ORIGINAL RESEARCH

HIV-Nef Protein Persists in the Lungs of Aviremic Patients with HIV and Induces Endothelial Cell Death

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Abstract

It remains a mystery why HIV-associated end-organ pathologies persist in the era of combined antiretroviral therapy (ART). One possible mechanism is the continued production of HIV-encoded proteins in latently HIV-infected T cells and macrophages. The proapoptotic protein HIV-Nef persists in the blood of ART-treated patients within extracellular vesicles (EVs) and peripheral blood mononuclear cells. Here we demonstrate that HIV-Nef is present in cells and EVs isolated from BAL of patients on ART. We hypothesize that HIV-Nef persistence in the lung induces endothelial apoptosis leading to endothelial dysfunction and further pulmonary vascular pathologies. The presence of HIV-Nef in patients with HIV correlates with the surface expression of the proapoptotic endothelial-monocyte–activating polypeptide II (EMAPII), which was implicated in progression of pulmonary emphysema via mechanisms involving endothelial cell death. HIV-Nef protein induces EMAPII surface expression in human embryonic kidney 293T cells, T cells, and human and mouse lung endothelial cells. HIV-Nef packages itself into EVs and increases the amount of EVs secreted from Nef-expressing T cells and Nef-transfected human embryonic kidney 293T cells. EVs from BAL of HIV$^+$ patients and Nef-transfected cells induce apoptosis in lung microvascular endothelial cells by upregulating EMAPII surface expression in a PAK2-dependent fashion. Transgenic expression of HIV-Nef in vascular endothelial–cadherin$^+$ endothelial cells leads to lung rarefaction, characterized by reduced alveoli and overall increase in lung inspiratory capacity. These changes occur concomitantly with lung endothelial cell apoptosis. Together, these data suggest that HIV-Nef induces endothelial cell apoptosis via an EMAPII-dependent mechanism that is sufficient to cause pulmonary vascular pathologies even in the absence of inflammation.

Keywords: EMAPII; emphysema; endothelial; extracellular vesicles; HIV-Nef

Clinical Relevance

This is the first report showing that the HIV virally encoded protein Nef persists in the lungs of antiretroviral therapy–treated patients with HIV even in the absence of viral replication. We show that Nef persistence leads to endothelial cell apoptosis and subsequent vascular damage and pulmonary pathologies. Targeting Nef-induced endothelial damage using endothelial-monocyte–activating polypeptide II–neutralizing antibody or PAK2 inhibitors could be a viable therapeutic strategy to combat HIV-induced pulmonary diseases.

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HIV-1 infection is poorly controlled by the immune system and requires combined antiretroviral therapy (ART) to avoid the development of AIDS in the infected population, which according to the Centers for Disease Control and the World Health Organization includes approximately 1.1 million people in the United States and 36.7 million worldwide. Although the effective deployment of ART has dramatically reduced the number of AIDS-related deaths, individuals chronically infected with HIV have an increased incidence of developing several lung complications, including emphysema, pulmonary hypertension, and lung cancer (1–6). Interestingly, development of emphysema often occurs at an earlier age in patients with HIV (i.e., in 20–40 yr-old patients, compared with 50–70 yr-old individuals without HIV), independently of cigarette smoking status (2, 3, 7). We have previously demonstrated that excessive endothelial apoptosis and inflammation, both of which significantly contribute to the development of cigarette smoke–induced emphysema in mice, are linked to the release of a proinflammatory cytokine, endothelial-monocyte–activating polypeptide II (EMAPII) (8). EMAPII is an intracellular protein that is transported to the cell surface and released in response to various stresses, including hypoxia, mechanical strain, apoptosis, TNF-α, cigarette smoke, and HIV envelope protein (9–13). In the context of the HIV virus, we have demonstrated that HIV-gp120 causes concerted upregulation of EMAPII and its CXCR3 receptor to induce apoptosis, suggesting a novel autocrine/paracrine mechanism of endothelial cell apoptosis in HIV infection (13).

Although most HIV$^+$ patients on ART have undetectable viral loads, the persistence of HIV viral proteins in multiple cell populations could be responsible for the higher incidence of vascular diseases. We have shown the presence of intracellular HIV-Nef protein in peripheral blood mononuclear cells from patients with HIV, including those on ART (14). Importantly, we found HIV-Nef in HIV-uninfected T cells and B cells, showing for the first time that HIV-Nef protein is able to transfer independently from cell to cell. The most likely mechanism of HIV-Nef transfer into circulating blood cells is through extracellular vesicles (EVs), which are transported in the blood plasma and can circulate rapidly through cells. In fact, several independent groups have identified HIV-Nef proteins in EVs from the plasma of HIV-infected patients, including those on ART (15–21). In this study, we sought to investigate whether HIV-Nef protein could be found in the lungs of patients with HIV on ART and thereby contribute to the development of pulmonary vascular diseases. We analyzed cells and acellular fluid derived from BAL in HIV$^+$ patients on ART for a minimum of 3 years with documented good viral control. Using flow cytometry, ELISA, and Western blotting, we stained for intracellular and extracellular vesicular HIV-Nef, and identified several patients who were positive for HIV-Nef even though they had undetectable viral loads. We further observed a strong correlation between HIV-Nef protein expression and EMAPII release, and identified EMAPII as the key mediator of HIV-Nef–induced vascular endothelial apoptosis leading to pulmonary pathology in both *in vitro* and *in vivo* models.

Methods

Tissue Culture

Human lung microvascular endothelial cells (HMVECs) were obtained from Lonza (CC2527) and cultured in microvascular endothelial cell growth medium-2. SupT1 and Nef-estrogen receptor (Nef-ER)–expressing SupT1 cells were obtained from the AIDS Reagent Repository and cultured in RPMI with 10% FBS. The following reagents were obtained through the National Institutes of Health AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: Nef-ER #31 clone from Drs. Scott Walk, Kodi Ravichandran, and David Rekosh; pcDNA3.1SF2Nef (catalog #11431) from Dr. J. Victor Garcia; anti–HIV-1 SF2 Nef monoclonal (EH1) from Dr. James Hoxie (catalog #2949); anti–HIV-1 Nef polyclonal from Dr. Ronald Swanstrom; and pNL4-3 from Dr. Malcolm Martin. Jurkat T cells and human-derived peripheral blood mononuclear cells (Indiana Blood Center) were cultured in RPMI with 10% FBS. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS. Primary alveolar macrophages were isolated from BAL fluid from healthy volunteers and cultured in RPMI with 10% FBS.

