HIV-1 Nef Is Associated with Complex Pulmonary Vascular Lesions in SHIV-*nef*-infected Macaques

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Rationale: HIV-infected patients with pulmonary arterial hypertension have histologic manifestations that are indistinguishable from those found in patients with idiopathic pulmonary arterial hypertension. In addition, the role of pleiotropic viral proteins in the development of plexiform lesions in HIV-related pulmonary hypertension (HRPH) has not been explored. Simian immunodeficiency virus (SIV) infection of macaques has been found to closely recapitulate many of the characteristic features of HIV infection, and thus hallmarks of pulmonary arterial hypertension should also be found in this nonhuman primate model of HIV.

Objectives: To determine whether pulmonary arterial lesions were present in archived SIV-infected macaque lung tissues from Johns Hopkins University and two National Primate Research Centers.

Methods: Archived macaque and human lung sections were examined via immunohistochemistry for evidence of complex vascular lesions.

Results: Complex plexiform-like lesions characterized by luminal obliteration, intimal disruption, medial hypertrophy, thrombosis, and recanalized lumina were found exclusively in animals infected with SHIV-*nef* (a chimeric viral construct containing the HIV *nef* gene in an SIV backbone), but not in animals infected with SIV. The mass of cells in the lesions were factor VIII positive, and contained cells positive for muscle-specific and smooth muscle actins. Lung mononuclear cells were positive for HIV Nef, suggesting viral replication. Endothelial cells in both the SHIV-*nef* macaques and patients with HRPH, but not in patients with idiopathic pulmonary arterial hypertension, were also Nef positive.

Conclusions: The discovery of complex vascular lesions in SHIV-*nef*-but not SIV-infected animals, and the presence of Nef in the vascular cells of patients with HRPH, suggest that Nef plays a key role in the development of severe pulmonary arterial disease.

Keywords: idiopathic pulmonary arterial hypertension; HIV-1; Nef; pulmonary hypertension; SHIV-*nef*

The first case of HIV infection associated with severe pulmonary hypertension appeared in 1987 (1) and confirmation by several follow-up studies (2–5) indicated that HIV infection may be

causally related to pulmonary hypertension, with histologic lesions that are indistinguishable from those present in idiopathic pulmonary arterial hypertension (IPAH) (6, 7). Although HIV-related pulmonary hypertension (HRPH) is still a rare disease, the incidence is many times higher than that of IPAH in the general population (approximately 1 in 4,000 vs. 2 in 1 million in the United States [N. Gallie, personal communication] or 1 in 200 in the Swiss or French populations [2, 8, 9]). Importantly, there is no evidence of viral particles in the complex pulmonary vascular lesions (3, 10) or bone morphogenetic protein receptor II mutations (11) in HRPH.

The mechanisms underlying HRPH may rely on indirect action of HIV proteins and/or the associated immune dysregulation present in patients with HIV infection. Among proteins of HIV that could participate in the pathogenesis of HRPH, the Tat protein causes increased endothelial cell permeability and injury via production of IL-6, shown to be present in lungs of patients with severe pulmonary hypertension (12). Nef, a 27-kD, *N*-myristoylated protein first misidentified as a negative regulatory factor for viral replication, is critical for the maintenance of high viral loads during the course of HIV infection *in vivo* (13, 14), and is an adaptor protein with multiple domains important for the interaction with the cellular signaling machinery. Its proline-rich (PxxP)₃ domain interacts with Src homology 3–containing signaling proteins (15, 16), while other Nef domains interact with the endocytotic cellular machinery responsible for the down-regulation of CD4 (17–19).

