



The Youngbloods. Get Together. Hypercoagulation, Complement, and NET Formation in HIV/SIV Pathogenesis

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Specialty section:

This article was submitted to
Virus and Host Immunity,
a section of the journal
Frontiers in Virology

Received: 15 October 2021

Accepted: 21 December 2021

Published: 18 January 2022

Citation:

Reno TA, Tarnus L, Tracy R,
Landay AL, Sereti I, Apetrei C and
Pandrea I (2022) The Youngbloods.
Get Together. Hypercoagulation,
Complement, and NET Formation in
HIV/SIV Pathogenesis.
Front. Virol. 1:795373.
doi: 10.3389/fviro.2021.795373

Chronic, systemic T-cell immune activation and inflammation (IA/INFL) have been reported to be associated with disease progression in persons with HIV (PWH) since the inception of the AIDS pandemic. IA/INFL persist in PWH on antiretroviral therapy (ART), despite complete viral suppression and increases their susceptibility to serious non-AIDS events (SNAEs). Increased IA/INFL also occur during pathogenic SIV infections of macaques, while natural hosts of SIVs that control chronic IA/INFL do not progress to AIDS, despite having persistent high viral replication and severe acute CD4⁺ T-cell loss. Moreover, natural hosts of SIVs do not present with SNAEs. Multiple mechanisms drive HIV-associated IA/INFL, including the virus itself, persistent gut dysfunction, coinfections (CMV, HCV, HBV), proinflammatory lipids, ART toxicity, comorbidities, and behavioral factors (diet, smoking, and alcohol). Other mechanisms could also significantly contribute to IA/INFL during HIV/SIV infection, notably, a hypercoagulable state, characterized by elevated coagulation biomarkers, including D-dimer and tissue factor, which can accurately identify patients at risk for thromboembolic events and death. Coagulation biomarkers strongly correlate with INFL and predict the risk of SNAE-induced end-organ damage. Meanwhile, the complement system is also involved in the pathogenesis of HIV comorbidities. Despite prolonged viral suppression, PWH on ART have high plasma levels of C3a. HIV/SIV infections also trigger neutrophil extracellular traps (NETs) formation that contribute to the elimination of viral particles and infected CD4⁺ T-cells. However, as SIV infection progresses, generation of NETs can become excessive, fueling IA/INFL, destruction of multiple immune cells subsets, and microthrombotic events, contributing to further tissue damages and SNAEs. Tackling residual IA/INFL has the potential to improve the clinical course of HIV infection. Therefore, therapeutics targeting

new pathways that can fuel IA/INFL such as hypercoagulation, complement activation and excessive formation of NETs might be beneficial for PWH and should be considered and evaluated.

Keywords: HIV, SIV, coagulation, complement, neutrophil extracellular traps

INTRODUCTION

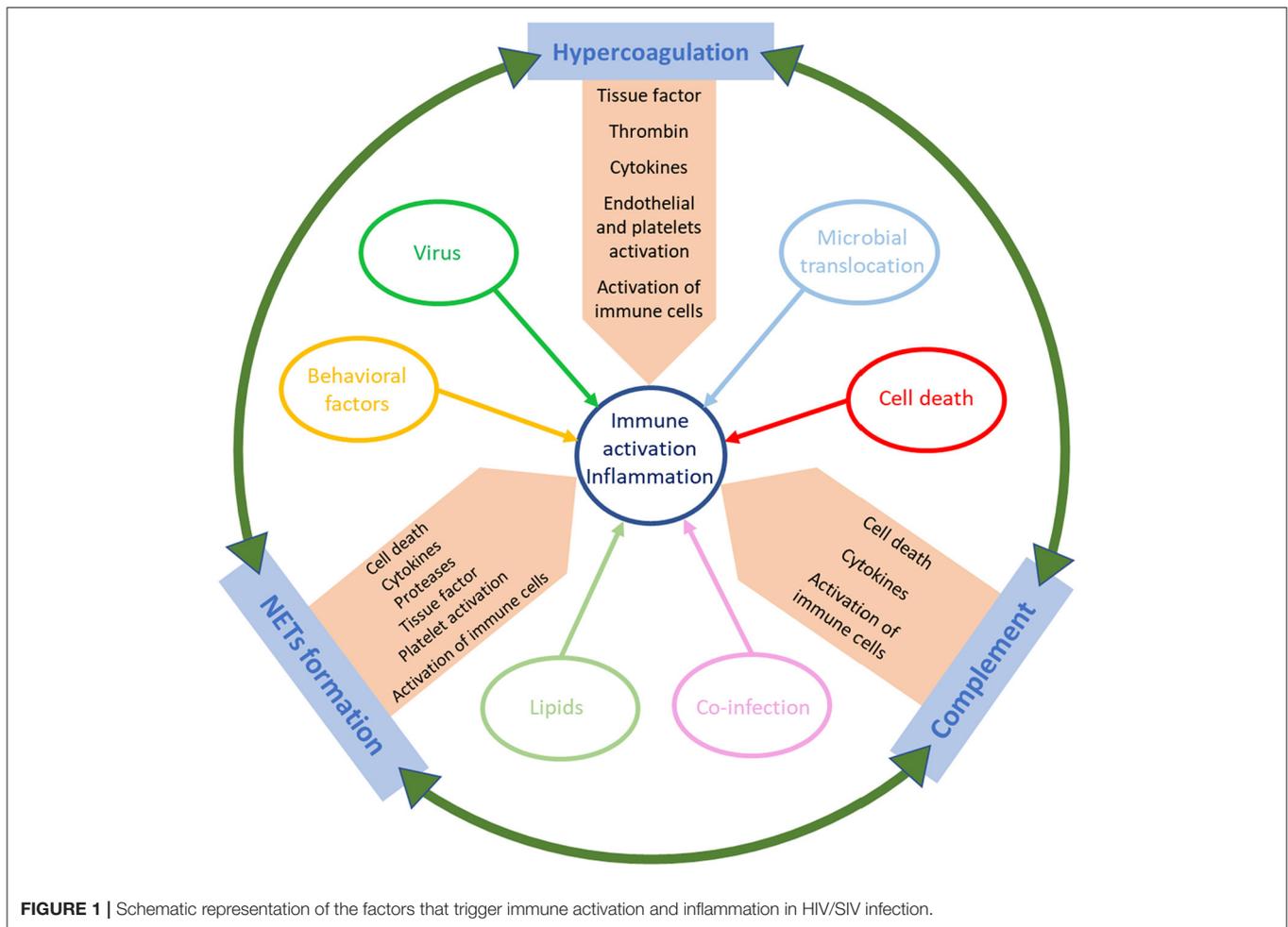
Antiretroviral therapy (ART) has increased the life expectancy and reduced morbidity and mortality for persons with HIV (PWH), thus becoming one of the most successful interventions of the twentieth century, that turned a virtually 100% deadly condition into a chronic disease (1). Yet, over the years, it became apparent that, despite successful suppression of HIV replication, PWH still experience higher rates of comorbidities and have a decreased survival compared to age-matched subjects (2). Even in cases presenting with complete, prolonged viral suppression, such as elite controllers or compliant PWH on ART with undetectable viremia for decades, the levels of T-cell activation and systemic inflammation remain higher than in HIV-uninfected individuals (3–5). Residual immune activation and inflammation lead to an incomplete immune restoration and a steady decline in the proficiency and functions of the immune system, similar to immunosenescence, i.e., progressive deterioration of the immune system by natural aging. As a result, in the recent years, a new paradigm of HIV pathogenesis emerged, in which persistent T-cell immune activation and inflammation are the key drivers of HIV pathogenesis and response to ART (6). The observations that CD8⁺ T cells from individuals with chronic HIV infection express high levels of CD38 and HLA-DR, indicating persistent, chronic, T-cell activation, and that these T cell activation levels are inversely correlated with CD4⁺ T cell counts, and are highly predictive for disease progression to AIDS were reported in the early days of the AIDS pandemic (7, 8). A fundamental role of T-cell immune activation for disease progression has also been suggested by studies in persons living with HIV-2 (PWH-2) and elite controllers (individuals naturally controlling their HIV-1 infection, i.e., with undetectable plasma viral loads in the absence of ART). In HIV-2 infection, the decline of circulating CD4⁺ T cell counts is slower than in HIV-1 infection, and patients develop AIDS after a median duration of incubation of 14 years of untreated infection, compared to only 6 years for HIV-1 (9). The levels of T-cell immune activation are lower in PWH-2 than in PWH-1 (10, 11). While the magnitude of T-cell immune activation is generally correlated with plasma viral load (12, 13), some PWH-2 with undetectable viremia present with persistent chronic immune activation (14). This indicates that viral replication is not the only source of chronic immune activation. Similar findings have been reported in elite controllers. Despite controlling viral replication, the levels of immune activation in these patients are higher than in uninfected subjects, albeit lower than in untreated HIV-1 progressors (15). The decline of CD4⁺ T cells was correlated to the levels of CD4⁺ and CD8⁺ T cell activation (16) and individuals without increased immune activation do not experience a CD4⁺ T cell

decline over time (17). As the absence of detectable viremia does not entirely avert the CD4⁺ T cell decline, nor the disease progression to AIDS, this strengthens the belief that dysfunctional, persistent immune activation is a key player in disease progression in humans.

