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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 were not yet assessed. We hypothesized that SIV infection and the related microbial translocation trigger NET activation and release (NETosis), and 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, liver, and in the blood vessels of kidney and heart. 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.

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Neutrophil Extracellular Trap Production Contributes to Pathogenesis in SIV-Infected Non-Human Primates

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Keywords

Simian immunodeficiency virus; CD4⁺ T-cells; neutrophil extracellular trap (NET); neutrophils; hypercoagulability; platelets; human immunodeficiency virus

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

25 **ABSTRACT**

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

42

43 INTRODUCTION

44 Neutrophils are central to the innate immune system, being involved in the
45 defense against bacteria and fungi (1), and even against viruses, as recently reported
46 (2). In addition to phagocytizing and killing microorganisms, neutrophils can control
47 infections through generation of extracellular chromatin fibers called neutrophil
48 extracellular traps (NETs) (3). Neutrophils that release NETs develop a unique cellular
49 morphology with decondensed nuclei that ultimately lose their DNA (4). NETs are
50 complex structures consisting of chromatin and proteins, such as lactoferrin,
51 myeloperoxidase (MPO), histones, and neutrophil elastase (NE) (5). In vitro generated
52 NETs are long, thin stranded, web-like, extracellular fibers (1). NETs with a thicker
53 morphology were identified in vivo in the gut, liver, skin and lung in numerous diseases
54 (4, 6, 7).

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

61 We thus studied the dynamics and functions of NETs during SIV infection, to
62 assess their contribution to disease progression and comorbidities. We report that: (a)
63 NET production increases throughout untreated SIV infection, being only partially
64 reduced by antiretroviral treatment (ART), (b) NETs may contribute to the indiscriminate
65 depletion of immune cells that are not direct virus targets, and to the incomplete CD4⁺

66 T-cell restoration observed in HIV-infected subjects on ART, and that (c) NETosis may
67 promote thrombosis in the thrombocytopenic environment of HIV infection by capturing
68 platelets and expressing tissue factor (TF).

69

70 RESULTS AND DISCUSSION

71 We assessed the role of NETs in the pathogenesis of HIV/SIV infection in thirty-
72 seven pigtailed macaques (*Macaca nemestrina*; PTMs). Ten PTMs were inoculated with
73 SIVsab92018 and used to assess NET dynamics during SIV infection. The impact of
74 ART on NET formation was evaluated in twelve additional SIVsab-infected PTMs
75 receiving coformulated ART for 10 months, initiated at 50 days postinfection (dpi), and
76 virologically suppressed below the detection limit (30 vRNA copies/ml). Ten uninfected
77 PTMs housed and followed in the same conditions as the SIV-infected ones were used
78 as controls. Five additional uninfected PTMs were used for apoptosis studies.
79 Peripheral blood mononuclear cells (PBMCs), neutrophils and platelets were isolated
80 from blood collected either prior to infection, or at critical time points pi and treatment.
81 Tissues from 25 chronically SIV-infected PTMs from previous studies were used for
82 histology.

83 Previous reports showed that neutrophils isolated from uninfected subjects can
84 release NETs that capture HIV (8); yet NET production in HIV-infected subjects has
85 never been demonstrated. Furthermore, the dynamics of NET production during HIV
86 infection, and the impact of ART on NETosis are so far unknown. To address these
87 questions, neutrophils isolated from untreated and treated SIV-infected PTMs were
88 incubated in the presence or absence of phorbol 12-myristate 13-acetate (PMA), and
89 stained for essential NET markers (Supplemental Figure 1). This strategy allowed
90 identification of the characteristic NET filaments, in which neutrophil-derived proteins
91 such as histone H3 (Supplemental Figure 1A), MPO (Supplemental Figure 1B), NE

92 (Supplemental Figure 1C), or lactoferrin (Supplemental Figure 1D) colocalized with
93 extracellular DNA (DAPI).

