

ORIGINAL RESEARCH

HIV-Nef Protein Transfer to Endothelial Cells Requires Rac1 Activation and Leads to Endothelial Dysfunction

Implications for Statin Treatment in HIV Patients

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RATIONALE: Even in antiretroviral therapy-treated patients, HIV continues to play a pathogenic role in cardiovascular diseases. A possible cofactor may be persistence of the early HIV response gene Nef, which we have demonstrated recently to persist in the lungs of HIV+ patients on antiretroviral therapy. Previously, we have reported that HIV strains with Nef, but not Nef-deleted HIV strains, cause endothelial proinflammatory activation and apoptosis.

OBJECTIVE: To characterize mechanisms through which HIV-Nef leads to the development of cardiovascular diseases using ex vivo tissue culture approaches as well as interventional experiments in transgenic murine models.

METHODS AND RESULTS: Extracellular vesicles derived from both peripheral blood mononuclear cells and plasma from HIV+ patient blood samples induced human coronary artery endothelial cells dysfunction. Plasma-derived extracellular vesicles from antiretroviral therapy+ patients who were HIV-Nef+ induced significantly greater endothelial apoptosis compared with HIV-Nef-plasma extracellular vesicles. Both HIV-Nef expressing T cells and HIV-Nef-induced extracellular vesicles increased transfer of cytosol and Nef protein to endothelial monolayers in a Rac1-dependent manner, consequently leading to endothelial adhesion protein upregulation and apoptosis. HIV-Nef induced Rac1 activation also led to dsDNA breaks in endothelial colony forming cells, thereby resulting in endothelial colony forming cell premature senescence and endothelial nitric oxide synthase downregulation. These Rac1-dependent activities were characterized by NOX2-mediated reactive oxygen species production. Statin treatment equally inhibited Rac1 inhibition in preventing or reversing all HIV-Nef-induction abnormalities assessed. This was likely because of the ability of statins to block Rac1 prenylation as geranylgeranyl transferase inhibitors were effective in inhibiting HIV-Nef-induced reactive oxygen species formation. Finally, transgenic expression of HIV-Nef in endothelial cells in a murine model impaired endothelium-mediated aortic ring dilation, which was then reversed by 3-week treatment with 5 mg/kg atorvastatin.

CONCLUSIONS: These studies establish a mechanism by which HIV-Nef persistence despite antiretroviral therapy could contribute to ongoing HIV-related vascular dysfunction, which may then be ameliorated by statin treatment.

VISUAL OVERVIEW: An online [visual overview](#) is available for this article.

Key Words: apoptosis ■ cardiovascular diseases ■ endothelial progenitor cells ■ extracellular vesicles ■ statin

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HIV-positive patients remain at higher risk for developing cardiovascular diseases (CVD) including acute myocardial infarction,¹ peripheral arterial disease,² and hypertension despite achieving circulating viral suppression with

antiretroviral therapy (ART).^{3–5} Importantly, CVD remains a major cause of death in HIV patients with undetectable viral loads and well-preserved CD4 counts.⁶ These data suggest that HIV infection increases the risk of vascular pathologies.

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The online-only Data Supplement is available with this article at <https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.119.315082>.

For Sources of Funding and Disclosures, see page 818.

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Novelty and Significance

What Is Known?

- HIV increases the risk of age-related disorders including cardiovascular disease.
- HIV encodes a protein, Nef, which can be found encapsulated in extracellular vesicles in the blood.
- HIV-Nef protein is necessary and sufficient to mediate HIV virus-induced death of vascular endothelial cells.

What New Information Does This Article Contribute?

- HIV-Nef protein is present in mononucleated peripheral blood cells of HIV+ individuals on antiretroviral therapy at similar levels to treatment naive patients.
- HIV-Nef protein in extracellular vesicles can result in arterial endothelial dysfunction; the latter can be reversed by treatment with statins.

HIV-Nef causes premature aging (senescence) in an endothelial progenitor cell population that helps regenerate blood vessels following injury, thus providing another novel mechanism of how HIV can contribute to vascular diseases. Over 1.1 million Americans are HIV positive. Although they are protected against

development of AIDS due to viral load suppressing anti-retroviral therapy (ART), these patients have a persistently increased risk for cardiovascular disease. In our study, we show that the HIV-secreted protein Nef remains present in aviremic HIV patients. Strategies are presented to extend these findings, that is, we demonstrate that the Rac1 signaling pathway plays an important role for the transfer of HIV-Nef via extracellular vesicles into endothelial cells and their progenitors (named endothelial colony forming cells or ECFC). This same pathway is also responsible for HIV-Nef-induced endothelial dysfunction in human coronary arterial endothelial cells and premature senescence in ECFC. Importantly, this study provides strong evidence that the Nef-Rac1-ROS pathway can be targeted using 2 different FDA approved statins (Atorvastatin and Pitavastatin) that have minimal drug-drug interactions with antiretroviral therapy and are routinely prescribed to HIV+ patients for dyslipidemia. This study provides a rationale to prescribe statins, even to HIV+ patients without dyslipidemia, to reduce the risk of HIV-associated cardiovascular diseases.

Nonstandard Abbreviations and Acronyms

ART	antiretroviral therapy
CFC	endothelial colony forming cell
CVD	cardiovascular disease
EV	extracellular vesicles
HCAEC	human coronary artery endothelial cell
PBMC	peripheral blood mononuclear cell
ROS	reactive oxygen species

A healthy endothelium is important for the maintenance of vascular tone and prevention of immune activation. Endothelial cell dysfunction impairs endothelium-mediated blood vessel function⁷ leading to dysregulation of vascular tone, arterial stiffness, and cardiovascular disease development. Endothelial dysfunction⁸ and increased arterial stiffening⁹ may contribute to greater risk of CVD.¹⁰ Identification of mechanisms underlying HIV-related endothelial dysfunction may lead to the development of therapeutic strategies against HIV-associated pathologies.

Continuous expression of HIV-encoded proteins in patients on ART with undetectable HIV RNA is a potential cause for HIV-related CVD. HIV-Nef is a myristoylated intracellular HIV protein adept at hijacking host cell signaling machinery and is released in circulating extracellular vesicles (EVs),^{11,12} thereby allowing widespread dissemination.

HIV-Nef's ability to persist in EV is conserved, with HIV-Nef+ EV found in the SIV macaque model.¹³ HIV-Nef persists in multiple immune cell types, including circulating CD4 T-cells, CD8 T-cells, and B cells¹⁴ and in lung alveolar macrophages.¹² HIV-Nef has been isolated in the extracellular compartment of plasma^{15,16} and bronchoalveolar lavage fluid.¹² HIV-Nef also shows persistence in several tissues including the heart,¹⁷ brain,¹⁸ and lung.¹² Thus, HIV-Nef is a likely etiologic agent for causing end-organ diseases linked to endothelial dysfunction because multiple studies have shown its capacity to induce endothelial cell apoptosis and mitochondrial dysfunction.^{19–21}

HIV-Nef directly activates small GTPase Rac1, which in turn promotes reactive oxygen species (ROS) production.^{22,23} Subsequently, ROS formation contributes to vascular dysfunction by promoting proapoptotic pathways and downregulating endothelial nitric oxide synthase (eNOS).¹⁵ Recent studies have suggested the use of statins, which by inhibiting the geranylgeranylation of Rac1,²⁴ can block NADPH oxidase-mediated ROS production. In fact, statin treatment protects HIV+ patients from the development of CVD to a larger extent than can be attributed to LDL reduction.^{25,26} Specifically, atorvastatin and pitavastatin have modest to no drug-drug interactions with ART, making them attractive drugs to prescribe to HIV+ patients.^{27,28} As such, pitavastatin is being studied in REPRIEVE (Randomized Trial to Prevent Vascular Events in HIV), an

ongoing randomized controlled study to prevent cardiovascular diseases in HIV+ patients.²⁹

In this current study, we have identified pathways by which HIV-Nef may exert negative effects on endothelial cells, with a focus on the role of Rac1 signaling in HIV-Nef dissemination and its downstream consequences. We further analyzed the role of endothelial expression of HIV-Nef on endothelial dysfunction and vascular pathologies in a murine model *in vivo*.

METHODS

The authors declare that all supporting data are available within the article and [Online Data Supplement](#).

Human Coronary Artery Endothelial Cells (HCAECs) were cultured in EGM2MV. HIV-Nef was expressed in cells via transfection or addition of HIV-Nef+ EVs. EVs were isolated from plasma or supernatants of HIV-Nef transfected cells using serial centrifugation. Peripheral blood mononuclear cells (PBMCs) and plasma was obtained from patients after acquiring informed consent in accordance with IRB approval. Transgenic endothelial HIV-Nef expression was under control of VE-Cadherin promoter. Animal experiments were performed after obtaining Institutional Animal Care and Use Committee approval.

Detailed methods are available in the [Online Data Supplement](#).

