1 **Plasma-derived HIV Nef+ exosomes persist in ACTG384 study participants despite successful**
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5
6 Short title: HIV Nef+ exosomes impact immune recovery

7 **Keywords:** Nef, exosomes, microvesicles, discordant CD count/viral load, CD4 recovery,
8 ACTG384 study

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Abstract

Human Immunodeficiency Virus (HIV) accessory protein Negative factor (Nef) is detected in the plasma of HIV+ individuals associated with exosomes. The role of Nef+ exosomes (exNef) in HIV pathogenesis is unknown. We perform a retrospective longitudinal analysis to determine correlative clinical associations of exNef plasma levels in ARV-treated HIV+ patients with or without immune recovery. exNef concentration in a subset of AIDS Clinical Trial Group (ACTG) 384 participants with successful virological suppression and with either high ($\Delta >100$ CD4 cell recovery/High Immunological Responders (*High-IR*) or low ($\Delta \leq 100$ CD4 cell recovery/ Low Immunologic Responders (*Low-IR*) immunologic recovery was measured and compared for study weeks 48, 96, and 144. CD4 recovery showed a negative correlation with exNef at study week 144 ($r = -0.3573$, $*p=.0366$). Plasma exNef concentration in high IRs negatively correlated with naïve CD4 count and recovery ($r = -0.3249$, $*p = 0.0348$ (*High-IR*); $r = 0.2981$, $*p = 0.0513$ (*Low-IR*)). However, recovery of CD4 memory cells positively correlated with exNef ($r = 0.4534$, $*p=.0358$) in *Low-IRs* but not in *High-IRs*. Regimen A (Didanosine, Stavudine, Efavirenz) lowered exNef levels in IRs by 2-fold compared to other regimens. Nef+ exosomes persist in ART-treated HIV+ individuals despite undetectable viral loads, negatively correlates with naïve and memory CD4 T cell restoration and may be associated with reduced immunological recovery. Taken together, these data suggest that exNef may represent a novel mechanism utilized by HIV to promote immune dysregulation.

40 Introduction

41 The prognosis for patients infected with HIV has improved significantly with the advent of
 42 combination antiretroviral therapy (cART) which can lead to suppression of plasma viremia
 43 usually associated with marked improvements in CD4⁺ T cell counts [1, 2]. However, a subset of
 44 patients do not experience a robust immune recovery despite viral suppression. While host
 45 factors such as age and baseline CD4⁺ T-cells with a naïve phenotype have been observed to be
 46 negatively associated with the magnitude of CD4⁺ T-cell count improvement [1, 3-6], virological
 47 factors that contribute to these immunologic outcomes are less well defined.

48 Immune reconstitution can be defined as an increase in the number of peripheral CD4 T-
 49 cells to greater than 350-500 cells/dL after 4 years of effective cART [3]. Discordant responses
 50 characterized by a lack of immune recovery despite viral suppression occur in 7-39% of
 51 participants receiving cART [4-7]. The reasons for this phenomenon are not understood.

52 The HIV-encoded Negative factor (Nef) has been implicated in pathogenesis based on
 53 studies examining HIV-infected long term non-progressors (LTNP) and elite controllers (EC).
 54 LTNPs remain asymptomatic for years with stable CD4 counts while ECs control plasma HIV
 55 RNA in the absence of ART [12]. Although several hypotheses have been presented to account
 56 for the protective factors leading to delayed disease progression, replication defective virions/*nef*-
 57 defective virions have been associated with these populations in some cases [9-11]. Recent
 58 reports have shown that the viruses within some LTNPs and ECs are actually replication
 59 competent and control of HIV may be due to host and/or viral factors that maintain low levels of
 60 viremia [11]. HIV-1 Nef may be integral to the control of viral replication and/or T-cell responses
 61 in an HIV-infected individual. Early *in vitro* studies have shown that soluble Nef is cytotoxic to

62 T-cells and is released from HIV-infected cells in plasma derived microvesicles that can be
63 detected in HIV-infected patients [13-23]. These studies suggest that Nef does not exist as a
64 soluble protein *in vivo* but instead non-virion associated Nef is found in microvesicles or
65 exosome-like microvesicles (exNef) [7, 8].