EV Isolation and Characterization

EVs were isolated from acellular BAL fluid and supernatant of control/Nef-expressing cells by ultracentrifugation. The number and size of the EVs released were assessed using NanoSight.

FACS

FACS was performed as previously described (22). Human-derived BAL cells and mouse lung cells were fixed in 1% paraformaldehyde for 15 min at room temperature. Cells were stained for surface markers for 45 min at room temperature, permeabilized using the FoxP3 intracellular staining kit (00-5523-00, eBioscience), stained for intracellular proteins, and acquired on a BD Fortessa cell analyzer. Data were analyzed with FlowJo v10.

Detection of Secreted Cytokines

Cytokine levels in acellular BAL fluid from patients with HIV and supernatants of alveolar macrophages and HIV-Nef–transfected HEK 293T cells were measured using the BD Cytometric Bead Array.

Apoptosis Detection

Apoptosis was measured in HMVECs using Flexstation (Molecular Devices) by detecting caspase 3 activity (APO Logix Caspase 3/7, Cell Technology) and mitochondrial depolarization (JC-1 Ab113850, Abcam). TUNEL staining was performed using the Apo-BRDU apoptosis detection kit (88-6671-88, Thermo Fisher) and analyzed using flow cytometry.

Volume-related Stereology for Calculation of the Total Number of Alveoli

To induce HIV-Nef protein in endothelial Nef transgenic (vascular endothelial [VE]-cadherin-Nef) progeny, neither the mothers nor the litters were given tetracycline. Lungs were fixed with 4.5% paraformaldehyde, volume was assessed by the water displacement method, and alveoli were quantified in 3-mm sections using resorcin/fuchsin and Nuclear Fast Red (Weigert's elastin staining).

Physiological Assessment of Lungs in Nef Transgenic Mice

Blood oxygenation levels were measured in alert animals using a MouseOx Plus neck sensor (Starr Life Sciences). Lung inspiratory capacity was measured with the

flexiVent system (Scireq) as previously described (8).

BAL Samples

Acellular BAL fluid and cells derived from BAL were obtained from HIV-1$^+$ patients and non-HIV–infected patients, and from the left lung of Nef transgenic mice.

Statistical Analysis

Samples were deidentified and the difference between groups was analyzed using Student's *t* test with Welch's correction, one-way ANOVA with Tukey's *post hoc* multiple comparison, and Mann-Whitney nonparametric tests as indicated. Spearman's nonparametric analysis was used to determine correlation. Additional information can be found in the data supplement.

Results

HIV-Nef Protein Persists in the Lungs of Patients with HIV on ART

To establish HIV-Nef protein persistence and distribution in the lungs of patients with HIV on ART, we analyzed cells and acellular fluid from BAL obtained from patients in a well-characterized cohort (Table 1). We first stained BAL-derived cells for intracellular HIV-Nef using three different anti-Nef monoclonal antibodies directed against three unique HIV-Nef epitopes (EH1, 3D12, and SN20; Figure E1A in the data supplement) to address the high mutation rate of HIV proteins. We also used a novel ultraviolet–nucleotide binding site (UV-NBS) antibody labeling method (Figures E1B and E1C) to enhance sensitivity. In our patient cohort, we detected HIV-Nef protein persistence in at least 2% of BAL cells in nine out of 15 HIV$^+$ patients on ART (Figures 1A and E2; Table 1). Using markers for alveolar macrophage and lymphocytes, we found that HIV-Nef$^+$ cells were distributed throughout alveolar macrophages, CD4 T cells, and CD8 T cells (Figure 1B), which is consistent with our previous finding that HIV-Nef protein is widespread in blood cells (14). In none of the examined samples was the HIV virus detectable based on staining for the HIV protein p24 (data not shown) or deep sequencing for HIV viral sequences (RNA and DNA) of BAL (data not shown). In concert with recent findings that HIV-Nef persists in plasma EVs of ART patients (15), we demonstrated Nef persistence in BAL-derived EVs (Figures 1C and 1D; Table 1). We followed up on this observation by studying the role of EVs in the lung microenvironment of patients with HIV. EVs isolated from BAL of three randomly selected HIV$^+$ and three HIV$^-$ patients with matched smoking status were evaluated for their effects on lung-derived primary cells, i.e., HMVECs. Indeed, these EVs from patients with HIV induced apoptosis in HMVECs (Figure 1E). These results highlight the fact that HIV-Nef protein is widely distributed in the lungs of patients with HIV even in the absence of detectable HIV RNA, and suggest that this persistence corresponds to a detrimental effect in patients' lungs by promoting a proapoptotic environment.

Table 1. Analysis of Cell and Acellular Fluid from Patient BAL

Patient ID	Nef$^+$ BAL Cells (%)	[Nef] in BAL Fluid–Derived Extracellular Vesicles (ng/3 ml)	EMAPII$^+$ BAL Cells (%)	Antiretroviral Therapy Start Date	CD4 Cell Counts	Smoking Status
8	0.63	N/A	1.41	N/A	HIV$^-$	Yes
10	9.86	0	6.62	7/1/92	709	Former
16	3.13	0	6.06	6/15/11	707	Former
18	2.22	1.5	2.99	10/15/06	688	Former
19	6.75	6.5	12.3	N/A (HIV2$^+$)	N/A	Former
20	8.24	1.7	0.55	7/1/95	985	No
21	2.6	0	0.54	12/10/08	904	No
22	0.15	3.8	0.03	10/9/07	771	Yes
23	2.31	0	0.02	7/1/96	158	Yes
24	0.03	0	0.00	12/15/11	1305	Yes
25	2.83	8.5	3.23	7/1/08	495	No
26	0.27	6.5	0.12	6/15/10	737	Yes
27	0.5	N/A	0.1	N/A	HIV$^-$	Yes
28	18.4	4.7	0.25	7/1/10	966	No
29	0.2	0.5	0.06	7/1/96	158	Yes
30	0.21	4.3	0.1	5/1/13	231	Former
31	0.98	N/A	0	N/A	HIV$^-$	Yes
32	0.1	1.6	0.03	11/15/07	332	Former

Definition of abbreviation: EMAPII = endothelial-monocyte–activating polypeptide II; N/A = not available. BAL fluid from 18 patients was analyzed for HIV-Nef persistence and EMAPII surface expression. Antiretroviral therapy start date, CD4 cell counts, and the smoking status of each individual were also characterized.