Non-human primate (NHP) models also contributed to this paradigm of T-cell immune activation and inflammation driving the outcome of HIV infection. SIV impact on the NHPs depends on species and nature of the infection (18). As Asian macaques are not natural hosts of SIV, lentiviral infections are pathogenic in those species. Similar to humans with untreated HIV infection, SIV-infected macaques have a severely damaged gastrointestinal tract, generalized immune dysfunction, persistent high levels of systemic immune activation and inflammation, and eventually progress to AIDS (19). In the models of HIV/SIV disease progression, dysregulation of the cell cycle (20), infection/dysfunction and loss of regulatory T cells (Tregs)/Th17 cells (21–23), dendritic cells (24–26), and/or B cells (27, 28) are all indicative of failure to control T-cell activation/proliferation and systemic inflammation, and contribute to poor clinical outcomes. Conversely, SIV infections of African NHP species, including the African green monkeys (AGMs), sooty mangabeys (SM) and mandrills, that are natural hosts of SIV, generally do not progress to AIDS, despite high levels of viral replication and massive acute CD4⁺ T cell depletion (29–32). The lack of disease progression in natural hosts likely rely on their ability to control gut damage (33, 34), and chronic systemic T-cell activation and systemic inflammation (30, 35, 36). This is supported by the fact that, over a 20-year follow-up of SIV infections in natural hosts, only a handful of cases of AIDS have been observed, and, in every case, disease progression was associated with increased chronic T cell immune activation and inflammation (37–40). Moreover, when inflammation and T-cell immune activation were experimentally induced in AGMs, they presented changes in biological parameters that are usually associated with disease progression (increased plasma viral loads, loss of mucosal CD4⁺ T cells and microbial translocation) (41–43).

Altogether, comparative pathogenesis studies in NHP species with different pathogenic outcomes of SIV infection decisively contributed to the current paradigm in which chronic inflammation and immune activation are key drivers of immunopathogenesis, progression to AIDS, and the HIV-associated comorbidities called residual immune dysregulation syndrome (RIDS) (44).

Previous studies have implicated multiple contributing factors as sources of the immune dysregulation characteristic to acute HIV infection (**Figure 1**). HIV virions can directly activate lymphocytes and macrophages, triggering the secretion of



inflammatory cytokines and chemokines, including type 1 IFNs, IP-10, TNF, IL-6, and IL-8 (45, 46). The production and release of these cytokines can be beneficial, as they contribute to suppression of viral replication (47–49). For example, IFN-1 expression causes a signaling cascade that leads to the production of restriction factors and thus establishes an antiviral milieu which inhibits viral replication (50). While the production of IFN-1 and other cytokines mediates the stimulation of innate and adaptive immune cell subsets that will fight the virus, the profound impact of these proinflammatory factors on the immune response can also be detrimental (51, 52), as demonstrated by experimental interventions in NHPs (53). IFN-1 also induces CD4⁺ T-cell loss by modulating the expression of TNF-related apoptosis-inducing ligand (TRAIL), which then binds to Death Receptor 5 (DR5) leading to apoptosis (53–56). T cell apoptosis during acute infection is also triggered by TNF binding to its cellular receptors (57, 58). Apoptosis of infected CD4⁺ T cells can initially curb viral propagation, yet uninfected T cells are not spared from destruction, which contributes to the massive T-cell loss observed during acute HIV infection. Consistent with observations in humans, in both non-pathogenic and pathogenic acute SIV infections, type 1

IFNs are highly upregulated through the activation of pDCs (59–62). The upregulation of IFN-1 leads to an increased expression of IFN-1-stimulated genes (ISG) that persists during chronic infection in pathogenic HIV/SIV infections but returns to baseline in natural hosts after a transient increase during acute infection (63, 64). TNF α enhancement by HIV proteins further fuels HIV replication in infected cells (65, 66). Meanwhile, the non-pathogenic SIV infection of AGMs causes induction of Forkhead Box P3 (FoxP3) (a transcription factor necessary for the development of Tregs), TGF- β , and IL-10, all of which contribute to the establishment of an anti-inflammatory environment (67). The maintenance of this anti-inflammatory milieu is enabled by the absence of persistent IFN expression (67). On the other hand, the pathogenic infection of macaques, associate a delayed IL-10 expression and only a slight increase in TGF- β levels, similarly to acute HIV infection (67). The late increase in IL-10 allows for inflammatory cytokines to go unchecked and may lead to the exhaustion of SIV/HIV-specific CD8⁺ T cells (68, 69).

As the virus begins to swiftly replicate, it disseminates to the lymphoid tissues throughout the body. One key location of the virus is the gut-associated lymphoid tissue (GALT). During acute infection, CCR5⁺ CD4⁺ memory T cells are depleted in the

GI tract. Inflammatory cells, including neutrophils, monocytes, and dendritic cells, accumulate in the mucosa, while Th17 cells, the CD4⁺ T cells that regulate epithelial homeostasis, are lost, creating an environment prone to alterations of the mucosal integrity (70–72). The damaged epithelial mucosa allows microbial translocation from the gut lumen first into lamina propria and then into the systemic circulation (73). Natural hosts of SIVs do not exhibit microbial translocation because they have the ability to maintain the mucosal integrity by rapidly repairing the damages (33, 74) inflicted to their mucosal barrier by the SIV infection (34). In SIVsub-infected AGMs, administration of lipopolysaccharide (LPS) (41), to model microbial translocation seen in PWH, resulted in increased systemic immune activation and inflammation, leading to a rise in viremia. The same was true when gut damage was directly induced through administration of dextran sulfate sodium (DSS) in chronically infected AGMs (43). These experiments directly probed the role of the gut damage in inducing immune activation and inflammation. The loss of mucosal integrity and subsequent microbial translocation in humans and pathogenic NHP models trigger inflammation and T-cell immune activation by enhancing the activity of pattern recognition receptors which normally recognize molecular patterns associated to foreign pathogens (75). The activation of the innate immune system unleashes a multitude of immune cells that trigger the production of inflammatory cytokines, including IFN- α , IFN- β , IL-6, and TNF- α (76, 77). Another consequence of microbial translocation is an increase in circulating levels of sCD14, a marker of monocyte activation and LPS bioactivity, which has been shown to predict mortality, and of the scavenger receptor sCD163, the levels of which are associated with unstable non-calcified coronary plaques (18, 70, 78–80). Increased plasma levels of the translocated fungal polysaccharide (1 \rightarrow 3)- β -D-Glucan (β DG) are also associated with immune activation (81). Microbial translocation is not the only way in which the gut microbiome contributes to the systemic immune activation, the composition of the gut microbiome can alter the degree of activation of monocytes and T cells (75). Furthermore, the composition of the translocated microbiome can impact the immune reconstitution in ART-treated PWH (82). Interventions aiming to introduce gut dysbiosis, e.g., through prolonged antibiotic administration, were not sufficient to lead to disease progression (83).

Meanwhile, microbiome alterations through antibiotics, such as cotrimoxazole and a combination of rifaximin and sulfasalazine, resulted in a transient reduction in systemic immune activation and inflammation (84, 85).

