Circulating Nef Induces Dyslipidemia in Simian Immunodeficiency Virus–Infected Macaques by Suppressing Cholesterol Efflux

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Human immunodeficiency virus (HIV) infection and subsequent antiretroviral therapy have been associated with an increased incidence of dyslipidemia and cardiovascular disease and has been shown to suppress cholesterol efflux from virus-infected macrophages by inducing Nef-dependent down-regulation of adenosine triphosphate–binding cassette transporter A1 (ABCA1). Here, the simian immunodeficiency virus (SIV)–infected macaque model was used to examine the consequences and mechanisms involved. SIV infection drove a significant remodeling of high-density lipoprotein profiles, suggesting that systemic inhibition of the ABCA1-dependent reverse cholesterol transport pathway occurred. The ABCA1 cholesterol transporter was significantly down-regulated in the livers of the SIV-infected macaques, and the viral protein Nef could be detected in the livers as well as in the plasma of infected animals. Extracellular myristoylated HIV Nef inhibited cholesterol efflux from macrophages and hepatocytes. Moreover, serum samples from SIV-infected macaques also suppressed cholesterol efflux in a Nef-dependent fashion. These results indicate that SIV infection is a significant contributor to primary dyslipidemia, likely through the ability of Nef to suppress ABCA1-dependent reverse cholesterol transport.

Cardiovascular disease contributes substantially to the overall morbidity of human immunodeficiency virus (HIV)–infected individuals. Epidemiological studies have connected cardiovascular disease and HIV infection, but the mechanisms of such connection remain elusive [1]. An important pathogenic factor contributing to cardiovascular disease in HIV-infected patients is dyslipidemia [2]. Disturbances of plasma lipoprotein metabolism associated with HIV infection and immune dysfunction are characterized by increased levels of triglyceride and hypocholesterolemia, with low levels of both low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) [3]. These changes are consistent with an atherogenic lipoprotein profile. Notably, low levels of HDL and high levels of triglycerides suggest that reverse cholesterol...
transport may be affected. Recently, it was demonstrated that the decline in HDL levels in treatment-naive patients correlated with HIV load [4] and that apolipoprotein A-I (apoA-I) levels correlated with CD4+ T cell count [5], supporting the notion that HIV infection plays a direct role in the pathogenesis of dyslipidemia and cardiovascular disease [6]. The question posed by these findings is how an infection that selectively targets monocytes, macrophages, and CD4+ T lymphocytes can have a systemic effect on lipid metabolism.

Our previous work demonstrated that HIV-1 infection induces impairment of both in vitro and in vivo reverse cholesterol transport [5, 7, 8]. In macrophages infected with HIV-1 in vitro, the virus inhibits cholesterol efflux by Nef-mediated down-regulation of adenosine triphosphate-binding cassette transporter A1 (ABCA1) [8]. ABCA1, also known as cholesterol efflux regulatory protein, is a protein encoded by the gene ABCA1 and is a major regulator of cellular cholesterol and phospholipid homeostasis as well as HDL metabolism. In human and animal models, ABCA1 mediates phospholipid and cholesterol efflux to several acceptors, but mainly to pre-β-1 (precursor) HDL particles [9].

In the present study, we examined HDL metabolism in simian immunodeficiency virus (SIV)–infected rhesus macaques (Macaca mulatta) receiving a diet high in fat and cholesterol. This animal model of HIV disease is widely used to study the pathogenesis of lentiviral disease and provides an opportunity to test the effects of viral infection in the absence of antiretroviral therapy (ART), which often affects lipid metabolism in HIV-infected patients [10]. Rhesus macaques fed an atherogenic diet developed alterations in serum lipid levels that parallel those observed in humans, including an increase in serum cholesterol, triglycerides, and LDL levels with a concurrent decrease in HDL level [11, 12]. We show here that SIV infection on the background of an atherogenic diet induces alterations of HDL remodeling that are consistent with impairment of ABCA1 function. Moreover, decreased expression of ABCA1 was observed in the livers of SIV-infected animals, and serum samples from these macaques suppressed ABCA1-mediated cholesterol efflux in a Nef-dependent fashion. These results indicate that SIV infection, via the activity of Nef released from infected cells, can affect cell-cholesterol efflux pathways, thus directly contributing to the pathogenesis of cardiovascular disease.