HIV-Nef Mediates Its Own Transfer into EVs

HIV-Nef has been suggested to exert its function through insertion into and transport through EVs (17–21, 23, 24). Here, we investigated how HIV-Nef impacts EV formation and mediates its own incorporation into EV. When HIV-Nef was induced in a 4-hydro-tamoxifen (4-HT)-inducible expression system using a SupT1 T-cell cell line, a strong increase in EV secretion was observed (Figure 2A). Likewise, HIV-Nef transfection into HEK 293T cells resulted in an increase in the number of secreted EVs (Figure 2B). This HIV-Nef–induced increase in EV secretion was abrogated when HEK 293T cells were transfected with HIV-Nef F195R, a mutant that is incapable of PAK2 activation (25, 26). This suggests that HIV-Nef modifies EV packaging and secretion contingent on PAK2 activation, a pathway that is implicated in exocyst complex–mediated vesicular transport and exocytosis. Similarly, PAK2 inhibition with 5 μM FRAX 597 reversed the HIV-Nef–induced increase in EV release (Figures 2C and E3). Interestingly, we found that PAK2 activation was also required for the incorporation of HIV-Nef into EVs (Figure 2D). HIV-Nef–containing EVs from transfected cells and from patient BAL samples induced the release of proinflammatory cytokines such as TNF-α, IL-6, MCP-1, IP-10, and RANTES (regulated upon activation, normal T cell expressed and secreted) (Figure 2E) when added to freshly isolated primary human alveolar macrophages. Our data suggest that HIV-Nef persistence in the lung involves packaging of HIV-Nef into EVs,

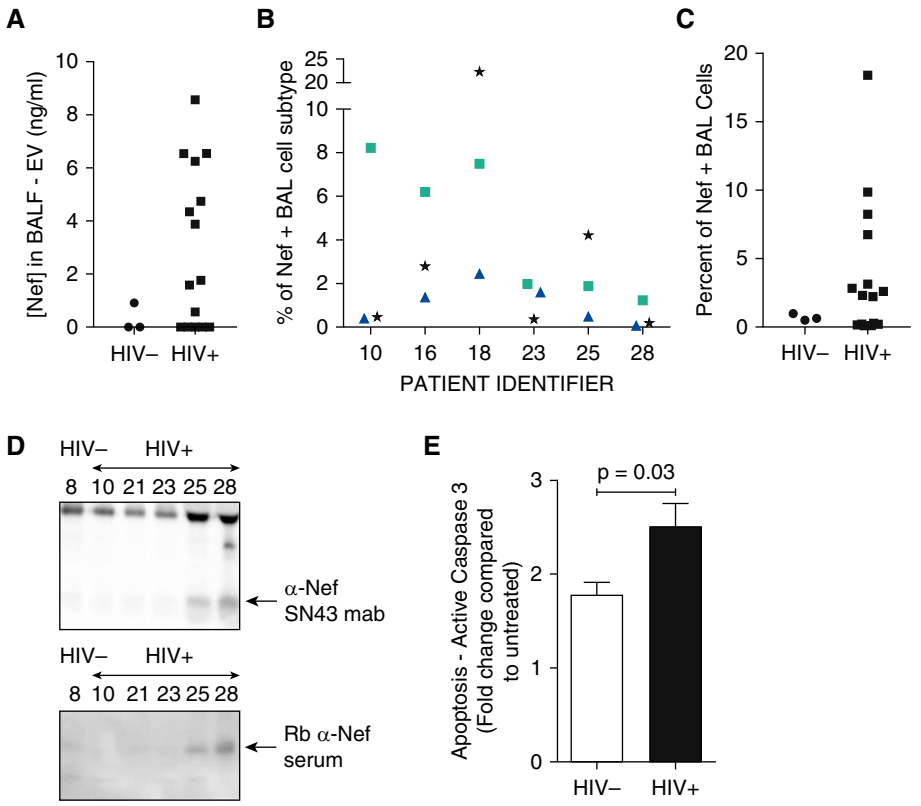


Figure 1. HIV-Nef protein persists in lungs of patients with HIV on antiretroviral therapy. (*A*) HIV-Nef protein was detected intracellularly in BAL-derived cells using flow cytometry (% Nef^+ cells). (*B*) BAL cells from six patients were costained with cell subtype markers to characterize HIV-Nef protein–positive cells as either CD4 T cells (blue triangles), CD8 T cells (green squares), or alveolar macrophages (black stars). (*C*) HIV-Nef in extracellular vesicles (EVs) isolated from acellular BAL fluid (BALF) was quantified by ELISA (ng/ml). (*D*) Western blot analysis of EVs isolated from acellular BALF (top: anti-Nef SN43ab; bottom: anti-Nef polyclonal Rb serum). (*E*) Apoptosis in human lung microvascular endothelial cells (HMVECs) was measured using caspase 3 activity upon addition of acellular BALF-derived EVs from three HIV^- and HIV^+ patients (with matched smoking status). The mean of the two groups was compared using the Mann-Whitney test.

and that these EVs are capable of promoting pathological activities *in vitro*.

HIV-Nef Protein Induces EMAPII Surface Expression

Based on our previous findings that the HIV envelope protein GP120 induces the surface expression and release of EMAPII, a central mediator of emphysema development and progression (10, 13, 27), we asked whether the persistent intracellular HIV viral protein HIV-Nef could also cause EMAPII release.

First, we investigated whether cells found in HIV-Nef–containing lung microenvironments were enriched for EMAPII surface expression. Indeed, we observed a strong correlation between the percentage of Nef^+ cells in the BAL and the percentage of $EMAPII^+$ cells (Spearman's correlation coefficient, 0.74; $P = 0.0006$) (Figure 3A).

To show that HIV-Nef expression can independently induce EMAPII trafficking to the cellular surface membrane, we transfected HEK 293T cells with cDNA to express HIV-Nef protein. Compared with mock-transfected HEK 293T cells, SF2 HIV-Nef–transfected cells showed increased EMAPII surface expression ($P < 0.001$) (Figure 3B). This EMAPII upregulation was dependent on PAK2 activation, as the Nef F195R mutant did not induce EMAPII surface expression (Figure 3B). Likewise, addition of 5 μM FRAX597, a PAK2 inhibitor, abrogated EMAPII surface expression in HIV-Nef–transfected HEK 293T cells (Figure E4B). Similarly, 4-HT activation of Nef-ER–expressing SupT1 T cells also induced an increase in surface EMAPII expression (Figure E4A).

