ and positive for CD14, CD16 or both.

A.1.4 PAR-1 expression assay

Fresh whole blood was obtained from healthy blood donors and PBMCs were isolated and resuspended in RPMI-1640 media supplemented with 10% human AB serum. Inhibition of PAR-1 was achieved using indicated doses of SCH79797 (Tocris, R&D Systems) or a blocking monoclonal antibody (WEDE15, Beckman Coulter), which blocks cleavage and activation of PAR-1 (60), prior to stimulation for 1hr at 37oC in 5% CO2. Cells were then stimulated for 6

hours with indicated doses of Thrombin (α- thrombin, factor IIa; Enzyme Research Laboratories, South Bend, IN) at 37oC in 5% CO2. Cells were stained for PAR-1 (surface staining) as described above.

A.1.5 Cytokine ELISA

To assess the impact of the anticoagulant treatment on inflammation in SIV-infected animals, inflammatory and anti-inflammatory cytokine levels in the plasma were measured as previously described (61) using the Cytokine Monkey Magnetic 29-Plex Panel (Invitrogen), as per the manufacturer's instruction. Results were read by a Bio-Plex reader (Bio-Rad Laboratories, Hercules, CA), using Luminex technology (Luminex Corporation).

A.1.6 D-dimer testing in NHP

DD was measured using a STAR automated coagulation analyzer, (Diagnostica Stago) and an immunoturbidimetric assay (Liatest D-DI; Diagnostica Stago) (25). The analytical coeficient of variation ranged from 5%-14%.

A.1.7 ELISA for TF

PBMC (106 cells/well) in 96 well plates were stimulated overnight with LPS (100 ng/mL). Wells were washed twice with HBSS and 50 μL of TBS containing Triton X- 100 (0.1%, v/v) was added to each well. After 30 minutes at RT, the plates were frozen at -80ºC and two cycles of freeze-and-thaw were carried out to lyse the cells. The cell lysates were transferred to Eppendorf

tubes and centrifuged for ten minutes at 14,000xg in a bench centrifuge at RT. Supernatants were used (without dilutions) to estimate TF protein with Immunobind tissue factor ELISA kit (Sekisui diagnostics, Lexington, MA) as described (62). A standard curve was carried out simultaneously in the same plate.

A.1.8 TF functional activity assay

TF functional activity was assessed using an assay designed to measure assembly of the extrinsic Xnase by PBMC. Cryopreserved PBMC were thawed, resuspended in complete RPMI media with 10% human AB serum, dispensed in 96-well plates (5x105 cells/well) and stimulated with indicated doses of LPS. Negative controls did not contain LPS. After 18 hours, wells were washed three times with 200 μL HBSS containing 0.3% BSA. Then, a mixture of 200 μL FX (50 nM) and FVIIa (5 nM) in HBSS-BSA 0.3% (no FBS or Single Quotes) was added. After 6h incubation at 37ºC, 5% CO2, 95 μL was removed, placed in another plate, and 5 μL S2222 (250 μM, final concentration) was added to start reactions. Hydrolysis was detected using a VersaMax ELISA microplate reader (Molecular Devices) equipped with a microplate mixer and heating system as described (62). Reactions were continuously recorded at 405 nm for one hour at 37ºC. Factor Xa concentration was estimated using a standard curve performed under identical conditions. Human recombinant factor X was from Hematologic Technologies (Essex Junction). Recombinant human Factor VIIa (NovoSeven) was from Novo Nordisk (Plainsboro). Chromogenic substrate S-2222 was purchased from Diapharma Group Inc. (Westchester).

A.1.9 Immunohistochemistry

Immunohistochemistry of TF was performed on formalin-fixed, paraffin-embedded intestinal tissues collected either during surgery or at necropsy of NHPs. For antigen retrieval, the sections were microwaved in Vector Unmasking Solution (Vector Laboratories) and treated with 3% hydrogen peroxide. Sections were incubated with TF primary antibody (Thermo Fisher) at 1:500 dilution. Secondary antibodies and Avidin/Biotin complex were from the Vector Vectastain ABC Elite Kit. Sections were visualized with 3,3-diamidino-benzidine (Dako Corporation) and counterstained with hematoxylin.

A.1.10 Quantification of gene expression

Total RNA was isolated from human monocytes sorted based on TF expression after LPS stimulation for 6h, using the RNeasy Mini Kit, and residual DNA was digested using RNase-free DNase (both from QIAGEN). The RNA samples were reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Gene expression was measured using SYBR Green–based real-time quantitative PCR, and 18S mRNA was used as the housekeeping gene. The oligonucleotide primers used in the experiments are listed in Table S5. Fold induction of a given gene expression was calculated using the delta delta threshold cycle method, normalizing mRNA levels for each sample to levels of GAPDH and comparing with mRNA levels in unstimulated cells.