**METHODS**

**Animals and diets.** Indian-origin rhesus macaques were maintained in accordance with the principles given in the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and all work was approved by Harvard Medical School’s Standing Committee on Animals. Macaques (n = 8) were initially fed a standard normal diet (no. 5038; LabDiet), which supplies 12.9% of calories through fat and is low in cholesterol and saturated fatty acids. The animals were changed to an atherogenic diet high in cholesterol and saturated fat (no. 57JI) for 6 months [13]. After a 6-month period receiving the atherogenic diet, animals were intravenously inoculated with 25 ng of SIVmac239 p27, and blood samples (n = 8) were obtained 2 months after inoculation. Additional archived serum samples from animals infected with SIVmac239 or SIVmac239ΔNef and from uninfected animals were evaluated to further investigate the contribution of Nef to HDL patterns. Immunohistochemical analysis for SIV Nef protein and in situ hybridization for SIV nucleic acid was performed as previously elsewhere [14].

**Nondenaturing 2-dimensional polyacrylamide gel electrophoresis.** ApoA-I–containing HDL subpopulations were determined by nondenaturing 2-dimensional gel electrophoresis, immunoblotting, and image analysis, as described elsewhere [15].

**Cells.** Monocyte-derived macrophages were prepared from peripheral blood mononuclear cells as described elsewhere [16]. HepG2, a human hepatocellular cell line [17], was obtained from the American Type Culture Collection (HB-8065).

**Immunoprecipitation and Western blotting.** To detect Nef in serum, samples were incubated in lysis buffer (1 mmol/L Tris-HCl [pH 7.5], 0.15 mmol/L MgCl₂, 1 mmol/L KCl, 1% Triton X-100, 150 mmol/L NaCl, 5 mmol/L 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid [CHAPS], and protease inhibitors) for 2 h on ice. Lysates were centrifuged at 10,000 g for 10 min and precleared with protein G sepharose for 1 h at 4°C. Anti–SIV Nef monoclonal antibody (17.2) was added (5 μg/mL), and samples were incubated overnight at 4°C. Immune complexes were precipitated with protein G sepharose (2 h at 4°C), beads were washed 3 times with washing buffer (10 mmol/L Tris-HCl [pH 7.5], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.3% Triton X-100, 75 mmol/L NaCl, 1.5 mmol/L CHAPS, and protease inhibitors), and then loaded on 18% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Western blot analysis was performed as described elsewhere [8], using the following antibodies: rabbit polyclonal anti-ABCA1 (Novus Biologicals), rabbit polyclonal anti–HIV-1 Nef (AIDS Research and Reference Reagent Program), mouse monoclonal anti–SIVmac251 Nef (AIDS Research and Reference Reagent Program), rabbit polyclonal anti–SIVmac239 Nef (ABR), mouse monoclonal anti–SIVmac p27 (AIDS Research and Reference Reagent Program), mouse monoclonal anti-AU1 (Covance), and mouse monoclonal anti–β-actin (Sigma). For ABCA1 analysis, macaque macrophages were either mock infected or infected with the macrophage-tropic SIV strain SIVmacMER or SIVmacMERΔNef, using 1.5 × 10⁶ cpmm of reverse-transcriptase activity per 1 × 10⁶ cells. At the peak of infection, cells were lysed in 1× phosphate-buffered saline con-
taining 1% Triton X-100 and 0.5% SDS and then analyzed by immunoblotting.

**ABCA1 immunohistochemistry.** Immunohistochemical analysis was performed with either an anti–ABCA1 mouse immunoglobulin G (IgG) 1 monoclonal antibody (GenWay) or with an isotype mouse monoclonal antibody (Covance) to serve as a negative control, which showed little or no staining. Slides were rinsed in Tris-buffered saline with Tween and then incubated for 0.5 h with a biotinylated goat antibody, rinsed and then incubated for 0.5 h with a strepABComplex/horseradish peroxidase (HRP) solution (DakoCytomation StreptABComplex/HRP Duet and Mouse/Rabbit kit), and incubated with the chromogenic substrate (DakoCytomation Liquid DAB Substrate Chromogen system). Images were recorded using an Olympus IX70 microscope, and Scanalytics IPLab image software was used to quantify the percentage of 3,3-diaminobenzidine (DAB) chromogen–positive area in each liver section. Mean values derived from 4 individual animals were compared.

**Detection of Nef in hepatic tissue.** Frozen liver samples (50 mg) were lysed in 500 μL of ice-cold lysis buffer (10 mmol/L Tris-HCl [pH 7.5], 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 150 mmol/L NaCl, 1% Triton X-100, and 5 mmol/L CHAPS, and protease inhibitor cocktail [Roche]) and sonicated on ice for 20 s 4 times, using a Tissumizer homogenizer (Tekmar). Fifty micrometers of protein was analyzed by Western blotting, using mouse monoclonal antibody against SIV Nef (AIDS Research and Reference Reagent Program).