66 Several *in vitro* studies have identified pathogenic activities of soluble Nef including its
67 capacity to reduce surface expression of CD4 and MHC class II, to increase HIV infectivity, to
68 stimulate primary macrophages to release pro-inflammatory cytokines and chemokines, and
69 induce apoptosis in CD4 T-cells [13, 18, 24-27]. We have previously shown that expression of *nef*
70 in HEK293 cells is sufficient to produce exNef, and the resultant exosomes can be absorbed by T-
71 cells and macrophage but only induces apoptosis in T-cells [14]. Taken together, these
72 observations suggest that Nef+ microvesicles/exosome could have an impact on CD4 T-cell
73 recovery. We hypothesize that Nef microvesicles/exosomes are released from HIV infected cells
74 despite successful cART-induced viral suppression and contribute to pathogenesis by impeding
75 CD4-T-cell recovery.

76 Exosomes range in size from 30 -100 nm and are released from hematopoietic (i.e. B-and T-
77 lymphocytes, dendritic cells, monocytes, mast cells, reticulocytes) and non-hematopoietic (i.e.
78 neurons, intestinal epithelial cells, and tumors) cells. Exosomes are detected in physiological
79 fluids such as plasma, urine, malignant effusions, and amniotic fluids. Several functions have
80 been attributed to exosomes/microvesicles including the modulation of cell signaling, cellular
81 homeostasis, intercellular communication, shuttling genetic material, establishment of tissue
82 polarity, regulation of immune responses, and enhancing the site of budding (i.e. HIV and

83 Murine Leukemia Virus) [28-32]. Given the immunomodulatory functions of exosomes, we
84 sought to explore whether exNef may selectively impair CD4⁺ T-cells recovery during cART.

85 For this study, Nef concentration was determined in plasma-derived exosomes isolated
86 from a subset of the ACTG-384 cohort with and without immune recovery post-cART. We show
87 that Nef⁺ exosomes persist and can be detected in study participants with undetectable viral
88 loads even after 144 weeks of therapy. This suggests that exNef production is independent of
89 plasma viral load. Interestingly, we demonstrate that *Low*-IRs have significantly higher levels of
90 exNef compared to *High*-IRs at 144 weeks post-treatment. Recovery of naive CD4 T-cells
91 (CD45RA⁺, CD62L⁺) and total CD4 cells negatively correlated with exNef. Overall, we report that
92 Nef⁺ exosomes are detected in the plasma despite viral suppression and that exNef is negatively
93 associated with changes in naïve and total CD4 count. Taken together these results suggest that
94 exNef may ultimately affect CD4 T-cell recovery and be a biomarker of immune recovery.

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96 **Material and Methods**

97 **Study Population.** A subset of cryopreserved plasma samples (n = 240) taken from participants
98 in ACTG 384 were used in this study [1] (Robbins, GK 2003; Shafer 2003). As previously
99 described ACTG 384 was a factorial multi-center randomized controlled trial conducted in the
100 United States and Italy that compared sequential three-drug regimens for treatment of HIV
101 infection. Nine hundred eighty ART-naïve HIV-1⁺ subjects were randomized and treated with
102 stavudine/didanosine or zidovudine/lamivudine with nelfinavir, efavirenz, or both nelfinavir
103 and efavirenz. If virological failure occurred, then participants were placed on another regimen

sequentially. In this study, samples from three distinct groups were obtained and assayed. The groups included 1) Treatment failure (TF) defined as those with a virologic failure with a detectable viral load (HIV RNA > 50 RNA copies) what at any time point before 144 weeks, 2). Immunologic responders defined as those with high CD4 improvement of > 100 cells/mm³ (achieved at any point post study initiation) and suppressed viremia (< 50 RNA copies) (High IRs) and 3) Immunologic non-responders defined as those with CD4 improvement of < 100 cells/mm³ and suppressed viremia (Low IRs) (Figure 1). To avoid confounding effects of multiple-treatment regimens, the samples utilized for this sub-cohort were derived from subjects on their first treatment regimen and those with samples from three- or four time intervals (0, 48, 96, and/or 144 weeks).