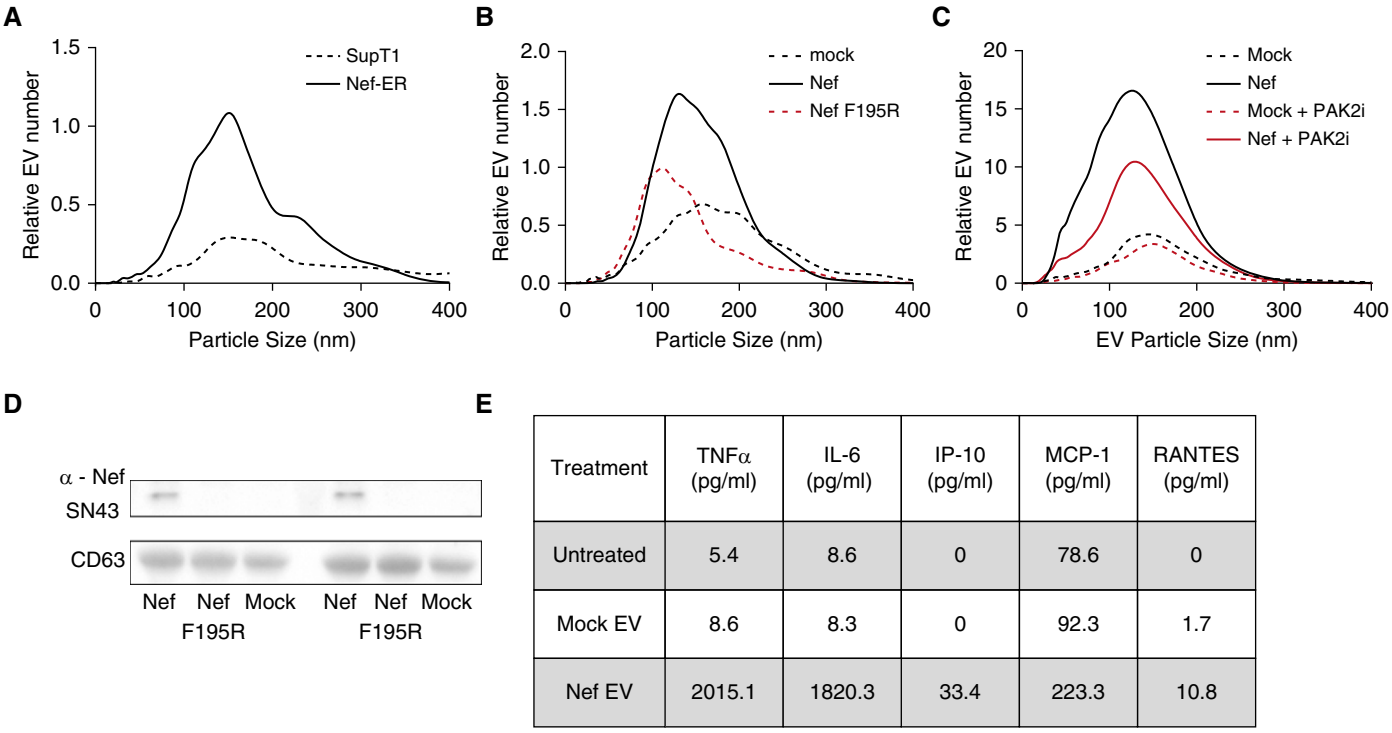
Next, we analyzed the impact of HIV-Nef protein persistence in the lung on EMAPII surface expression in the lung endothelium. We tested the hypothesis that Nef-containing EVs or Nef-expressing T cells can induce EMAPII expression in HMVECs. The addition of Nef-ER–expressing SupT1 cells (Figure 3C) and EVs from HIV-Nef–transfected HEK 293T cells (Figure 3D) significantly induced EMAPII surface expression in HMVECs. Again, the use of a PAK2 activation-incapable mutant (Figure 3D) abrogated EMAPII surface expression in EV-treated HMVECs and pulmonary arterial endothelial cells (Figure E4C).

Other investigators and we have reported that HIV-Nef protein can be transferred to endothelial cells and that its transfer stresses endothelial cells, which is a precursor for the development for vascular lesions (28–30). Based on these reports, we wanted to know whether HIV-Nef in endothelial cells is sufficient to cause EMAPII upregulation *in vivo*. To this end, we crossed HIV-Nef transgenic mice containing the tetracycline-responsive element with the VE-cadherin promoter–controlled tet-off-transactivator mouse line (31). The resulting offspring were kept for the duration of life without tetracycline to induce endothelial HIV-Nef, which led to a statistically significant rise in EMAPII surface expressing endothelial cells isolated from the lungs of VE-cadherin-Nef double-transgenic versus wild-type (WT) littermates (Figure 3E).

These data suggest that HIV-Nef persistence in the ART-treated HIV population leads to EMAPII upregulation *in vitro* and *in vivo*.

HIV-Nef Protein Induces Lung Endothelial Cell Apoptosis in an EMAPII-Dependent Fashion

We have previously shown that HIV-Nef protein can independently mediate its transfer from T cells to endothelial cells. We also found that HIV-Nef transfer is necessary and sufficient to induce endothelial cell reactive oxygen species production and apoptosis (30). Here, when we investigated the role of EMAPII surface expression in HIV-induced endothelial cell apoptosis, we observed a significant increase in apoptosis of HMVECs when they were cocultured with



Treatment	TNFα (pg/ml)	IL-6 (pg/ml)	IP-10 (pg/ml)	MCP-1 (pg/ml)	RANTES (pg/ml)
Untreated	5.4	8.6	0	78.6	0
Mock EV	8.6	8.3	0	92.3	1.7
Nef EV	2015.1	1820.3	33.4	223.3	10.8

Figure 2. Analysis of HIV-Nef protein expression in EVs. (*A–C*) NanoSight-based size and number analysis of EVs secreted from (*A*) Nef-ER–expressing SupT1 T cells and transfected human embryonic kidney (HEK) 293T cells compared with (*B*) PAK2 mutant or (*C*) the PAK2 inhibitor FRAX597. (*D*) Representative Western blot of EVs isolated from HEK 293T cells transfected with empty vector (mock), wild-type (WT) Nef, or a Nef mutant that is incapable of activating PAK2 (Nef F195R). CD63 antibody was used to show EVs in all lanes. (*E*) The addition of Nef-containing EVs isolated from transfected HEK 293T cells to primary BAL-derived alveolar macrophages induced the secretion of proinflammatory cytokines after 4 hours, as measured using BD Cytometric Bead Array. ER = estrogen receptor; RANTES = regulated upon activation, normal T cell expressed and secreted.