94 To determine the effect of gram positive and gram negative bacteria on NETosis,
95 we incubated neutrophils with *Staphylococcus aureus* (1) (Supplemental Figure 2A), or
96 *Escherichia coli* (Supplemental Figure 2B). Both conditions elicited NET formation, with
97 bacteria being trapped in the NETs. We also observed SIV virion capture in the NETs
98 (Supplemental Figure 2C), in agreement with studies showing similar HIV trapping (8).
99 This suggests that indeed, neutrophils from HIV-infected subjects and SIV-infected
100 NHPs are particularly prone to NET overproduction through excessive stimulation by the
101 virus, and by bacterial products translocated from the gut (11).

102 We monitored NET dynamics by comparing and quantifying NETs at critical time
103 points pre- and post-SIV infection using immunofluorescence staining and picogreen
104 dsDNA quantification. In SIV-uninfected NHPs, both unstimulated and stimulated
105 neutrophils produced minimal levels of thin NETs (Figure 1A, E; Supplemental Figure
106 3). Neutrophils isolated during acute SIV infection (14 dpi) showed a dramatic increase
107 of NET production (Figure 1B, F). This early NET increase occurring prior to the major
108 alterations in gut integrity, we concluded that SIV itself contributes to NET formation
109 (Supplemental Figure 2C). A progressive and significant increase of NET production by
110 neutrophils isolated throughout the follow-up (90 dpi) (Figure 1 C, G), was documented
111 by immunofluorescent staining (Figure 1I) and picogreen dsDNA quantification (Figure
112 1J) in both unstimulated and stimulated PMNs. The increased NETosis in unstimulated
113 neutrophils isolated during chronic infection likely occurs as a consequence of SIV-
114 induced severe gut damage and microbial translocation, which release potent NET

115 triggers (11). ART suppressed the virus and reduced NET production by isolated
116 neutrophils, but did not normalize it to preinfection levels in all the SIV-infected PTMs
117 (Figures 1D, H, I, J). This is likely due to incomplete healing of the intestine in virus-
118 suppressed macaques, leading to incomplete resolution of microbial translocation (12).
119 The dynamics of NETosis was confirmed in vivo with a NET ELISA on plasma samples
120 from the same SIV-infected PTMs (13) (Figure 1 K).

121 CD4⁺ T-cell depletion, the hallmark of HIV/SIV infection, occurs mainly by direct
122 virus cytopathic effect and bystander effects of excessive immune activation (14).
123 However, these mechanisms do not fully explain the magnitude of CD4⁺ T-cell loss
124 observed during SIV/HIV infection. We hypothesized that immune cell trapping in the
125 NETs may also account for CD4⁺ T-cells loss during HIV/SIV infection. We therefore
126 incubated PBMCs with neutrophils from SIV-infected PTMs in the presence or absence
127 of a NET stimulus and observed CD4⁺ T-cell capture (Figure 2A; Supplemental Figure
128 4) and destruction (Figure 2B, C) in the NETs, as demonstrated by the T-cell
129 morphological changes (membrane bleb formation, cell membrane disintegration, nuclei
130 irregularities and fragmentation) (Figure 2B, C). These changes involved only the CD4⁺
131 T-cells caught in the NETs, and not those free in media (Figure 2B insert).

132 To confirm that cell capture by NETs is indeed deadly, we performed a series of
133 experiments in which we first incubated PBMCs and neutrophils isolated from
134 uninfected PTMs in the presence or absence of NET stimuli and then treated cultures
135 with nucleases, to break the NETs (Figure 3 A-D). Only the cells recovered from the
136 stimulated cell cultures showed increases in ANNV apoptosis marker (Fig 3C, D), in
137 accordance with the NET presence in cultures (Figure 1 E), as opposed to minimal NET

138 production in unstimulated samples (Figure 1A). Cell death in the NETs was also
139 directly confirmed by in situ quantification of active caspase 3 IHC staining in these cell
140 cultures from uninfected animals (Figure 3E, F; Supplemental Figure 5), as well as in
141 cocultures of nonstimulated PBMCs and neutrophils from SIV-infected PTMs (Figure
142 3G-H).

143 We thus directly proved that capture by the NETs may represent a previously
144 unidentified mechanism of CD4⁺ T-cell depletion during HIV/SIV infection. Furthermore,
145 since CD4⁺ T-cell trapping by NETs persists in ART-treated SIV-infected NHPs (Figure
146 2C), residual NETosis may be a significant factor behind the incomplete CD4⁺ T-cell
147 recovery observed in HIV-infected subjects on ART.