Nonhuman primate models of HIV-1 infection with either SIV or genetically manipulated pathogenic viruses containing HIV-1 genes recapitulate many of the end-organ pathologies and immune deficiencies seen in humans (20). Only a few investigations using this model for noninfectious lung complications in SIV infection have been done. However, one report by Chalifoux and coworkers described a pulmonary arteriopathy in the lungs of 19 of 85 macaques infected with SIV at the New England National Primate Research Center (NENPRC, Southborough, MA) (21). As HIV modeling in monkeys has significantly evolved with the use of recombinant, chimeric SIV vectors containing potentially pathogenetic HIV genes (SIV/HIV, or SHIV constructs), or SIV with tropism to organs such as brain or lung, we therefore investigated whether SIV-infected macaque lungs displayed the characteristic complex vascular lesions, including the characteristic plexiform lesions, present in HRPH. We further extended the scope of our study to address whether the HIV-1 Nef protein might be detected in the lungs of patients with HRPH and in macaques infected with a chimeric viral construct containing the HIV *nef* gene in an SIV backbone (SHIV-*nef*), but not in normal or uninfected IPAH lungs. Our findings indicate that HIV-1 Nef may be involved in the development of vascular lesions found in HRPH. Some of the results of these studies have been previously reported in the form of an abstract (22).

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METHODS

Animal Selection

From the NENPRC, we obtained eight animals: two infected with a chimeric SHIV-*nef* virion (23, 24) and six infected with SIV_{mac251} or SIV_{E660} containing the native SIV *nef* allele. From the California National Primate Research Center (CNPRC; University of California at Davis, Davis, CA) we obtained five macaque lungs: one infected with SIV_{mac239} and four infected with SHIV-*nef* chimeric viruses (25). From the Retrovirus Laboratory, Department of Comparative Medicine, Johns Hopkins University (JHU; Baltimore, MD) we obtained 12 lungs from macaques infected with a mixture of lymphocytotropic and monocyctotropic SIV strains (26). Table 1 describes the different animals and their known characteristics. No hemodynamic parameters were available from any of the macaque cohorts. All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved before implementation by the Institutional Animal Care and Use Committees at the University of California at Davis, JHU, and Harvard University.

Human Tissues

Archived, paraffin-embedded lung tissue sections were obtained from two patients with IPAH, two patients with HRPD, and one patient with no clinical or histologic evidence of IPAH. These tissues were used for immunohistochemistry of the HIV-1 Nef protein. Patient characteristics have already been described by Cool and coworkers (6, 27).

Tissue and Histologic Examination

All tissues were obtained at necropsy, and were fixed and embedded in paraffin. Serial sections were prepared and shipped to the University

of Colorado at Denver and Health Sciences Center (Denver, CO) for hematoxylin and eosin (H&E) staining and immunohistochemistry as indicated. The sections were masked concerning the identity of the experimental groups and examined independently by two pulmonary pathologists (C.D.C. and R.M.T.) for the presence of pulmonary vascular lesions as recommended by Pietra and coworkers (28). A total of 10 and 12 serial sections from a single lung block were obtained from the NENPRC and CNPRC, respectively. From JHU, we received one H&E-stained section for each animal (n = 12). Representative sections were performed on the basis of random selection by the respective institutions. Therefore, we had to limit our analyses to the archived material already collected for other studies. Nevertheless, we are confident that the random nature of the archival process allowed us an analysis of representative samples from many different areas of the lung.

Immunohistochemistry

Identification of vascular cells. Sections were immunostained at the Histology Laboratory (University of Colorado at Denver and Health Sciences Center) with a HexES automated apparatus (Ventana Medical Systems, Tucson, AZ), using the following antibodies: rabbit polyclonal anti-human von Willebrand factor (factor VIII; A0082; Dako, Carpinteria, CA), mouse monoclonal anti- α -smooth muscle actin (α -SMA; A5228; Sigma-Aldrich, St. Louis, MO), and mouse monoclonal anti-muscle specific actin (MSA; C34931; Enzo Life Sciences, Inc., Farmingdale, NY). Immunodetection was performed with the Ventana avidin-biotin peroxidase system with anti-mouse or anti-rabbit IgG. Both positive and negative control tissues were provided with each antibody.