An additional contributor to the SNAEs may be the altered lipid profiles in PWH, which can also contribute to immune activation and inflammation (86). The SMART study established that varying sizes of HDL particles, but not other lipoproteins, were associated with an increased risk of cardiovascular disease (CVD) events. Meanwhile, the levels of the inflammatory and coagulation markers CRP, D-dimer, IL-6 and sCD14 could predict mortality from CVD in PWH (78, 87). Furthermore, the oxidation levels of HDL and LDL are modified in PWH, and HDLox levels positively correlate with the increases in IL-6 and sCD163, contributing to persistent immune activation for

patients on ART (88) who are at an increased risk of serious non-AIDS events (SNAEs), including CVD (5). Administration of a diet rich in saturated fats and cholesterol to SIV-infected NHPs triggered immune activation and inflammation, thus confirming the importance of lipids as inducers of inflammation. In the NHPs fed with high fat diet, the increases in immune activation associated elevated levels of other cardiovascular biomarkers and a high incidence of cardiovascular lesions. Administration of the unhealthy diet also accelerated disease progression in the SIV-infected macaques and led to disease progression in one SIV-infected African green monkey (89).

Chronic coinfection with other viruses, notably human cytomegalovirus (hCMV), but also hepatitis B (HBV) and hepatitis C (HCV) viruses, can exacerbate HIV pathogenesis (90, 91). Exhaustion of virus-specific CD8⁺ T cell populations that regulate viral replication results in the reactivation of latent viruses and allows their active replication, boosting in turn systemic immune activation (92, 93). For example, coinfection with hCMV is associated with increased inflammatory and coagulation markers, including sCD14, IFN- γ -induced protein (IP-10), and D-dimer (94, 95). In PWH, chronic hCMV coinfection also exacerbates immunosenescence and the severity of CD4⁺ T cell depletion due to progressive thymic dysfunction (96). Individuals on ART still experience asymptomatic hCMV shedding, which triggers T-cell activation, proliferation, and exhaustion (97). Furthermore, an increased number of CD4⁺ T cells from the HIV/hCMV-coinfected subjects express the cellular senescence marker CD57, compared to individuals infected with either HIV or hCMV only (98). The increased pool of dysfunctional CD4⁺ and CD8⁺ T cells is associated with a loss of the effective control of hCMV and triggers a reversal of the CD4⁺/CD8⁺ ratio leading to an increased risk of morbidity and mortality (99, 100). This pathologic feature also occurs during the HIV/HCV coinfection, through the loss of CD4⁺ T cells and alterations of the Kupffer cells, the macrophage population resident in the liver, leading to elevated levels of circulating LPS and sCD14 (101–103). The loss of HCV-specific CD4⁺ T cells also results in an inadequate HCV control and reactivation (104, 105), which creates a positive feedback loop of viral replication fueling immune activation (102). Patients with HIV/HCV coinfection who received direct-acting antiviral agents for HCV eradication had significant decreases in the markers of immune activation and of increased HIV mortality: HIV DNA, sCD14, LPS, and D-dimers, indicating that coinfection suppression or eradication indeed help curb immune activation and inflammation (106).

As stated previously, ART is not able to completely reverse immune activation and inflammation, and low-level immune stimulation persists despite undetectable viremia, highlighting the importance of identifying and therapeutically targeting different mechanisms causing immune activation. In the following sections, we will focus on the role of additional factors that can fuel residual immune activation and inflammation under ART (**Figure 1**). We will thus discuss the relative contribution of hypercoagulation, complement pathway activation, and NET formation in driving systemic immune activation and inflammation in PWH

and how can these pathways be therapeutically targeted to reduce SNAEs.

HYPERCOAGULATION

The coagulation cascade is composed of a series of rapid and sequential enzymatic reactions leading to the generation of thrombin and eventually a fibrin clot (107). Among the positive and negative regulators of the coagulation cascade are immune activation and inflammation, the levels of which can trigger coagulation (108). The interplay between immune activation, inflammation and coagulation is critical for infection control, as the activation of coagulation can restrict pathogens movement, thus helping pathogen elimination (109). While this is beneficial for controlling infection, persistent inflammation can also trigger thrombosis and may result in blood vessel occlusion, causing pathologies, including peripheral thrombosis, pulmonary embolism, myocardial infarction, or stroke.

Contributing to the increased risk of thrombosis and CVD are deficiencies in proteins C and S, which hinder the inactivation of factors Va and VIIIa, and promote a procoagulant state (110, 111). Though immune activation and inflammation can initiate a procoagulant milieu, this interaction is now seen as a feedback loop where each can trigger the other (87, 112, 113) (Figure 2). This may be the reason for which the prevalence of CVDs and venous thromboembolism (VTE) is increased in untreated PWH and it is also not entirely abated by ART even when HIV replication is completely suppressed (114–116).

The altered coagulation associated with HIV infection was confirmed in NHPs (42), in which comparative studies of progressive and non-progressive SIV infections clearly demonstrated that the hypercoagulable state is specifically associated with the pathogenic, progressive SIV infections (42), providing strong validation of the SMART study data (87) and demonstrating the usefulness of animal models for the study of HIV-related comorbidities (117).