**Statistical analysis.** Statistical analysis was performed using commercially available software (Prism for Windows, version 5.00; GraphPad Software).

## RESULTS

**Similarity of HDL profile changes in response to an atherogenic diet between macaques and humans.** We and others have previously described dyslipidemia after initiation of an atherogenic diet in nonhuman primates [13, 18]. Changes in lipid composition included marked increases in total cholesterol (708 mg/dL), triglyceride (149.3 mg/dL) and LDL (641.0 mg/dL) levels, with no changes in the concentration of HDL (67.0 mg/dL), and the animals developed atherosclerotic lesions that mimic those observed in humans [13]. Examination of serum samples from rhesus macaques receiving a normal diet revealed an HDL profile similar to that observed in humans (Figure 1A), except that macaques had a prominent particle, termed VL–α, that was larger than α-1. The change from a normal diet to an atherogenic diet was associated with statistically significant decreases in levels of the mature, lipid-rich VL–α and α-1 particles and significant increases in levels of the small, lipid-poor α-3 and pre–α-3 HDL particles (Table 1), suggesting that maturation of HDL particles was blocked. An increase in the pre–β-1:α-1 ratio has been associated with an elevated risk of coronary artery disease in human clinical studies [19, 20]. Further analysis of the group receiving the atherogenic diet demonstrated increases in the pre–β-1:VL–α HDL ratio from 1.56 (normal diet) to 5.86 (atherogenic diet) (P = .017, t test) and in the pre–β-1:α-1 HDL ratio from 1.59 (normal diet) to 4.2 (atherogenic diet) (P = .010, t test). In contrast, no significant effect was observed on the pre–β-1:α-4, pre–β-1:α-3, or pre–β-1:α-2 ratios, again indicating that an atherogenic diet may affect the late steps of HDL particle maturation.

**Exacerbation after SIV infection of the derangement in the HDL population caused by an atherogenic diet.** After 6 months of receiving an atherogenic diet, animals were inoculated with SIV and monitored prospectively for another 2 months. Samples were obtained at viral set point (mean, 6.19 log₁₀ RNA copies/mL of plasma and 679 CD4 cells/mL). A decrease in the levels of the mature large HDL particles (VL–α and α-1) was maintained after infection (Table 1). Moreover, we observed a statistically significant decrease in the proportion of the small α-mobility particles (α-3 and α-4) (Figure 1B) and a further increase in the proportion of the pre–β-1 particles (Figure 1C), compared with preinfection levels. Thus, SIV inoculation exacerbates the derangement in the HDL population profile caused by an atherogenic diet, primarily by further increasing the concentration of the pre–β-1 subfraction and decreasing the concentration of the α-mobility subfractions (Figure 1B and 1C). These changes are consistent with an impairment of cellular cholesterol efflux and/or a remodeling of small nascent HDL particles and a decreased flow of cholesterol through the reverse cholesterol transport pathway. We observed a specific increase in pre–β-1:α-4 (atherogenic diet, 4.53; atherogenic diet plus SIV, 10.15) and pre–β-1:α-3 (atherogenic diet, 2.89; atherogenic diet plus SIV, 5.93) ratios in SIV-infected but not in uninfected animals receiving an atherogenic diet (Figure 1D and Table 1), suggesting that an SIV-specific block of HDL remodeling occurred at the step of conversion of pre–β-1 to the immature α-mobility particles.

Importantly, the effect of SIV infection on HDL remodeling was observed only when animals were fed an atherogenic diet. Archived samples from animals fed a normal diet (n = 12) and inoculated with SIVmac239 (n = 5) or SIVmac239ΔNef (n = 7) were analyzed 14 weeks after infection. No statistically significant difference was observed between the groups in the pre–β-1:α-4 (normal diet, −2.69; normal diet plus SIVmac239ΔNef, −3.57; normal diet plus SIVmac239, −1.60; P = .074) and pre–β-1:α-3 (normal diet, −0.32; normal diet plus SIVmac239ΔNef, −0.50; normal diet plus SIVmac239, −0.42; P = .308) ratios. This result is consistent with the requirement of an atherogenic diet for the development of atherosclerosis in SIV-infected macaques [21].