A.1.11 Statistical analysis

For the in vivo experiments in NHP, we compared parameters (e.g., cytokines, chemokines) between Ixolaris-treated and control animals at two time periods separately: acute phase and post-acute phase, because the profiles of change were very different in these two periods. To improve the power of these analyses, we used linear mixed-effects models (63). In this approach, we use all the measurements available together, and use macaque as the grouping (or random) factor to account for the repeated measurements made in each animal. We tested multiple models with fixed effects for time and treatment, with or without interactions. In this way, we are analyzing not only differences in the levels of the variable between treated and control monkeys, but also whether there is a difference in the variability of those levels over time (corresponding to the interaction term). During the acute phase the behavior of the different parameters assayed over time is variable, so to allow for a general pattern of dependency of the variable on time, we

considered the number of days since infection (between day 1 and day 21, as the acute phase) as a categorical factor (akin to a repeated ANOVA analysis) (63). For this, we need to have measurements on the same days for controls and treated animals. Therefore, we did not consider time points when there was only data for one of the groups. During the postacute phase, from day 42 p.i. onwards, the values of all parameters tended to be more constant or changed monotonically, therefore, we considered the number of days since infection as a continuous variable (akin to an ANCOVA analysis). In all cases (except viral loads), we analyzed changes from baseline, i.e., we normalized the variables by the baseline value within each animal. Assumptions on the distribution of residuals and appropriateness of the fitted values were checked by visual inspection of residual and fitted plots for the significant results. The best

model for the data (with or without the interaction term) was chosen by comparing the log likelihood. For these analyses we used the lme function of the nlme package (63) of R (http://cran.r-project.org/). We adjusted the resulting p-values due to multiple comparisons using the Holm-Bonferroni correction. We evaluated correlations between markers of interest using generalized estimating equations (gee) using an exchangeable working correlation structure. For these analyses, we used the geepack package of R.

A.2 SUPPLEMENTARY FIGURES

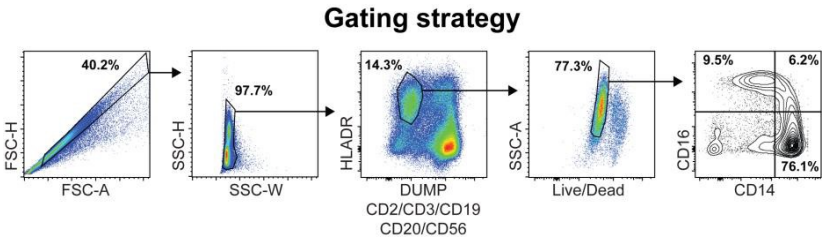


Figure 16. Gating strategy used to evaluate monocytes in PBMCs.