D-Dimer

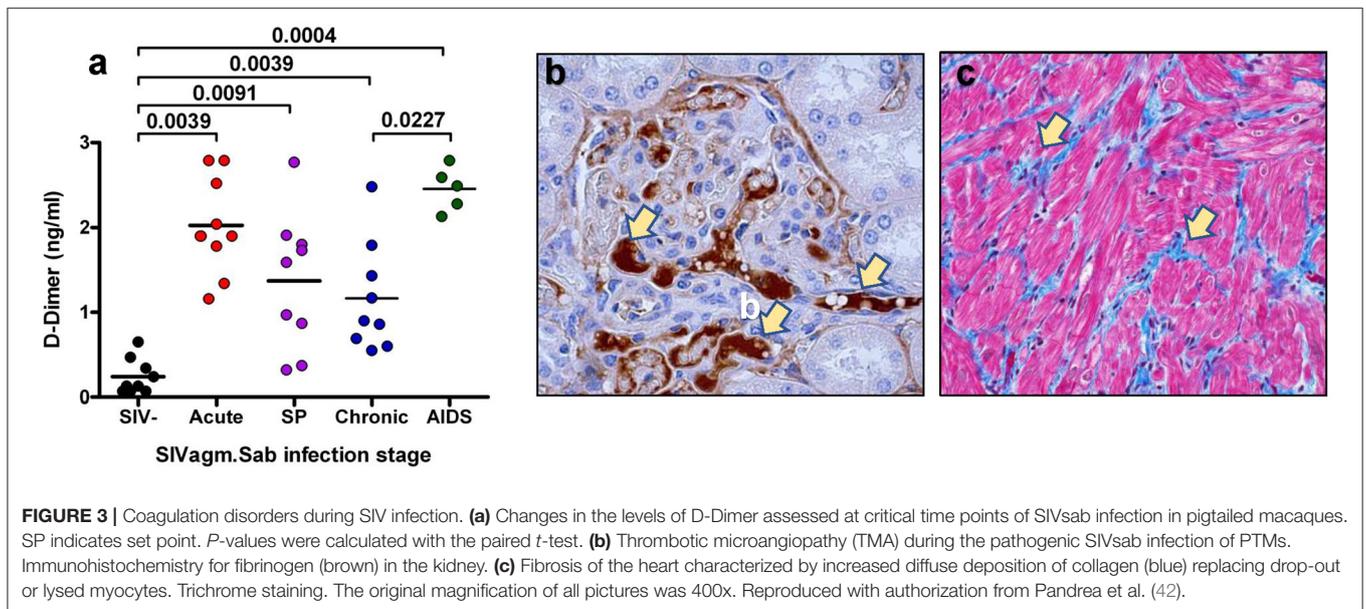
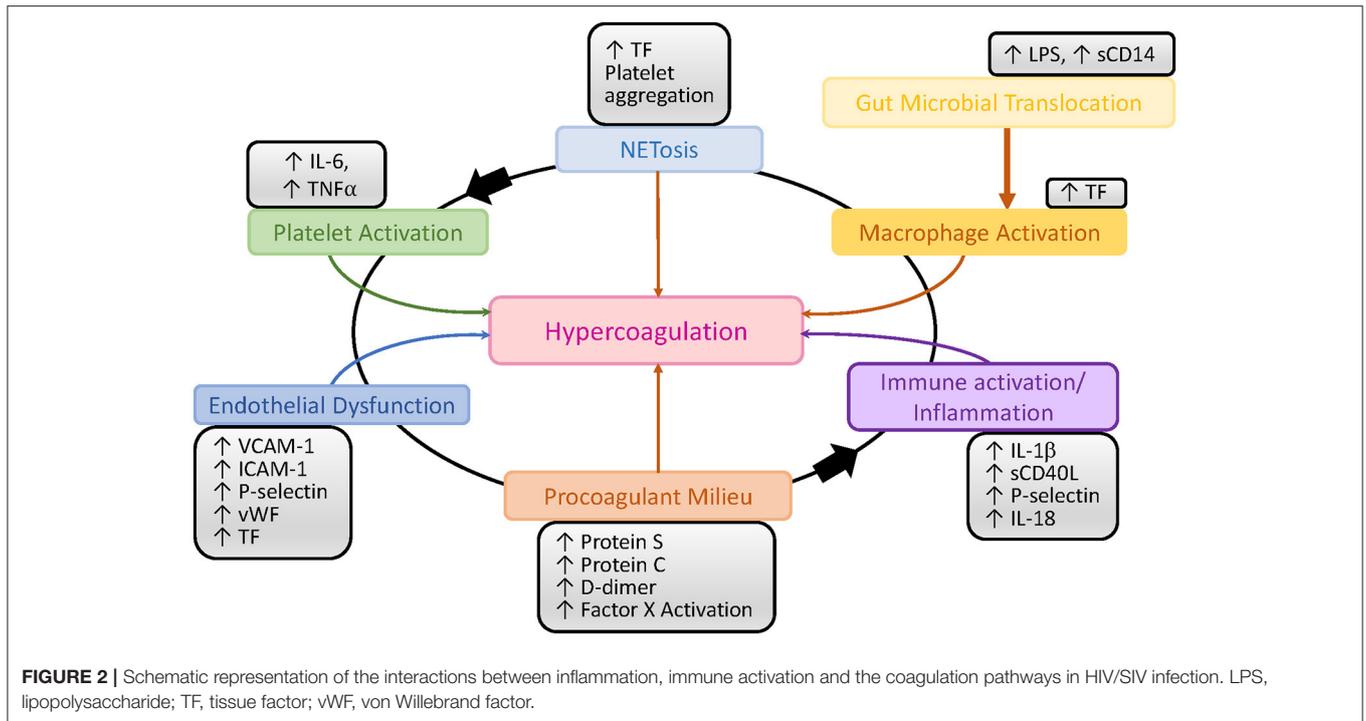
One of the best biomarkers to assess the hypercoagulable state and the risk of death in the PWH and SIV-infected monkeys is D-dimer (42, 43, 87, 118–120), a byproduct of fibrin cleavage by plasmin. D-dimer plasma levels correlate with the total thrombolytic activity. The levels of D-dimer in the PWH included in the SMART cohort were 94% higher than in uninfected donors recruited in the MESA cohort (121). Two independent retrospective case control studies further confirmed that the increased levels of D-dimer in PWH were associated with CVD and VTE (122, 123). The elevation of D-dimer is not entirely correlated with plasma viral load, as virologically suppressed PWH still present increased levels of D-dimer (124). Further studies have confirmed that D-dimer levels were reduced but not normalized in ART-treated patients with undetectable viremia, and that higher levels of D-dimer in those patients were associated with an increased risk of SNAEs (87, 125, 126). Indeed, the frequency of myocardial infarction in PWH is significantly

higher than in their non-infected peers (127). High D-dimer levels in both PWH and their uninfected counterparts have been shown to lead to immune activation and inflammation through the increase in cytokines, such as IL-6 (128, 129). Finally, high D-dimer levels are associated with immune reconstitution inflammatory syndrome (IRIS) and death, even after adjusting for other factors (130). The importance of D-dimer has also been shown in pathogenic SIV infections (42, 89, 117, 120), in which D-dimer increased early after infection (Figure 3) and correlated with immune activation and inflammation. Furthermore, in SIV-infected pigtailed macaques (PTM), the increases in D-dimer levels are associated with the presence of microthrombi in tissues (Figure 3). These microthrombi may promote hypoxia and tissue fibrosis (Figure 3) and thus significantly contribute to the lack of immune reconstitution and to the development of numerous comorbidities. This may provide a new mechanism responsible for the development of concomitant comorbidities and of end stage organ diseases in PWH. Conversely, non-progressive SIV infections of the natural hosts, in which D-dimer levels remain at the preinfection levels during chronic infection, do not develop CVD or thrombotic lesions (42, 131, 132).

These data show the importance of D-dimer levels as a biomarker of morbidity and mortality in PWH, and underline the strong relationship between coagulation and inflammation.

Tissue Factor

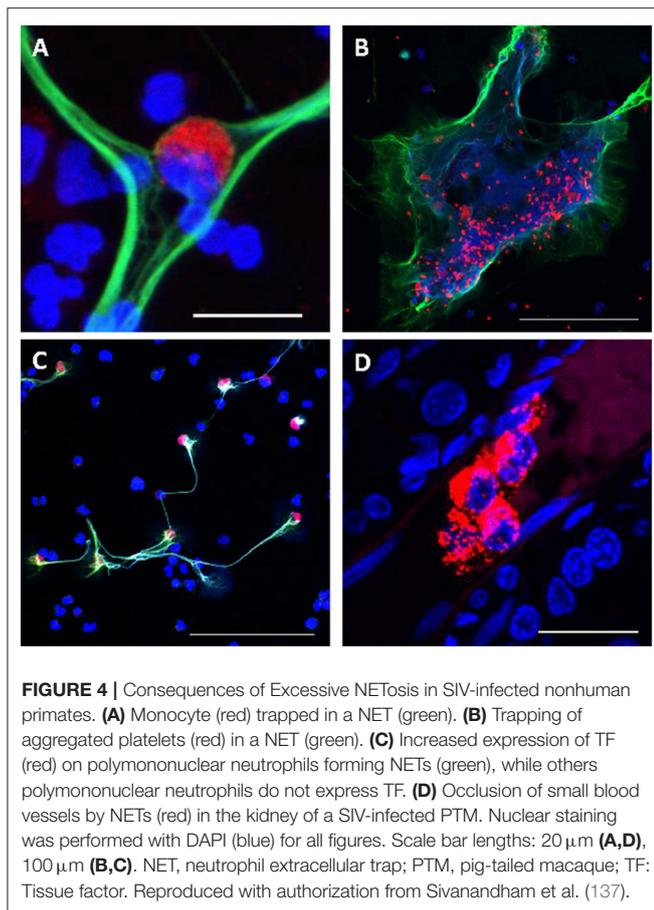
TF initiates the extrinsic clotting pathway of the coagulation cascade (Figure 2), playing a pivotal role in thrombosis (133). TF is expressed on the surface of monocytes in PWH (134) and SIV-infected NHPs (135), and it is thought to be at least partly induced by the inflammation triggered by microbial translocation (134, 135) (Figure 2). Increased expression of TF on the surface of monocytes likely contributes to the atherosclerotic plaques that lead to CVD in PWH and to increased clot formation and thrombosis. These TF-expressing monocytes persist even when PWH are virologically suppressed with ART, and they can trigger both coagulation *via* the activation of factor X (135) and production of inflammatory cytokines, including interleukin-1 β , IL-6, and TNF α . TF has also been found to be expressed on CD8⁺ and CD4⁺ T cells during HIV infection and its levels correlated with those of the T-cell immune activation marker CD38 (136). Similar findings have been observed in NHP models: in SIV-infected macaques, TF-expressing monocytes were crucial to the development of coagulopathy and its associated proinflammatory environment (135). Recently, we showed that in SIV-infected PTMs, TF is also expressed by neutrophils that release NETs and by the NETs themselves, predisposing these animals to pathological clotting (137) (Figure 4). Altogether these results place TF at the intersection between coagulation and inflammation, suggesting that TF inhibition may represent a valuable therapeutic strategy to reduce the risk of comorbidities in PWH (138).