HIV-NL43–infected Jurkat T cells (Figure 4A), which was abolished in cells treated with anti-EMAPII antibodies. We previously showed that HIV-induced human coronary artery endothelial cell apoptosis was HIV-Nef dependent (30). Supporting this finding, HIV-induced HMVEC apoptosis was also HIV-Nef dependent. Jurkat cells infected with HIV-NL4–3 mutant with defective Nef (NL4–3 ΔNef) had no significant upregulation of HMVEC apoptosis compared with uninfected Jurkat cells (Figure 4A).

To examine the role of EMAPII in HIV-Nef–induced endothelial apoptosis, we analyzed endothelial cell apoptosis in the coculture model of Nef-ER–expressing SupT1 cells (Nef-ER) and HMVECs. Coincubation with 4-HT–activated Nef-ER increased endothelial cell apoptosis as measured by TUNEL staining ($P < 0.05$) (Figure 4B) and promoted mitochondrial depolarization, an early marker for apoptosis as measured by JC-1 staining ($P < 0.01$) (Figure 4C). The addition of neutralizing antibodies against EMAPII and the EMAPII receptor (CXCR3) abolished Nef-ER cell–induced HMVEC apoptosis (Figure 4B), and anti-EMAPII antibodies prevented HMVEC mitochondrial depolarization (Figure 4C), indicating that EMAPII surface expression plays an important role in mediating HIV-Nef–induced endothelial apoptosis. Furthermore, secreted EMAPII may potentiate HIV-Nef–induced endothelial apoptosis, as the addition of recombinant EMAPII to a coculture of Nef-ER SupT1 T cells and endothelial cells further enhanced endothelial cell apoptosis (Figure E5).

Finally, when HIV-Nef^{+} and HIV-Nef^{-} EVs (Figure 2B) were added to endothelial cells, only the HIV-Nef–containing EVs induced dose-dependent (Figure E6A) apoptosis in HMVECs after 24 hours, but not after 4 hours (Figure E6B). This HIV-Nef-EV–induced apoptosis was prevented with EMAPII-neutralizing antibody treatment (Figure 4D). Taken together, these data clearly demonstrate that HIV uses HIV-Nef–induced and EMAP-mediated pathways to induce apoptosis in endothelial cells.

Endothelial HIV-Nef Expression in Transgenic Mice Induces Pulmonary Pathology

To test whether Nef transfer to the endothelium is sufficient to cause pulmonary pathologies, we analyzed mice in which Nef was specifically expressed in the endothelium under control of the VE-cadherin promoter. At the age of 4 months, Nef-expressing mice did not manifest significant changes in weight compared with their Nef^{-} WT littermates (data not shown). However, pulse oximetry showed a statistically significant reduction in blood oxygenation ($P = 0.016$) in VE-cadherin-Nef double transgenics (Figure 5A). Pulmonary function tests in 5-month-old animals showed a significant increase in inspiratory capacity as measured using a deep inflation maneuver (gray bars) and extrapolated from a pressure–volume loop (red bars) (Figure 5C), indicating lung