RESULTS

PBMC and EV From HIV+ Patients on ART Induce Endothelial Dysfunction

Earlier, we published that cocultivation of HIV-infected T cells induce ROS formation and apoptosis in HCAEC.²¹ Here, we investigated whether HIV+ patient-derived PBMC also induce endothelial dysfunction in HCAEC. Overnight incubation of PBMC from HIV+ patients prominently increased caspase 3 activation (Figure 1A) and promoted mitochondrial depolarization (Figure 1B) when compared with control PBMC from HIV− patients.

Using the same assays, we next questioned whether EVs isolated from HIV+ patients can damage HCAEC. Indeed, overnight incubation of HCAEC with HIV+ plasma EV significantly increased caspase 3 activation (Figure 1C) and induced mitochondrial depolarization (Figure 1D) compared with EV from healthy individuals. When EV from ART naïve (ART−) and ART-treated (ART+) HIV+ patients were compared, no significant differences in HCAEC caspase 3 activation or mitochondrial depolarization were observed (Online Figure 1B).

PBMC and EV From HIV+ Patients on ART Contain HIV-Nef Protein

Previously,^{12,21} we have shown that HIV-Nef protein persists in PBMC and BAL cells from HIV+ patients in CD4 T cells, CD8 T cells, and alveolar macrophages.

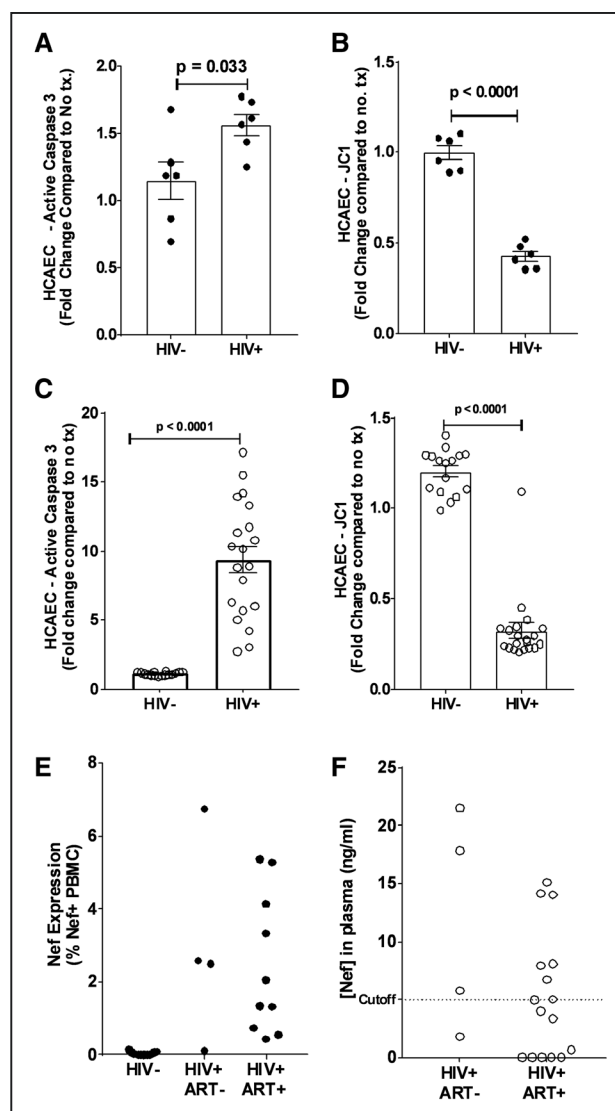


Figure 1. HIV-Nef protein persists in virologically suppressed HIV+ patients.

Addition of peripheral blood mononuclear cells (PBMCs; **A** and **B**, closed circles) and extracellular vesicles (**C** and **D**, open circles) from HIV+ patients to human coronary artery endothelial cell (HCAEC)-induced endothelial cell apoptosis as measured by active caspase 3 levels (**A** and **B**) and mitochondrial depolarization using JC-1 (**C** and **D**). HIV-Nef protein persists in PBMCs (**E**) and plasma (**F**) of HIV+ patients either treatment naïve or on antiretroviral therapy. Dotted line indicates cutoff (5 ng/mL) based on HIV-control plasma and previously published results.³⁰ Statistical significance between groups was determined using Student *t* test with Welch correction. Raw *P* is indicated in graphs.

Therefore, we queried whether the effects of PBMC and EV on HCAEC could be explained by the presence of HIV-Nef protein. To detect HIV-Nef protein, we employed an approach we recently developed to cope with the high rate of mutation of the HIV using 3 monoclonal antibodies (SN20, EH1, and 3D12) targeting different HIV-Nef epitopes.¹² Fluorescence-activated cell sorting analysis of PBMC from 14 HIV+ patients demonstrated persistent HIV-Nef protein in 3 out of 4 treatment-naïve and 9 out of 10 ART-treated HIV patients (Figure 1E). All

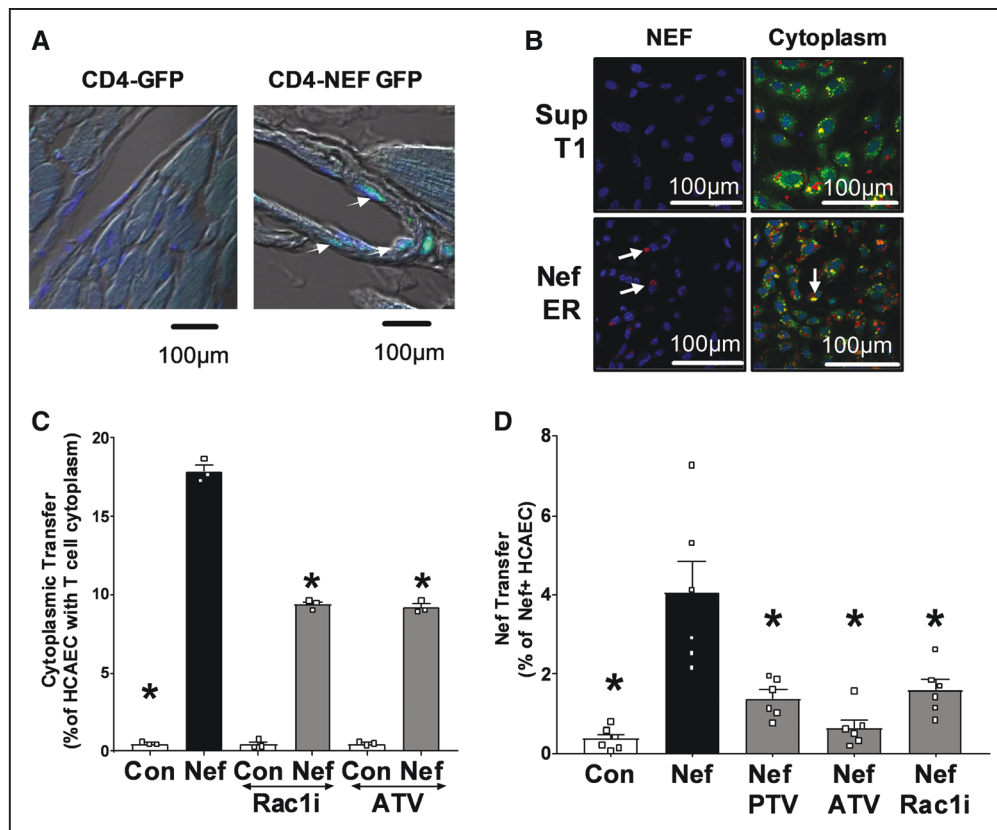


Figure 2. HIV-Nef protein promotes cytoplasmic transfer from T cells to coronary artery endothelial cells.

Heart sections of single CD4-GFP and double CD4-Nef-GFP transgenic mice were stained with α GFP mAb (green). White arrows indicate T-Cell cytoplasm within endothelial lining (**A**). HIV-Nef protein transfer from SupT1 or Nef-ER T cells to human coronary artery endothelial cell (HCAEC) is visualized using anti-Nef EH1 mAb (monoclonal antibody; left). Cytoplasmic transfer of Cell Tracker Deep Red Dye from T cells is seen in Calcein AM-stained endothelial cells (right; **B**). Quantification of cytoplasmic transfer (**C**) or HIV-Nef protein transfer (**D**) from T-Cells to HCAEC was quantified using fluorescence-activated cell sorting. ATV=5 μ mol/L atorvastatin; PTV=100 nmol/L pitavastatin; Rac1i=5 μ mol/L NSC23766. Groups were compared using 1-way ANOVA followed by Tukey Post hoc test. *Denotes adjusted $P < 0.001$ compared with HIV-Nef group (black bar). Scale bars denote 100 μ m. EV indicates extracellular vesicle.

13 HIV- patients had undetectable HIV-Nef showcasing the specificity of our assay. Interestingly, HIV-Nef was detected in HIV+ patients on various ART regimens (Online Table I) suggesting that HIV-Nef persistence in PBMC may be independent of ART strategy.

We detected HIV-Nef protein in plasma from ART naïve (ART-, $n=4$) and ART treated (ART+, $n=16$) HIV patients. Analysis of patients on ART revealed that 7 were HIV-Nef-positive whereas 9 were not (Figure 1F), which agrees with a previous study showing 50% of HIV+ patients on ART have HIV-Nef-positive plasma samples.¹⁶ Interestingly, EV from HIV-Nef positive plasma induced more endothelial caspase-3 activation than EV from HIV-Nef negative plasma (Online Figure 1C). This suggests that the proapoptotic activity in HIV patients is mediated by HIV-Nef.