Endothelial Dysfunction

Endothelium integrity is quintessential to hemostasis and its disruption leads to an inflammatory response that can promote VTEs, including platelet aggregation on the exposed subendothelial structures and clotting activation (139). HIV proteins can also cause endothelial dysfunction by reducing antioxidants through limiting nitric oxide bioavailability and increasing cell permeability (140). The association between HIV and endothelial dysfunction was reported early in the

epidemic and has been confirmed in multiple studies which reported increases in VCAM-1, ICAM-1, P-selectin, sTF, and Von Willebrand factor in chronically HIV-infected individuals (140–142) (**Figure 2**). Studies have reported a short-term improvement of the endothelial dysfunction after initiation of ART (143, 144), while others reported that prolonged exposure to certain antiretrovirals has a detrimental effect on the integrity of the endothelium, leading to CVD and VTEs (145, 146).

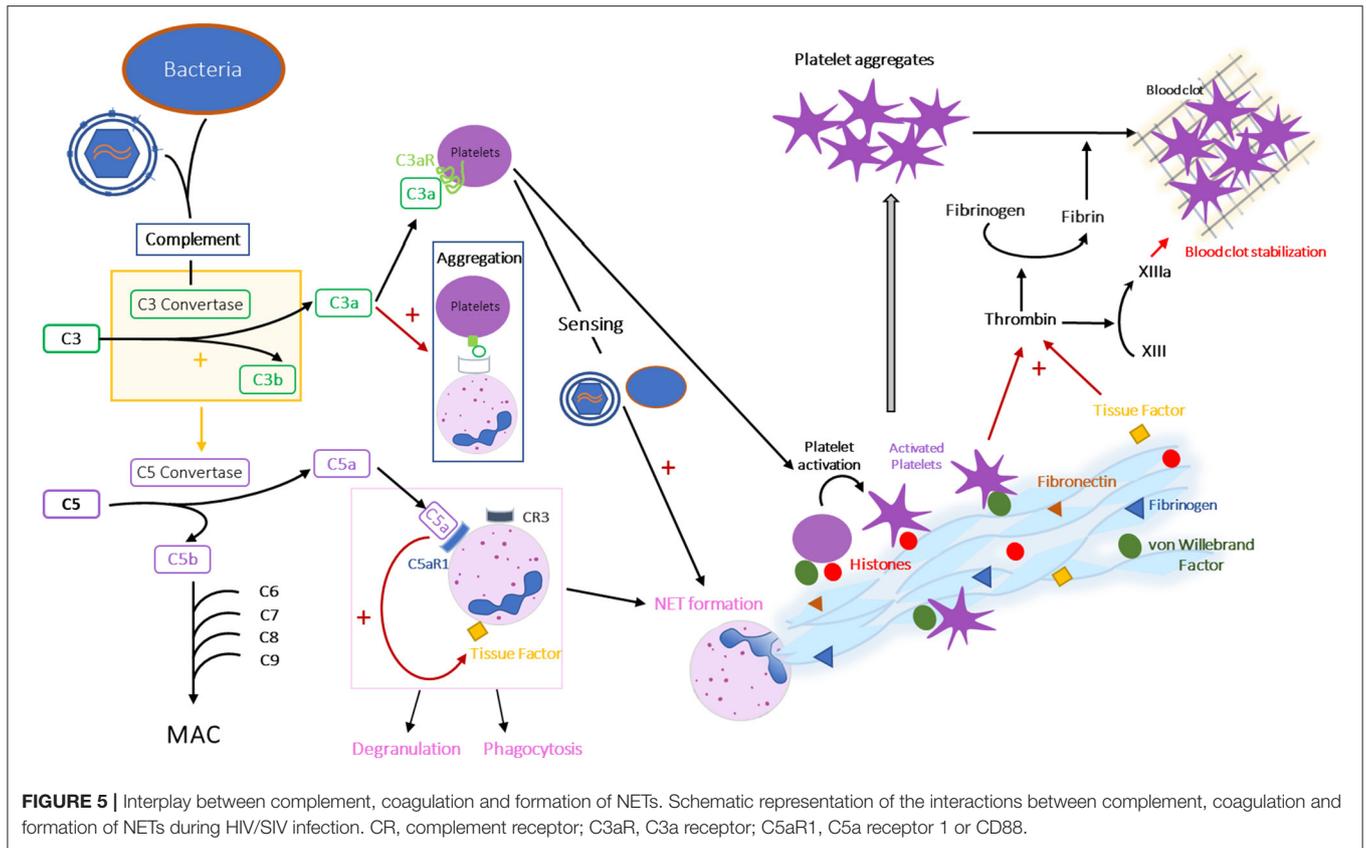


Platelets

Platelets also play a significant role in HIV-induced hypercoagulation (Figures 2, 4). Platelet aggregation can limit pathogens access to the vascular system and they can also directly interact with pathogens and alter the inflammatory processes triggered by infection (147). Several studies have confirmed the interactions between platelets and HIV and have shown that these interactions lead to virus internalization and platelet activation which, in turn, can propagate the virus and lead to a thrombocytopenia (148, 149). Another plausible reason for the paradoxical thrombocytopenia associated with the SIV-induced hypercoagulable state is platelets being trapped and destroyed in NETs that are excessively released by neutrophils during SIV infection (137) (Figure 4). Platelet activation contributes to the inflammatory environment seen in PWH, which is characterized by increases in IL-1 β , sCD40L, P-selectin, and IL-18 (150). Despite undetectable viremia, ART-treated PWH continue to present increased platelet activation and spreading (i.e., the process of platelet flattening at sites of vascular injury to increase their contact area) (151). Meanwhile, platelets from PWH also experience apoptosis and mitochondrial dysfunction (151), suggesting that monitoring platelet functions might be clinically relevant in PWH to assess their overall risk for VTE and CVD (152).

Targeting Hypercoagulation in PWH as an Adjunct Intervention to Control Immune Activation and Inflammation

Attempts have been made to reduce hypercoagulation in PWH. (i) The use of warfarin in PWH is limited by many drug-drug interactions that result in changes in warfarin metabolism, notably by inhibiting cytochrome P450 3A4, as well as the need to monitor INR levels through regular blood tests (153, 154). Therefore, its use has decreased sharply over the recent years in favor of direct oral anticoagulants (DOACs). (ii) Aspirin is one of the most widely used and well-studied anticoagulants that reduces platelet activity and aggregation. Aspirin also significantly reduced platelet activation and platelet hyperactivity in PWH (155). In addition, aspirin showed beneficial effects in reducing the levels of T-cell activation (CD38 and HLA-DR), monocyte activation (sCD14), platelet activation (P-selectin), and responsiveness of leukocytes to TLR agonist stimulation after only 1 week of treatment (156). Yet, no significant impact of aspirin on VTEs and inflammation has been reported in PWH (157, 158). Aspirin was reported to be less effective for the primary prevention of myocardial infarction in PWH compared to the normal population (159). It has also been shown that the risk of a recurrent acute coronary event was 6.5 times higher in ART-treated PWH than in matched, uninfected patients (160). These studies suggest that the current strategies for primary and secondary cardiovascular disease prevention in PWH should be tailored to this patient population, to include more potent anticoagulants, as well as other therapeutic targets, notably regulating lipids levels. Important insights could be obtained through the large, ongoing REPRIEVE clinical trial that evaluates the efficacy of statins for the primary prevention of cardiovascular events in PWH. This cohort confirmed the high incidence of coronary atherosclerosis in PWH on ART, the presence of plaques being associated with increased immune activation (161). (iii) Angiotensin receptor blockers have also been evaluated in PWH, to assess their potential benefits in reducing endothelial injury. After a 3-month prospective study, telmisartan administration led to an increase in endothelial progenitor cells, but had no effect on immune activation (162–164). (iv) Ixolaris, a TF inhibitor that blocks the extrinsic coagulation pathway at its origin, is a promising candidate for the treatment of hypercoagulation in PWH. In chronically SIV-infected pigtailed macaques, Ixolaris decreased D-dimer expression and TF activity, and reduced monocyte activation (135). Inflammation was not impacted in this study, except for IL-17, which was slightly reduced by Ixolaris administration. (v) Several studies assessed the benefits of DOAC administration in PWH. A longitudinal cohort study assessed DOAC safety among PWH who were undergoing ART (165) and concluded that there were similar effects compared to those taking warfarin, i.e., that these patients should be monitored as drug-drug interactions are likely, especially when DOACs are coadministered with protease inhibitors. This can lead to an increased risk of bleeding. Meanwhile, association with non-nucleoside reverse transcriptase inhibitors (NNRTIs), such as nevirapine and



etravirine, can lead to reduced plasma DOAC levels and increase the risk of VTE (166). When dabigatran, a direct thrombin inhibitor, was administered to SIV-infected pigtailed macaques, it did not normalize coagulation parameters and resulted in a cardiovascular event in the study group. Edoxaban, a factor Xa inhibitor, significantly decreased the D-Dimer and TAT levels among PWH, but did not impact inflammation (167). Vorapaxar, a PAR-1 inhibitor did not impact HIV-related hypercoagulation, in PWH, as shown by the lack of effect on D-Dimer levels (168). In SIV-infected pigtailed macaques, Vorapaxar administration had only a modest impact on T-cell immune activation and did not normalize coagulation, nor did it impact disease progression.