TABLE 1. ANIMALS WITH PULMONARY VASCULAR ARTERIOPATHY FROM NEW ENGLAND NATIONAL PRIMATE RESEARCH CENTER, CALIFORNIA NATIONAL PRIMATE RESEARCH CENTER, AND JOHNS HOPKINS UNIVERSITY

Animal	Viral Strain*	Survival	Pulmonary Vascular Lesions	Lung Pathologies
New England National Primate Research Center				
NENPRC1	SIV _{E660}	1 yr, 3 mo	None	Hemorrhagic necrotizing bronchopneumonia, pulmonary alveolar proteinosis
NENPRC2	SIV _{mac251} , BCG, SEB	1 yr, 3 mo	None	Pulmonary edema, thromboemboli
NENPRC3	SIV _{mac251}	1 yr, 3 mo	None	Hyperplasia of BALT
NENPRC4	SIV _{mac251}	1 yr, 4 mo	None	Organizing thromboemboli, hyperplasia of BALT, hemosiderin
NENPRC5	SIV _{mac251}	3 yr, 4 mo	None	Nonnecrotizing vasculitis (predominantly lymphocytic)
NENPRC6	SIV _{mac251}	1 yr	None	Nonnecrotizing vasculitis (predominantly lymphocytic), pulmonary edema, fibrin exudates
NENPRC7	SHIV- <i>nef</i> _{1486D95}	1 yr, 9 mo	EC proliferation, medial hypertrophy, plexiform-like lesions	Nonnecrotizing vasculitis, (predominantly lymphocytic), focal thromboemboli
NENPRC8	SHIV- <i>nef</i> _{1486D95}	N/A	EC proliferation, medial hypertrophy, intimal hyperplasia, plexiform-like lesions	Hyperplasia of BALT
California National Primate Research Center				
CNPRC1	SIV _{mac239}	1 yr	None	Alveolar proteinosis (<i>Pneumocystis carinii</i>), type II pneumocyte hyperplasia, multinucleated giant cells, neutrophilic infiltration
CNPRC2	SHIV- <i>nef</i> _{SF33}	2 yr, 3 mo	Mild vascular remodeling of medium to large vessels	Intraepithelial infiltrates of neutrophils and lymphocytes
CNPRC3	SHIV- <i>nef</i> _{SF33A}	2 yr, 3 mo	Significant vascular remodeling, abnormal EC layers in the vasculature	Multifocal lymphoid hyperplasia, mild invading mononuclear cells (neutrophilic and lymphocytic)
CNPRC4	SHIV- <i>nef</i> _{SF33A}	1 yr, 6 mo	Significant vascular remodeling, abnormal EC layers in the vasculature	Subpleural alveolar proteinosis (<i>P. carinii</i>), bronchopneumonia, <i>Cryptosporidium</i>
CNPRC5	SHIV- <i>nef</i> _{SF33A}	3 mo	Pulmonary arteries normal, unorganized architecture	Alveolar proteinosis (<i>P. carinii</i>), neutrophilic and lymphocytic alveolitis, septal damage
Johns Hopkins University				
JHU1-JHU12	SIV/ΔB670 and SIV/17E-Fr	N/A	None	Perivascular lymphocytic infiltrates, interstitial pneumonitis, septal damage with airspace enlargement

Definition of abbreviations: BALT = bronchial-associated lymphoid tissue; BCG = *Mycobacterium bovis* bacillus calmette-gaérin; EC = endothelial cells; N/A = not available; SEB = staphylococcal enterotoxin B superantigen.

* Subscript refers to the viral strain.

Detection of HIV-1 Nef. A mouse monoclonal antibody to amino acids 153–158 of HIV-1 Nef (Advanced Biotechnologies, Inc., Columbia, MD) was used to stain sections with a Zymed AEC (aminoethyl carbazole) immunohistochemical staining kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols (for further details, *see* the online supplement). Negative controls for the immunohistochemistry were performed using secondary antibody alone.

Statistics

A Fisher's exact test of a 4×4 matrix was used to analyze the observations, with $p < 0.05$ considered significant.

RESULTS

Pulmonary Pathologies in SIV- and SHIV-*nef*-infected Macaques

We examined a total of 24 different monkey lungs from two separate primate centers based in New England (NENPRC), California (CNPRC), and Johns Hopkins University (JHU), with a total of 18 monkeys infected with SIV and six infected with SHIV-*nef* (Table 1). The SIV strains contained the simian *nef* allele whereas the SHIV-*nef* strains were chimeric viruses with HIV *nef* from various HIV isolates cloned in place of the native SIV *nef* sequence. Figure E1 of the online supplement shows a diagrammatic depiction of the genomic structures of parental and chimeric SIV strains.

