Specific Aims

Neutrophils are the first responders to infection and are crucial for defending against pathogens. Despite their crucial role in immunity, we know relatively little about neutrophil diversity, dysfunction, or how they contribute to HIV-related comorbidities. One of neutrophils' defense mechanisms is the release of a web-like chromatin structure alongside antimicrobial granules, so called "Neutrophil Extracellular Trap" (NET)[1]. Far beyond their antimicrobial role, a growing body of evidence over the last decade has demonstrated the clinical association between NETs and thrombotic complications[2-6]. However, the current understanding of NETs in HIV has mainly focused on their role in immune defense[7-9], leaving the clinical relevance of NETs in HIV-associated thrombosis largely unknown.

Studies have demonstrated that low-density granulocytes (LDGs), a neutrophil subset in peripheral blood mononuclear cells (PBMCs), display a proinflammatory phenotype in diseases, such as autoimmune and infectious diseases[10-12]. Moreover, LGDs are a potent producer of NETs[13, 14]. However, it remains unknown whether HIV+ neutrophils preferentially allow NET formation and what effect they have on platelet activation and coagulation. In this study, we will investigate the functional characteristics of LDGs and their ability to form NETs compared with normal-density granulocyte (NDGs) in HIV+ and healthy individuals. Also, we will evaluate what role aberrant NET formation plays in pathological thrombosis in HIV+ individuals.

Specific Aim I. Compare neutrophil subtypes, activation status, and NET forming efficiency in HIV+ and healthy individuals.

To compare neutrophil heterogeneity in healthy and HIV+ individuals, we will analyze the proportion of two neutrophil subsets, NDGs and LDGs, present in the circulating blood of both healthy and virally suppressed HIV+ individuals by flow cytometry (FACS) analysis. To define unique functional characteristics of LDGs in HIV+ individuals, we will analyze expression of neutrophil activation (CD11b, CD66b, CD63, Arginase-1, and CD62L) and migration markers (CXCR2 and CD62L) on LDGs isolated from HIV+ (HIV LDGs) and healthy individuals (HC LDGs) by FACS analysis. These neutrophil markers will also be analyzed in HIV NDGs to determine whether NDGs and LDGs are differentially activated in HIV+ individuals. Also, differences in NET formation between two neutrophil subsets in HIV+ individuals will be evaluated.

Specific Aim II. Determine the association between NET formation and thrombosis in HIV+ individuals.

Our *in vitro* assay has shown that plasma collected from HIV+ individuals triggers neutrophils to form NETs and that this effect was abrogated when platelets were removed from plasma. These data suggest that a direct platelet-neutrophil interaction could facilitate NET formation. To examine the basal level of platelet activation and the proportion of activated platelets in HIV, we will analyze platelets expressing CD62P (P-selectin), CD63, and PAC1 (an epitope of glycoprotein IIb/IIIa) by FACS analysis. To investigate whether NETs play a role in the thrombotic predisposition in HIV, we will assess platelet-neutrophil aggregates (PNAs) in circulation and expression of coagulation factors (prothrombin, fibrinogen, and Factor X) present in NETs. Finally, plasma levels of NET and coagulation markers, such as thrombin-antithrombin complex (TAT), D-dimer, and plasmin and antiplasmin (PAP) as well as platelet activation markers, including P-selectin, platelet-derived microparticles (PMPs), and CD40L will be measured. We will then determine if NET levels are correlated with thrombotic markers in HIV.

The proposed work will advance our current knowledge of neutrophils in HIV and define the potential pathologic function of NET in HIV. In particular this work shed light on the role of neutrophils and NET in the increased risk of thrombosis in people living with HIV.

Significance

Antiretroviral therapy (ART) prolongs life expectancy and prevents AIDS-related complications. Nevertheless, people living with HIV (PLWH) have a high prevalence of non-AIDS related comorbidities, including cardiovascular disease (CVD), diabetes mellitus, malignancies, and thrombotic diseases[15-18]. HIV-associated thrombosis occurs largely in the absence of traditional thrombophilic risk factors (such as advanced age, family history of thrombosis, malignancies, and immobilization)[19, 20]. This clinical manifestation is thought to result from coagulation dysregulation[21]. Epidemiologic studies have demonstrated that coagulation biomarkers are elevated with HIV infection and generally their levels are positively corelated with inflammatory markers[22]. Thus, chronic immune activation and inflammatory state in HIV+ individuals may be related to abnormalities in coagulation pathway. However, the mechanisms behind the risk for dysregulated coagulation in HIV infection have not been

fully elucidated. Therefore, **a better understanding of the drivers of inflammation and coagulation in PLWH on ART is important, and by elucidating the mechanisms that contribute to abnormal coagulation, effective therapeutic intervention to ameliorate that risk can be revealed.**