In summary, the anticoagulant interventions performed so far resulted in some improvements in the levels of T-cell activation but did not normalize the inflammation biomarkers that are routinely measured in clinical trials to predict an improved clinical outcome.

Interventions aimed at blocking the coagulation cascade upstream seem to be more efficient than interventions targeting the extrinsic or common coagulation pathways. It can be hypothesized that, in HIV/SIV infections, once several coagulation factors have been activated and feedback loops are established between different pathways of the coagulation cascade, it is extremely difficult to counter the hypercoagulable state and its related inflammation.

HIV/SIV-INDUCED COMPLEMENT PATHWAY ACTIVATION

The complement system is a key effector of the innate immune system, which is also involved in the adaptive immune responses. It is composed of over 30 proteins, interacting in three pathways: classical, alternative and lectin pathways, all converging toward the production of C3 convertases that cleave C3 into C3a and C3b (Figure 5). The terminal, lytic phase is common to all pathways and is initiated by the C5 convertase that severs C5 into C5a and C5b. The latter combines with C6, C7, C8, and C9 to form the membrane attack complex (MAC) (Figure 5).

Complement activation fights microorganisms through multiple effector functions: (i) MAC is inserted into the membrane of the pathogen or infected cell that has triggered complement activation, leading to the formation of pores in the membrane and causing osmotic lysis; (ii) C3b binds to microorganisms insuring their opsonization and phagocytosis; (iii) C3a, C4a, and C5a induce modifications in vascular permeability, facilitate and attract immune cell subsets that fight against the microorganisms and induce histamine release from mast cells (169).

Two types of regulators of complement activation (RCA) are described, soluble factors and membrane-bound regulators. The later play an important role in clearing immune complexes from circulation (e.g., CR1) and protecting host cells from

the deleterious consequences of complement activation on self-cells (170). Those membrane-bound RCA include CD46, the membrane cofactor protein (MCP), a cofactor for complement factor I, which degrades C3b and C4b; CD55, the decay-accelerating factor (DAF), which accelerates the degradation of C3 and C5 convertases; and protectin (CD59), which limits C9 incorporation and thus MAC formation (170).

There are numerous triggers of the classic pathway, notably antibody-antigen complexes, apoptotic cells, C-reactive protein, beta-amyloid, and LPS (171, 172). The lectin pathway is activated by carbohydrates structures, such as oligosaccharides on bacterial membranes, as well as IgA or IgM (173, 174). The alternative pathway is activated by molecules expressed on non-self surfaces (microorganisms) or self surfaces (apoptotic and necrotic cells) (172). The alternative pathway is usually spontaneously activated at low levels, but can be further amplified after the activation of the classical pathway through interactions *via* C3b.

In HIV-1 infection, complement activation can be triggered through multiple mechanisms: (i) activation of classical pathway by C1q binding to anti-HIV antibodies (175) and C1s binding to gp41 (176); (ii) activation of lectin pathway through gp120 recognition by the mannose binding lectin (MBL) (177); and (iii) an amplification loop through the alternative pathway; can also boost this complement activation. Furthermore, the microbial translocation observed during pathogenic HIV/SIV infection can lead to the activation of all complement pathways: (i) the lectin pathway *via* the recognition of Pathogen Associated Molecular Patterns (PAMPs), (ii) the alternative pathway *via* LPS, (iii) the classic pathway, which is triggered by the inflammation through LPS and the production of CRP, that can bind to C1q (178).

Through these multiple pathways, increased activation of the complement system occurs in PWH, as indicated by the increased levels of the complement cleavage products (179). Interestingly, complement activation increases with disease progression to AIDS, as well as in PWH developing IRIS (180–183).

While lower levels of the classic pathway activation were observed in PWH initiating ART, as emphasized by a modest increase in classic total complement activity and a 30% increase in intact C3 and C4 proteins after 12 weeks of ART, the levels of factor B (a factor involved in the formation of the alternate C3 convertase) are not completely restored on ART, suggesting that the alternative pathway is still activated (184). The residual complement activation in virologically-suppressed PWH was also observed in other studies reporting a 64% increase in C3a levels (185) and a 9% increase in soluble MAC (186), compared to uninfected subjects. Note that the levels of soluble MAC were particularly increased in virologically suppressed subjects with CD4⁺ T cell counts below 200/mm³ (186). Furthermore, while the levels of mannose-binding lectin increased in untreated PWH, they did not decrease over a 2-year period of ART (187). This residual complement activation may have multiple sources: (i) limited, but persistent viral replication in blood and/or lymphoid tissues leading to the activation of the classical and lectin pathways by gp41 and gp120 binding to C1s and MBL, respectively, as well as by anti-HIV antibodies triggering classic pathway, and (ii) residual microbial translocation causing an activation of all pathways (188).

Yet, despite this steady complement system activation, the complement-mediated HIV/SIV virolysis is limited (189). This high resistance of HIV and SIV to complement-mediated virolysis is due to the presence on viral membranes of membrane-bound RCA, such as CD46, CD55, and CD59, that are acquired during the budding from the cellular membrane (189–191). In addition to this viral escape mechanism, HIV and SIV can also hijack complement-induced opsonization. Immune cells recognize opsonized viruses through their FcR or complement receptors (CRs), and HIV and SIV can either infect these cells (192, 193) or use them as vehicles for transport to the germinal centers of lymphoid tissues (194). For example, follicular dendritic cells (FDC) exhibit CR1, CR2, and CR3, allowing them to bind opsonized viruses. In the follicles, viruses can either infect nearby T lymphocytes, or remain trapped by FDC, enabling affinity maturation of B cells. However, when trapped by FDC, viruses remain intact and infectious for months, constituting one of the most important viral reservoirs in the organism (195). Infection of the T CD4⁺ lymphocytes and viral trapping in the lymphoid tissues fuel immune activation in PWH and lead to histological alterations of the lymphoid tissues (196, 197). Intriguingly, while the pathogenic HIV and SIVmac are trapped in germinal centers of humans and macaques, respectively, this phenomenon is less frequent in non-pathogenic SIV infections of the natural African NHP hosts (32, 198–201), indicating that deposition of complement-opsonized viral particles in the lymphoid tissues might be critical for disease progression.

Complement may also impact HIV pathogenesis by fueling immune activation and inflammation. C3a is a proinflammatory factor that attracts macrophages, monocytes and mast cells, triggers histamine release by mast cells, induces the production of proinflammatory cytokines, such as IL-1 β , IL-6, and TNF- α by monocytes and macrophages, induces eosinophil degranulation, prompts platelet activation, and increases vascular permeability (202). C3a is also an immunomodulatory agent as it inhibits neutrophil mobilization (203). C5a is a potent inducer of inflammation after binding to its receptors, C5aR1 (CD88) or C5aR2. The inflammatory effects of C5a include, but are not limited to, recruitment of the immune effectors (macrophages, monocytes, dendritic cells, neutrophils, lymphocytes), activation of neutrophils, which result in enhanced production of the inflammatory cytokines IL-6 and TNF- α , increases in vascular permeability and tissue factor expression on endothelial cells, and suppression of Treg. Interestingly, CRP, IL-6, and TNF- α , whose expressions are increased during complement activation, have all been linked to disease progression and comorbidities in PWH (87, 126, 204–206). Thus, it is not surprising that complement activation itself has been linked to different comorbidities, notably neurological, kidney, and cardiovascular diseases, both in PWH and in the general population.