148 A key unsolved aspect of HIV/SIV pathogenesis is that, in addition to the
149 depletion of the virus targets, other immune cell subsets (i.e., CD8⁺ T-cells, B-cells, and
150 even neutrophils) are also massively lost, without a clear cause. Bystander apoptosis is
151 accepted as the main cause of death for these immune effectors (14), however other
152 unknown factors may be involved. We thus investigated whether NET capture is
153 responsible for the loss of these immune cells. PBMCs and neutrophils were incubated
154 with or without a NET stimulus and stained for CD8, CD20, or CD163 and lactoferrin.
155 Indeed, CD8⁺ T-cells (Figure 2D; Supplemental Figure 4), B cells (Figure 2E), and
156 neutrophils were trapped in the NETs, similar to the CD4⁺ T-cells (Figure 2A-C) and
157 monocytes (Figure 2F). The three-dimensional confocal microscopy views clearly
158 showed that we are dealing with true cell capture and not merely superposition of the
159 immune cells and NETs in cultures (Supplemental Videos 1, 2). Quantification of the
160 cells captured by NETs failed to identify preferential targeting of a particular immune cell

161 subset (Figure 2G). Through combined immunofluorescence for lactoferrin and
162 RNAscope in situ hybridization with an SIVsab probe, we also showed that capture of
163 both infected (Figure 2H, yellow arrow) and uninfected lymphocytes (Figure 2H, white
164 arrow) occurred in the NETs.

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

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

182 In addition to acting as mechanical barriers leading to platelet aggregation, NETs
183 may impact coagulation through other pathways. Both neutrophils and NETs can

184 express TF (17, 18) an essential activator of coagulation (19). By culturing unstimulated
185 and stimulated neutrophils from chronically SIV-infected PTMs, we found that those
186 generating NETs preferentially express high levels of TF (Figure 2J). The same was
187 true for the NETs themselves (Figure 2J). In high contrast, the surrounding neutrophils
188 were negative for TF (Figure 2J). TF expression by NETs and their ability to capture
189 platelets could thus potentiate each other and promote an environment favorable to
190 platelet aggregation and activation, leading to a hypercoagulable state.

191 To strengthen our data with more in vivo observations, we next analyzed tissues
192 collected from chronically SIV-infected PTMs, and similar to previous studies from other
193 research areas (4, 6), we found NETs (Figure 4; Supplemental Figure 7). To accurately
194 identify the NETs in tissues, we first assessed their presence in crypt abscesses in the
195 gut (Figure 4 A). Previous studies reported that NET density is high in pathological
196 conditions associated with abscess formation, such as psoriasis, bronchopneumonia
197 and ulcerative colitis (6, 20, 21). In tissues, NETs had a slightly different morphology
198 than ex vivo: they were thicker and with a more granular, “bead on a string” appearance
199 (22). Similar structures occurred in the liver, in the SIV-infected PTMs with liver
200 granulomas induced by atypical *Mycobacteria* (Figure 4 B). As described for the ex vivo-
201 generated NETs, tissue NETs captured immune cells, such as CD3⁺ lymphocytes and
202 macrophages (Figure 4 C, D; Supplemental Videos 3, 4). Interestingly, the animals with
203 a high frequency of crypt abscesses had the lowest CD4⁺ T-cell counts in the gut
204 (Supplemental Table 2). Around the crypt abscesses there were a large number of
205 neutrophils that directly interacted with T cells (Figure 4 C), potentially contributing to
206 their destruction. The intensive tissue damage induced by neutrophils and their NETs