Thrombosis is the most common pathology underlying major cardiovascular diseases (CVDs): ischemic heart disease, ischemic stroke, and venous thromboembolism (VTE)[23]. Notably, the proportion of mortality attributable to CVD in PLWH in United States increased more than doubled from 1999 to 2013[24], reinforcing the importance of primary prevention of CVD in PLWH. During thrombosis, innate immune cells such as monocyte and neutrophils, are involved in this process[25]. Neutrophils are rapidly recruited to the microvasculature of inflamed tissue and incite cell-specific prothrombotic pathways during thrombosis. NET formation not only serves antibacterial functions[7-9], but also induces procoagulation responses and facilitates platelet aggregation[25, 26]. Indeed, numerous studies have found that NETs are a major contributor to thrombogenesis in various diseases, such as atherosclerosis[4], coronary artery disease[27], sepsis[28, 29], and cancers[2, 3].

Despite the known contribution of neutrophils and NETs to thrombogenesis in general, it is less clear whether neutrophil activation and/or neutrophil-derived product are relevant for the formation of thrombi in HIV. To our knowledge, our preliminary data is the first time to show clinical evidence of the potential importance of NETs in HIV-associated thrombosis. Therefore, **the proposed study will provide previously undefined neutrophil function in chronic HIV as an effector of thrombosis and mechanism of coagulation augmentation by neutrophils.**

The functional characteristics of neutrophils in HIV are even less understood. Studies have suggested that neutrophils isolated from HIV+ individuals exhibit impaired chemotaxis, bactericidal activity, and oxidative burst abilities functionality[30-32]. Although the higher frequency of LDGs have been correlated with the severity of HIV[12], it remains unknown whether LDGs are a major neutrophil subtype that contribute to HIV-associated thrombosis. Also, a comprehensive and comparative immunophenotyping of activation and NET formation on the LDGs and NDGs has yet to be performed which is needed to link these cells to the risk of HIV-associated thrombosis and CVDs. Thus, **the proposed study will give a better understanding of neutrophil subtypes and their ability to contribution to the hypercoagulable state in HIV. This will enable us to potentially design an effective targeted therapy for HIV-associated thrombosis and CVD focused on eliminating the detrimental side effects of neutrophils.**

Innovation

NETs can have detrimental consequences for disease progression by promoting inflammation[34] or thrombosis[2, 5, 26], or supporting tumor cells to metastasize[35, 36]. Thus, it is widely accepted that NETs have both a protective and a pathological impact on various diseases. However, the existence of NETs and the biological and functional implications of NETs in chronic HIV remain mostly unclear. The proposed study will specifically focus on understanding their biological aspects leading to coagulation dysregulation in virally suppressed chronic HIV. Generating data from this study will provide clues for better prediction of non-AIDS complications associated with thrombosis and may lead to new therapeutic opportunities to minimize such risks.

Research on neutrophils is often challenging due to their short life cycle. To determine whether the systemic environment in chronic HIV infection can facilitate NET formation, we will utilize flow cytometry to detect NET forming neutrophils without setting up the in vitro culture. While studies have examined the LDG subset in various diseases, their function and role vary depending on the context of the diseases. Parallel analysis of NDGs and LDGs lends important context for the analysis and interpretation of immunophenotypic and functional data.

Approach

Specific Aim I. Compare neutrophil subtypes, activation status, and NET forming efficiency in HIV+ and healthy individuals.

Rationale: LDGs are proposed to be involved in thrombosis development by spontaneously releasing NET[14, 25]. Although it has been reported that the frequency of LDGs correlate with the severity of HIV[12], it remains unclear whether LDGs are more prone to release NETs than NDGs in HIV. Therefore, this aim is to a better understand how LDGs contribute to dysregulation of innate immunity and whether LDGs are the primary source of NETs.