Therefore, both the cellular and extracellular fractions of HIV+ patient blood contain HIV-Nef protein despite suppression of viral replication using ART. As such, blood HIV-Nef-positivity could be used to predict endothelial cell dysfunction.

HIV-Nef Increases Cytosolic Transfer From T Cell to the Endothelium

HIV-Nef protein promotes its own transfer from HIV-infected CD4 T cells to the endothelium in vitro and ex vivo.^{14,21} Therefore, we investigated the mechanism used by HIV-Nef to facilitate the transfer of cytosol to endothelial cells. We confirmed our previous finding³¹ of cytosol transfer to the endothelium in a murine transgenic model expressing HIV-Nef but no other HIV protein. HIV-Nef and GFP are bidirectionally expressed in CD4 T cells allowing the use of GFP staining as a surrogate for cytoplasmic transfer from T cells to the endothelium. We observed endothelial GFP only in heart sections of the HIV-Nef-GFP transgenic mice but not in those mice only expressing GFP (Figure 2A, right).

To analyze the mechanism of how HIV-Nef induces cytoplasmic transfer from HIV-Nef-expressing T cells to endothelial cells, we cocultured Cell Tracker Deep Red-labeled SupT1 T Cells with Calcein AM-labeled HCAEC (Figure 2B). We used a tamoxifen-inducible HIV-Nef estrogen receptor model to mediate HIV-Nef-dependent

activities.^{12,32} HIV-Nef activation in Nef estrogen receptor expressing SupT1 T cells increased cytoplasmic transfer (Figure 2B, right) in a T cell concentration-dependent fashion (Online Figure IIA) and was attenuated by both Rac1 inhibitor and atorvastatin (Figure 2C). Using confocal microscopy, we observed a significant increase in the uptake of vesicular structures containing T cell cytoplasm into HCAEC (Figure 2B, right; Online Figure IIB). Concomitant with the cytoplasmic transfer from T cells, HIV-Nef was detected in HCAEC cocultured with HIV-Nef-expressing T cells (Figure 2B, left), which was significantly reduced with Rac1 inhibitor as well as pitavastatin and atorvastatin (Figure 2D).

This suggests that HIV-Nef expression enhances transfer of cytoplasm from T cells to endothelial cells in a process mediated by the small GTPase Rac1.

HIV-Nef Expressing T Cells Induce Endothelial Cell Dysfunction Dependent on Rac1 Activation

HIV-Nef protein is necessary and sufficient to induce ROS production, which results in endothelial cell apoptosis.²¹ To gain insight into the mechanism mediating this process, we analyzed the importance of HIV-Nef-induced Rac1 activation previously shown to cause NADPH-dependent ROS production in cells of the monocytemacrophage lineage.³³ Both transfection of HCAEC with HIV-Nef expressing plasmids (Online Figure III) and overnight coculture with HIV-Nef-expressing SupT1 T cells (Figure 3A) induced ROS production in HCAEC, which could be ameliorated by Rac1 inhibition using both the small molecule selective inhibitor NSC23766 (that blocks Rac1 activation) and atorvastatin (reduces Rac1 trafficking to plasma membrane via inhibition of Rac1 geranylgeranylation³⁴; Figure 3A). As we had shown earlier,²¹ this HIV-Nef-induced ROS production was dependent on the NADPH oxidase complex as an inhibitory peptide against the GP91 subunit prevented caspase 3 activation after coculture with HIV-Nef+ PBMC (Figure 3B). Following ROS imbalance, mitochondrial depolarization is considered a pathway toward apoptosis in cells. Rac1 inhibition (with both NSC23766 and statins) and NADPH oxidase inhibition (with the GP91 inhibitory peptide) ameliorated HIV-Nef-induced HCAEC mitochondrial dysfunction in endothelial cells after coculture with HIV-Nef+ T cells (Online Figure IVA). These treatments also blocked HIV-Nef-induced Caspase 3 activation (Figure 3C) and DNA fragmentation (Online Figure IVC and IVD), thereby implicating this signaling pathway in endothelial cell apoptosis.

We next attempted to replicate our findings in Figure 1 of endothelial apoptosis induced by PBMC from HIV+ patients on ART with using a model of HIV-Nef+ PBMC. We treated PBMC from healthy donors with EV from HIV-Nef transfected (Nef) or mock transfected (Con) HEK293T cells for 2 hours to create HIV-Nef+ PBMC (Figure 3C). These PBMCs were cocultured

overnight with HCAEC resulting in Rac1-dependent (using Rac1 inhibitor NSC23766 and pitavastatin) endothelial ROS formation (Figure 3D), which was accompanied by increased proapoptotic signaling (Figure 3E) and mitochondrial depolarization (Online Figure IVB). Blocking endothelial ROS formation via inhibition of the gp91 subunit of NADPH oxidase complex also prevented HCAEC apoptosis (Figure 3E).

HIV-Nef-positivity in T cells and PBMC potentially induce endothelial cell dysfunction by upregulating endothelial ROS production in a Rac1 activation-dependent fashion.

HIV-Nef+ EVs Are Key Mediators of HIV-Nef-Induced Cytosolic Transfer to and Dysfunction in Endothelial Cells

As, EV have been suggested to mediate the transfer of HIV-Nef protein and cytoplasm to the endothelium,¹² we hypothesized that Rac1 inhibition could block HIV-Nef-increased EV release. Rac1 inhibition by a chemical inhibitor (NSC23766), a dominant negative Rac1 (RacT17N), Rac1 siRNA (Online Figure V), and pitavastatin all impeded HIV-Nef's ability to increase EV release from HIV-Nef-expressing HEK293T cells (Figure 4A; Online Figure VIA). The presence of EVs was characterized using transmission electron microscopy (Figure 4B), Nanoparticle Tracking Analysis, and Western blotting for membrane-associated protein, HIV-Nef and the tetraspanin CD9 (Online Figure VIB). Therefore, HIV-Nef utilizes a Rac1 activation-dependent pathway to increase communication between T cells and the endothelium.

We have previously demonstrated that direct contact between T cells²¹ or PBMC¹⁴ from HIV+ patients and HCAEC facilitated enhanced transfer of HIV-Nef protein into endothelial cells. Here, we tested whether EV could mediate this effect. First, we isolated HIV-Nef protein in the EV fraction of HIV-Nef transfected human embryonic kidney (HEK) 293T (Online Figure VIB) and showed that these HIV-Nef+ EV could transfer HIV-Nef protein into HCAEC after 2 hours (Figure 4C). We had earlier shown that transfection of HEK293T with wild-type (WT) SF2 HIV-Nef but not Pak2 activation deficient mutant produced HIV-Nef+ EV¹². Using this model, we could show that HIV-Nef presence in EV cargo was necessary to induce HCAEC apoptosis (Online Figure VII). The uptake of HIV-Nef+ EV resulted in increased ROS production in HCAEC after further incubation overnight, which could be blocked with Rac1 inhibition. NADPH oxidase mediated this effect since inhibition with GP91 inhibitory peptide abrogated HIV-Nef induced ROS production (Online Figure VIIIA).

Blocking Rac1 activity using transfection with dominant negative mutant RacT17N and Rac1 siRNA also nullified HIV-Nef EV-induced endothelial ROS production (Online Figure VIIIA). Furthermore, incubation of HCAEC cells with HIV-Nef+ EV resulted in endothelial