From the NENPRC, we obtained eight animals: two infected with a chimeric SHIV-*nef* virion that contained the SIV_{mac251} backbone and the HIV *nef* gene, and six infected with SIV_{mac251} or SIV_{E660} containing the native SIV *nef* allele. We found that the two SHIV-*nef* animals (designated NENPRC7 and NENPRC8 in Table 1) showed pulmonary vessels with medial smooth muscle cell hypertrophy and luminal obstruction with endothelial cells (Figures 1B and 1C). The lesions were histologically similar to the plexiform lesions seen in patients with HRPD. One animal infected with SIV_{mac251} (Figure 1A and Table 1; designated as NENPRC1), showed significant perivascular cellular infiltration, but little disruption of the intimal or medial layers of the pulmonary vasculature. Furthermore, none of the animals infected with parental SIV strains (SIV_{mac251} or SIV_{E660}, NENPRC1–NENPRC6 in Table 1) had any evidence of endothelial layer disruption or smooth muscle cell proliferation. Several of the prominent histopathologic features in these lungs included pulmonary edema, organizing thromboemboli, nonnecrotizing perivascular lymphocytic infiltrates, neutrophilic infiltration, and hyperplasia of bronchial-associated lymphoid tissue (Table 1). In four animals, alveolar proteinosis from *Pneumocystis carinii* was also evident, suggesting severe immunodeficiency with opportunistic infections.

No measurements of pulmonary artery pressure or cardiac output were taken in the NENPRC early study. On the other hand, examination of echocardiographic data from monkeys infected with the parental SIV strains showed no evidence of right ventricular changes in these monkeys (i.e., no echocardiographic evidence of right ventricular hypertrophy in approximately 30 SIV-infected animals examined [R.P.S., unpublished data]), despite evidence of widespread myocarditis and left ventricular changes.

We then compared the findings in the studies from the NENPRC with those in five additional monkey lungs obtained from the CNPRC (animals designated as CNPRC1–CNPRC5 in Table 1). Animal CNPRC1 had been infected with SIV_{mac239} and animal CNPRC2 had been infected with a chimeric SHIV-*nef* containing the HIV-1 *nef*_{SF33} allele in the SIV_{mac239} backbone. CNPRC1 had no involvement of the pulmonary vasculature but

showed neutrophilic and lymphocytic infiltration of the perivascular space (Figure 1D). One SHIV-*nef*-infected monkey (CNPRC2) showed mild vascular remodeling (*see* Figure E2). Another animal infected with SHIV-*nef*_{SF33} (tissues unavailable) died of immunodeficiency, but its SHIV-*nef* virus was isolated, recovered, and used to infect three additional macaques (CNPRC3, CNPRC4, and CNPRC5 in Table 1). The *nef* allele, which had acquired four amino acid mutations during passage *in vivo*, was designated as Nef_{SF33A} (A, for adapted) (25). Two animals (CNPRC3 and CNPRC4; Figures 1E and 1F, respectively) showed abnormal vascular lesions with medial hypertrophy and a disrupted, cuboidal endothelial cell layer. Pentachrome staining corroborated both medial and intimal hypertrophy of the pulmonary arteries (*see* Figure E3). CNPRC5 died of severe wasting and opportunistic infections 3 mo postinfection, with lung samples showing extensive inflammation, but no vascular remodeling (*see* Figure E2). As with animals in the NENPRC cohort, no evidence of cardiac abnormalities was found in young animals infected with parental SIV_{mac251} at the CNPRC (A.F.T., unpublished data).