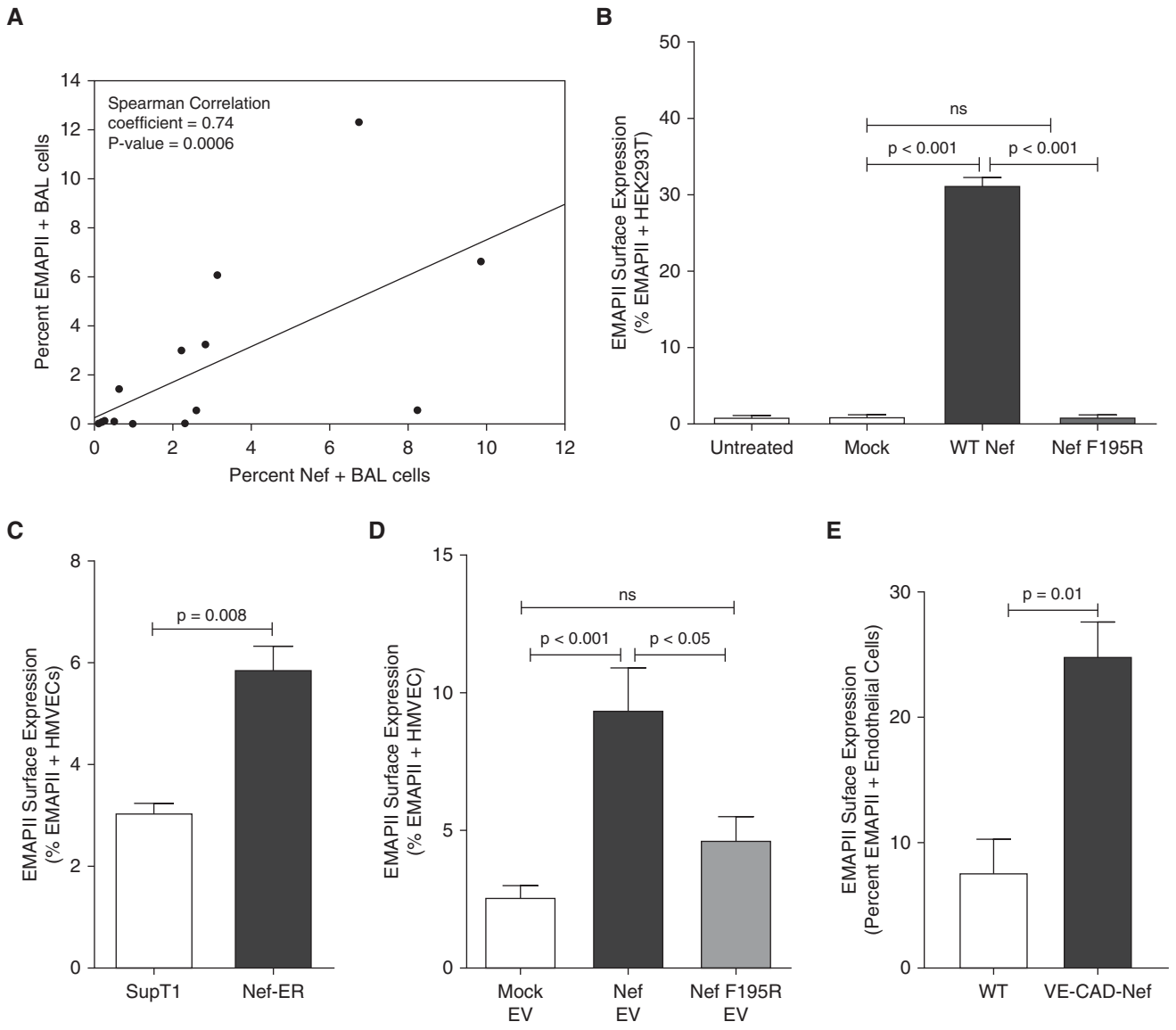


Figure 3. HIV-Nef protein induces endothelial-monocyte–activating polypeptide II (EMAPII) surface expression. (*A*) BAL-derived cells from 17 patients in the INHALD (Investigating HIV Associated Lung Disease) cohort were stained for intracellular Nef and surface EMAPII. The percentage of Nef^{+} and $EMAPII^{+}$ cells was quantified by flow cytometry. (*B*) EMAPII surface expression was measured in HEK 293T transfected with empty vector (mock), WT Nef, or a Nef mutant that is incapable of activating PAK2 (Nef F195R). (*C*) EMAPII surface expression in HMVECs 24 hours after the addition of tamoxifen (4-HT) activated Nef-expressing SupT1 T cells (Nef-ER) or 4-HT–activated control SupT1 T cells (SupT1). (*D*) EMAPII surface expression in HMVECs 24 hours after the addition of EVs isolated from either WT Nef (Nef) or Nef-mutant (Nef F195R) transfected HEK 293T supernatants. (*E*) EMAPII surface expression was measured in $CD45^{-}/CD326^{-}/CD31^{+}$ cells isolated from lungs of HIV-Nef transgenic mice and their littermates. The means of each group were compared using Student's *t* test or one-way ANOVA with Tukey's *post hoc* comparison. ns = not significant; VE-CAD = vascular endothelial-cadherin.

tissue rarefaction (32). Indeed, stereological analysis demonstrated a decrease in alveoli numbers in HIV-Nef transgenic animals compared with their Nef^{-} WT littermates (Figures 5B and E7). Importantly, endothelial cells from lungs of Nef-expressing mice manifested a higher percentage of cleaved caspase 3^{+} cells (Figure 5D), suggesting a possible role of Nef-induced apoptosis in alveolar loss. However, we failed to observe significant changes in the wet/dry lung ratio, white blood cell count and only a trend towards protein infiltration in the lungs (Figures E8A–E8C). These data highlight the notion that HIV-Nef causes EMAPII-mediated endothelial apoptosis, followed by lung tissue rarefaction, and are in line with our previous findings in lungs of EMAPII transgenic mice (8).

Discussion

In this study, we report for the first time that the virally encoded HIV-Nef protein persists in the lungs of patients with HIV on ART in both cells and EVs, as determined using three different techniques (flow cytometry, ELISA, and Western blot). In addition to Nef's persistence in the blood (16, 20), our discovery highlights a likely mechanism for

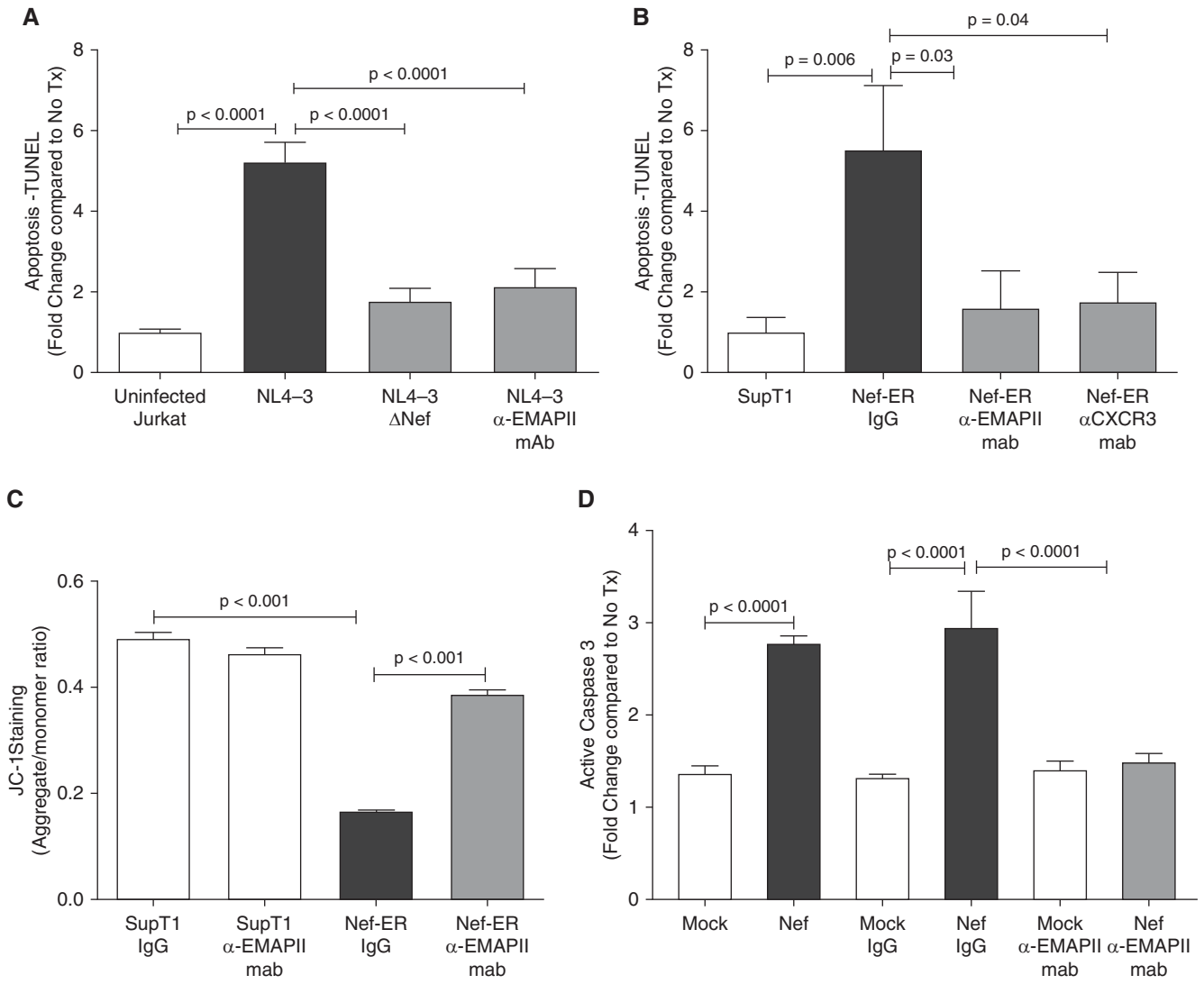


Figure 4. EMAPII mediates HIV and HIV-Nef–induced endothelial apoptosis. (*A*) Effect of HIV-NL4-3–infected Jurkat T cells on apoptosis as determined by TUNEL. (*B*) Coculture of 4-HT–activated Nef-ER–expressing SupT1 T cells (with/without EMAPII and CXCR3 neutralizing antibodies), and 4-HT–activated control SupT1 T cells induced HMVEC apoptosis as measured by TUNEL staining. (*C*) Confirmation of apoptosis staining through TUNEL by JC-1 staining, which is indicative for mitochondrial function. Note the decreased staining of JC-1 after exposure to 4-HT–activated Nef-ER SupT1 cells. (*D*) Effects on HMVEC apoptosis (determined by staining for active caspase 3) after addition of HIV-Nef–containing EVs from HEK 293T cells transfected with WT HIV-Nef plasmid or HIV-Nef F195R mutant. The groups were compared using one-way ANOVA with Tukey's *post hoc* comparison. Tx = treatment.

