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Neutrophil extracellular traps (NETs) are involved in the pathogenesis of many infectious diseases, yet their dynamics and impact on HIV/SIV infection have not yet been assessed. We hypothesized that SIV infection and the related microbial translocation trigger NET activation and release (NETosis), and we investigated the interactions between NETs and immune cell populations and platelets. We compared and contrasted the levels of NETs between SIV-uninfected, SIV-infected, and SIV-infected antiretroviral-treated nonhuman primates. We also cocultured neutrophils from these animals with either peripheral blood mononuclear cells or platelets. Increased NET production was observed throughout SIV infection. In chronically infected animals, NETs were found in the gut, lung, and liver, and in the blood vessels of kidney and heart. Antiretroviral therapy (ART) decreased NETosis, albeit above preinfection levels. NETs captured CD4+ and CD8+ T cells, B cells, and monocytes, irrespective of their infection status, potentially contributing to the indiscriminate generalized immune cell loss characteristic to HIV/SIV infection, and limiting the CD4+ T cell recovery under ART. By capturing and facilitating aggregation of platelets, and through expression of increased tissue factor levels, NETs may also enhance HIV/SIV-related coagulopathy and promote cardiovascular comorbidities.
Neutrophil extracellular trap production contributes to pathogenesis in SIV-infected nonhuman primates

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Introduction

Neutrophils are central to the innate immune system, being involved in the defense against bacteria and fungi (1), and even against viruses, as recently reported (2). In addition to phagocytizing and killing microorganisms, neutrophils can control infections through generation of extracellular chromatin fibers called neutrophil extracellular traps (NETs) (3). Neutrophils that release NETs develop a unique cellular morphology with decondensed nuclei that ultimately lose their DNA (4). NETs are complex structures consisting of chromatin and proteins, such as lactoferrin, myeloperoxidase (MPO), histones, and neutrophil elastase (NE) (5). In vitro–generated NETs are long, thin-stranded, web-like extracellular fibers (1). NETs with a thicker morphology were identified in vivo in the gut, liver, skin, and lung in numerous diseases (4, 6, 7).

NETs can capture bacteria (1), fungi (5), and viruses, promoting their elimination (8). For example, HIV-1 stimulates neutrophils to produce NETs, through TLR7/TLR8. NETs can then capture HIV-1 virions and inactivate them via MPO and α-defensins (8). NETs are not always beneficial: they promote thrombosis (9), being involved in the pathogenesis of cardiovascular and autoimmune diseases. In cancers, NETs facilitate metastasis by sequestrating circulating tumor cells (10).

We thus studied the dynamics and functions of NETs during SIV infection, to assess their contribution to disease progression and comorbidities. We report that (a) NET production increases throughout untreated SIV infection, being only partially reduced by ART, (b) NETs may contribute to the indiscriminate depletion of immune cells that are not direct virus targets, and to the incomplete CD4+ T cell restoration observed in HIV-infected subjects on ART, and (c) NETosis may promote thrombosis in the thrombocytopenic environment of HIV/SIV infections by capturing platelets and expressing tissue factor (TF).

Results and Discussion

We assessed the role of NETs in the pathogenesis of HIV/SIV infection in 37 pigtailed macaques (Macaca nemestrina; PTMs). Ten PTMs were inoculated with SIVsab92018 and used to assess NET dynamics during SIV infection. The impact of ART on NET formation was evaluated in 12 additional SIVsab-infected PTMs receiving coformulated ART for 10 months, initiated at 50 days postinfection (dpi), and virologically suppressed below the detection limit (50 vRNA copies/ml). Ten uninfected PTMs housed and followed in the same conditions as the SIV-infected ones were used as controls. Five additional uninfected PTMs were used for apoptosis studies. Peripheral blood mononuclear cells (PBMCs), neutrophils, and platelets were isolated from blood collected either prior to infection or at critical time points after infection and treatment. Tissues from 25 chronically SIV-infected PTMs from other studies were used for histology.

Previous reports showed that neutrophils isolated from uninfected subjects can release NETs that capture HIV (8), yet NET production in HIV-infected subjects has never been demonstrated. Fur-
We monitored NET dynamics by comparing and quantifying NETs at critical time points before and after SIV infection, using immunofluorescence staining and picogreen dsDNA quantification. In SIV-uninfected NHPs, both unstimulated and stimulated neutrophils produced minimal levels of thin NETs (Figure 1, A and E, and Supplemental Figure 3). Neutrophils isolated during acute SIV infection (14 dpi) showed a dramatic increase of NET production (Figure 1, B and F).