To further confirm that SIV alone is not associated with complex pulmonary vascular lesions, we examined an additional 12 monkeys infected with an immunosuppressive swarm SIV- Δ B670 and a macrophage-tropic molecular clone SIV/17E-Fr mixture (JHU1–JHU12; Table 1) from the Retrovirus Laboratory, Department of Comparative Medicine, Johns Hopkins University. These macaques developed a rapid and lethal immunodeficiency within 3 mo of infection (26). Although significant inflammatory lung involvement with inflammation and alveolar simplification was observed, no complex vascular lesions were detected (*see* Figure E4).

Combining the results from the three geographically separate primate centers, four of the six SHIV-*nef*-infected animals were found to have complex vascular lesions, and one additional animal had evidence of less prominent pulmonary vascular remodeling. Thus, five of the six SHIV-*nef*-infected animals (83%) with remodeling were chronically infected (more than 12 mo), whereas none of the animals infected with the parental SIV strains containing the SIV *nef* allele ($n = 18$) developed complex pulmonary vascular lesions. Taken together, there is a statistically significant association between SHIV-*nef* infection in macaques and the presence of pulmonary vascular lesions (Fisher's exact test, $p < 0.0001$).

Characterization of Pulmonary Vascular Lesions in SHIV-*nef*-infected Animals

To further characterize the lesions, we immunostained the sections with the endothelial cell marker factor VIII, and the smooth muscle markers muscle-specific actin (MSA) and α -smooth muscle actin (Figures 2 and 3). Animals NENPRC7 and NENPRC8 showed marked medial hypertrophy along with a mass of endothelial cells at a site of bifurcation (Figures 2D–2F). The pattern of factor VIII staining (Figures 2B, 2E, 2H, and 2K) highlighted the endothelial cell layer and intimal thickening, and the muscle-specific actin staining pattern (Figures 2C, 2F, 2I, and 2L) indicated medial hypertrophy. In the SHIV-*nef* animals CNPRC3 and CNPRC4 (Figure 2, rows 3 and 4), the pattern of factor VIII staining highlighted the disrupted, cuboidal endothelial cells, and the muscle-specific actin staining indicated medial hypertrophy. In animal NENPRC7, in a vessel at a bifurcation point, the lesion showed a core of factor VIII-positive endothelial cells obliterating the lumen (Figures 3A and 3B). Furthermore, at higher magnification, the smooth muscle actin-positive cells

Figure 1. Pulmonary vasculature and plexiform lesions in macaques infected with simian immunodeficiency virus (SIV) or SHIV-*nef* (a chimeric viral construct containing the HIV *nef* gene in an SIV backbone). Paraffin-embedded sections from the lungs of macaques infected with SIV_{mac251}-NENPRC1 (A), SHIV-*nef*_{1486D95}-NENPRC7 (B), SHIV-*nef*_{1486D95}-NENPRC8 (C), SIV_{mac239}-CNPRC1 (D), SHIV-*nef*_{SF33A}-CNPRC3 (E), or SHIV-*nef*_{SF33A}-CNPRC4 (F) were stained with hematoxylin and eosin (H&E) and examined for vascular pathologies by two independent pathologists. Arrows indicate vessels with lymphocytic infiltration (SIV) and plexiform-like lesions (SHIV-*nef*). Scale bars: 100 μ m.

(Figures 3C and 3D) can be seen as interdigitating strings surrounded by an endothelial cell core.

Presence of Nef in the Lungs of HIV-1-infected Patients and SHIV-*nef*-infected Macaques

The presence of plexiform lesions in the pulmonary vasculature of SHIV-*nef* but not in the SIV-infected animals suggested a role

for HIV-1 Nef in the pathogenesis of HRPD. As the presence of Nef in the lungs of patients with HRPD has not been reported previously, we examined paraffin-embedded lung sections from a normal human lung, two patients with IPAH, two patients with HRPD, and two SHIV-*nef*-infected macaques (CNPRC3 and CNPRC4) for the presence of the HIV-1 Nef protein. Figure 4 (first column) shows some representative lung sections stained

Figure 2. Characterization of vascular cells in the lesions of SHIV-*nef* macaques from the New England National Primate Research Center (NENPRC) and the California National Primate Research Center (CNPRC) cohorts. Paraffin-embedded serial sections from animals NENPRC7 (A–C) and NENPRC8 (D–F), and from animals CNPRC3 (G–I) and CNPRC4 (J–L), were stained with H&E (A, D, G, and J), factor VIII (B, E, H, and K), and mouse monoclonal anti-muscle specific actin (MSA; C, F, I, and L) for visualization of endothelial cells and smooth muscle cells in the plexiform lesions. Scale bars: 50 μ m.