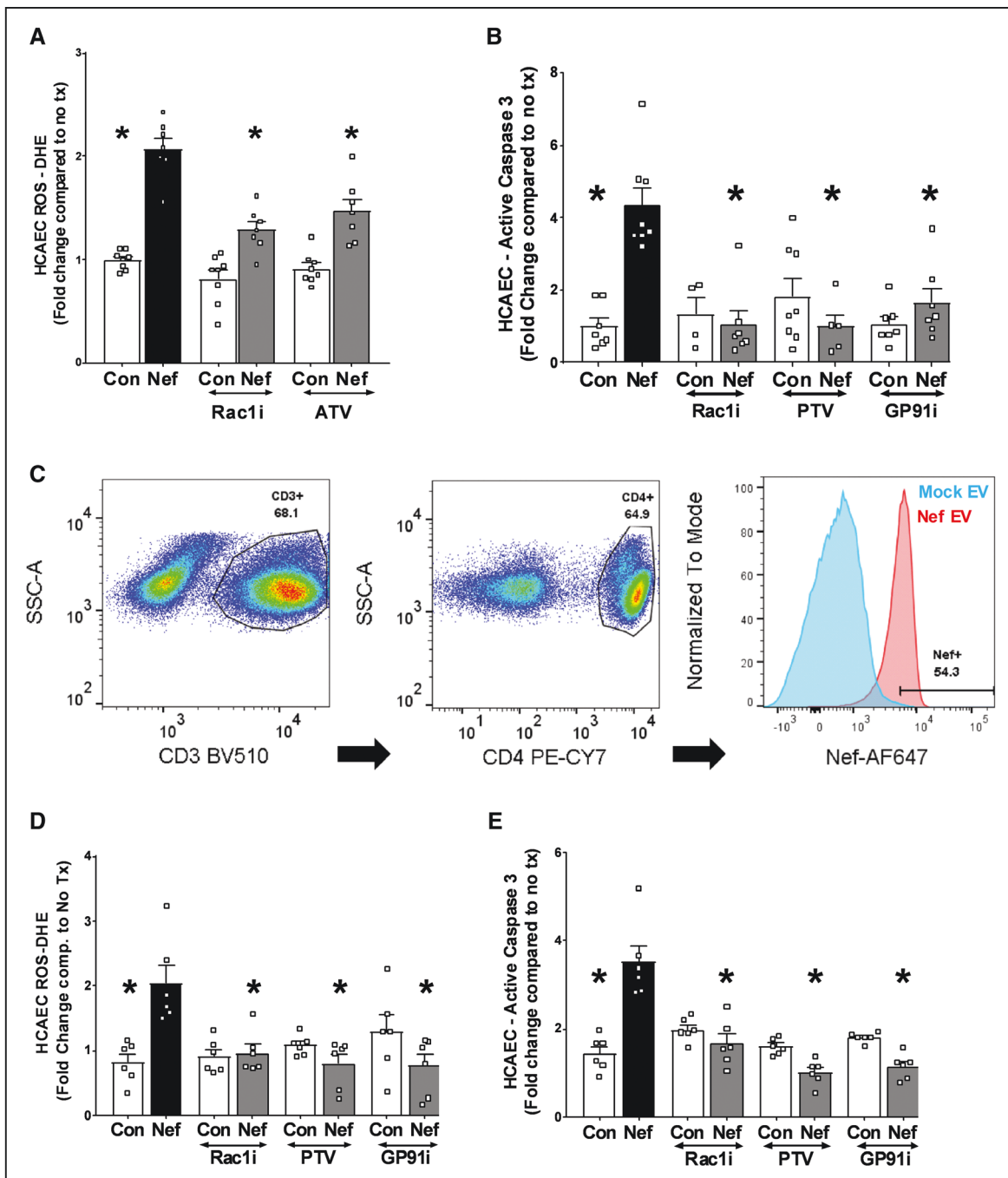


Figure 3. Rac1 inhibition and statin treatment block HIV-Nef protein-induced endothelial cell stress.

Coculture of HIV-Nef expressing T cells (Nef) induced reactive oxygen species (ROS) production (A) and apoptosis (B) in human coronary artery endothelial cell (HCAEC). HIV-Nef protein was taken up by CD45+/CD3+/CD4+ T cells when peripheral blood mononuclear cells (PBMCs) were treated with mock extracellular vesicle (EV) or HIV-Nef EV (Nef) for 2 h (blue=mock, red=Nef; C). HIV-Nef EV-treated PBMC induced ROS production (D) and caspase 3 activation (E) in HCAEC. ATV=5 μ M/L atorvastatin; PTV=100 nmol/L pitavastatin; Rac1i=5 μ M/L NSC23766. GP91i, inhibitory peptide 1 μ M/L. Groups were compared using 1-way ANOVA followed by Tukey Post hoc test. *Denotes adjusted $P < 0.001$ compared with HIV-Nef group (black bar).

apoptosis (Figure 4E and 4F) and mitochondrial depolarization (Online Figure VIIIB). HIV-Nef induced Caspase-3 activation could be prevented by both atorvastatin and pitavastatin (Figure 4E). Of note, statins were comparable to the Rac1 activation inhibitor-NC23766 (Online Figure VIIIC) and Rac1 geranylgeranylation inhibitor- GGTI-298

(Figure 4D) in their ability to block HIV-Nef-induced HCAEC ROS production. Thus, statins, which also inhibit Rac1 geranylgeranylation, is an effective treatment to block HIV-Nef induced endothelial stress.

HIV-Nef-induced PAK2 activation is an important element for endothelial dysfunction that follows uptake of

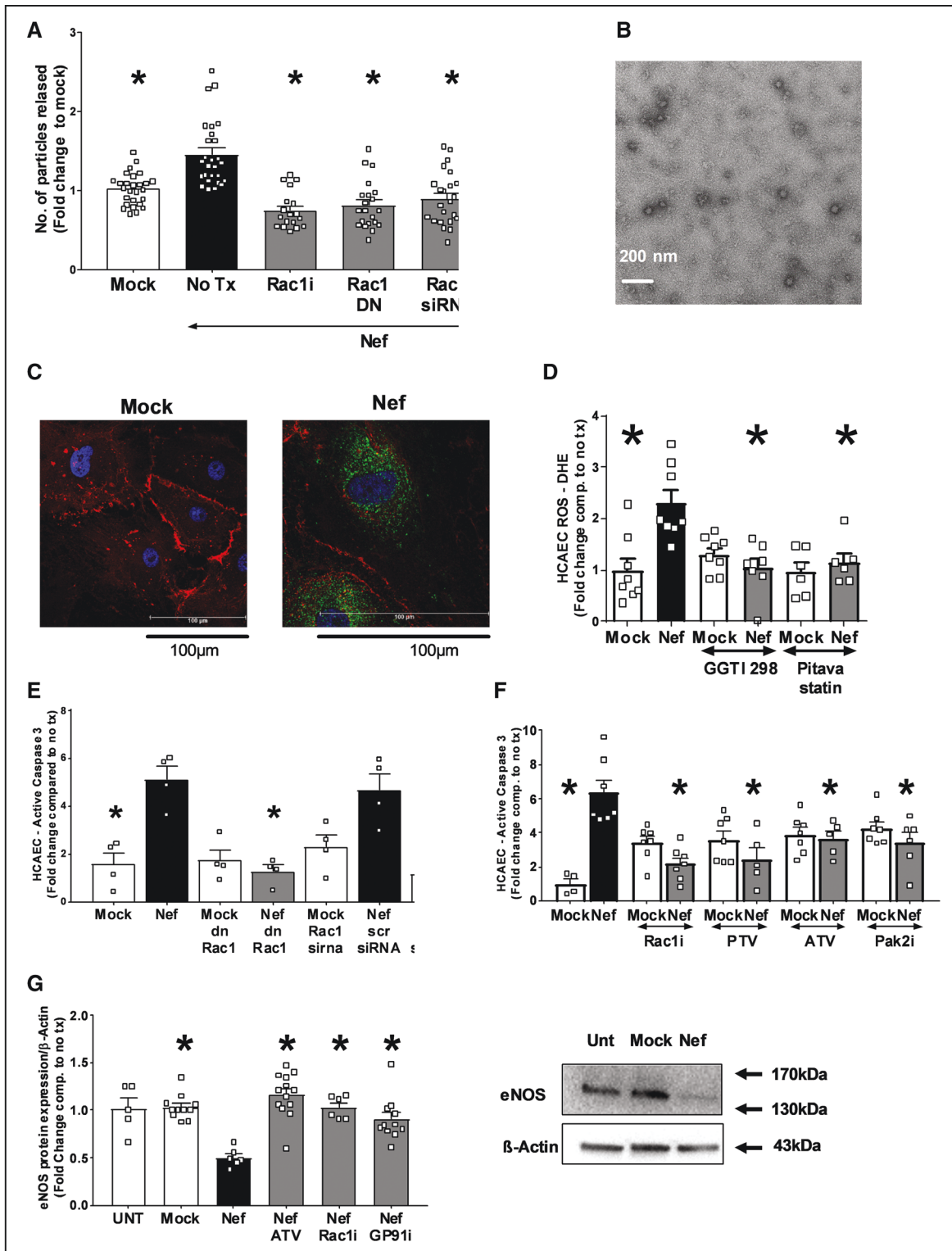


Figure 4. HIV-Nef extracellular vesicle (EV) induce endothelial cell apoptosis.

A, HIV-Nef transfection into HEK293T increases the amount of EV released. EV morphology was analyzed using transmission electron microscopy (**B**) with scale bar denoting 200 nm. **C**, Addition of HIV-Nef+ EV transfected HIV-Nef protein to human coronary artery endothelial cell (HCAEC; green= α Nef EH1 mab; red=PECAM; blue=4',6-diamidino-2-phenylindole [DAPI]; scale bars=100 μ m). HIV-Nef+ EV promotes reactive oxygen species (ROS) production (**D**), apoptosis (**E** and **F**) and downregulation of endothelial nitric oxide synthase (eNOS; **G**) in HCAEC. ATV=5 μ mol/L atorvastatin; PTV=100 nmol/L pitavastatin; Rac1i=5 μ mol/L NSC23766. GP91i, inhibitory peptide 1 μ mol/L; Pak2i=5 μ mol/L FRAX597 GGTI-298=5 μ mol/L. Groups were compared using 1-way ANOVA followed by Tukey Post hoc test. *Denotes adjusted $P<0.001$ compared with HIV-Nef group (black bar). #Denotes $P<0.0001$ compared with HIV-Nef scr siRNA. UNT indicates untreated.