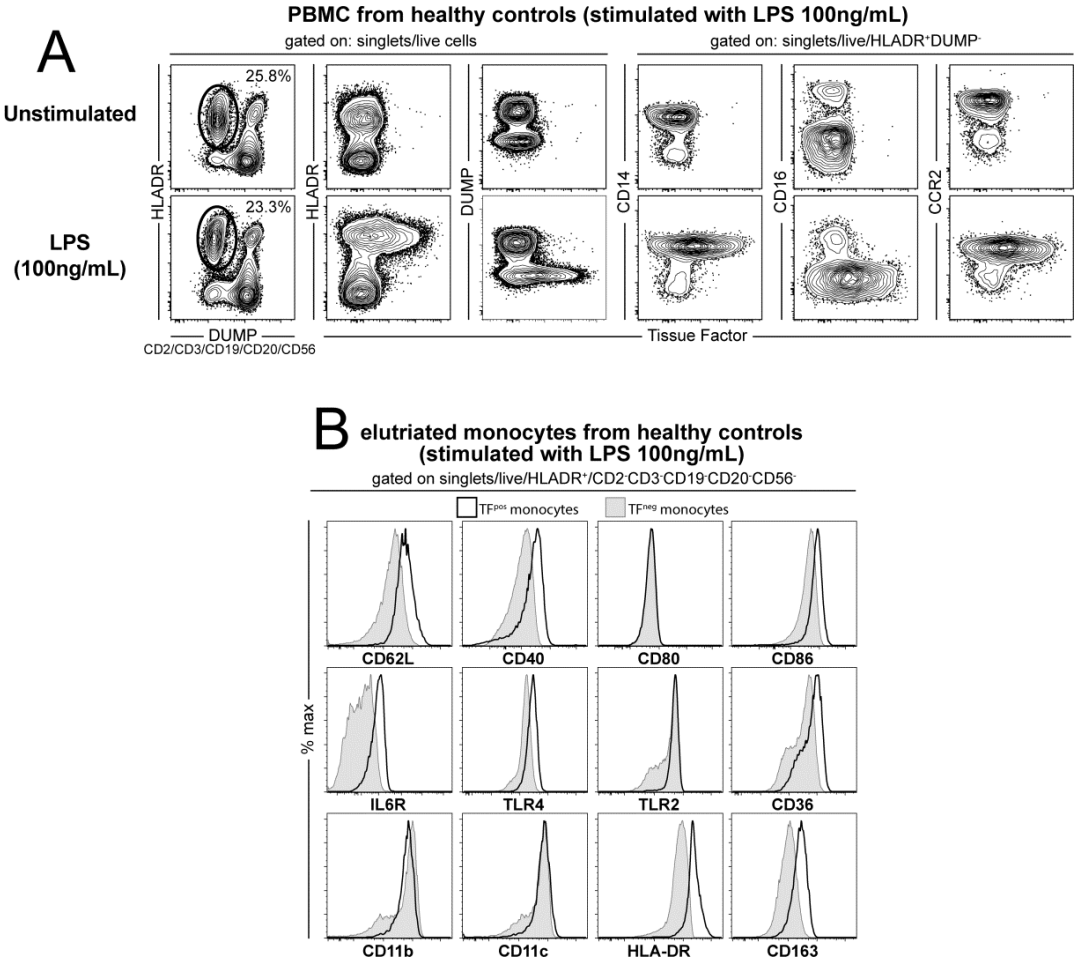


Figure 17. Detailed phenotyping of TF-expressing monocytes
(A) Representative plots show detailed phenotypic analysis of TF expressing cells upon LPS stimulation. (B) Histograms show various phenotypic markers on TFpos and TFneg subsets of stimulated elutriated monocytes from healthy donors.

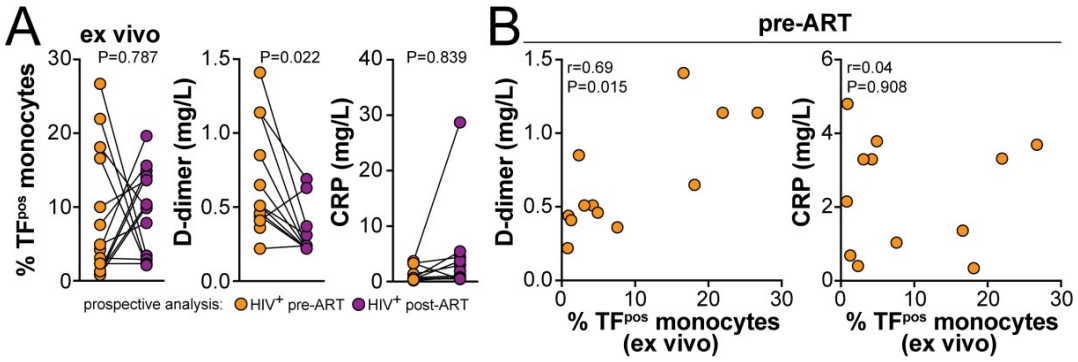


Figure 18. Frequency of TF-expressing monocytes in patients prospectively undergoing ART and its relationship with CRP and D-dimer

(A) In a parallel longitudinal analysis of a cohort of ART-naïve HIV+ patients before therapy and after ART-induced virological suppression (n=15; paired samples), frequency of TFpos cells as well as circulating levels of D-dimer and C-reactive protein (CRP) were compared using the Wilcoxon matched pairs test. (B) Spearman rank correlations were employed to test associations between frequency of TFpos monocytes ex vivo and plasma levels of D-dimer and CRP in ART-HIV+ individuals.

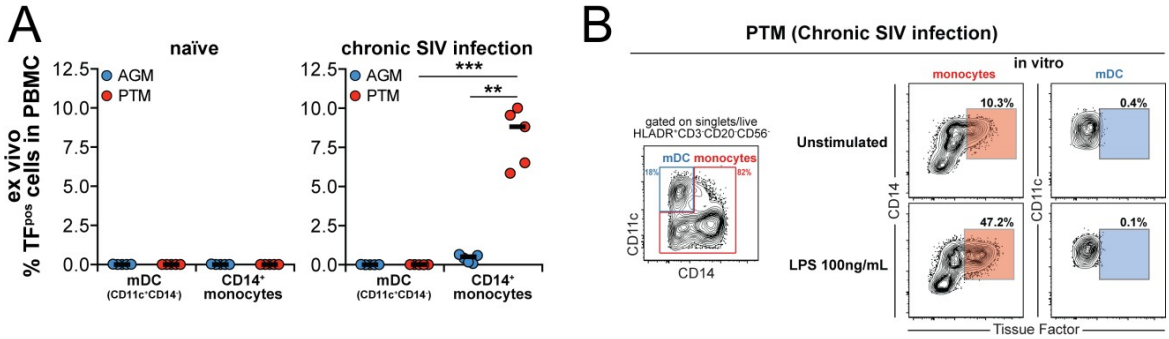


Figure 19. Circulating CD14+ monocytes, but not myeloid dendritic cells, express TF in chronically SIV-infected PTMs

(A) Frequency of TF expressing myeloid CD11c+ dendritic cells (mDC, left panel) or CD14+ monocytes ex vivo was compared between naïve and chronically infected pigtail macaques (PTM; n=6) and African green monkeys (AGM; n=6). Data were analyzed using the Mann-Whitney U test. **P<0.01, ***P<0.001. (B) Representative plot from a chronically-SIV infected PTM shows gating of mDC and monocytes (left panel) and expression of TF on these cell types in unstimulated and LPS-stimulated conditions (right panel).