207 may thus contribute to the severe CD4⁺ T-cell depletion and early death observed in
208 these animals (Supplemental Table 2). The correlations between the frequency of crypt
209 abscesses and the number of CD4⁺ T-cells or survival support this hypothesis
210 (Supplemental Figure 8). In SIV-infected animals, NETs were also present in the lung
211 (Figure 4E), lamina propria of the gut, distant from crypt abscesses (Figure 4F), in blood
212 vessels from heart (Figure 4G) and kidneys, in both glomerular capillaries (Figure 4H)
213 and in the small blood vessels outside glomeruli (Figure 4I). The T cells trapped in the
214 NETs had morphological changes suggestive of apoptosis, such as irregular shapes
215 and fragmented nuclei (Figure 4E, Supplemental Figure 7E). Also, in small blood
216 vessels, the NETs and the neutrophils producing them formed small obstructive barriers
217 (Figure 4I), supporting our ex vivo findings. These observations, together with NET
218 ability to capture platelets (Figure 2I) and activate them *via* TF expression (Figure 2J),
219 may provide a valid explanation for the high frequency of kidney microthrombi observed
220 in chronically SIV-infected PTMs (23).

221 Excessive NET production during SIV infection may thus provide a dual
222 mechanism for enhanced thrombi formation in the context of low platelet counts.
223 NETosis might thus decisively contribute to both the high risk of cardiovascular events
224 observed in HIV/SIV-infected subject/NHPs (15, 23), and to the development of
225 thrombotic microangiopathy, which may be at the origin of multiple HIV-related
226 comorbidities.

227 Altogether, our results point to a new paradigm of SIV/HIV pathogenesis, in
228 which neutrophils attempting to phagocytize translocated microbes are overwhelmed
229 and driven to excessive suicidal NET formation. The beneficial effects of NETs, such as

230 the elimination of free virions and of HIV-infected CD4⁺ T-cells, are then gradually and
231 largely outweighed by multiple collateral damages, such as indiscriminate trapping and
232 destruction of immune cells in the NETs, and excessive platelet capture and
233 aggregation. Excessive NETosis characteristic to HIV infection can thus contribute to
234 immune failure post-ART, and to the development of both non-HIV-associated
235 comorbidities and end-stage organ disease characteristic to SIV/HIV infection. Adjuvant
236 therapies to eliminate NETs may thus be beneficial for HIV-infected patients.

237

238 **METHODS**

239 **Study approval.** The animals were housed at the Plum Borough Research Center,
240 University of Pittsburgh, where they were monitored, as per the Association for
241 Assessment and Accreditation of Laboratory Animal Care (AAALAC) International, and
242 the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health
243 recommendations. The study was approved by the Institutional Animal Use and Care
244 Committee (IACUC), University of Pittsburgh (Protocols 15045829, 17040178, 0911844,
245 0907039, 12121250, 12040408).

246

247 **AUTHOR CONTRIBUTIONS**

248 RS, EBC, CA, IP designed these studies. RS, EBC, SMKV conducted the experiments.
249 RS, EBC, EF, NK, SMKV acquired the data. ES, EBC, NK, CA, IP analyzed and
250 interpreted the data. RMR performed advanced data analyses. RS, EBC, RMR, CA, IP,
251 wrote the manuscript.