HIV-Nef–induced pulmonary vascular pathologies.

We also observed that HIV-Nef protein could be found in lung macrophages and $CD4^+$ and $CD8^+$ T cells in addition to BAL-derived EVs (33, 34). Our hypothesis that HIV-Nef mediates its transfer through EVs is in line with findings showing the persistence of HIV-Nef in plasma (16, 20) and our previous discovery that this protein is present in HIV^- T cells and B cells in the blood (35). Although we do not have any viremic patients with HIV in our cohort, Lee and colleagues (20) noted that Nef persists in plasma EVs of both viremic and aviremic patients with HIV. They also showed that both groups have similar EV profiles, as characterized by an increase in EV quantity and changes in microRNA and protein cargo compared with noninfected individuals.

The detection of HIV-Nef protein in patients on ART has the potential to act as a biomarker for HIV reservoir activity and explain the inability of ART to prevent the development of HIV-associated cardiopulmonary vascular diseases. Hence, we used a strict validation approach deploying three monoclonal antibodies directed against three distinct epitopes (SN20, 3D12, and EH1; *see* Figures 1A and E1A) to combat the high mutation rate of the HIV virus. We also conjugated our anti-Nef antibodies using a novel ultraviolet–nucleotide binding site conjugation system to enhance the

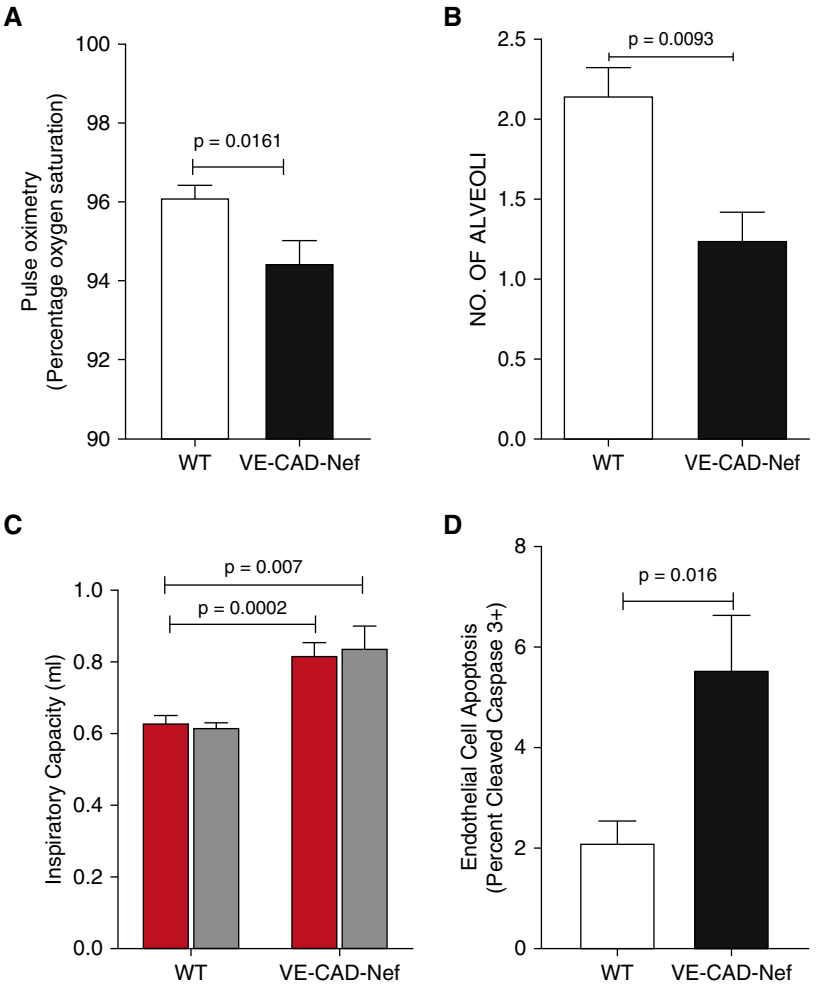


Figure 5. HIV-Nef expression in endothelial cells leads to emphysema-like lung tissue rarefaction in transgenic mice. Transgenic mice expressing HIV-Nef in VE-CAD$^+$ cells display lung tissue rarefaction. (*A*) Oximetry showed reduced oxygenation in the blood of HIV-Nef transgenic mice. (*B*) Stereological analysis revealed a corresponding reduction in alveoli numbers in VE-CAD-Nef–expressing lungs compared with WT littermates. (*C*) Inspiratory capacity was quantified using a deep inflation maneuver (gray bars) and extrapolated from the pressure–volume loop (red bars) using flexiVent data analysis. (*D*) Cleaved caspase-3 staining in CD45$^-$/CD326/CD31$^+$ cells from lungs. The means were compared using Student's *t* test with Welch's correction; $n > 5$.

sensitivity and specificity of HIV-Nef detection. We used a similarly rigorous approach in our discovery of HIV-Nef protein persistence inside EV isolated from BAL. We validated our findings from a commercially available anti-Nef ELISA kit that was previously used to detect HIV-Nef in plasma EV (16) with Western blot using two different monoclonal antibodies against Nef.

HIV-Nef is known to have the ability to interact with vesicular trafficking proteins and regulate EV secretion (36). Similarly to previous studies in plasma (24), we could not distinguish between microparticles and exosomes, and hence decided to use the term "extracellular vesicles." To the best of our knowledge; this is the first report to demonstrate Nef in EVs isolated from HIV-infected patients' BAL fluid. These EVs could originate from latently HIV-infected T cells and macrophages from HIV reservoirs in the lymphatic system, as Nef-containing EVs were shown to be CD45$^+$ (16). Another possible cellular source for Nef-containing EVs is the liver, based on microRNA and effector protein profiling of Nef-containing EVs isolated from plasma (21).