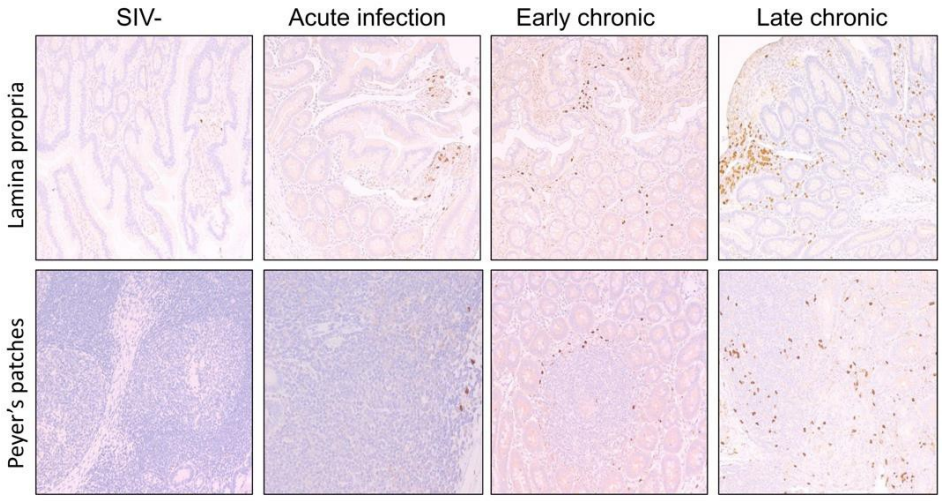


Figure 20. Induction of TF expression in the gut of PTMs infected with SIVsab
Immunohistochemistry of TF was performed on formalin-fixed, paraffin-embedded intestinal tissues and lymph nodes collected by resection or at the necropsy of NHPs according to Methods. Data are representative from three different animals.

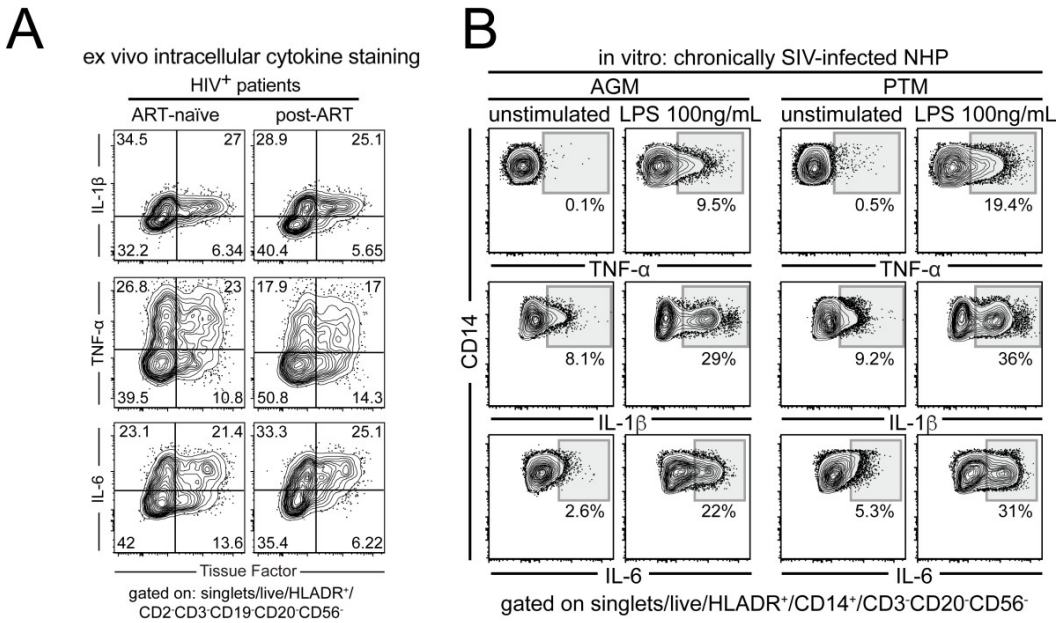


Figure 21. Intracellular cytokine production and TF expression in HIV-infected patients and in SIVsab-infected NHPs
(A) Representative plots (from 15 different individuals) show ex vivo cytokine production (IL-1β, TNF-α and IL-6) and tissue factor expression in ART-naïve HIV+ individuals at before therapy and at the time of ART-induced virological suppression. (B) Representative plots show intracellular production of TNF-α, IL-1β and IL-6 in stimulated monocytes of chronically-SIV infected PTM (n=6) and AGM (n=6).