Figure 3. Identification of endothelial and smooth muscle cell markers in a lesion from an SHIV-*nef* animal (NENPRC7). Endothelial cells stained with factor VIII (A and B) and smooth muscle cells stained with α -smooth muscle actin (α -SMA; C and D) in lesions from the SHIV-*nef* animal NENPRC7. Arrows indicate regions of mixed endothelial and smooth muscle cell staining in the lesion. (B) and (D) are magnifications of the lesion in (A) and (C), respectively. Scale bars: (A and C) 50 μ m; (B and D) 100 μ m.

with a monoclonal HIV-1 Nef antibody, whereas a separate section and region of the same subject in the remaining panels show dual-immunofluorescence stains for factor VIII and Nef. We detected no Nef staining in an uninfected control (Figure 4A; and see Figure E5A) or in patients with IPAH (Figure 4E; and see Figure E5B), illustrating the specificity of the antibody and the absence of any molecular mimicry (29). On the other hand, the lungs from patients with HRPD showed distinct positive staining in alveolar mononuclear cells (most likely lymphocytes and macrophages) as well as in endothelial cells lining the vessels (see Figures E5C and E6). The occluding cells and some luminal cells found in the complex lesion in the patient with HRPD also stained positive for Nef (Figure 4I), and Nef-positive cells were observed clustering at sites adjacent to the vessel walls. In addition, Nef-positive cells were observed in lymphoid-associated tissues (see Figure E6), suggesting a potential source for the viral protein.

Figures 4 and 5 show immunofluorescence staining for factor VIII and Nef in human and macaque lungs, respectively. The pattern of staining in the merged images clearly indicates that Nef colocalizes with endothelial cells. This pattern in the SHIV-*nef* macaque lungs recapitulated that found in the human lungs, with staining in several endothelial cells within the vasculature as well as in perivascular sites (Figure 5I). We also found factor VIII-negative, Nef-positive medial and parenchymal cells. Interestingly, some adventitial cells in HRPD stained positive for both factor VIII and Nef (see Figure E6). As expected, lymphoid tissue showed staining for Nef, but no factor VIII positivity (Figure 5). These findings provide additional evidence that HIV-1 Nef may be a critical player in the development of complex pulmonary vascular lesions.

DISCUSSION

The main findings of our study are that complex vascular structures resembling plexiform lesions of IPAH are found in the lungs of macaques infected with a chimeric virus with the SIV backbone and the HIV *nef* gene. None of the monkeys infected with the parental SIV *nef* showed pulmonary vascular lesions, suggesting that there are functional differences between HIV and SIV *nef*. These differences may result in a protein that can act either alone or in conjunction with other viral or cellular factors in promoting alterations of pulmonary arteries characteristic of severe pulmonary hypertension. These findings were somewhat unanticipated, because HIV *nef* can complement *nef* deletions in SIV (24, 25), suggesting that at least in terms of immunodeficiency alleles from both lentiviruses are functionally equivalent. We found that Nef was present in endothelial and in factor VIII-negative cells, including the infected mononuclear cells. To our knowledge, this is the first time that any group has identified plexiform lesions in macaque lungs infected with a lentivirus.