252

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261

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333

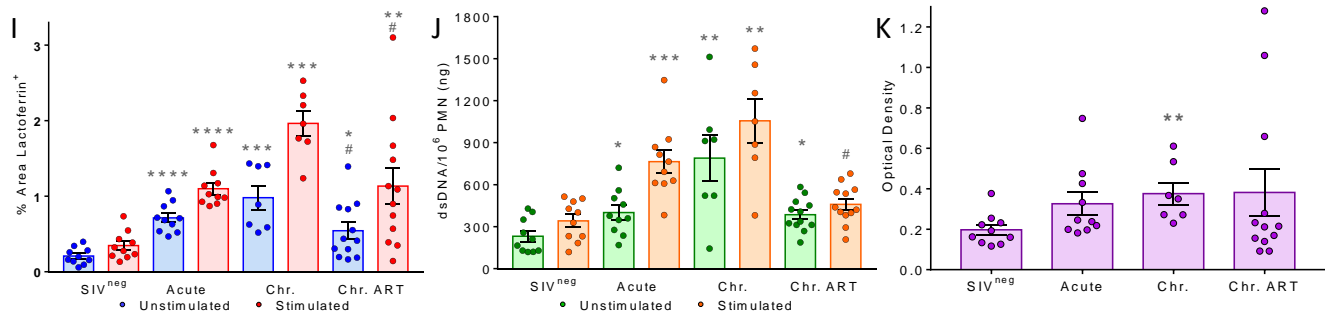
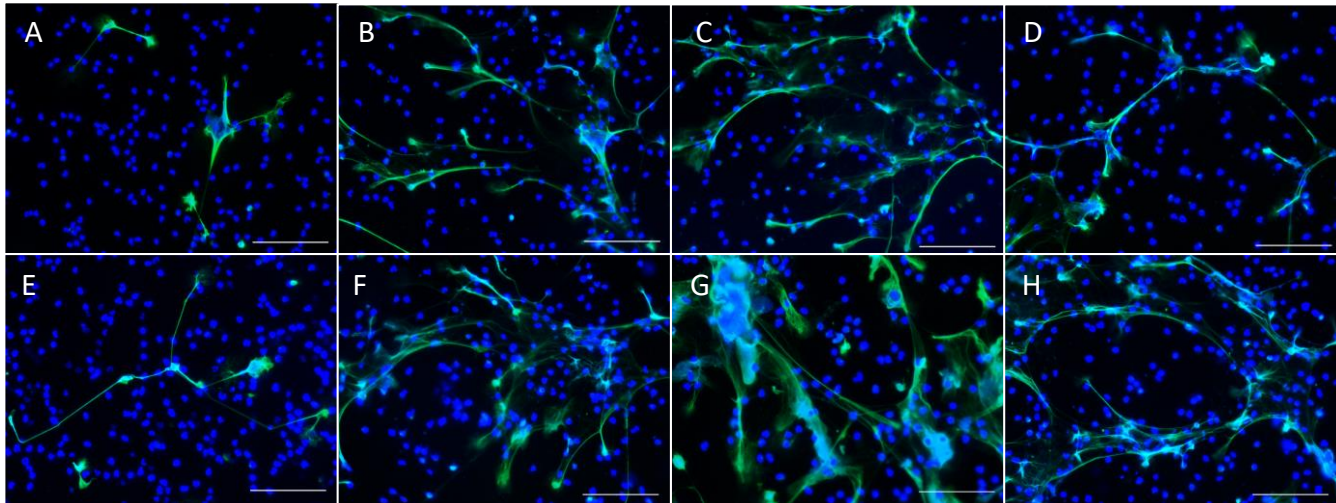


Figure 1. NET dynamics in SIV infection. NET production in unstimulated (panels A-D) and stimulated PMNs (panels E-H): prior to infection (n=10) (A, E), during acute SIV infection (n=10) (B, F); during chronic infection (Chr.) (n=7) (C, G); and in SIV-infected PTMs receiving ART (Chr. ART) (n=12) (D, 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). Picrogreen dsDNA quantification in unstimulated (green) and stimulated (orange) samples (J). Dynamics of NETs, in plasma, assessed by ELISA (K). Scale bars are 100 μ m in length. Two-tailed Mann-Whitney *U* test was used for statistical analyses, significance being defined as compared to baseline preinfection values after 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 shown in Supplemental Table1.