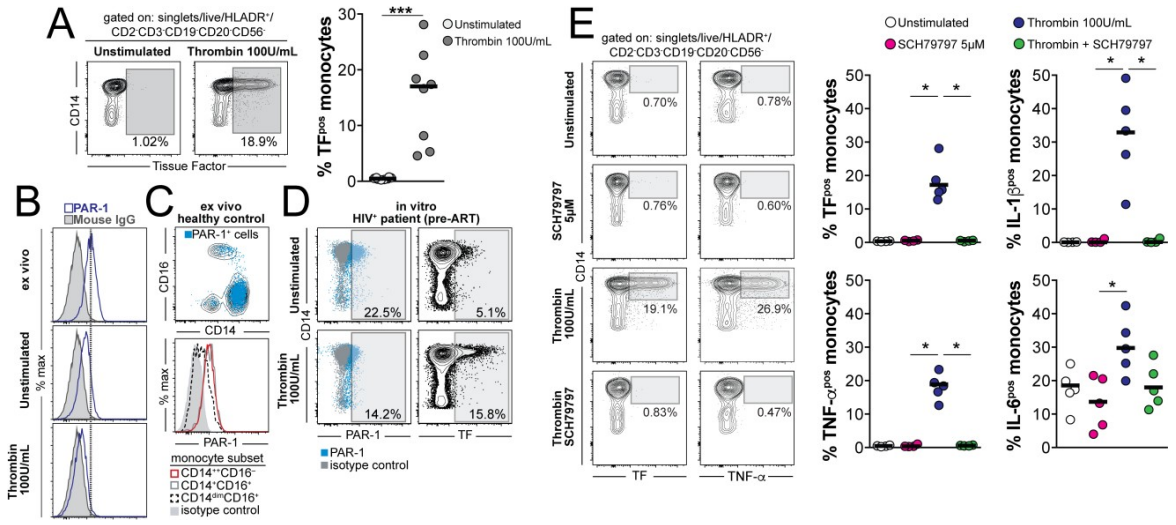


Figure 22. Thrombin induces TF expression on CD14^{high} monocytes via PAR-1
(A) Representative plot shows TF expression on monocytes from healthy controls induced by thrombin stimulation in vitro (left panel). Right panel shows summary data comparing percentage of TFpos monocytes between unstimulated and thrombin-stimulated conditions (n=8). Lines represent median values. Data were analyzed using the Mann- Whitney U test. (B) Histograms show expression of uncleaved PAR-1 on monocytes ex vivo and upon thrombin stimulation in a representative healthy control. (C) Representative plot shows co-localization of PAR-1 and CD14 in a healthy control ex vivo (upper panel). Histograms of PAR-1 expression in monocyte subsets reveals increased expression on CD14++CD16- and CD14+CD16+ cells. (D) Representative plots of PAR-1 expression (left panel) and TF expression (right panel) on monocytes from an ART-naïve HIV+ patient upon thrombin stimulation. (E) Representative plots show TF expression and TNF-α expression on monocytes upon thrombin stimulation in the presence of the PAR-1 inhibitor, SCH79797 (left panel). Right panels show a summary of the data comparing the frequency of TFpos monocytes, IL-1β, TNF-α and IL-6 in healthy donors upon thrombin stimulation in the presence or absence of SCH79797 (n=4). Lines represent median values. Data were analyzed using the Mann-Whitney U test. * P<0.05, *** P<0.001.

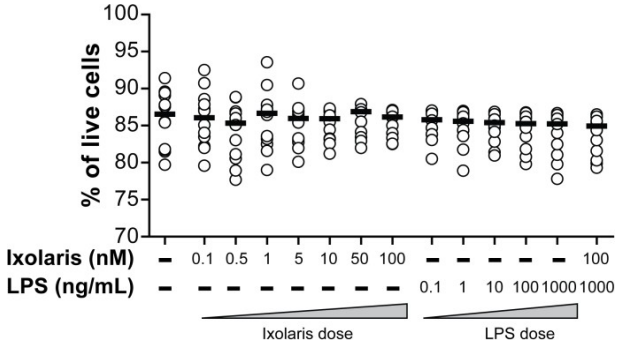


Figure 23. Cell viability upon treatment with Ixolaris and/or LPS in vitro
PBMC from healthy controls (n=12) were stimulated with LPS in the presence or absence of indicated doses of Ixolaris and cell viability was assessed by quantifying frequency of live cells (Live/Dead negative cells in flow cytometry). Results shown are measured in cells gated on singlets/HLA-DR+/CD2-CD3-CD19-CD20-CD56-. Data were compared using the Kruskal-Wallis test.