We propose to analyze the proportion of NDGs and LDGs in healthy and HIV+ individuals. We will also conduct comparative immunophenotyping to determine activation state and assess NET formation capabilities on the LDGs and NDGs isolated from HIV+ individuals.

Preliminary data:

Our preliminary data showed the proportion of LDGs ($CD11b^{+}CD14^{-}CD15^{+}$) was significantly decreased in HIV+ individuals, compared to healthy individuals (Fig 1A and D). However, LDGs from HIV+ individuals displayed significantly higher CD11b expression, but lower CD16 expression, compared to healthy LDGs (**Fig 1E and F**). In addition, intracellular granularity of LDGs was significantly decreased in HIV+ individuals (**Fig 1B**). These data suggest that HIV+ LDGs have a more activated phenotype and the functional behaviors of LDGs can be altered by the systemic environment in chronic HIV infection.

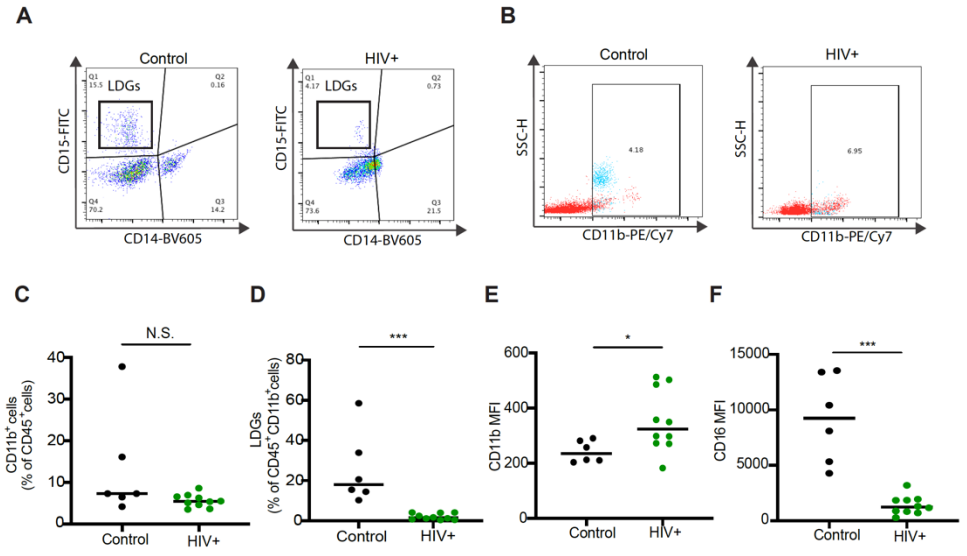

Figure 1. HIV+ low-density granulocytes observed increased CD11b and reduced CD16 expression.
(A) LDGs ($CD11b^{+}CD14^{-}CD15^{+}$) presence in healthy (control, n=6) and HIV+ individuals (HIV+, n=10).
(B) Blue dots indicate LDGs and their granularity was assessed by side scatter,SSC-H (B).
(C-F) Flow cytometry analyzed the percentage of the $CD45^{+}CD11b^{+}$cells (C) and LDGs (D) and the mean fluorescence intensity (MFI) of CD11b and CD16 expression levels on LDGs in healthy and HIV+ individuals. Mann–Whitney U test; * $P < 0.05$, ***$P < 0.001$.

Experimental design:

To analyze the differences between neutrophil subtypes in healthy and HIV+ individuals, we will utilize clinical samples obtained from the Ola Hawaii Program Project (U54MD007601) entitled: *"Role of Monocyte/Macrophages in HIV-related Cardiovascular Risk"*. This study is a cross sectional study enrolling 72 aviremic older (age>40 years) individuals with chronic HIV on ART and 72 HIV uninfected individuals of similar age, gender, and ethnic and socio-demographic composition. The HIV+ cohort is composed of fully viral suppressed individuals, defined by plasma HIV RNA<50 copies/ml. We can also obtain clinical laboratory data, including HIV CD4 nadir, and ART medication and immunologic data to include CD4, CD8 T cell, monocyte subsets, and soluble markers.