Because this early NET increase occurred prior to the major alterations in gut integrity, we concluded that SIV itself contributes to NET formation (Supplemental Figure 2C). A progressive and significant increase of NET production by neutrophils isolated throughout the follow-up (90 dpi) (Figure 1, C and G) was documented by immunofluorescence staining (Figure 1I) and picogreen dsDNA quantification (Figure 1J) in both unstimulated and stimulated neutrophils. The increased NETosis in unstimulated neutrophils isolated during chronic infection likely occurs as a consequence of SIV-induced severe gut damage and microbial translocation, which release potent NET triggers (11). ART suppressed the virus and reduced NET production by isolated neutrophils, but did not normalize it to preinfection levels in all the SIV-infected PTMs (Figure 1, D and H–J). This is likely due to incomplete healing of the intestine in virus-suppressed macaques, leading to incomplete res-

Figures 1. NET dynamics in SIV infection. NET production in unstimulated (A–D) and stimulated PMNs (E–H): prior to infection (SIVneg; n = 10; A and E), during acute SIV infection (Acute; n = 10; B and F), during chronic infection (Chr.; n = 7; C and G), and in SIV-infected PTMs receiving ART (Chr. ART; n = 12; D and H). NETs were identified by immunohistochemical staining for lactoferrin (green); neutrophils were stained with DAPI (blue). Quantitative image analyses showing the percentage of the area positive for lactoferrin as a NET marker in unstimulated (blue) and stimulated (red) samples (I). Picogreen dsDNA quantification in unstimulated (green) and stimulated (orange) samples (J). Dynamics of NETs, in plasma, assessed by ELISA (K). Scale bars: 100 μm. A 2-tailed Mann-Whitney U test was used for statistical analyses, significance being defined as compared with baseline preinfection values after Bonferroni’s correction for multiple comparisons: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, or to chronic infection #P < 0.05, ##P < 0.01. Actual P values are shown in Supplemental Table 1.
recovered from the stimulated cell cultures showed increases in annexin V apoptosis marker (Figure 3, C and D), in accordance with the NET presence in cultures (Figure 1E), as opposed to minimal NET production in unstimulated samples (Figure 1A). Cell death in the NETs was also directly confirmed by in situ quantification of active caspase-3 IHC staining in these cell cultures from uninfected animals (Figure 3, E and F, and Supplemental Figure 5), as well as in cocultures of nonstimulated PBMCs and neutrophils from SIV-infected PTMs (Figure 3, G and H).

We thus directly proved that capture by the NETs may represent a previously unidentified mechanism of CD4+ T cell depletion during HIV/SIV infection. Furthermore, since CD4+ T cell trapping by NETs persists in ART-treated SIV-infected NHPs (Figure 2C), residual NETosis may be a significant factor behind the incomplete CD4+ T cell recovery observed in HIV-infected subjects on ART.

A key unsolved aspect of HIV/SIV pathogenesis is that, in addition to the depletion of the virus targets, other immune cell subsets (i.e., CD8+ T cells, B cells, and even neutrophils) are also massively lost, without a clear cause. Bystander apoptosis is accepted as the main cause of death for these immune effectors (14); however, other unknown factors may be involved. We
thus investigated whether NET capture is responsible for the loss of these immune cells. PBMCs and neutrophils were incubated with or without a NET stimulus and stained for CD8, CD20, or CD163, and lactoferrin. Indeed, CD8+ T cells (Figure 2D and Figure 3A–C) and monocytes (Figure 2F) were trapped in the NETs, similar to the CD4+ T cells (Figure 2, A–C) and neutrophils (Figure 2E). The 3D confocal microscopy views clearly showed that we are dealing with true cell capture and not merely superposition of the immune cells and NETs in cultures (Supplemental Videos 1 and 2). Quantification of the cells captured by NETs failed to identify preferential targeting of a particular immune cell subset (Figure 2G). Through combined immunofluorescence for lactoferrin and RNAscope in situ hybridization with an SIVsab probe, we also showed that capture of both infected (Figure 2H, yellow arrow) and uninfected (Figure 2H, white arrow) lymphocytes occurred in the NETs.