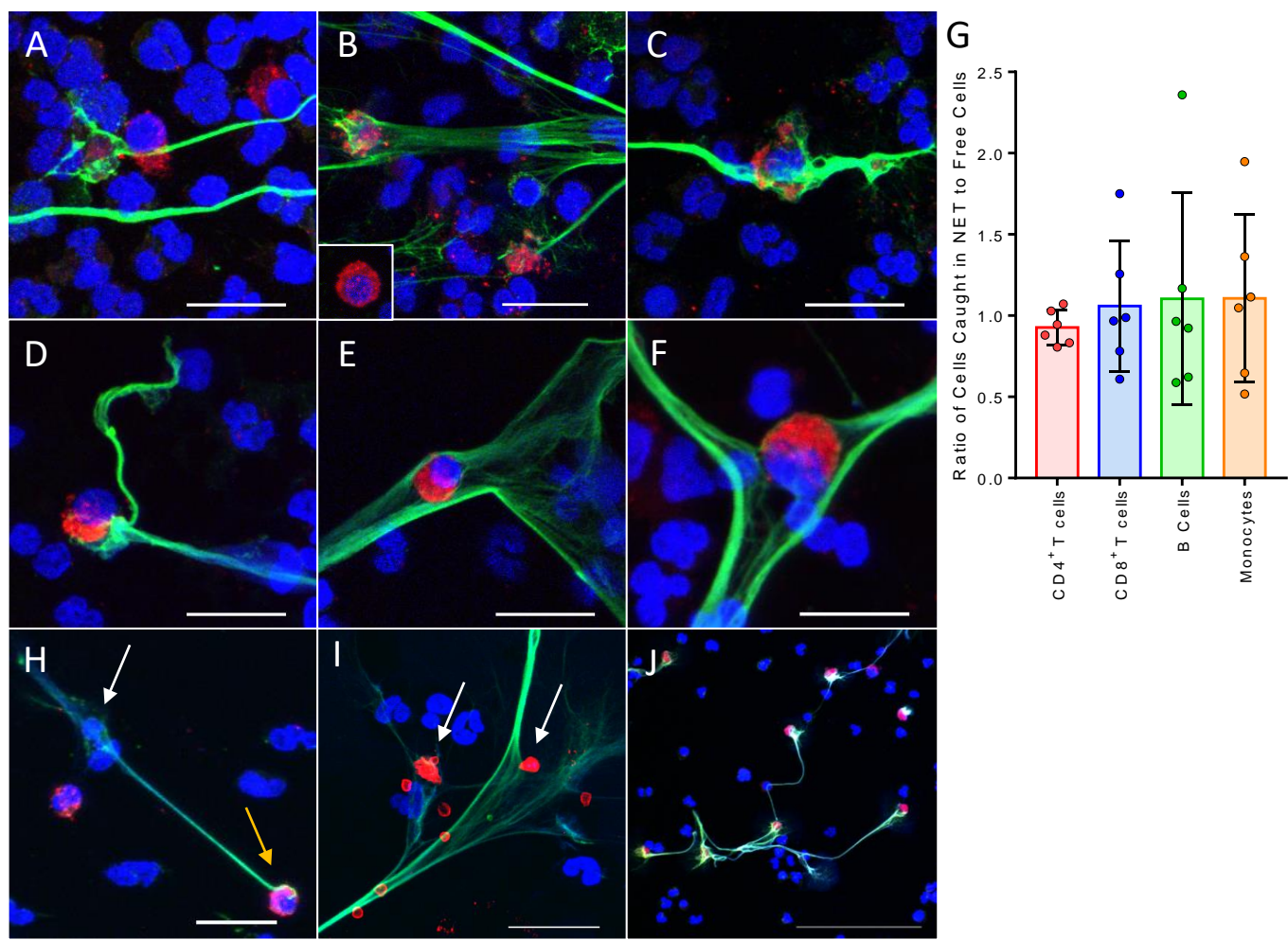


Figure 2. Key collateral damage associated with excessive NET formation during SIV infection. CD4⁺ T cell (red) capture (A) and destruction (B) in the NETs; the insert in B shows a free CD4⁺ T cell for comparison. CD4⁺ T cell capture and destruction in chronically-infected PTMs on ART (C). Other immune cells (red) caught in NETs: CD8⁺ T cells (D); B cells (E); and monocytes (F). The ratios of cells caught in NETs versus those outside of NETs were similar for the various cell types, indicating no preferential capture (n=6) (G). Combined SIV RNAScope (red), lactoferrin immunofluorescence (green), and nuclear staining with DAPI (blue) showed both infected (orange arrow) and uninfected (white arrow) cells caught in NETs (H). Individual platelets (red circles) caught in NETs (I). Aggregated platelets (red) in the NETs (arrow) (I). Expression of TF (red) by neutrophils undergoing NETosis and their NETs (green) (J), and lack of TF expression by normal neutrophils (blue) (J). Stimulated neutrophils (A-F, H, I); Unstimulated neutrophils (J). Scale bar lengths: 20 μ m (A-I); 100 μ m (J).