We will utilize a sample size of 12 HIV+ and 12 healthy individuals based on achieving 80~90% power by power calculation. Blood samples will be utilized for LDG ($CD11b^{+}CD14^{-}CD15^{+}$) and NDG ($CD11b^{+}CD16^{+}CD66b^{+}$) analysis. Then, we will compare the proportion of LDGs and NDGs between groups. To define functional characteristics of LDGs in HIV+ individuals, we will compare the mean fluorescence intensity (MFI) of neutrophil activation markers (CD11b, CD66b, CD63, Arginase-1, and CD62L) and migration markers (CXCR2 and CD62L) between HIV NDGs, HIV LDGs, and/or HC LDGs. Then, we will evaluate whether HIV LDGs are pre-activated and display phenotypic differences compared to NDGs from the same donor. To further determine whether HIV LDGs have a higher capacity to produce NETs, NET forming neutrophils will be identified as citrullinated Histone H3 (CitH3) and myeloperoxidase (MPO) expressing cells. CitH3 and MPO double positive events on neutrophils will be analyzed by FACS analysis and the proportion of NET forming neutrophils will be compared between neutrophil subsets.

For group comparisons, we will determine the mean ± standard deviation (SD) and compare a proportion of LDGs and activated LDGs between HIV+ individuals and healthy individuals using Mann-Whitney U test. In addition, group difference will be further investigated using generalized linear regression models fitted for two outcome variables: LDGs and MFI of neutrophil activation or migration markers, by filtering out effects of other potential factors that may affect the outcome. Possible covariates such as age and gender will be included in the analysis. The link function for such regression models will be selected upon the distribution of outcome.

Anticipated results, potential problems, and alternative approaches:

In our preliminary data, we found the percent of LDGs was significantly decreased in HIV+ individuals compared to that in healthy individuals although HIV LDGs display activated phenotype (**Fig 1**). The observed decrease in neutrophil counts in HIV+ individuals versus healthy controls is a possible explanation for decreased proportion of HIV LDGs. Therefore, we anticipate that both the proportion of NDGs and LDGs in HIV+ individuals are decreased, compared to healthy individuals. In chronic HIV, LDGs were shown to have less granularity (**Fig 1B**). Indeed, HIV+ neutrophils were able to form NET spontaneously in vitro, while control neutrophils did not, (**Fig 2**) suggesting that neutrophils in chronic HIV infection are highly susceptible to forming NETs. Thus, we expect to see increased expression of activation markers in HIV+ neutrophils. Study have shown the phenotypic difference between NDGs and LDGs in HIV+ treatment-naïve individuals[12]. LDGs represent immature and degranulated neutrophils. We anticipate to also find that neutrophils from HIV+ individuals on ART are hyperactivated, with reduced CD62L and CD16 and increased CD11b expression. Such results would suggest that LDGs have proinflammatory activity that could contribute to persistent inflammation in virally suppressed chronic HIV.

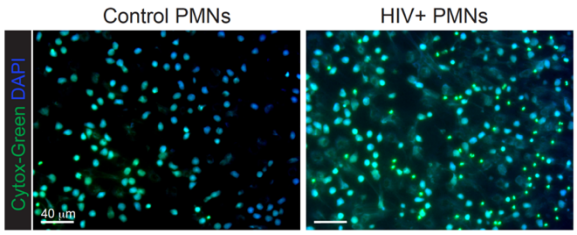

Figure 2. HIV+ neutrophils showed spontaneous NET formation
Representative image of NETs formation (stained with the nucleic acid dye Sytox-Green) on neutrophils isolated from healthy or HIV+ individuals. polymorphonuclear cells (PMN).

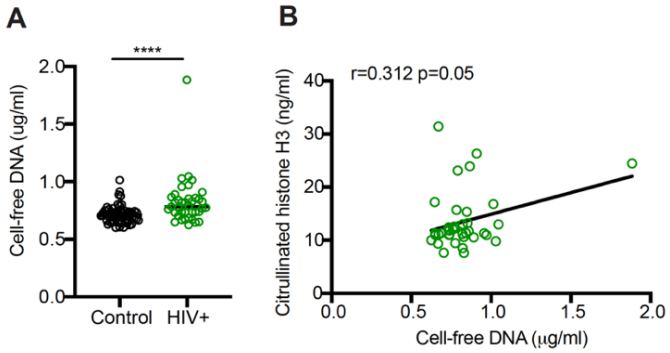

Figure 3. ART-treated HIV+ individuals had higher levels of plasma NETs compared to healthy individuals
(A) Plasma from healthy donors (control, n = 58) and HIV+ individuals (HIV+, n=39) were assessed for cell-free DNA (B) Correlation between Cit-H3 levels and cell-free DNA in HIV+individuals. Pearson's correlation coefficients were calculated. Mann-Whitney U test; ****P < 0.001.