HIV-Nef+ EV into human lung microvascular endothelial cells.¹² In this regard, Rac1 inhibition showed potency in abrogating HIV-Nef+ EV induced HCAEC apoptosis comparable to the PAK2 inhibitor, FRAX597. We confirmed the importance of the Rac1-Pak2 signaling axis in HCAEC apoptosis using a second source of HIV-Nef+ EV, namely HIV-Nef estrogen receptor expressing SupT1 T cells (Online Figure IX).

The uptake of HIV-Nef potentially downregulated eNOS expression in HCAEC (Figure 4G), suggesting that transfer of HIV-Nef to HCAEC could impair endothelium mediated vasodilation. Of note, atorvastatin treatment blocked HIV-Nef-induced eNOS downregulation.

Our data strongly suggest that treatment with HIV-Nef+ EV may cause endothelial cell dysfunction contingent on Rac1 activation-dependent ROS production by the NADPH oxidase complex.

HIV-Nef+ EVs Enhance T Cell Adhesion to the Endothelium

HIV-Nef protein persists in a variety of immune cell subsets, including circulating CD4 T cells, despite ART-induced virological suppression.¹⁴ As HIV-Nef expression has been shown to regulate cellular motility,³⁵ we hypothesized that uptake of HIV-Nef+ EV from plasma would enhance CD4 T cell adhesion to an endothelial monolayer. SupT1 T cells expressing HIV-Nef showed increased adhesion to HCAEC monolayer compared with control T cells (Figure 5A) under static conditions. HIV-Nef is known to activate the small GTPase Rac1, a regulator of cellular adhesion.³⁶ Figure 5A shows that inhibition of Rac1 activation using chemical inhibitor NSC23766 and statin treatment blocked the ability of HIV-Nef to increase T cell adhesion to endothelial cells. Because T cell interaction with the endothelium in vivo is subject to flow, we studied adhesion of T cells to the endothelial monolayer under conditions that recapitulate the shear stress observed in coronary arteries (Figure 5B). Using fluorescence microscopy, we quantified the number of cells that adhered to human umbilical vein endothelial cells (HUVEC) monolayer over 1 hour. Similar to static conditions, HIV-Nef-expressing T cells displayed increased attachment to endothelial monolayer compared with control T-cells, which was suppressed by atorvastatin.

Following the transfer of HIV-Nef protein to endothelial cells when HIV-Nef+ EV are added to HCAEC (Figure 4C), we explored whether exposure to HIV-Nef+ EV enhance adhesion of lymphocytes to the endothelium. First, we assessed the expression of adhesion protein being responsible for rolling (E-selectin, P-selectin) and firm adhesion (VCAM1 [vascular cell adhesion molecule 1], ICAM1 [intercellular adhesion molecule])³⁷ in HCAEC after treatment with HIV-Nef+ EV. We found that addition of HIV-Nef+ EV (Figure 5C and 5D; Online Figure XA and XB) to HCAEC upregulated all 4 adhesion mediators.

This in turn enhanced adhesion of control SupT1 T cells (Figure 5E). Importantly, Rac1 inhibition with NSC23766 and pitavastatin inhibited HIV-Nef-induced upregulation of adhesion markers as well as adhesion of CD4 T cells to HIV-Nef EV-treated HCAEC. These data demonstrate that HIV-Nef expression promotes T cell adhesion to endothelial cells in a Rac1-dependent fashion regardless of whether the HIV-Nef protein is present on T cells or endothelial cells.

HIV-Nef EV Induce Premature Senescence in Endothelial Colony Forming Cells due to Cleavage of Transmembrane TNF α

Recently, we have shown that plasma of HIV+ patients independent of ART can impair endothelial cell function in terms of network formation of cord blood-derived endothelial colony forming cells (ECFC).³⁸ ECFCs were chosen as these are highly proliferative endothelial cells that play an important role in the repair of damaged vasculature.³⁹ We investigated whether HIV-Nef in EV could serve as a factor in acellular plasma that is capable of inducing ECFC dysfunction. Indeed, HIV-Nef+ EV induced the expression of 2 cellular senescence markers, namely the cyclin-dependent kinase inhibitor p16INK4a (Figure 6C) and lysosomal β -galactosidase activity (Figure 6D). To address the underlying mechanism through which HIV-Nef induces premature senescence in this endothelial progenitor population, we analyzed the ability of HIV-Nef+ EV to regulate tmTNF α (transmembrane tumor necrosis factor alpha), which we had earlier shown to be critical for preserving the replicative potential of ECFC through TNFR2 signaling.⁴⁰ We observed a strong down-regulation of tmTNF α in ECFC treated with HIV-Nef+ EV (Figure 6A). This could be attributed to the increased activity of ADAM17/TNF α -converting enzyme on treatment of ECFC with HIV-Nef+ EV (Figure 6B). As previously analyzed in depth,⁴⁰ tmTNF α protects ECFC from premature senescence. Therefore, we evaluated ECFC senescence in the presence of TNF α -converting enzyme inhibitor TNF-alpha processing inhibitor (TAPI). Indeed, TAPI treatment effectively blocked HIV-Nef's ability to promote premature senescence in ECFC as evidenced by reduced p16INK4a expression (Figure 6C) and lysosomal β galactosidase activity (Figure 6D). These results support potential links between vascular inflammation, HIV-Nef activity, and exhaustion of vascular repair cells.

HIV-Nef EV Induce Endothelial Dysfunction in Endothelial Colony Forming Cells via Induction of Rac1-Dependent ROS Production

We hypothesized that HIV-Nef+ EV addition would lead to ECFC dysfunction by upregulating Rac1-dependent ROS production. Indeed, HIV-Nef+ EV increased ROS

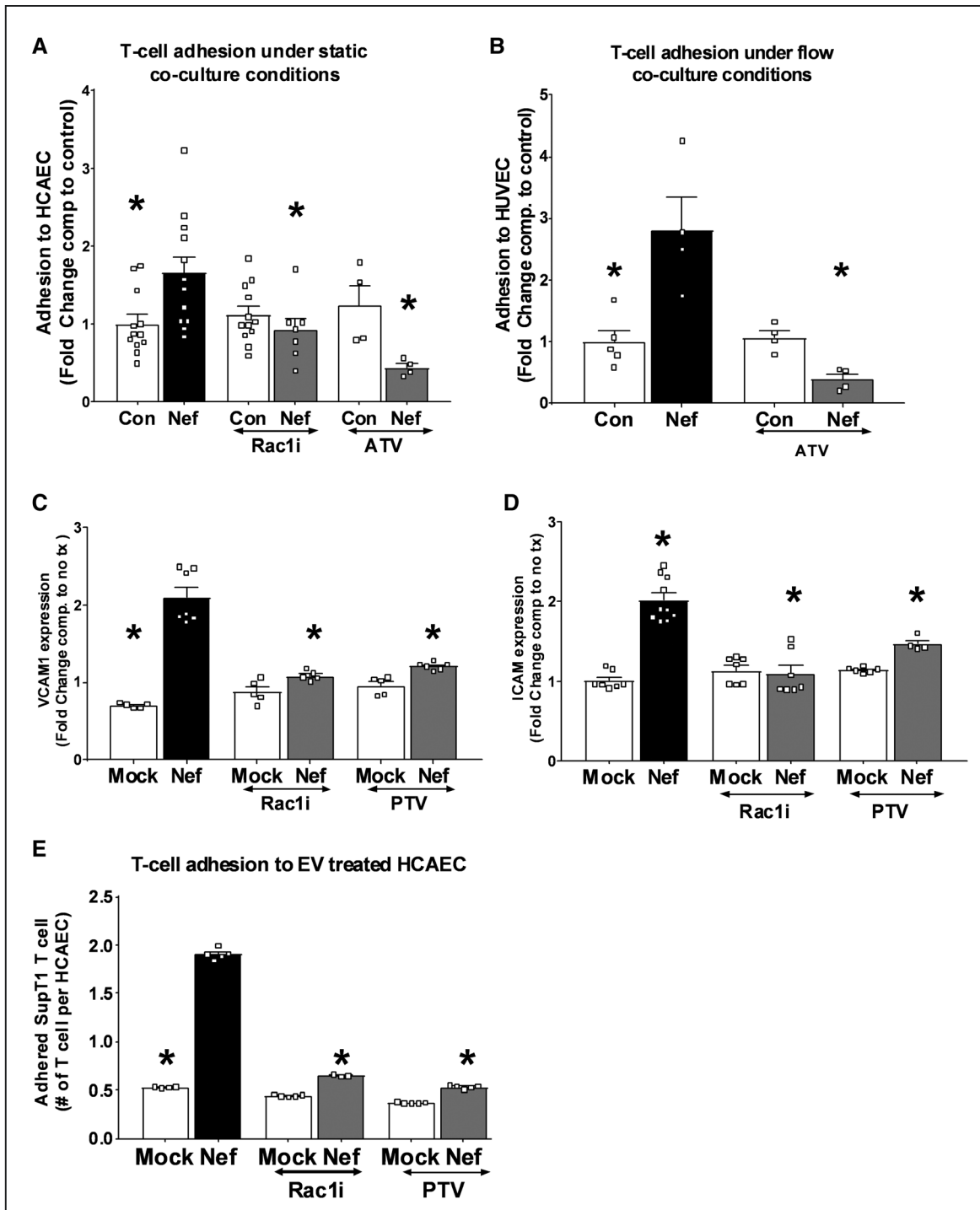


Figure 5. HIV-Nef expression enhances interaction between T-cell and endothelial cells.