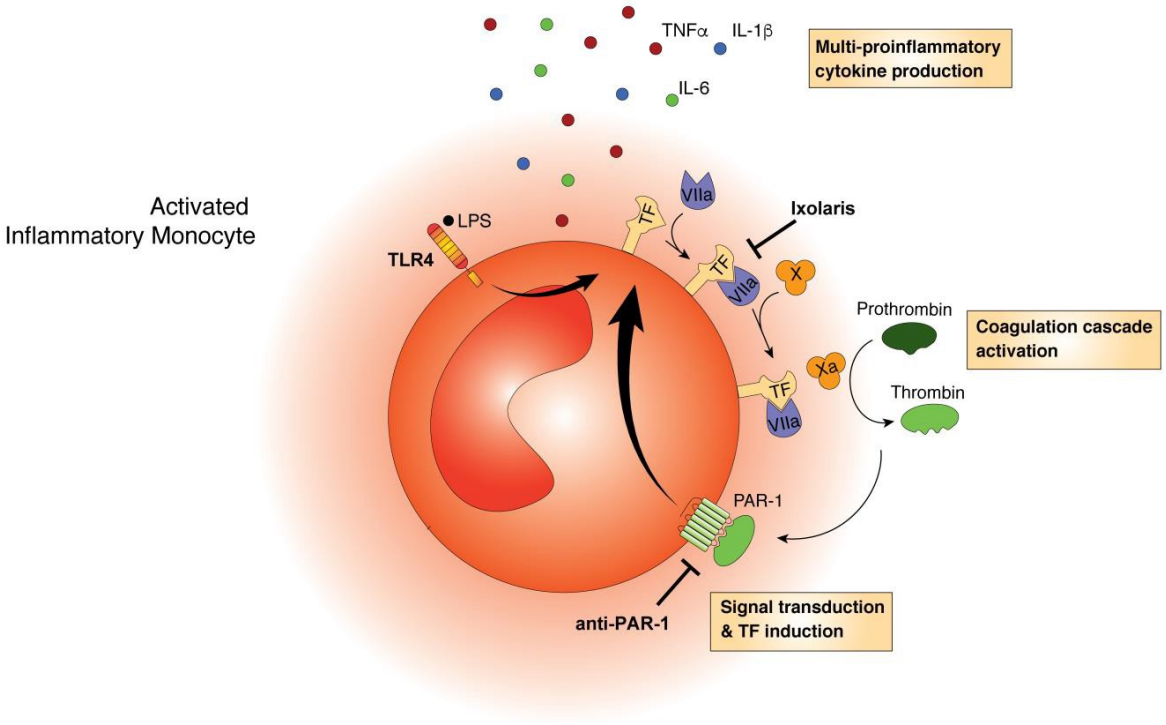


Figure 24. Activated monocytes expressing TF represent a link between coagulation and inflammation
We have shown that TF is preferentially expressed in activated CD14++CD16- monocytes. This monocyte subset is capable of producing multiple pro-inflammatory cytokines upon LPS stimulation. Expression of TF in vivo ultimately results in activation of the coagulation cascade and formation of thrombin. Our results indicate that the same subpopulation of monocytes that express TF is capable of sensing thrombin via PAR-1. PAR-1 signaling driven by thrombin triggers further TF expression and production of pro-inflammatory cytokines. We hypothesize that during chronic viral infection, induction of TF caused by thrombin on monocytes already activated by TLR stimulation might perpetuate a vicious cycle promoting persistent inflammation and coagulation. Inhibition of TF activity with Ixolaris or blockage of PAR-1 signaling could interrupt this pathological cycle and may serve as a target for future host-directed therapies in chronic viral infection.

A.3 SUPPLEMENTARY TABLES

Table 5. Characteristics of the NHPs used for the in vitro studies

Parameter	PTM (n=6)			AGM (n=6)		
	Pre-infection	Chronic infection	P-value	Pre-infection	Chronic infection	P-value
$CD4^+$ T cell count (cells/µL)	1070±283	336.26±299.5	<0.001	365.23±120.64	345.45±88.26	0.624
Log10 HIV RNA (copies/mL)		5.44±1.24			4.20±1.05	
CRP (mg/L)	7.56±4.52	63.12±23.87	<0.001	30.7±24.72	32.13±21.74	0.602
D-dimer (µg/mL)	0.25±0.05	2.3±0.16	<0.001	0.18±0.03	0.23±0.05	0.488

Data represent median and IQR. Data were analyzed using Wilcoxon matched pairs test. AGM, African green monkeys; PTM, pigtail macaques.

Table 6. Characteristics of HIV-infected individuals included in the cross-sectional analysis

Characteristic	Study population (N=20)		
	ART-naïve (n=10)	post-ART (n=10)	P-value
Male sex, no. (%)	8 (80%)	9 (90%)	1.0
Race/ethnicity, no. (%)			0.185
African American	6 (60%)	5 (50%)	
Latino/Other	2 (20%)	5 (50%)	
White	2 (20%)	0 (0%)	
Age, y	38 (26-46)	37 (32-48)	0.926
$CD4^+$ T-cell count, cells/µL	375 (38-802)	282 (108-350)	0.807
HIV RNA, log_{10} copies/mL	4.3 (3.6-4.9)	1.7 (1.7-1.7)	<0.001
CRP, mg/L	2.0 (0.38-4.8)	1.9 (0.62-2.7)	0.780
D-dimer, µg/mL	0.90 (0.54-1.4)	0.35 (0.27-0.39)	<0.001

Two distinct groups of ART-naïve HIV-infected individuals before ART initiation and post ART-induced HIV suppression (post-ART) were compared in a cross-sectional analysis. Data represent frequency or median and interquartile ranges. Continuous variables were compared using the Mann- Whitney test whereas percentages were compared using the Fisher's exact test.