Specific Aim II. Determine the association between NET formation and thrombosis in HIV+ individuals.

Rationale:

Increasing evidence points to a bidirectional relation between inflammation and coagulation and their extensive crosstalk plays a pivotal role in the pathogenesis of vascular disease[26, 37, 38]. Persistent NET formation is associated with inflammation and thrombus formation[4, 6, 25, 34]. Nevertheless, no specific investigative data is available to understand how NETs contribute to the risk of thrombosis in chronic HIV infection. Therefore, in this aim, we will assess potential mechanisms underlying the role NET formation can play in coagulation activity in HIV-infected individuals. Also, we will assess the relationship between NET markers and platelet activation in chronic HIV and the potential clinical implication of NET in HIV-associated thrombosis.

Preliminary data:

We found increased plasma levels of cell-free DNA (cfDNA), a known NET marker in chronic HIV+ individuals on ART relative to healthy individuals (Fig 3A). cfDNA levels correlated with CitH3 indicating NET formation (**Fig 3B**). In addition, plasma levels of CitH3 were associated with inflammatory markers (IL-6 and CRP) and thrombosis markers (Fibrinogen and D-dimer) in HIV+ individuals (**Fig 4**).

Experimental design:

To examine whether platelets are preactivated in HIV+ individuals, we will use the same clinical samples analyzed in *Aim 1*, and the basal level of platelet activation will be analyzed by FACS analysis. We will compare MFI of platelet activation markers (CD62P, CD63, and PAC) between heathy control platelets (HC Pla) and HIV+ individual platelets (HIV Pla), and also analyze percentage of positive events in the platelets. To assess neutrophils' procoagulant activity in HIV+ individuals, we will assess platelet-neutrophil aggregates (PNAs) in circulation. For this analysis, LDGs will be further gated into CD41a-positive (platelet-bound, $CD41a^{+}CD66b^{+}$) and CD42a-negative (platelet-free, $CD41a^{-}CD66^{+}$).

To determine whether NETs provide an assembly site for the prothrombinase complex, neutrophils will be isolated from HIV+ individuals (n=6) and then placed onto a glass coverslip in a 24-well plate with serum-free RPMI-1640 media. After incubation with either HIV+ or control plasma for 3 h, the binding of coagulation factors to NET structure, such as fibrinogen, prothrombin, and Factor X will be visualized by immunofluorescence staining and adhesion of coagulation factor adhesion will be quantified.

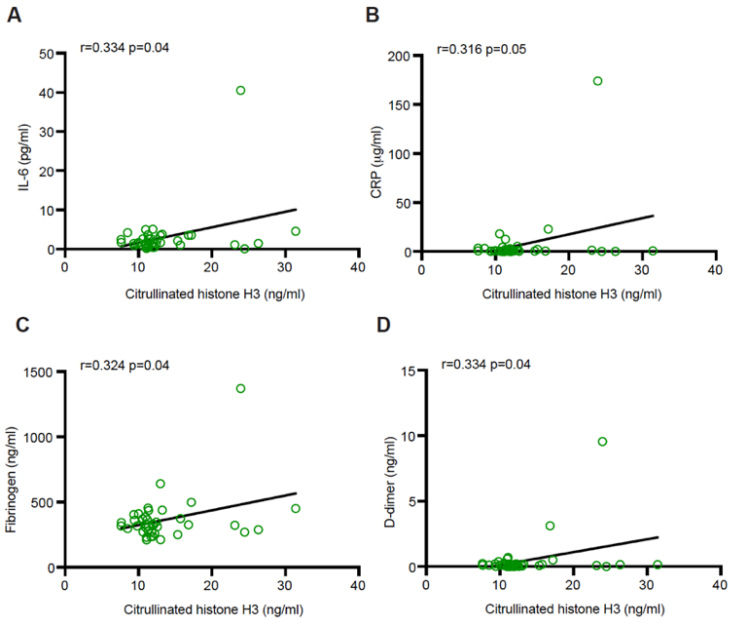

Figure 4. Plasma NET levels were correlated with inflammatory and thrombosis biomarkers
(A-D) Citrullinated histone H3 (Cit-H3) levels were compared with indicated biomarkers and pearson's correlation coefficients were calculated for IL-6 (A), CRP (B), Fibrinogen (C), and D-dimer (D). N=39.