Compared with control SupT1 T cells (SupT1), HIV-Nef expressing T cells (Nef-ER) displayed increased adhesion to human coronary artery endothelial cell (HCAEC) under static (A) and to human umbilical vein endothelial cells (HUVEC) under flow (B) conditions. HCAEC treated 24 h with 3 $\mu\text{g/mL}$ Nef extracellular vesicle (EV) showed increased VCAM1 (vascular cell adhesion molecule 1; C) and ICAM1 (intercellular adhesion molecule; D) surface expression as determined by fluorescence-activated cell sorting. T-cell adhesion to HCAEC pretreated with 3 $\mu\text{g/mL}$ of mock EV or HIV-Nef+ EV in the presence of Rac1 inhibitors (E). ATV=5 $\mu\text{mol/L}$ atorvastatin; PTV=100 nmol/L pitavastatin; Rac1i=5 $\mu\text{mol/L}$ NSC23766. Groups were compared using 1-way ANOVA followed by Tukey Post hoc test. *Denotes adjusted $P<0.001$ compared with HIV-Nef group (black bar).

production in ECFC, which could then be completely abrogated with Rac1 inhibition using the chemical inhibitor NSC23766 and both atorvastatin and pitavastatin

(Figure 7A). EV isolated from cells transfected with the HIV-Nef PAK2 activation deficient mutant (F195R) and treatment with Rac1 geranylgeranylation inhibitor

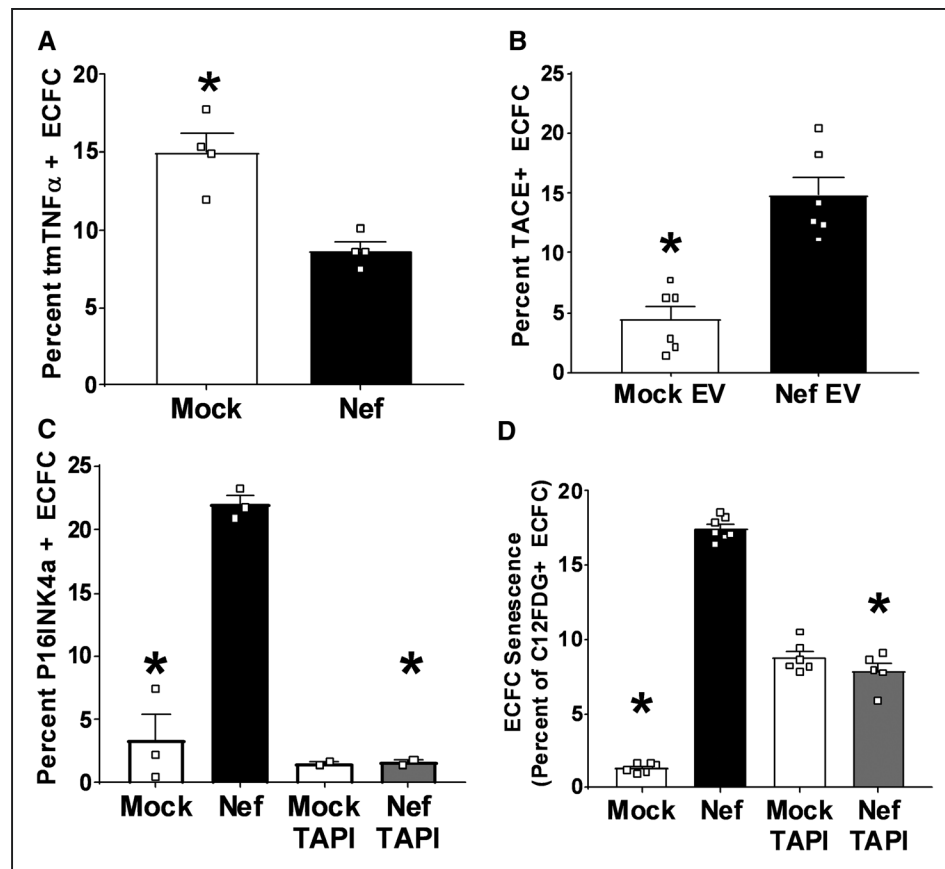


Figure 6. HIV-Nef extracellular vesicle (EV) downregulates tmTNF α (transmembrane TNF α) to induce premature endothelial colony forming cell (ECFC) senescence.

Addition of HIV-Nef+ EV downregulates surface expression of transmembrane TNF α (A) via upregulation of TNF α -converting enzyme activity (B). HIV-Nef+ EV induced ECFC senescence as evidenced by p16INK4a induction (C) and lysosomal β -galactosidase activity (D). TNF- α processing inhibitor (TAPI)=10 μ mol/L. Groups were compared using 1-way ANOVA followed by Tukey Post hoc test or Student *t* test with Welch correction. *Denotes adjusted *P*<0.05 compared with HIV-Nef group (black bar). TACE indicates TNF- α -converting enzyme.

GGTI-298 did not induce ECFC ROS production (Online Figure XIA), thereby providing support for a Rac1-dependent mechanism for HIV-Nef+ EV induced ROS production as PAK2 is a Rac1 downstream effector. Following increased ROS production, we quantified DNA damage in ECFC by measuring phosphorylated histone 2Ax using both fluorescence-activated cell sorting (Online Figure XII) and western blot. Histone 2Ax is phosphorylated at Ser139 upon double-stranded DNA breaks. Rac1 inhibition successfully blocked HIV-Nef+ EV-induced DNA damage (Figure 7B).

We hypothesized that ROS formation and DNA damage would decrease ECFC proliferative capacity by inhibiting cell cycle progression. We observed G1 cell cycle arrest as evidenced by decreased cell cycle checkpoint regulator—cyclin D3 (Figure 7C). Cyclin D3 downregulation resulted in the lack of phosphorylation of Rb protein at sites Ser795 (Online Figure XIII) and Ser807 (Figure 7D), which controls cell cycle progression from G1 to S-phase. Rac1 inhibition prevented HIV-Nef EV-induced cell cycle arrest. Cell cycle arrest in senescent cells is associated with increased lysosomal accumulation. In

this regard, we measured cellular senescence using lysosomal β -galactosidase activity using C12FDG dye staining. HIV-Nef EV potently induced ECFC senescence, which was abrogated with Rac1 inhibition and statin treatment (Figure 7E). Similarly, EV isolated from HIV-Nef F195R mutant transfected HEK did not induce premature senescence (Online Figure XIB). Finally, Nef-induced ECFC dysfunction was also confirmed by finding reduced protein expression of eNOS, which could also be rescued with Rac1 inhibition using both NSC23766 and pitavastatin (Figure 7F).

Therefore, HIV-Nef+ EV induce ECFC dysfunction by promoting ROS-mediated DNA damage that results in cell cycle arrest and cellular senescence.

Endothelial HIV-Nef Expression Leads to Vascular Pathologies in a Transgenic Murine Model

While HIV infection is limited to CD4 T cell and macrophages, HIV-Nef protein is capable of mediating its own transfer to endothelial cells.²¹ To determine if