Unstimulated

Stimulated

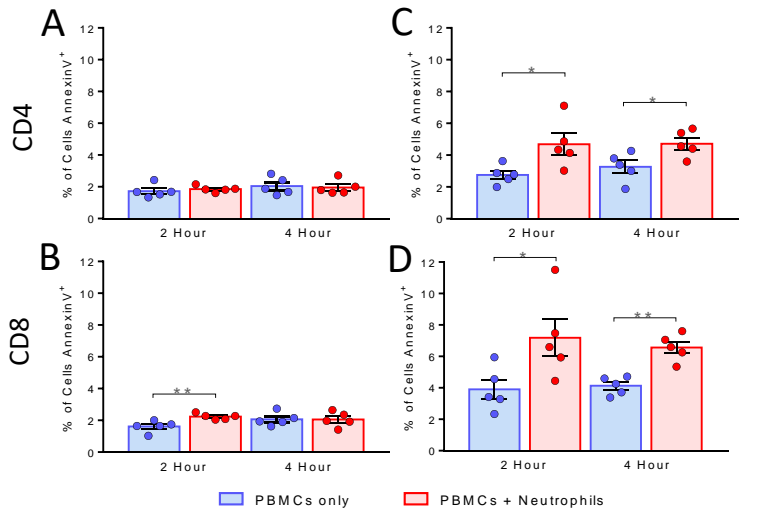
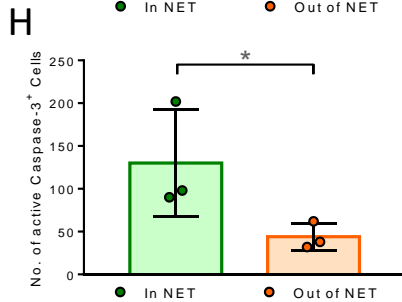
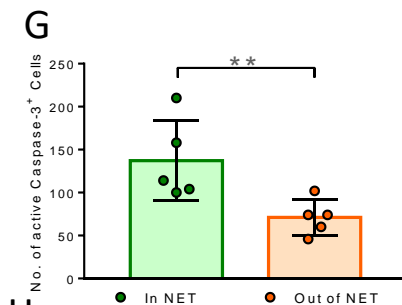
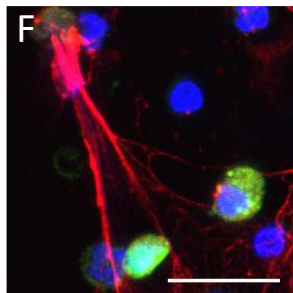
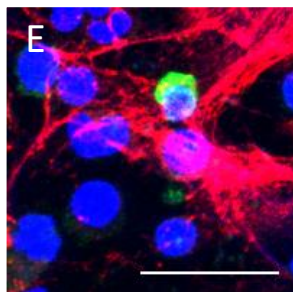


Figure 3. Cell death in NETs. No increase of CD4⁺ T cell apoptosis (A); and minimal increase of CD8⁺ T cell apoptosis upon incubation with neutrophils (B) without PMA stimulation. Significant increase of CD4⁺ T cell apoptosis (C) and CD8⁺ T cell apoptosis (D) occurred upon incubation with neutrophils after PMA stimulation. Two-tailed Mann-Whitney *U* test was performed, significance being defined as: *: p<0.05, **: p<0.01. Stimulated cocultured PBMCs and neutrophils from uninfected PTMs (n=5) stained for active caspase-3 (green) and NE (red) showed that cells trapped in the NETs undergo apoptosis (E). Unstimulated cocultured PBMCs and PMNs from chronically infected PTMs (n=3), stained for active caspase-3 (green) and NE (red) showed that cells trapped in the NETs undergo apoptosis (F). Quantification of apoptotic cells in the NET vs out of the NET in stimulated uninfected cells (G) and unstimulated infected cells (H) confirmed increased cell death in the NET. Scale bars length: 20 μm. One-tailed Mann-Whitney *U* test, with significance being defined as: *: p<0.05, **: p<0.01.