Although all the animals in cohorts from the New England and California National Primate Research Centers have abnormal endothelial cell morphology, the characteristics of the vasculopathies are different. Vascular lesions in the NENPRC SHIV-*nef*_{1486D95} cohort more closely resemble the plexiform lesions seen in HRPD patients with medial smooth muscle cell hypertrophy and multiple luminal obstructions containing factor VIII-positive endothelial cells. Interestingly, lesions in the SHIV-*nef*_{SE33A} animals from the CNPRC cohort were dominated by medial hypertrophy and a disrupted, cuboidal endothelial cell layer. The different pathologies in the two cohorts may be related to the stage of plexiform lesion formation in SHIV-*nef* animals, the rate of progression of lesion development, or the specific *nef* isolates

Figure 4. Colocalization of HIV-1 Nef in pulmonary arterial endothelial cells in human patients with HIV-related pulmonary hypertension (HRPH), but not in normal humans or in patients with idiopathic pulmonary hypertension (IPAH). Paraffin-embedded lung sections from normal human lung (A–D), human IPAH lung with plexiform lesions (E–H), and human HRPH lung (I–L) were stained with a monoclonal antibody to HIV-1 Nef and counterstained with methyl green (A, E, and I). Separate sections from the same patients were dual-stained with a rabbit polyclonal antibody to factor VIII and detected with an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (B, F, and J) and a mouse monoclonal antibody to HIV-1 Nef and detected with a goat anti-mouse antibody, using Vector red (C, G, and K). The factor VIII and Nef images were digitally merged (D, H, and L) to demonstrate colocalization of Nef in endothelial cells. Human lungs from normal subjects and patients with IPAH

lacked staining in either infiltrating mononuclear cells, plexiform lesions, or the unaffected vasculature. The lung from a patient with HRPH showed distinct positive staining in the occluding cells of a plexiform lesion (I, arrows) as well as localization to the endothelial cell layer (K and L, arrows) in an uninvolved vessel from the same section. N = normal patient; I = patient with IPAH; H = patient with HRPH; L = lumen.

in the chimeras. Without prospective studies using both of the constructs in cohorts infected in parallel, we are unable to provide direct evidence for the underlying mechanistic reasons for the differences. Nevertheless, the appearance of a disrupted endothelial cell layer containing the HIV-1 Nef protein suggests a role for Nef in the development of the complex severe pulmonary vascular lesions.

Weesner and Kaplan (30) found elevated pulmonary artery pressures in a subset of SIV-infected stump-tailed macaques (*Macaca arctoides*) and only moderate medial thickening of the pulmonary arteries. Chalifoux and coworkers (21) described an arteriopathy characterized by intimal and medial thickening in 19 of 85 macaques infected with SIV at the NENPRC. The medial thickening was seen only in large and medium-sized pulmonary arteries, and was reported to be the result of extracellular matrix deposition. Thirty-seven percent of the macaques with arteriopathy contained lumina with thrombi in various stages of organization and recanalization. In fact, after exhaustive examination, the authors did not find evidence of plexogenic lesions of small pulmonary arterioles and mention that the endothelial layer was always intact in these small arterioles. Infection of C57BL/6 mice with the LP-BM5 retroviral mixture, a murine model of immunodeficiency, leads to a diffuse interstitial pneumonitis similar to AIDS-associated interstitial pneumonitis, characterized by lymphocytic and macrophage infiltration in a setting of immunodeficiency (31). Although these mice develop right ventricular hypertrophy and show an increase in the percentage of muscularized pulmonary arteries with increased perivascular

collagen deposition, they are remarkable for not developing plexiform lesions or intimal fibrosis of the pulmonary arteries (32). Transgenic mice expressing the entire HIV-1-coding sequence under the control of the CD4 promoter develop a severe AIDS-like disease and a lymphoid interstitial pneumonitis as well (33). These studies highlight the relevance of our identification of HIV-1 Nef as a potential contributor to advanced pulmonary vascular remodeling and plexiform lesions in HIV-associated pulmonary hypertension. The present study, in combination with the observation that lung tissue samples from many patients with pulmonary hypertension were positive for human herpesvirus type 8 (27), suggests a central role for viral gene products and their influence on endothelial cell biology.