Isolation of plasma-derived exosomes. Plasma exosomes were isolated as previously described [14, 23, 36]. Plasma was pre-cleared by centrifugation at 10,000g for 30 minutes to remove particulates. Microvesicles were pelleted from pre-cleared plasma via ultra-centrifugation for 1 hour at 300,000 x g and then re-suspended in 250 µl of phosphate buffered saline (PBS). Immune complexes within the re-suspended exosomes were removed using acid-dissociation prior to Nef measurement similar to p24 antigen measurements from plasma. Exosomes/high speed pellets (100 µl) were treated with 100 µl of 0.3N hydrochloric (HCl, Sigma) and allowed to incubate for 1 hour at 37 °C. The acid mixture was neutralized with 100 µl of 0.3N sodium hydroxide (NaCl, Sigma) prior to assaying for Nef.

Nef Enzyme-linked Immunosorbent Assay (ELISA). Nef concentration in acid-dissociated microvesicle/exosome preparations was measured using a commercially available anti-Nef

sandwich ELISA kit (Immunodiagnostics, Bedford, MA) according to the manufacturer's instructions. Briefly, the neutralized microvesicle preparations were diluted 1:1 with sample diluent (Component C) and added to ELISA plates coated with anti-Nef (Component A). Following 1-hour incubation at room temperature the plates were washed three times with wash buffer (Component B), and 100 µl of anti-Nef-HRP labeled antibody solution (component E) was then added to each well. Plates were incubated for 1 hour at room temperature, washed three times with component B, and developed by adding 100 µl of alkaline phosphatase substrate (component F) per well. Plates were developed for approximately 10 minutes after which 100 µl of stop solution (Component G) was added to each well and the absorbance at 450 nm determined using a Spectramax spectrophotometer.

Statistical Analysis. Exosomal Nef concentrations were correlated with various immunologic outcomes (immune recovery, change in cell populations, etc.) in each group using the Spearman rank test. The Kruskal-Wallis test was used to compare continuous outcomes between these three groups. The Mann-Whitney rank-sum test or the Dunn's multiple comparison tests were used appropriately to compare Nef levels in Treatment failures and immunological responders. Statistical analysis was performed using MYSTAT software version 12 (Systat software, Inc. 2007).

Results

Baseline characteristics of ACTG384 sub-cohort. This sub-cohort consisted primarily of males (88.4%) (**Table 1**). Plasma HIV RNA levels at baseline were not significantly different between

the three groups (range). Baseline CD4 counts were significantly different between groups (137 cells/mm³ in TF, 60 cells/mm³ in High IRs, 38 cells/mm³ in Low IRs) (as previously described that CD4 count at the initiation of therapy may not play a role in immune recovery [3]. Activated CD4 and CD8 T-cells were not significantly different between the *High*- and *Low*-IRs

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Characteristics	Complete subcohort	Treatment Failures	>200 ΔCD4 cell count	<200 ΔCD4 cell count
sex[n,%]	(n=47)	(n=14)	(n=15)	(n=18)
Male	41,(88.4%)	10(71%)	13(87%)	18(100%)
Female	6,(11.6%)	4(29%)	2(13%)	0
Age (Baseline)	37	36	37	39
Viral load(log10)				
Baseline	5.447	4.998	5.563	5.425
96 weeks	1.68	3.32	1.699	1.699
CD4 Count	***	***	***	*
Baseline	116	252	61	87
96 weeks	330	372	409	253.5
Activated CD4/CD38/HLA(%)	***	***	***	***
Baseline	28	21	22	31
96 weeks	7	11	7	6.5
Activated CD8/CD38/HLA(%)	***	*	***	***
Baseline	53	52	57	53
96 weeks	20	29	22.5	18.5
Naïve CD4 (cells/mm3)	***	ns	***	***
Baseline	6.4	42.7	9.9	5.3
96 weeks	102.3	58.5	151.8	60.9
Memory CD4	***	ns	***	***
Baseline	24.5	78.9	22.5	33.9
96 weeks	205.1	161.7	271.4	174.9