Complement and Neurological Comorbidities

HIV-associated neurocognitive disorders (HAND) have been described since the dawn of the AIDS pandemic (207). HAND aggregates wide clinical presentations ranging from HIV-related

dementia, mostly characteristic to the pre-ART era, to milder neurocognitive (NC) impairments (208). With the advent of ART, incidence of HIV-related dementia has dramatically declined, but 30–50% of PWH still present NC disorders (209), with mild NC impairments now accounting for most NC diseases among PWH (210). There are multiple mechanisms through which HIV can induce neurocognitive disorders: neurotoxicity of viral proteins produced by infected cells (macrophages or glial cells, including astrocytes) (211), death of the productively-infected cells (212), brain damage (edema, weakening of the blood-brain barrier) induced by the immune and/or inflammatory responses (213), and ART neurotoxicity (214). These mechanisms are non-exclusive. Complement could also play a role in HIV neurotoxicity, as it leads to apoptosis and increased production of inflammatory cytokines. This is not unexpected as complement is involved in synapse pruning *via* C1q during brain development (215). Furthermore, complement might also play a role in some autoimmune neurological diseases, Alzheimer's disease, and multiple sclerosis (216).

In PWH that progress to AIDS, complement activation can be observed in the cerebrospinal fluid (CSF), with increased levels of C4 indicative of intrathecal synthesis (217). While most complement proteins are synthesized by hepatocytes, they can also be produced by other cells, notably astrocytes (218). Increased levels of C3 and C4 have been found in the CSF of PWH with NC dysfunction (219), and HIV-1 infection induces *in vitro* expression of C2 and C3 in infected-astrocytes (220, 221). This effect was observed in cell cultures treated with both whole virions and after incubation with only viral proteins gp41 or Nef (221, 222). HIV-1 also induces C3 expression in neurons (221, 222). In macaques with SIV encephalitis, high C3 and C1q expression on astrocytes and neurons was immunohistochemically detected, while the same cells were rarely positive in uninfected animals (223). Interestingly, C1q expression increased throughout the course of SIV infection and was correlated to CSF viral load (224), a marker of HIV-related dementia (225). Overexpression of C3 was confirmed in postmortem brain tissues from patients with HAND (226). Upregulation of the genes involved in the interferon responses and the complement pathway was higher in PWH with NC impairment and HIV encephalitis compared to those without NC impairment (227). Interestingly, a longitudinal study of PWH with NC impairment found that patients with improved neurocognition presented a down-regulation of the complement system, and *vice versa* (228). Another potential explanation for the neurological dysfunction in PWH is that neurons and astroglia of the PWH may be more susceptible to complement-mediated lysis, as the expression by neuronal and astroglial cell lines of CD59, one of the membrane-bound RCA, is down-regulated after incubation with gp41 peptides or inflammatory cytokines (229).

Despite a clear association between complement activation and NC impairment, the mechanism(s) through which complement activation impacts neurocognitive functions is not yet fully defined. As inhibitors of complement activation are developed for non-HIV related neurological diseases, this

could pave the way for their evaluation in PWH presenting with HAND or with other NC disorders.

Complement and Renal Comorbidities

Increased incidence of kidney diseases was reported early in PWH (230). In addition to the HIV-associated nephropathy (HIVAN), other kidney diseases are described in PWH, notably HIV-associated immune complex kidney diseases (HIVICD) (231). HIVICD are glomerulopathies with glomerular deposition of immune complexes. Kidney involvement in PWH varies according to the location of the deposits of immune complexes. Positive staining of renal biopsies for IgG, IgM, IgA, C3, C4, and C1q, and hypocomplementemia is frequently reported (232). The high frequency of the immune complexes in the kidney may play a role in the complement activation and/or in tissue damage in PWH (231). Unlike HIVAN, HIVICD is not significantly impacted by ART in PWH (233).

Complement and Other Comorbidities

Recently, complement activation, particularly the increased C3 levels, has been associated with various metabolic and CVD in the general population. In a large cohort of patients followed over 6 years, patients with high C3 levels at inclusion had an increased risk of developing diabetes (234). Increased C3 levels were also associated with insulin resistance and non-alcoholic fatty liver disease (NAFLD), after adjusting for other variables (235, 236). The role of C3a in NAFLD and insulin resistance has also been demonstrated in animal studies, with C3aR^{-/-} mice being resistant to diet-induced diabetes (237, 238). Prospective studies reported an association of increased C3 or C4 levels with higher risks of myocardial infarction (239, 240).

In HIV-uninfected subjects with advanced atherosclerosis, increased C5a was linked to an exacerbated CVD risk (241). This might be explained by the proinflammatory and procoagulant properties of C3a and C5a, notably platelet activation and TF induction on endothelial cells, respectively. An increase in C5 and factor H-related protein 5 levels in PWH on ART and higher levels of C5 in patients with non-AIDS comorbidities were also recently reported (242).

Higher C3 levels were associated with metabolic syndrome in PWH on ART (243), even after adjusting for confounding factors, but no data are available regarding association of complement proteins with NAFLD or CVD among PWH. Thus, the involvement of complement activation in driving the high frequency of CVD in PWH remains to be determined.

TARGETING COMPLEMENT PATHWAYS

Currently, only four drugs targeting the complement system are approved by the FDA: two C1 esterase inhibitors and two C5 inhibitors (244). The C1 esterase inhibitors hinder C1s, C1r and mannan-binding lectin-associated serine protease (MASP), and they are used to treat patients with hereditary angioedema caused by a deficiency in C1 (SERPING1) inhibitor. The C5 inhibitors, eculizumab and ravulizumab, are monoclonal antibodies that bind to C5 and prevent its cleavage, and thus the formation of MAC. Eculizumab was developed for paroxysmal nocturnal

hemoglobinuria, but its off-label use for kidney diseases of PWH has been reported (245). Numerous complement inhibitors are currently under development, including: a MASP-2 inhibitor (narsoplimab), C3 inhibitors (compstatin and its analogs, such as AMY-101, and pegcetacoplan), a C5aR1 antagonist (avacopan), an anti-C5aR1 monoclonal antibody (avdoralimab) and a factor B inhibitor (244). Their evaluation in PWH in clinical trials aiming to limit development of comorbidities may therefore be considered.

NET FORMATION

Generation of neutrophil extracellular traps (NETs) by polymorphonuclear neutrophils is a recently described mechanism of the innate immune response (246). Neutrophils are key effectors of the innate immune system, being traditionally considered to be involved in the defense against bacteria and fungi (246). More recently, they have been reported to be involved in the innate immune response against viruses (247). The principal mechanism of action of neutrophils is phagocytosis and killing of microorganisms through degranulation and production of reactive oxygen species. It was recently discovered that they can also control infection through generation of extracellular chromatin fibers called NETs (248). Neutrophils that release NETs develop a unique cellular morphology with decondensed nuclei, and ultimately lose their DNA (249). The extracellular chromatin fibers of the NETs are decorated with numerous granule proteins, such as lactoferrin, myeloperoxidase (MPO), histones, and neutrophil elastase (NE) (250). NETs can capture and kill bacteria, fungi, and viruses (246, 250, 251). *In vitro* generated NETs are long, thin stranded, web-like, extracellular fibers (246). NETs with a thicker morphology were identified *in vivo* in the gut, liver, skin, and lung in numerous diseases (249, 252, 253).

NETs are beneficial for the organism by eliminating microorganisms and preventing their spread, yet long-lasting or excessive NET formation can be deleterious, as they induce a proinflammatory environment, damage the tissues and can trigger immunothrombosis (254).

The involvement of NETs in the pathogenesis of HIV and SIV has only been studied recently. NETs can capture and eliminate HIV-1 *in vitro* (255, 256) and their production seems to be regulated by IL-10 (255). Formation of NETs by neutrophils isolated from SIV-infected macaques, upon stimulation with phorbol myristate acetate (PMA), is enhanced compared to neutrophils isolated from uninfected non-human primates (137).