Our results suggest that, at least in part, bystander death of immune cells that are not directly targeted by the virus results as a pure mechanical effect of NETosis, and occurs as collateral damage rather than a targeted killing of a particular immune cell subset. Our data thus provide a plausible explanation for the loss of multiple immune cells during HIV/SIV infection, irrespective of their ability to support virus replication.

HIV infection associates a hypercoagulable state, directly linked to both a high risk of cardiovascular events and death (15). The causes of HIV-related hypercoagulability are not completely elucidated, preventing appropriate interventions to alleviate this root cause of multiple comorbidities. Since platelet trapping in the NETs may promote thrombosis (9), we posited that NETosis can lead to hypercoagulopathy in SIV/HIV infection. We incubated platelets and neutrophils from SIV-infected PMTs in the presence or absence of a NET stimulus. A large number of platelets were indeed caught in the NETs (Figure 2I and Supplemental Figure 6), explaining, at least in part, the thrombocytopenia associated with SIV/HIV infection (16). Meanwhile, aggregation of platelets in the NETs (Figure 2I) may trigger thrombi formation, thus obstructing small blood vessels or complicating atherosclerotic lesions (9).

In addition to acting as mechanical barriers leading to platelet aggregation, NETs may impact coagulation through other pathways. Both neutrophils and NETs can express TF (17, 18), an essential activator of coagulation (19). By culturing unstimulated and stimulated neutrophils from chronically SIV-infected PMTs, we found that those generating NETs preferentially express high levels of TF (Figure 2J). The same was true for the NETs themselves (Figure 2J). In high contrast, the surrounding neutrophils were negative for TF (Figure 2J). TF expression by NETs and their ability to capture platelets could thus potentiate each other and promote an environment favorable to platelet aggregation and activation, leading to a hypercoagulable state.

To strengthen our data with more in vivo observations, we next analyzed tissues collected from chronically SIV-infected PMTs, and similar to previous studies from other research areas (4, 6), we found NETs (Figure 4 and Supplemental Figure 7). To accurately identify the NETs in tissues, we first assessed their presence in crypt abscesses in the gut (Figure 4A). Previous studies reported that NET density is high in pathological conditions associated with abscess formation, such as psoriasis, bronchopneumonia, and ulcerative colitis (6, 20, 21). In tissues, NETs had a slightly different morphology than they did ex vivo:
Excessive NET production during SIV infection may thus provide a dual mechanism for enhanced thrombi formation in the context of low platelet counts. NETosis might thus decisively contribute to both the high risk of cardiovascular events observed in HIV/SIV-infected subjects/NHPs (15, 23), and to the development of thrombotic microangiopathy, which may be at the origin of multiple HIV-related comorbidities.

Altogether, our results point to a new paradigm of SIV/HIV pathogenesis, in which neutrophils attempting to phagocytize translocated microbes are overwhelmed and driven to excessive suicidal NET formation. The beneficial effects of NETs, such as the elimination of free virions and of HIV-infected CD4+ T cells, are then gradually and largely outweighed by multiple collateral damages, such as indiscriminate trapping and destruction of immune cells in the NETs, and excessive platelet capture and aggregation. Excessive NETosis characteristic to HIV infection can thus contribute to immune failure after ART, and to the development of both non-HIV-associated comorbidities and end-stage organ disease characteristic to SIV/HIV infection. Adjuvant therapies to eliminate NETs may thus be beneficial for HIV-infected patients.

Methods

For a complete description of the methods, see Supplemental Methods.

Study approval. The animals were housed at the Plum Borough Research Center, University of Pittsburgh, where they were monitored as per the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, and the NIH Guide for the Care and Use of Laboratory Animals (National Academies Press, 2011). The study was approved by the IACUC of the University of Pittsburgh (protocols 15045829, 17040178, 0911844, 0907039, 12121250, 12040408).
Author contributions
RS, EBC, CA, and IP designed these studies. RS, EBC, and SMKV conducted the experiments. RS, EBC, EF, NK, and SMKV acquired the data. RS, EBC, NK, CA, and IP analyzed and interpreted the data. RMR performed advanced data analyses. RS, EBC, RMR, CA, and IP wrote the manuscript.

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