Table 1. Statistical analysis – Intergroup analysis (Column) compared gender and group association using chi-square; ethnicity and group composition at baseline, using Chi-square analysis, #Trend *p-value* <0.1; Median age of groups compared using ANOVA – Kruskal-Wallis, *ns*-not significant; Viral load at baseline between groups at baseline and 96 weeks, Kruskal-Wallis, *ns*- not significant.; Baseline CD4 count, *ns*-not significantly different between groups; Post-96 weeks CD4 count significantly different between Low and High Immunological responders (IR); Kruskal-Wallis, Dunnett's Multiple comparisons, ****p-value*<.001; CD4 count different between baseline and 96 weeks for each group Mann-Whitney ****p-value* <.0001; Activated CD4 and CD8 T-cell counts are *ns*-(not significant) between groups at baseline and 96 weeks, Kruskal-Wallis, Dunnett's Multiple; Naïve CD4 counts is significantly different between high and low IRs at baseline but at 96 weeks differ significantly, Kruskal-Wallis, Dunnett's Multiple comparisons ****p-value*<.001; Intra-group difference in IR memory differ significantly, Mann-Whitney ****p-value* <.0001

groups, either at baseline or post 96 weeks of treatment. (**Table 1**). Other cell subsets B-cells and natural killer (NK) cells were not significantly different between *High*-IRs and *Low*-IRs (data not shown). However, naïve and memory CD4 T-cell subsets were significantly different between *High*-and *Low*_IRs (Table 1) suggesting that these cell subsets are integral to immune recovery.

CD4 recovery among immunological responders negatively correlates with plasma exNef concentration. ART-induced decrease in viral load (VL) to <50 RNA copies did not necessarily reduce Nef concentration in plasma or result in CD4 recovery for all HIV+ patients within the sub-cohort. We examined study participants receiving anti-retrovirals with successful viral suppression that had CD4 recovery above or below 300 cells/mm³ at study week 144 (**Fig 1A**). At study week 144, 40% of the sub-cohort exhibited discordant VL and CD4 cell recovery along with increased exNef level. In the absence of detectable viral replication, exNef could be detected in the plasma and the levels correlated with total CD4 recovery (**Fig 1B**).

187 **Figure 1: ARV-treated HIV+ patients exhibit differential CD4 T-cell recovery, which negatively correlates with**
 188 **exNef level despite successful viral suppression. (A)** At study weeks 48 (n=38), 96(n=32), and 144 (n=25) the
 189 change in CD4 count (as measured by flow cytometry) was tracked in in the participants with VL >50 RNA copies
 190 and compared to baseline CD4 count. High = change in CD4 count >100; Low= change in CD count <100 cells.
 191 Statistical significance determined by Kruskal-Wallis test statistic, ***P<.001, Dunn's Multiple comparison's **P<.01.
 192 **(B)** Plasma-derived exNef concentration negatively correlates with the change in CD4 count. *P<0.05, Spearman r.

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 194 **Trend in exosomal Nef correlates with immunological response status.** cART initiation reduces
 195 viral load to undetectable levels over time but the effects of ART on exNef production is
 196 unknown. Cross-sectional reports have demonstrated that Nef can be detected in the plasma in
 197 the absence and presence of ART [36]. Since exNef may play a role in HIV immunopathogenesis
 198 and impact CD4 recovery we examined the longitudinal changes of exNef in High IRs.
 199 Interestingly, exNef was detected in the plasma of these patients despite suppression of viral
 200 replication (**Fig 2A**). A retrospective longitudinal analysis shows that at the initiation of therapy,
 201 Nef levels were not significantly different between the *Low_IRs* and *High_IRs* (**Fig 2A**, upper
 202 panel). However, by study week 144, the *Low-IRs* had significantly higher levels of plasma exNef
 203 than the High IRs (**Fig 2A**, lower panel). The median baseline plasma Nef level in the sub- cohort
 204 at 48 weeks was approximately 2.5 ng per 1 ml plasma and by 144 weeks Nef levels increased to
 205 almost 5 ng per 1 ml plasma in the Low IRs. In fact *Low-IRs* exhibit an increasing trend in exNef
 206 levels from study weeks 48 through 144 while in *High-IRs* exNef level decreased over the study
 207 (**Fig 2B**). This finding suggests that plasma exNef may play a role immunological recovery.