Table 7. Characteristics of HIV-infected individuals included in the prospective analyses

Characteristic	Study population (n=15)		
	ART-naïve	post-ART	P-value
Male sex, no. (%)	12 (80%)		
Race/ethnicity, no. (%)			
African American	7 (47%)		
Latino/Other	8 (53%)		
Age, y	33 (30-47)	37 (32-48)	
$CD4^+$ T-cell count, cells/μL	39 (19-72)	350 (183-448)	<0.001
HIV RNA, log_{10} copies/mL	4.8 (4.5-5.1)	1.7 (1.6-1.7)	<0.001
CRP, mg/L	2.7 (0.78-3.6)	1.5 (0.55-4.6)	0.831
D-dimer, μg/mL	0.49 (0.38-1.1)	0.23 (0.22-0.33)	0.020

A group of ART-naïve HIV-infected individuals were evaluated at before ART initiation and post ART-induced HIV suppression. Data represent frequency or median and interquartile ranges. Data were analyzed using Wilcoxon matched pairs test.

Table 8. List of antibodies used in the flow cytometry experiments in both human and NHP samples

Marker	Clone	Company
BDCA-1 (CD1c)	L161	Biolegend
CD2	RPA-2.10	eBioscience
CD3*	UCHT1	eBioscience
CD3¶	SP34-2	BD Biosciences
CD4*	OK-T4	BD Biosciences
CD4¶	L200	BD Biosciences
CD8*	MHCD0828	BD Biosciences
CD8¶	RPA-T8	BD Biosciences
CD11b*	M1/70	Biolegend
CD11c*	3.9	Biolegend
CD13	WM15	eBioscience
CD14*	Tük4	Invitrogen
CD14¶	M5E2	BioLegend
CD16*¶	3G8	Biolegend
CD19*	HIB19	eBioscience
CD20*¶	2H7	eBioscience
CD36	CB38	BD Biosciences
CD38*	HB7	BD Biosciences
CD40	5C3	eBioscience
CD56*	B159	eBioscience
CD62L	DREG-56	BD Biosciences
CD80¶	MEM-233	Invitrogen
CD86¶	2331 (FUN-1)	BD Biosciences
CD142 (Tissue factor)*¶	HTF-1	eBioscience
CD163	GHI/61	Biolegend
CD206	19.2	eBioscience
HLA-DR*¶	L243	BD Biosciences
IL-1β*	JK1B-1	Biolegend
IL-6*	MQ2-13A5	eBioscience
IL-6R	BR-6	eBioscience
Ki-67*¶	B56	BD Biosciences
PAR-1	WEDE15	Beckman Coulter
TLR2	TL2.1	Biolegend
TLR4	HTA125	Biolegend
TNF-α*	MAb11	Biolegend
Glut-1¶	Polyclonal	Novus Biologicals

*All Abs were validated and titrated using PBMCs from NHP.

¶ Abs used for ex vivo flow cytometry assays in the Ixolaris study in NHPs.

Table 9. List of human primers

cDNA	Forward (5'- 3')	Reverse (5'- 3')
CCR2	CCACATCTCGTTCTCGGTTTATCAG	CGTGGAAAATAAGGGCCACAG
CCR5	GGAGCCCTGCCAAAAAATC	CTGTATGGAAAATGAGAGCTGC
CD163	CCAGTCCCAAACACTGTCCT	ATGCCAGTGAGCTTCCCGTTCAGC
CX3CR1	TTGCCCTCACCAACAGCAAG	AAGGCGGTAGTGAATTTGCAC
CXCL10	CCAGAATCGAAGGCCATCAA	CATTTCCTTGCTAACTGCTTTCAG
F3	CAGACAGCCCGGTAGAGTGT	CCACAGCTCCAATGATGTAGAA
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
IFNGR1	CATCACGTCATACCAGCCATTT	CTGGATTGTCTTCGGTATGCAT
IL10RA	TTCTTTGCCTTTGTCCTGCT	GCAGGTCCAAGTTCTTCAGC
IL1R1	TGGAGCAGGGATGTCACGTCTT	TTCCTCCACCCACGCTTATCCA
IL1R2	TGTGTTGTCCATAATACCCTGAGTT	TTGGGATAGGATTGAAAGTCTTGA
IL6R	GAGGGCTTCTGCCATTTCTGAG	CCAGGTTCAGCTGACAACAAACA
MYD88	GAGCGTTTCGATGCCTTCAT	CGGATCATCTCCTGCACAAA
PAR1	CAGTTTGGGTCTGAATTGTGTCG	TGCACGAGCTTATGCTGCTGAC
PAR2	GGGTTTGCCAAGTAACGGC	GGGAACCAGATGACAGAGAGG
PAR3	TCCCCTTTTCTGCCTTGGAAG	AAACTGTTGCCCACACCAGTCCAC
PAR4	AACCTCTATGGTGCCTACGTGC	CCAAGCCCAGCTAATTTTTG
STAT3	ACCTGCAGCAATACCATTGAC	AAGGTGAGGGACTCAAACTGC
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT
TLR4	CCAGTGAGGATGATGCCAGAAT	GCCATGGCTGGGATCAGAGT
TLR5	TGCCTTGAAGCCTTCAGTTATG	CCAACCACCACCATGATGAG
TRIF	ACGCCATAGACCACTCAGCTTTCA	AGGTTGCTCATCATGGCTTGGTTC