To determine the potential involvement of NETs in thrombotic complications in HIV, plasma collected from 144 subjects (72 HIV+ and 72 HIV- [age > 40 years]) will be utilized. The presence of NETs in plasma from HIV and healthy individuals will be examined with specific NETosis markers: cell-free DNA (cfDNA), MPO-DNA, and citrullinated histone H3 (CitH3). Plasma levels of *in vivo* coagulation markers; and platelet activation markers; P-selectin, platelet-derived microparticles (PMPs), platelet factor 4 (PF4), and CD40L, will be quantified using commercially available kits. We will determine the correlation between circulating NET markers and markers of coagulation and platelet activation. For in vitro experimental variable, data will be analyzed with an unpaired Student t test (comparison of 2 groups) or a 1-way analysis of variance (ANOVA) with Tukey's multiple-comparisons post hoc testing (comparison of > 2 groups). All tests will be 2 sided, and P<0.05 will be considered statistically significant. The relationship among variables will be assessed using Pearson or Spearman correlation.

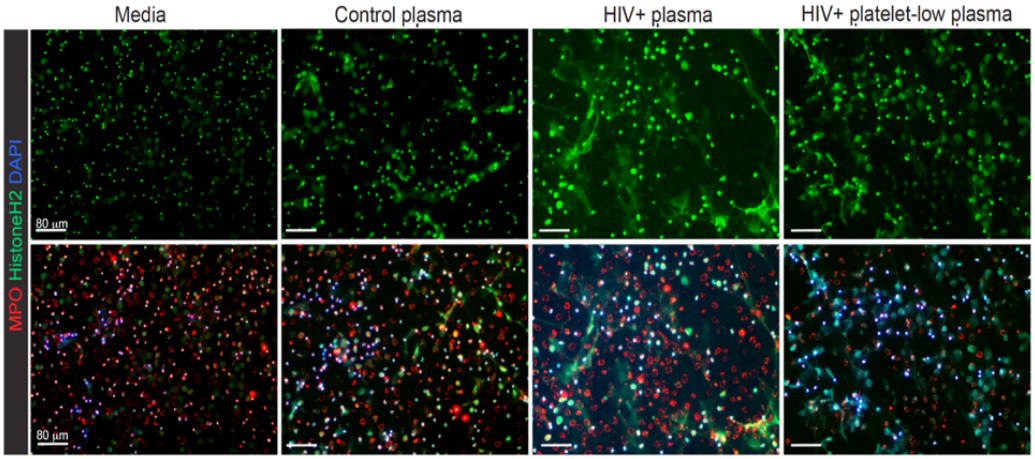

Figure 5. Platelets associated with NET formation in HIV patients
Representative immunofluorescence images of NET formation in control neutrophils treated with HIV+ plasma w/wo platelet removal or control plasma. Neutrophils were stained with Histone H2 (green)and MPO (red). DNA was expelled to the extracellular space (yellow arrows) from neutrophils were exposed to plasma

<u>Anticipated results, potential problems, and alternative approaches:</u>
While no significant difference of plasma D-dimer was found in our preliminary data between HIV+ and healthy individuals, there was positive correlation between D-dimer and citH3 in levels in HIV+ individuals (**Fig 4**). This data suggest that elevated NET could enhance thrombosis in chronic HIV. In addition, NET formation was facilitated by HIV+ platelet-rich plasma (PRP) treatment, indicating that the bidirectional platelet-neutrophil interaction is crucial for their activation (**Fig 5**). Therefore, we anticipate positive associations between NETs and soluble markers for platelet activations and platelet-neutrophil aggregates. Previous study showed that HIV+ individuals exhibited higher platelet reactivity[39], suggesting that HIV+ individuals have spontaneously activated platelets and substantial alterations in their hemostatic system. Thus, we expect to observe increased expression of coagulation factors when neutrophils are treated with PRP.