208 **Figure 2: Exosomal Nef is significantly different between low and high immunological responders and**
 209 **trends upward in low immunological responders. (A)** exNef concentration as measured by anti-Nef ELISA is
 210 increased in Low responders at study weeks 48(Low-n=25, High=5 , 96(Low-n=16; High-n=14), and 144 weeks (Low-

211 n=10 ;High n=16). Statistical significance determined via Mann-Whitney, *p-value <0.05, Mann-Whitney. **(B)** High
212 and low immunological responders defined in sub-cohort defined by change in CD4 count from baseline. High –
213 change ≥ 100 (cells/mm³); Low change <100 cells/mm³) (B) Median Nef concentration in exosomes isolated from
214 plasma of subjects with Treatment Failure (n=10) or Success (with high, and low CD4 recovery; n=30) subjects. Nef
215 quantified by anti-Nef ELISA.

216 **Naïve CD4 count and recovery inversely correlate with exNef.** We then investigated whether
217 Nef+ exosome levels correlated with recovery of naïve CD4 cell counts specifically.
218 Immunological recovery (as defined in Methods) 48 weeks post treatment and close to 90% (18
219 out of 20) by week 96 of treatment (**Fig 3A**). However, none of the *Low*-IRs exhibited increases in
220 CD4 T-cell count close to 350 cells/mm³ by 144 weeks (**Fig 3A**). Although naïve T-cells do recover
221 in both *High*- or *Low*-IR the IRs have appreciably less naïve CD4 cells than the IRs 96- and 144
222 weeks post treatment initiation (**Fig 3C**). Notably the changes in both CD4 T-cell count and CD4
223 naïve T-cells negatively correlated with the Nef concentration in plasma-derived microvesicles
224 (**Fig 3, B and D**) suggesting that *in vivo* microvesicular Nef may be associated with immune
225 recovery/ CD4 T-cell rebound.

226 **Figure #3: Naïve CD4 counts inversely correlate with exNef.** (A) Naïve counts (as measured by flow cytometry)
227 significantly higher in the High_IR group at study week 48 and (B) study week 96. (C) Nef-level inversely correlates
228 with both Naïve CD T-cell count and the (D)change in Naïve CD4 count post ARV-treatment. Correlation determined
229 by Spearman r, *p-value<.05, #p-value <.1 (trend). Statistical significance determined by One-Way ANOVA Kruskal-
230 Wallis test statistic and Dunn's Multiple comparison * P-value<0.05, **p-value<.01, and ***p-value<.001.

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232 **Memory CD4 T-cells are significantly different in high low and responders.** Given the role of
233 memory cells in immunological recovery, we sought to determine whether exNef also impacted
234 CD4 memory cell recovery. Interestingly at weeks 48 and 96 post-therapy *High*-IRs exhibited

235 appreciably more CD4 memory cells than the *Low_IRs* (**Fig 4**, panel A). The degree of CD4
236 increase directly correlated with exNef in the *Low_IRs* (**Fig 4**, panel B), suggesting that CD4
237 memory cells could be one of the sources of exNef during anti-viral suppression.

238 **Figure #4: Memory CD4 T-cells are significantly different in high low and responders. (A)** CD4 memory cell
239 counts (as determined by flow cytometry) in high-immunological responders are significantly increased compared to
240 low responders at study weeks 48 (Low-n=25; High-n=5); and 96 (High- n=14; Low-n=16). Significance from baseline
241 determined via Kruskal-Wallis, intergroup differences compared via Dunn's Multiple comparison *p-value<0.05, **p-
242 value<.01, and ***p-value<.001. **(B)** The change in CD4 memory count from baseline(n=33) to study week 96 (n=30)
243 directly correlates with exNef level in low- immunological responders (n=18). Correlation determined using
244 Spearman r, *p-value<0.05, ns=not significant.

245 **PI-sparing regimen significantly reduced plasma exNef.** ACTG 384 was a prospective double-
246 blinded study using a factorial design to compare sequential three-drug regimens. Study arms
247 are depicted in **Table 2**. Basically, two NRTIs zidovudine and lamivudine or didanosine and
248 stavudine followed by either efavirenz or nelfinavir were compared (**Fig 5A**, upper panel).
249 Previous results from the ACTG-384 cohort demonstrated that the combination of zidovudine,
250 lamivudine, and efavirenz lead to the shortest time to viral suppression suggesting that this
251 combination was the most efficacious combination [2]. However, we are still able to detect exNef
252 in the sub-cohort of ACTG384 participants with successful viral suppression. If exNef negatively
253 affects immunological recovery, then we must identify a treatment regimen that suppresses both
254 viral replication and exNef release. To determine how treatment regimen impacts exNef
255 production we stratified participants by treatment regimen and compared their respective exNef
256 level. By weeks 96 and 144 exNef was reduced 2-fold only in participants (with undetectable
257 viral load) that received regimen A (Didanosine, Stavudine and Efavirenz) (**Fig 5**, lower panel).