APPENDIX B

SUPPLEMENTARY MATERIALS FOR CHAPTER 4.0

This appendix includes the supplementary materials for Chapter 4.0, which are unpublished data.

B.1 SUPPLEMENTARY FIGURES

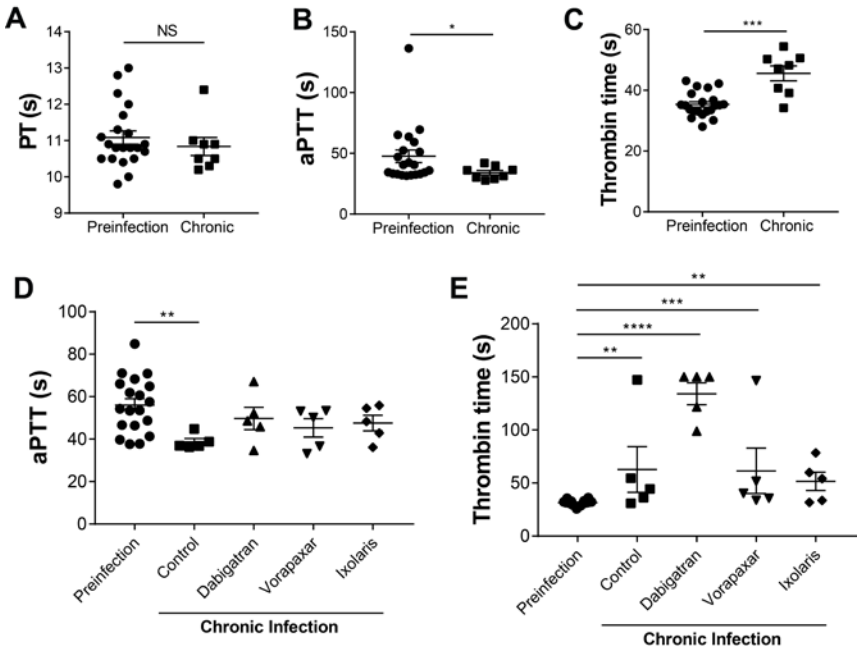


Figure 25. Anticoagulant therapies moderately increased aPTT in chronically-infected PTMs but no significant improvement on PT or thrombin time

Results gathered from untreated SIV-infected PTMs in other studies in our laboratory showed no change of PT (A), a decrease in aPTT (B) and an increase in thrombin time (C) during chronic SIV infeciton. In the current study,

aPTT (D) and thrombin time (E) were measured and compared between dabigatra-, vorapaxar-, Ixolaris-treated groups and controls. Values between groups were compared using Mann Whitney U test. Data are presented as mean±SEM. P values are presented as: * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

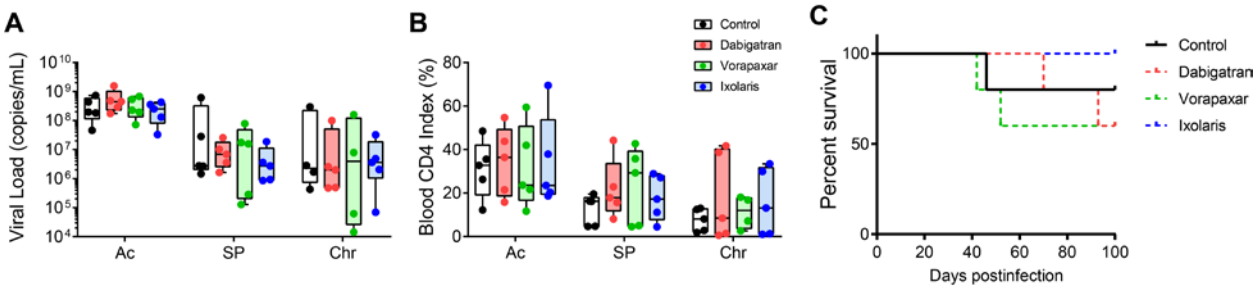


Figure 26. Anticoagulant therapies did not alter the natural history of SIV infection in PTMs
Plasma viral load (A) and CD4 Index (B) in control PTMs, dabigatran-, vorapaxar- and Ixolaris-treated PTMs during acute (Ac), set point (SP), and chronic (Chr) infection were compared using Mann Whitney U test. Data are presented as min to max, with the line representing the median. (C) The Kaplan-Meier survival curves between groups were compared using Mantel-Cox method.

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