Interestingly, HIV-Nef persistence in the lungs of patients with HIV corresponds to an increased presence of proinflammatory chemokines in the lung milieu (Table 1). Although it was not significant given the relatively small number of patients analyzed, there was a trend toward higher chemokine levels in the HIV-Nef$^+$ group versus the HIV$^-$ control and HIV-Nef$^-$ groups, which was especially remarkable for IP-10. It would be interesting to address in longitudinal studies the relevance of these differences in the context of chronic obstructive pulmonary disease (COPD) development, given that we have available pulmonary function test data (forced expiratory volume in 1 second and D_{LCO}) and computed tomography scans of the lung. It would be desirable to test whether blood samples also reflect the changes observed in the lung (BAL). However, considering the dilution factor of BAL, all of the chemokines except for RANTES are found in much higher concentrations in the lung compared with the blood. Furthermore, the effects of Nef are more pronounced in the lung than in the blood compartment, indicating that the observed changes in chemokines and possibly other cytokines are restricted to the lung.

The ability of HIV-Nef to alter the chemokine profile of alveolar macrophages is important. These changes contributing to T cell dysfunction are believed to be one of the major mechanisms leading to consistent imbalance of the pulmonary microbiome (37–39). This imbalance both contributes to chronic inflammation in the lung and acts as a potential source for increasing the risk for lung cancer. Using alveolar macrophages, we highlight the ability of HIV-Nef–containing EVs to promote an inflammatory phenotype in a lung-specific lens. These findings reflect the ability of HIV-Nef–containing EVs to promote activation of blood-derived monocytes and macrophages, a common feature in HIV-associated end-organ diseases, including neurocognitive disorders and atherosclerosis (17, 20, 21, 24, 40, 41). However, the lung pathologies we characterized in our endothelial-specific Nef-expressing mice can be specifically attributed to increased endothelial cell apoptosis (Figure 5D). Because in this model we focused on the endothelium as the target tissue, the absence of inflammation in the lungs of these mice is

not surprising (Figure E8A). Further studies including injections of Nef-containing EVs and/or transgenic mouse studies with Nef–expressing alveolar macrophages are required to evaluate the entire spectrum of immunological and pathological consequences of HIV-Nef-EV uptake by alveolar macrophages.

Our finding that the persistence of HIV-Nef protein in latent infection is relevant with regard to end-organ diseases reflects previous findings in other viral infections; for example, adenoviral protein E1A was shown to persist in latent infections and to prime the lungs for the development of COPD/emphysema (42). Similarly, the risk of developing a pulmonary disease like COPD is heightened in patients with HIV on ART. Here, we show that the persistence of HIV-Nef protein in end organs such as the lung could induce secretion of proapoptotic EVs and thus act as a precursor for the development of pulmonary pathologies. In our extensively characterized cohort of patients with HIV on ART (Figures 1 and 2; Tables 1 and E1), we discovered a close correlation between HIV-Nef and EMAPII in BAL cells. This is in line with our previous demonstration that cigarette smoke also induces upregulation of EMAPII, thus leading to endothelial apoptosis (8, 43). Our findings showing that HIV-Nef can use an EMAPII-mediated pathway to induce endothelial cell apoptosis may help explain lung pathologies in patients with HIV because endothelial cell death has been suggested as an important mechanism for inducing lung emphysema in rodents (44–47) and humans (48). HIV-Nef–induced cytotoxic lymphocyte activation and lung capillary endothelial cell apoptosis may explain the increased incidence of emphysema in HIV-infected patients (6). Our results demonstrate that transgenic expression of HIV-Nef in the endothelium causes remodeling resembling emphysematous changes (Figures 5B–5D) in the lungs of these mice. This effect was mediated by a HIV-Nef–induced induction of endothelial apoptosis rather than inflammation, as we did not observe a significant increase in inflammatory immune cells in the BAL (Figure E8A), edema (Figure E8B) and only a trend for protein content (Figure E8C). Hence, in this study we focused on the contribution of endothelial cell apoptosis to the development of pulmonary pathology. This observation is consistent with previous data showing that apoptosis of lung endothelial cells driven by vascular endothelial growth factor receptor inhibitors is sufficient to cause emphysematous changes (47).

Although we did observe an increase in surface EMAPII expression in lung endothelial cells of Nef transgenic mice (Figure 3E), we only detected a slight, nonsignificant trend toward an increase in secreted EMAPII in the BAL (Figure E8D). Previously, we have shown that EMAPII, which is secreted from the lung epithelium, induces an emphysema-like phenotype in mice with both increased apoptosis and inflammation in the lungs (8). It is possible that endothelial cell–only expression of Nef causes EMAPII surface expression restricted to the lung endothelium and local apoptosis, whereas released EMAPII by proteases also contributes to inflammation. In support of this, our *in vitro* transmigration experiments showed no significant increase in transmigration of human monocytic cell line (THP-1) toward HMVECs treated with Nef-EVs (data not shown), whereas alveolar macrophages treated with Nef-EVs secreted proinflammatory cytokines (Figure 2E). Future studies expressing HIV-Nef in other cell types, including alveolar macrophages, can examine whether HIV-Nef can also upregulate EMAPII secretion that in turn drives inflammation.

Targeting an HIV-induced proapoptotic pathway in endothelial cells via blocking EMAPII with neutralizing antibodies may be sufficient to treat HIV-associated pulmonary diseases. Our data suggest that transfer of HIV-Nef to the vascular endothelium can cause pulmonary emphysema–like changes (Figure 5). HIV-Nef has already been associated with another pulmonary vascular disease: pulmonary hypertension (28, 29). Here, we show that BAL fluid–derived EVs contain HIV-Nef, and that HIV-Nef–containing EVs induce EMAPII surface expression in primary human pulmonary artery endothelial cells (Figure E4C). It is possible that HIV-Nef–containing EVs from the lung act as the initial insult that results in the loss of endothelium that is characteristic of pulmonary arterial hypertension. Given the recent proposition that endothelial cell apoptosis may be a common denominator for the development of both emphysema and pulmonary arterial hypertension (49, 50), the mechanism suggested here to explain how HIV contributes to endothelial apoptosis should be of general interest. In this regard, targeting PAK2 activation–dependent EMAPII pathways could be of therapeutic interest in combating HIV-associated pulmonary vascular diseases. ■

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