258 This suggests that drug regimen may also dictate exNef levels and that PI-sparing regimens
259 reduce both viral load and exNef level.

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Initial	Initial NNRTI	Initial PI	Initial PI +NNRTI
NRTI Factor	Efavirenz (EFV)	Nelfinavir (NFV)	NFV + EFV
DDI+D4T	A (5)	B (3)	E (11)
ZDV /3TC	C (10)	D (7)	F(14)

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262 **Table 2: Study arms of ACTG384.** Drug regimen groupings as described by Smeaton et al 2001
263 followed by the number of participants (in parentheses) within regimen group.

264 **Figure #5: PI-sparing regimen significantly reduced plasma exNef by study weeks 96 and 144. (Upper panel)**
265 Schematic of drug regimens administered to sub-cohort in the first tier of the ACTG384 study. Participants on
266 regimens A (n=5) or C (n=10) received efavirenz with DDI/4TC or ZDV/4TC while those given regimen D(n=7)
267 received ZDV/4TC and Nelfinavir(**Lower Panel**). Longitudinal comparison of plasma exNef level as measured by
268 anti-Nef ELISA in participants taking regimen A,C, and D. Statistical significance determined via Kruskal-Wallis, and
269 Dunn's multiple comparison *p-value <0.05, **p-value<.01, ***p-value<.001, and ns=not significant.

270

271 Discussion

272 This study shows that exNef is detected in the plasma of cART-treated HIV+ patients with
273 successful viral suppression. Immunological recovery does not occur in 40% of cART-treated
274 HIV+ patients. Understanding the factors involved in poor immunological recovery could lead to
275 the development of novel therapeutics that inhibit viral replication while promoting

immunological recovery. We posit that exNef may represent a novel mechanism utilized by HIV to promote viral replication in resting CD4 T-cells. The impact of exNef on HIV pathogenesis and CD4 T-cell recovery during cART is unknown. Additionally, we demonstrate that exNef level was not only elevated in cART-treated HIV+ patients with low immunological recovery but also negatively correlated with CD4 count recovery in these participants. This clearly suggests a role for exNef in immunological recovery.

Plasma exNef could theoretically determine immune recovery potential. Detection of Nef microvesicles is not unexpected since several studies have shown that in order to lower surface expression of CD4 and MHC class II Nef interact with component of the endocytic and exocytic machinery [13, 22]. Nef has no enzymatic activity and functions primarily as an adaptor within an infected cell. The function of extracellular Nef is unclear. However, several *in vitro* studies have demonstrated that extracellular soluble Nef is cytotoxic to cells, induces cytokine and chemokine release from macrophage activation, increases viral infectivity and alters innate immune signaling pathways [13, 18, 27, 37-40]. We know from *in vitro* and *ex vivo* studies that Nef is released in exosome-like vesicles from *nef*-transfected and HIV-infected cells and that these vesicles are detected in the plasma of HIV+ patients [14, 23]. Although the biological role of Nef+ exosomes is still unknown we expected exNef to have similar cellular and functional affects ascribed to soluble Nef.

It has recently been reported that released Nef microvesicles/exosomes similar to soluble Nef can trigger activation induced cell death (apoptosis) in peripheral blood leukocytes thus promoting the depletion of CD4+ T-cells [21]. Another report indicated that Nef induces massive

297 secretion of microvesicle clusters in HIV-infected T-cells and that extracellular Nef is then passed
298 to uninfected bystander by cell-to-cell contact via an ERK-1/2 dependent mechanism [41]. Other
299 studies have shown that extracellular Nef vesicles are taken-up/absorbed by T-cells and that
300 these released Nef microvesicles cause activation-induced cell death in primary peripheral blood
301 leukocytes [14, 21]. Primary leukocytes exposed to exNef as observed with soluble Nef release
302 chemokines MIP-1a and MIP-1b (unpublished result).