How could Nef exert its effects on endothelial cells? HIV does not appear to infect endothelial cells directly, but extracellular Nef has been found in HIV-infected patients at a level of approximately 10 ng/ml (34). We also found Nef-positive cells in medial layers as well in the parenchyma (Figures 4K and 4L, arrows; and see Figure E6), and the cell morphology suggests that these may be smooth muscle and epithelial cells, respectively. As with endothelial cells, productive infection of these non-CD4⁺ cells has not been described. Acheampong and coworkers (35) demonstrated that either exogenous or intracellular Nef induced apoptosis of human brain microvascular endothelial cells. In lymphocytes, Nef dose- and time-dependently induced apoptosis; however, infected cells were protected from death through down-regulation of class I major histocompatibility complex (36). Because Nef has been shown to enter lymphocytes via

Figure 5. Colocalization of HIV-1 Nef in pulmonary arterial endothelial cells in SHIV-*nef*-infected macaques, but not in SIV-infected macaques. Paraffin-embedded lung sections from SIV-infected macaque lung (A–D), SHIV-*nef*_{5F33A}-CNPRC3 lung (E–H), and SHIV-*nef*_{5F33A}-CNPRC4 lung (I–L) were stained with a monoclonal antibody to HIV-1 Nef and counterstained with methyl green (A, E, and I). Separate sections from the same animals were dual-stained with a rabbit polyclonal antibody to factor VIII and detected with an Alexa Fluor 488–conjugated goat anti-rabbit secondary antibody (B, F, and J) and a mouse monoclonal antibody to HIV-1 Nef and detected with a goat anti-mouse antibody, using Vector red (C, G, and K). The factor VIII and Nef images were digitally merged (D, H, and L) to demonstrate colocalization in vascular cells. SIV-infected lungs showed no evidence of Nef staining. The staining pattern in the SHIV-

nef macaque lungs mimicks the findings in human tissues, with staining in the endothelial cell layer (E, G, and H, arrows) as well as in perivascular sites with massive lymphocytic infiltration (I, K, and L, arrows). S = SIV; SH = SHIV-*nef*.

the chemokine receptor CXCR4 (37, 38) which is expressed on endothelial cells in the lung vasculature, active infection is not a prerequisite for HIV-induced pulmonary vascular injury. Furthermore, HIV-infected alveolar macrophages and lymphocytes may act as Nef reservoirs and increase angiogenic factors such as platelet-derived growth factor- α (39) to induce endothelial and smooth muscle cell proliferation in the lung vasculature.

Thus, the viral replication-independent effects of Nef may directly affect uninfected cells or indirectly activate alveolar macrophages to induce a proangiogenic response. On the basis of all the known properties of Nef, our own studies with cultured endothelial cells, and the presence of Nef in HRPD and SHIV-*nef* lungs, we propose the model shown in Figure 6. Infected lymphocytes or macrophages can either secrete Nef into the

Figure 6. Hypothetical relationship between HIV-1 Nef and the development of severe angioproliferative pulmonary hypertension. The accessory HIV-1 protein Nef (solid triangles) is expressed early during infection in HIV-infected resident alveolar macrophages and circulating lymphocytes, providing proximate sources for viral proteins for surrounding cells and the circulation. The uptake of Nef into vascular endothelial cells (ECs), in conjunction with immune insufficiency, leads to endothelial cell dysfunction and apoptosis. The engulfment of apoptotic cells by surrounding endothelial cells leads to an

apoptosis-resistant population with increased secretion of cytokines and growth factors (GFs). The uncontrolled endothelial cell proliferation leads to complex vascular remodeling, plexiform lesions, and HRPD.

extracellular compartment or transfer Nef to surrounding cells via cell-to-cell contact (40). Nef may then induce changes in the uninfected cells that may include apoptosis and growth factor release, proliferation and/or transdifferentiation, and formation of obliterative vascular lesions. In this model, an initial insult leads to subsequent selection of an apoptosis-resistant angioproliferative phenotype (41, 42). Whether this effect is mediated by the direct action of Nef on the target cells or indirectly through angiogenic growth factors remains to be determined.

In summary, we describe the associations between HIV-1 Nef and complex pulmonary vascular lesions in SHIV-*nef*-infected monkeys, which recapitulate the alterations seen in HRPD and IPAH. Our findings should lead to future investigations of the monkey model to elucidate the pathogenesis of these vascular lesions, and molecular examination of how the Nef protein might alter pulmonary vascular cells.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the manuscript.

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