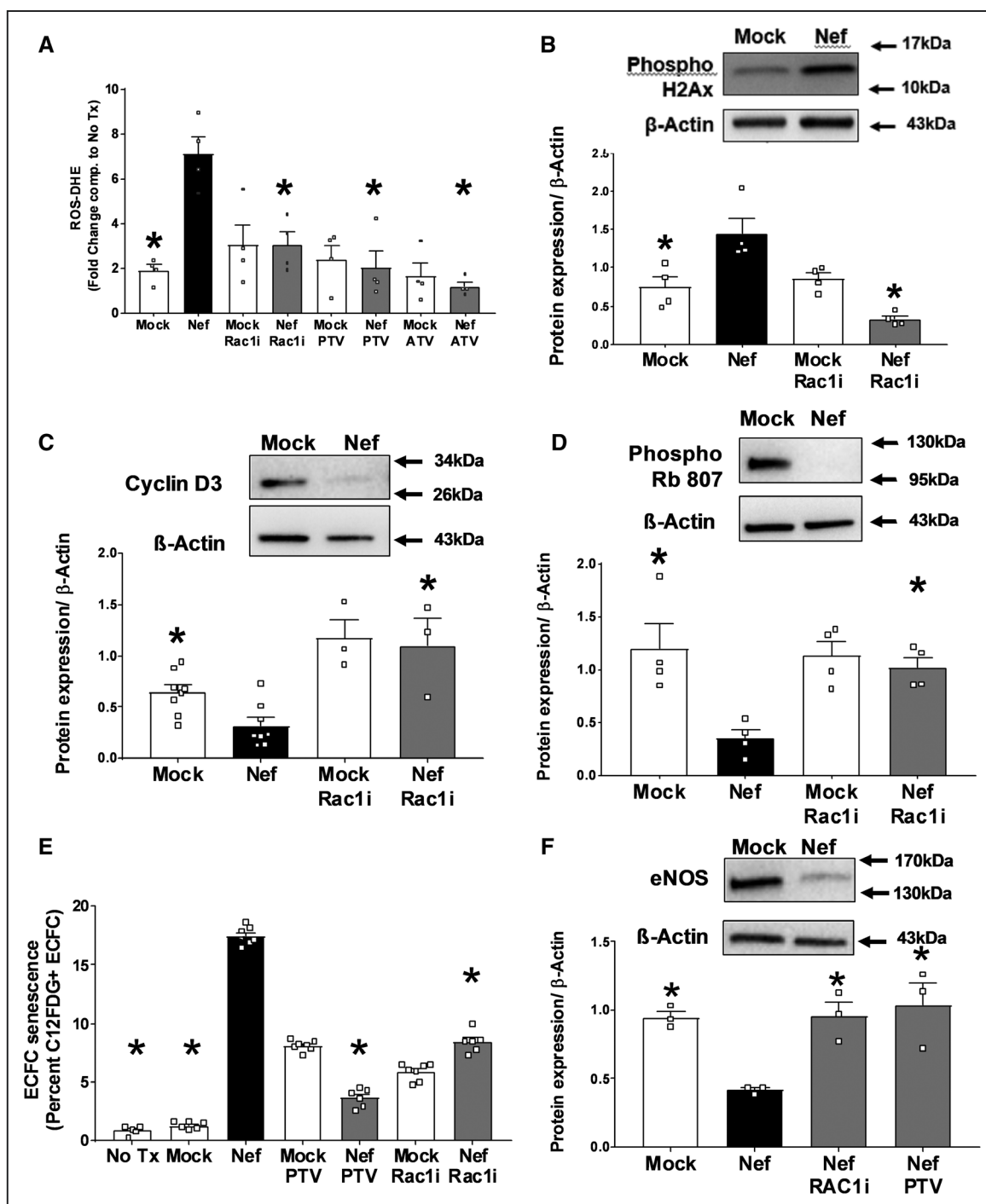


Figure 7. HIV-Nef+ extracellular vesicle (EV) induce endothelial colony forming cell (ECFC) dysfunction.

Addition of HIV-Nef EV induce reactive oxygen species production (A) and double-stranded DNA breaks (B) in ECFC quantified using dihydroethidium (DHE) staining and phosphorylated histone 2Ax, respectively. HIV-Nef+ EV downregulates Cyclin D3 (C) and Phospho Rb 807 (D). HIV-Nef+ EV induces premature senescence in ECFC quantified using C12FDG staining to measure lysosomal β -galactosidase activity (E). HIV-Nef+ EV downregulates endothelial nitric oxide synthase (eNOS) protein expression in ECFC (F). Atorvastatin (ATV)=5 μ mol/L atorvastatin; PTV=100 nmol/L pitavastatin; Rac1i=5 μ mol/L NSC23766. Groups were compared using 1-way ANOVA followed by Tukey Post hoc test or Student *t* test with Welch correction. *Denotes adjusted *P*<0.05 compared with HIV-Nef group (black bar).

endothelial HIV-Nef is sufficient to cause vascular pathologies, we generated transgenic mice—expressing HIV-Nef in endothelial cells under control of the VE-Cadherin promoter.¹²

There were no apparent changes in heart/body weight ratio at 4 and 5 months of age in these transgenic mice. However, we did observe a statistically significant decrease in cardiac output (Figure 8A) in HIV-Nef

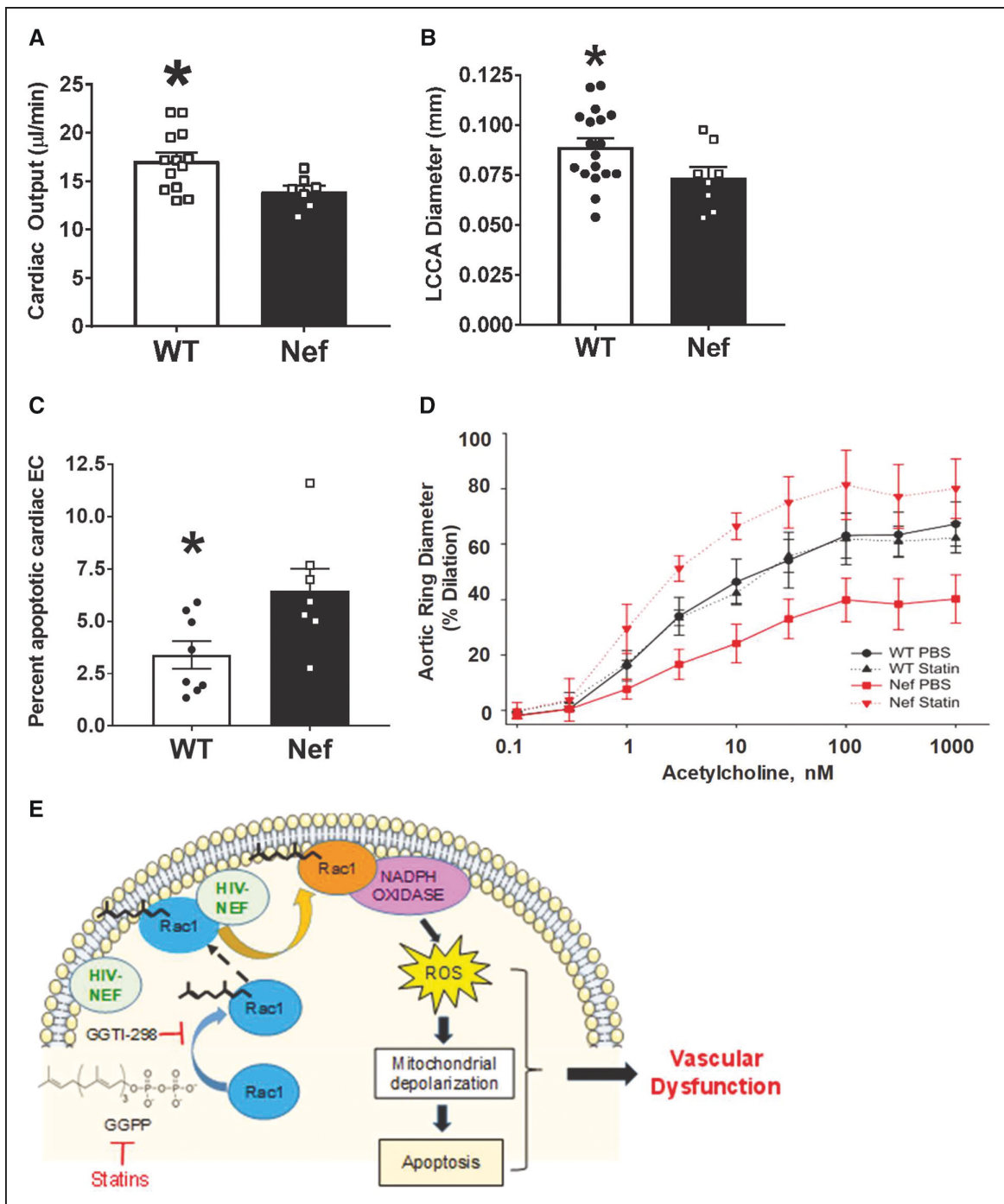


Figure 8. Endothelial expression of HIV-Nef in VE-Cadherin promoter driven HIV-Nef transgenic mice induces cardiovascular dysfunction.

Echocardiography was used to evaluate Left Circumflex Coronary Artery (LCCA) diameter (**A**) and cardiac output (**B**). Endothelial cells in the heart showed increased apoptosis measured through cleaved caspase 3 staining in CD31+/CD45− endothelial cells (**C**). Aortic rings isolated from wild-type (WT) and HIV-Nef expressing mice ($n=4-6$) treated with vehicle or 5 mg/kg atorvastatin (daily for 3 wk) were precontracted using phenylephrine and endothelium dependent vasodilation was measured in response to increasing concentration of acetylcholine (**D**). Schematic describes proposed mechanism of HIV-Nef induced endothelial dysfunction (**E**). Groups were compared using Student *t* test with Welch correction. *Denotes adjusted $P<0.05$ compared with HIV-Nef group.

transgenic mice as compared with WT littermates. In addition, the diameter of the left circumflex carotid artery (Online Figure XIV) was significantly smaller in HIV-Nef transgenic mice (Figure 8B). This finding suggests that

endothelial dysfunction accompanied the reduction in cardiac output.

Consistent with our in vitro data on HIV-Nef-induced endothelial dysfunction, we observed an elevated level of

endothelial cell apoptosis in the heart of HIV-Nef transgenic mice when compared with HIV-Nef⁻ littermates (Figure 8C). We then assessed endothelial-specific vascular function in HIV-Nef transgenic mice by measuring the dilation of precontracted aortic rings in response to acetylcholine. Aortas from 3-month-old HIV-Nef transgenic animals showed dramatically impaired ability to dilate in an endothelial-dependent manner. Importantly, aortas from HIV-Nef transgenic animals treated for 3 weeks with atorvastatin showed normalized dilation in response to acetylcholine (Figure 8D).