303 Here we show that plasma-derived microvesicles/exosomes from participants within the
304 ACTG384 cohort contain Nef even in the presence of successful HAART therapy and are elevated
305 in low immunological responders. This suggests that exNef may play a role in preventing
306 sufficient CD4 T-cell recovery thereby promoting immunodeficiency despite successful HAART
307 outcomes. Taken together, these studies show that extracellular Nef exosomes function similar to
308 soluble Nef in that exNef is absorbed by T-cells, induce apoptosis and may play a role in
309 discordant successful viral suppression and CD4 T-cell recovery.

310 Most interestingly Nef+ microvesicles/exosomes persist and are detectable over a 144-week
311 period suggesting that exNef may simply be a product of chronic HIV infection. We observed
312 that significant reduction of viral load correlated with an increase in exNef level suggesting that
313 exNef may regulate viral replication and/or cell function. These findings are novel in HIV-
314 pathobiology but may not be a unique to HIV. Indeed, recent reports demonstrated that EBV-
315 infected nasopharyngeal cells release inhibitory exosomes containing the EBV-encoded protein
316 latent membrane protein-1 (LMP-1) [42]. These LMP-1+ exosomes inhibited T-cell activation and

317 anti-EBV immune responses [42]. So exNef released from HIV-infected cells may have a similar
318 effect on anti-HIV immune responses.

319 Aside from the ACTG384, several studies have shown HIV+ patients with successful virological
320 responses to HAART yet incomplete CD4 recovery have increased mortality [43-45]. Age and
321 prolonged periods of immunodeficiency prior to successful HAART are risk factors for
322 incomplete/insufficient CD4 T-cell recovery [46]. We provide evidence in this study that plasma
323 derived Nef+ microvesicles may be associated with immune recovery. We detected increased in
324 exNef only in *Low*_IRs. In these *Low*-IRs exNef concentration correlated negatively and positively
325 with the recovery of CD4 Naïve and memory T-cell counts, respectively. This result suggests that
326 naïve CD4 cells are a potential exNef targets while the CD4 memory cells could be a source of the
327 Nef+ exosomes.

328 Treatment regimens not only directly impact viral replication but also appear to affect the
329 generation of Nef+ exosomes. PI-sparing HAART regimens (e.g. Regimen A: Didanosine,
330 Stavudine, and Efavirenz) reduced both viral load and exNef level. This result suggests that
331 treatment regimen may dictate exNef level and exNef in turn may be developed as a prognostic
332 indicator of CD4 immune recovery during HAART.

333 We posit that the increased level of Nef+ exosomes early in *High*_IRs (by week 48) are
334 indicative of reduced virus production and suggests that in the absence of productive viral
335 replication HIV-infected cells release more Nef+ exosomes. If these exosomes affect T-cell
336 activation or viability, then this may impact immune recovery. By 144 weeks however, exNef is
337 significantly reduced in *High*-IRs participants relative to *Low*-IRs participants. Taken together

our study suggests that extended use of combination therapy lacking a HIV protease inhibitor impairs the release of Nef+ exosomes while NRTI/NNRTIs have no long-term effects on exNef.

Overall, these data also suggest that increased Nef levels maybe a double-edged sword – in terms of viral suppression- high exNef is associated with decreased viral load but in regard to immune recovery- elevated exNef is associated with reduced CD4 T-cell rebound/immune recovery. Recently, regions within Nef important for secretion have been identified. Disruption of the secretion modification region (SMR) within the Nef gene appears to abolish its release in microvesicles/exosomes [61]. Peptide-targeted disruption of the SMR also inhibited Nef release suggesting that SMR peptides could be developed as a therapeutic agent.

Ultimately, we provide evidence of exNef as a novel virological factor contributing to the dissociation of viral load and immunological recovery. Since, drug regimens can alter exNef levels, therapies need to be developed that both successfully lower viral load and Nef+ exosomes levels. Our findings also suggest that clinicians could monitor exNef level along with CD4 T-cell count in patients undergoing ARV-treatment to assess the effectiveness of therapeutic regimens.

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Treatment Arm:

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