Increased NET formation occurs early during the acute SIV infection, probably triggered directly by the high virus replication. Interestingly, NET formation by neutrophils is higher during chronic infection than during acute infection. This is not unexpected, as the triggers for NET formation diversify and increase in magnitude during later stages of infection. They include bacterial products translocated from the gut, and opportunistic pathogens, in addition to the retrovirus. NETosis is reduced by ART, but the number of NETs remains higher than in uninfected animals (137). Besides capturing virions and

SIV/HIV infected cells, NETs can, especially when they are released in excess, also indiscriminately trap other immune cells (such as neutrophils, T, B cells, macrophages, etc.) and induce their apoptosis or lysis (**Figure 4**) (137). Thus, NETs might play a significant role in the destruction and/or lack of recovery of numerous immune cell subsets during SIV/HIV infection and thus provide a plausible explanation for a key unsolved aspect of HIV/SIV pathogenesis: the bystander loss of cells that are not directly infected with HIV/SIV. Besides the negative consequences related to the death of multiple immune cells, the overly stimulated neutrophils may represent a neglected source of inflammation in SIV-infected macaques and PWH. As neutropenia is often observed during SIV/HIV infection, the impact of neutrophils on SIV/HIV pathogenesis could be questioned but, similarly to CD4⁺ T cells, the issue might not rely on the absolute neutrophil counts, but rather on the activation status and the production of inflammatory cytokines by this immune cell subset. By trapping immune cells in the NETs, the neutrophils may contribute to their activation and death, both processes being associated with release of inflammatory cytokines or chemotaxis of other cells resulting in a fueling of inflammation. As such, normalizing NET formation may be critical to avoid tissue damages and comorbidities characteristic to HIV/SIV infection (117).

NETs can also trap and destroy platelets (**Figure 4**), contributing to platelet loss. Interestingly, while this observation provides a plausible explanation for the thrombocytopenia associated with SIV/HIV infection, it also links NETs to the coagulation issues seen in PWH and SIV-infected macaques (**Figure 4**) (137). It was recently showed that TF expression by NETs (**Figure 4**) and their ability to capture platelets potentiate each other to promote a proaggregative environment and platelet activation, leading to a hypercoagulable state. TF production by neutrophils may also play an important role in triggering systemic and tissue INFL, as does the TF expression by monocytes.

The contribution of NETs to the pathogenesis of HIV/SIV infection was recently supported by multiple findings in other infectious diseases. Thus, excessive formation of NETs is detected in severe influenza and SARS-CoV-2 infections (257, 258), where it is correlated with an higher mortality (259). Meanwhile, NETs also play a deleterious effect in cancer by promoting metastasis (260), and in autoimmune diseases and atherosclerosis by promoting macrophage-mediated inflammation (261). NETs can also facilitate venous thrombosis (262, 263), through expression of tissue factor (TF) (264, 265), platelet deposition, aggregation and activation (262), activation of factor XII (266), and cleavage of TF pathway inhibitor (267).

TARGETING NET FORMATION

No drug inhibiting NET formation is currently FDA-approved. However, several clinical trials are ongoing, most of them using nebulized DNase that degrades NETs. Other compounds are currently investigated for autoimmunity diseases, infectious diseases or cancers, either in humans or in animal models.

This includes PAD-4 inhibitor (Cl-amidine), neutrophil elastase inhibitor (GW311616A), serine protease inhibitor (camostat mesylate), gasdermin D inhibitor (disulfiram) and C3 inhibitor (Cp40) (268–272). If any of these drugs is proven to be efficient in reducing NET formation in other pathologic conditions, it could be interesting to evaluate them in the context of HIV/SIV infection. However, preliminary studies using NHP models will be needed, both to confirm their efficacy on reducing the virus-driven NETosis and to collect pharmacokinetics data on those different compounds. Indeed, based on their administration route (per os, intravenous or subcutaneous), tissue distribution of those drugs could vary and modulate their efficacy in preventing NET formation.

INTERACTIONS BETWEEN COMPLEMENT, COAGULATION AND NETS

It is now well-understood that complement, coagulation, and formation of NETs are intertwined mechanisms (Figures 1, 5). Indeed, complement protein C3a can activate platelets, by binding to its receptor C3aR present on platelets (273), and platelet-bound C3a can promote the platelet aggregation to neutrophils by binding to complement receptor 3 (CR3) expressed on the neutrophil membrane (274). Meanwhile, platelets are involved in the NET formation, as they can sense LPS through TLR4, which leads to their activation and adhesion to neutrophils, eventually inducing NETs (275). C5a is a neutrophil chemoattractant, leading to neutrophil recruitment and activation (276). C3a and C5a have also been linked to an induction of NETs, C3a through a mechanism remaining to be determined (277) and C5a by increasing TF expression and upregulating complement receptors on neutrophil surface (278, 279). Conversely, complement activation can also be triggered by activated neutrophils and their NETs, as complement proteins can deposit on their surface and form C3 convertases, leading to the production of new anaphylatoxins (280). Note that regulatory mechanisms can also be involved in these interactions. For example, factor H, a known complement inhibitor, can also bind to neutrophils and inhibit NET formation (281).

In addition to these interactions between complement proteins and NET formation, both complement and NETs interact with the coagulation cascade. Thus, both C3a and C5a participate in thrombus formation (282, 283). C3a is linked to platelet activation, while C5, after binding to its receptor C5aR1 (CD88), induces tissue factor expression on endothelial cells and neutrophils (284, 285). C5a is also thought to play a role in TF activation (282). Multiple effectors of coagulation are detected on the extracellular fibers of the NETs, including von Willebrand factor, fibrinogen, fibronectin (262) and TF (137). Besides, some proteins associated with NETs (neutrophil elastase and myeloperoxidase) can inhibit anticoagulant factors, such as thrombomodulin (286). When platelets adhere to NETs by binding to histones or to von Willebrand factor, they are activated and form platelet aggregates (137, 262) (Figure 3). Thrombin is then generated by platelets trapped in NETs after the activation of the coagulation cascade by the TF exposed by

NETs and/or by NET-damaged endothelial cells (287), while the contact coagulation pathway can be initiated by the activation of factor XII by NETs and platelets (266). Transformation of fibrinogen by thrombin leads to fibrin deposition on the NETs, and to the formation of an immunothrombus (262). During infections, NETs and immunothrombosis prevent the spread of microorganisms throughout the body. As this process is constrained to microvessels, it prevents deleterious effects. However, in some cases, an uncontrolled immunothrombosis can lead to formation of pathological thrombi. It is therefore conceivable that such a scenario is present during HIV/SIV infection, especially in PWH off ART. Immunothrombosis in small blood vessels (Figure 4D) could drive the development of comorbidities and end-stage organ diseases.

CONCLUSION

Numerous mechanisms are involved in the persistence of low levels of inflammation and T-cell immune activation in ART-treated PWH. The intertwined roles of hypercoagulation, complement system and NET formation has recently emerged, highlighting different pathways that could be targeted to reduce the risk of SNAEs that remains elevated in ART-treated PWH.

Anticoagulant drugs have been available for decades, with DOACs recently expanding the panel of drugs, even though drug-drug interactions remain an issue for PWH. Meanwhile, drugs targeting the complement system and/or NET formation are either newly approved or still in research or industry pipelines. Interestingly, some drugs, such as C3 inhibitors, can inhibit two pathways (complement and NET formation) *in vitro*. Evaluating these compounds in NHP models, then in PWH if deemed promising, would be of great interest, as they might be able to thwart different mechanisms involved in the residual inflammation in ART-treated PWH. Studies gathering both clinical and biological data would be necessary, to observe if those drugs effectively reduced inflammation and immune activation, and if this translates into a reduction in the risk of SNAEs.

AUTHOR CONTRIBUTIONS

TR, LT, RT, AL, IS, CA, and IP wrote, edited the manuscript, prepared, and selected the figures. All authors contributed to the article and approved the submitted version.

FUNDING

TR, LT, CA, and IP were supported by grants from the National Institutes of Health/ National Institute of Diabetes and Digestive and Kidney Diseases/National Heart, Lung and Blood Institute/National Institute of Allergy and Infectious Diseases: RO1 HL117715 (IP), RO1 HL123096 (IP), RO1 DK130481 (IP) RO1 DK113919 (IP/CA), RO1 DK119936 (CA), RO1 AI119346 (CA), RO1 DK131476 (CA), RO1 HL154862 (MF/IP). The work of IS was supported by the intramural research program of NIAID/NIH. AL was supported by

UM1-AI106701 AIDS Clinical Trials Group Immunology Support Laboratory. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We would like to thank Adam Kleinman for helpful discussion and critical reading of the manuscript.

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