Similarly, HIV-Nef expression by CD4⁺ cells impacted the endothelium. These CD4-Nef tg mice also displayed impaired endothelium mediated vasodilation as evidenced by small responses to acetylcholine in aortic rings isolated from HIV-Nef tg mice but not their WT littermates (Online Figure XV).

Therefore, transfer of HIV-Nef to the endothelium is sufficient to induce a variety of vascular pathologies clinically relevant to those observed in HIV+ patients on ART (Figure 8E).

DISCUSSION

In this study, we demonstrated that PBMC and plasma EV isolated from HIV+ patients induced apoptosis in HCAEC. Moreover, HIV-Nef+ EV from plasma obtained from patients virally suppressed on ART-induced HCAEC dysfunction, thereby supporting a role for persistent HIV-Nef on vascular pathology. Although we did not analyze factors like other viral proteins and inflammatory cytokines as reviewed by Marincowitz et al⁴¹ that could influence vascular dysfunction in HIV+ patients on ART, the following lines of evidence suggest that HIV-Nef may contribute to the persistently heightened cardiovascular risk observed in ART-treated HIV patients: (1) compared with the other early response HIV genes *rev* and *tat*, HIV-Nef continues to be produced at high levels even when patients are receiving virologically suppressive ART⁴²; (2) HIV-Nef is the only HIV protein that has been found in the blood circulation of patients on ART,^{15,16} and (3) transgenic endothelial HIV-Nef expression causes similar endothelial dysfunction in vivo (this study and Chelvanambi et al¹²).

While HIV itself only infects a few human cell types, the ability of Nef+ EV to transfect other cell types could possibly explain the widespread identification of HIV-Nef in PBMC,¹⁴ the heart,²¹ and endothelial cells.^{30,43} As we have shown that HIV-Nef transfer to endothelial cells cause their dysfunction, this mechanism may lead to various end-organ pathologies in the aging HIV+ population. Interestingly, we also demonstrate in vitro that HIV-Nef transfer to endothelial cells is prevented by statins, which suggests that these currently available drugs could help prevent HIV-Nef-dependent endothelial dysfunction in ART-treated HIV+ patients.

Immune cell-mediated endothelial cell activation is a major contributor to endothelial dysfunction in HIV+ patients.⁴⁴ HIV-Nef is capable of increasing endothelial cell adhesion markers such as ICAM-1 in an ERK signaling dependent fashion.⁴⁵ Here, we show that Nef+ EV independently upregulate T cells adhesion to a HCAEC monolayer via increased surface expression of adhesion markers (Figure 5). HIV-Nef+ EV may be a major factor in inducing lymphocyte adhesion to the endothelium as a secreted protein from T cells in patients on ART. Our demonstration that Rac1 inhibition using statins was capable of preventing upregulation of these adhesion markers to block HIV-Nef-induced adhesion of T cells to the endothelium suggests that statin treatment could be a viable strategy to reduce vascular inflammation in HIV+ patients.

Cardiovascular dysfunction⁴⁶ impaired endothelium-mediated vasodilation⁸ and arterial stiffening⁴⁷ are well-established phenotypes in the HIV patient population. Similarly, endothelial dysfunction has been characterized in mammals including transgenic rats-expressing HIV proteins,⁴⁸ porcine pulmonary arterial rings treated ex vivo with HIV-Nef⁴⁹ and SHIV-infected macaques.⁵⁰ Our study shows that endothelial HIV-Nef independently impaired endothelium-mediated aortic ring vasodilation in 2 different HIV-Nef transgenic murine models, even in the absence of other HIV-associated confounding variables, including direct ART drug effects, immune cell activation, and viral replication.

The first murine model expressed endothelial HIV-Nef under the control of CD4 promoter elements (CD4c) and is already well described for its induction of AIDS and cardiac pathology.⁵¹ We used this model to address production of HIV-Nef-induced EV. Reproducing observations from our previous publication, we observed cytosol from CD4 T cells to be present within the endothelium of the cardiac vasculature (Figure 2A). These mice display endothelial dysfunction when tested in our ex vivo arterial dilatation model (Online Figure XV). This cardiovascular phenotype could also be attributed to the transfer of HIV-Nef protein directly to cardiac myocytes as described by Gupta et al.¹⁷ To specifically address the effects of HIV-Nef transfer into endothelial cells, we expressed HIV-Nef under control of the endothelial-specific VE-cadherin promoter.¹² Using this mouse model, we could replicate the most important cardiovascular findings with CD4c-Nef transgenic mice, namely vascular dysfunction and impaired cardiac output (Figure 8). Importantly, this model reflects our in vitro findings that HIV-Nef expression in the endothelium is sufficient to initiate vascular dysfunction and that statins can reverse these effects.

We found that HIV-Nef protein can induce endothelial cell apoptosis because of increased ROS production by the NADPH Oxidase complex because of Rac1 activation. Of clinical relevance is our finding that statins also mitigated this endothelial pathology both in vitro and in vivo.

One of the limitations of our study was the small number of treatment naïve patients included (n=4). Therefore, we could not detect any potentially statistically significant differences between plasma EV from these patients with those from the ART+ patients to induce HCAEC apoptosis (Online Figure 1A). However, we did find statistically significant differences between the HIV+ Nef+ and HIV+Nef− samples in their ability to induce HCAEC apoptosis (Online Figure 1C). When examining for potential associations between the collected medical histories of these patients (Online Table 1) with the HIV-Nef-related results or which patients would be HIV-Nef+ versus HIV-Nef−, we could not identify any (though we acknowledge we did not have data on route of HIV acquisition nor illicit drug use). We could not assess the relationship of smoking on our results because only non-smokers were purposefully enrolled. Larger studies are necessary to identify clinical factors predicting HIV-Nef persistence in HIV+ patients. However, our study lays the foundation that HIV-Nef persistence in plasma of HIV+ patients on ART could be used as a predictor for risk of developing endothelial dysfunction.

We have recently shown that plasma from both ART treated and untreated HIV+ patients impair angiogenic properties of ECFC.⁵² This current study is the first to identify a virally encoded protein being capable of reducing eNOS and inducing premature senescence in this population of progenitor cells, which may thus further impair vascular function and repair. Future studies can study how chronic infection by other viruses impacts the endothelial progenitor cell population. The ability of statins to reverse HIV-Nef-induced effects on ECFC in the current study is in line with other studies showing the benefits of statin treatment on endothelial progenitor populations in murine models⁵³ and in clinical trials of statin intervention.³¹

These findings are of particular interest as premature senescent cells, which are positive for p16ink4A and senescence associated β -galactosidase (Figures 6 and 7) have been demonstrated to cause many features of human aging including cardiovascular dysfunction.⁵⁴ Our observation of NADPH oxidase-mediated endothelial ROS production followed by decrease in eNOS protein expression was also reported in aging HUVEC.^{15,30} Combined with our observation that HIV-Nef induces premature senescence in endothelial progenitor cell populations, our study lends credence to the theory that end organ failure in HIV patients on ART is a disease of accelerated aging.⁵⁵

In conclusion, HIV-Nef protein persists in HIV patients on ART at levels comparable to ART naïve patients. Our findings suggest that HIV-Nef uses a Rac1-mediated pathway to induce endothelial cell stress, which in turn leads to endothelial dysfunction. The ability of statin treatment to block HIV-Nef-mediated Rac1 signaling could help limit vascular dysfunction in HIV patients on

ART and potentially delay the development of cardiovascular diseases.

ARTICLE INFORMATION

Received March 26, 2019; revision received August 16, 2019; accepted August 26, 2019.

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Acknowledgments

We appreciate the help of Dr Ting Wang, Dr Linden Green, and Noelle Dahl. The following reagents were obtained through the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH: Nef-ER #31 Clone from Drs Scott Walk, Kodi Ravichandran, and David Rekosh; Anti-HIV-1 SF2 Nef Monoclonal (EH1) from Dr James Hoxie; Cat #2949; Anti-HIV-1 Nef Polyclonal from Dr Ronald Swanstrom; pcDNA3.1SF2Nef (Cat #11431) from Dr J. Victor Garcia; 1SF2NefF195R (Cat#11430) from Dr J. Victor Garcia; and pNL4-3 from Dr Malcolm Martin.

Sources of Funding

This publication was made possible with support from the American Heart Association (16PRE27260181 to S. Chelvanambi), the National Institutes of Health (NIH: 5R21HL120390-02 (M. Clauss, S.K. Gupta) 1R01HL129843-01 (M. Clauss, A.G. Obukhov, P. Jolicœur), R01 HL141909-01A1 (P. Zorlutuna), NSF-CAREER Award No 1651385 (P. Zorlutuna), and the Indiana Clinical and Translational Sciences Institute funded, in part by Award Number UL1TR002529 from the NIH, National Center for Advancing Translational Sciences, and Clinical and Translational Science Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Disclosures

S.K. Gupta obtained travel support and personal/advisory fees from Gilead Sciences and GSK/ViiV Healthcare. The other authors report no conflicts.

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