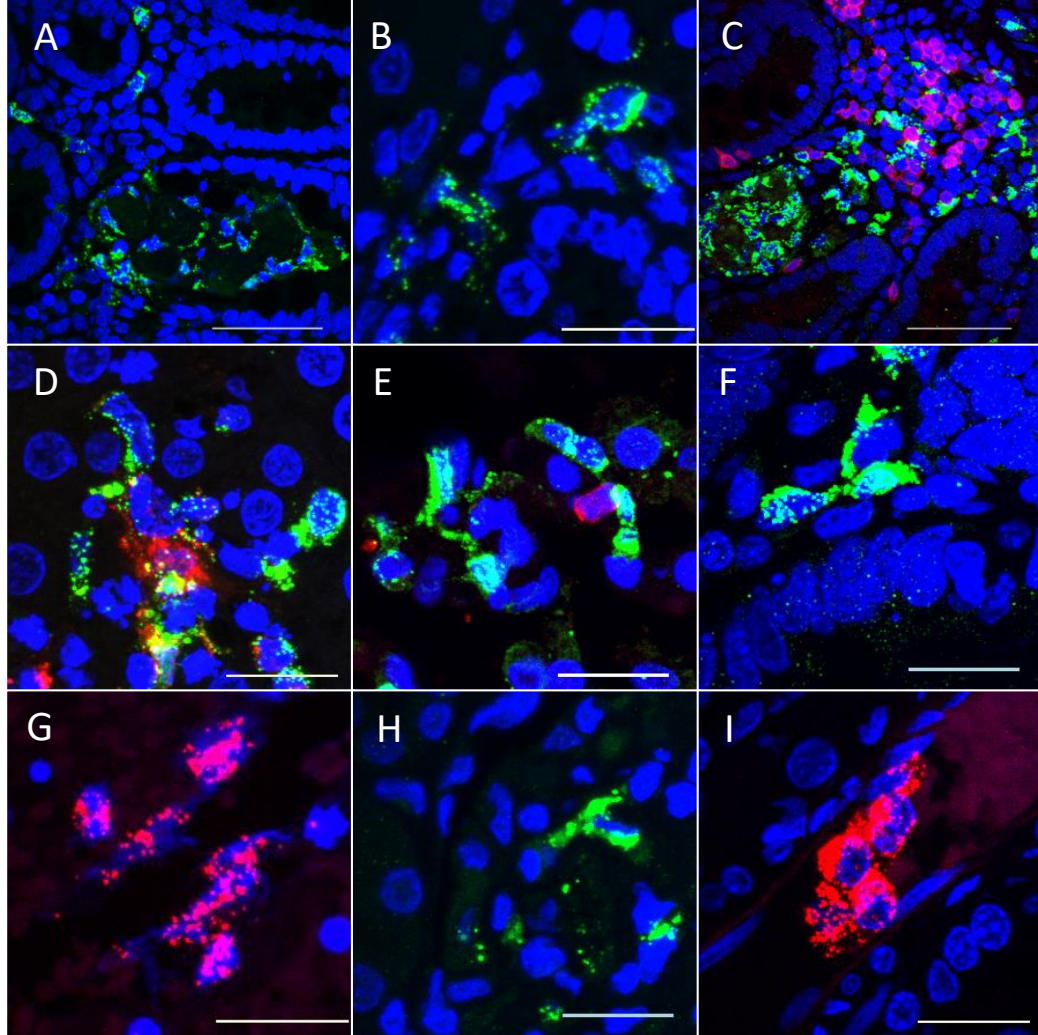


Figure 4. Assessment of NETs in tissues. In chronically SIV-infected PTMs NETs (green) were found in crypt abscesses in the gut (A), and in liver granulomas (B). Infiltration with neutrophils that form NETs (green) able to trap CD3⁺T cells (red) occurred around crypt abscesses (C). In liver granulomas, NETs (green) captured CD68⁺ macrophages (red) (D). In the lung, NETs (green) captured CD3⁺T cells (red). NETs (green) were found in the lamina propria of the gut distant from crypt abscesses(F); in the large vessels of the heart (G); in the glomerular capillaries in the kidney (green) (H); and appear to occlude small blood vessels in the kidney (red) (I). Scale bars lengths: 50 μ m (A, C); 20 μ m (B, D-I). NET identification by staining for: MPO (A-F and H); NE (G and I). Nuclear staining: DAPI (blue).