**A mechanism for SIV-induced impairment of reverse cholesterol transport.** Changes in HDL subfractions due to SIV...
Figure 1. Production of a proatherogenic lipid profile in rhesus macaques by simian immunodeficiency virus (SIV) infection and a high-fat, high-cholesterol diet. A, Apolipoprotein A-I (apoA-I)–containing high-density lipoprotein (HDL) subpopulations in a human and a rhesus macaque sample, as determined by 2-dimensional non-denaturing gel electrophoresis and immunoblot analysis. B, Analysis of small pre-β-1 (a and b) particles. C, Analysis of large VL-α and α-1 particles. D, Analysis of pre-β-1:α-4 ratio. ApoA-I–containing HDL particles were determined from whole plasma by 2-dimensional non-denaturing gel electrophoresis and immunoblot analysis. Five microliters of plasma was separated into pre-β-, α-, and pre-α-mobility particles by agarose electrophoresis, on the basis of surface charge in the first dimension. A slice of the agarose gel was transferred to the top of 3%–35% concave-gradient polyacrylamide gel and separated by size in the second dimension. Gel was electrotransferred to nitrocellulose membrane followed by incubation of monospecific apoA-I antibody and 125I-labeled secondary antibody. 125I signal was evaluated using a Storm 860 phosphorimager (Molecular Dynamics). Data are mean values ± standard errors. Statistical analysis is presented in Table 1; P values were determined by 1-way analysis of variance (*) or Kruskal-Wallis 1-way analysis of variance on ranks (†) with Tukey posttest comparison (**) AD, atherogenic diet; ND, normal diet.

infection are consistent with the impairment of an early step of reverse cholesterol transport [22]. The ABCA1 cholesterol transporter plays a critical role in mediating phospholipid and free cholesterol efflux to pre-β-1 particles, enabling their conversion into small α-migrating particles [23]. We tested whether an alteration of the HDL subpopulation profile of SIV-infected macaques could be explained by a systemic repression of ABCA1 expression. We first examined the expression of ABCA1 in the livers of infected macaques, because ABCA1 expression in this organ controls the circulating level and composition of HDL [24]. Liver samples obtained from SIV-infected and uninfected macaques while they were receiving the atherogenic diet were immunostained for ABCA1 expression. In uninfected samples, ABCA1 was prominently expressed in the hepatocytes but not on the endothelium of the portal vessels (Figure 2A), as has been previously reported in the mouse liver [25]. In contrast, livers from the infected macaques showed a statistically significant decrease in staining for ABCA1 (Figure 2B and 2C). Importantly, hepatic expression of ABCA1 was negatively correlated with log_{10} viral load (r = -0.9945; P < .006). Both in situ hybridization and immunohistochemistry did not detect any viral replication in the liver (data not shown). This result
indicated that active viral replication in the liver could not explain the repression of ABCA1 in this organ and suggested that systemic circulating factors may be responsible.

One such candidate factor could be the viral protein Nef, because it mediates the ability of HIV to down-regulate ABCA1 and cholesterol efflux to apoA-1 [8]. We found Gag and Nef in forming atheromas in the atherogenic diet–fed SIV-infected macaques at the time of necropsy (Figure 2D), suggesting that active viral replication occurred in these cells. Although the presence of Nef in infected macrophages may exacerbate the progression of atheroma, we questioned whether Nef may also have a more global effect on lipoprotein metabolism. Nef is shed by dead or dying virus-infected cells and can be detected in the circulation of infected individuals [26]; extracellular Nef has been shown to affect the activity of bystander cells [27–29]. Approximately 50–100 ng/mL Nef was detected by immunoblotting in the blood of SIV-infected macaques (Figure 2E), consistent with previously reported results from a study of HIV-infected individuals [26]. Furthermore, analysis of the liver samples from SIV-infected macaques demonstrated the presence of Nef (Figure 2F).

**Systemic effects of circulating Nef on reverse cholesterol transport.** The above results suggest that Nef could be responsible for the observed repression of ABCA1 in the liver. To further explore this possibility, we first examined whether SIV Nef could, like HIV-1 Nef [8], repress ABCA1-mediated cholesterol efflux when transfected into HeLa cells that stably express ABCA1 [30]. For this experiment, we used AU1-tagged Nef constructs [31] derived from previously described HIV and SIV isolates. The lengths of the Nef amino acid sequences varies between 206 (HIV-1) and 263 (HIV-2 and SIVmac). Furthermore, Nef is posttranslationally modified by myristoylation, phosphorylation, and ubiquitination [32, 33]. Thus, the different sizes of the Nef proteins account for the different migration in SDS-PAGE observed in Figure 3A. For some Nef constructs (HIV-2 BEN, SIVmac239, and SIVcpz GAB2), we observed several forms of the protein in cellular extracts (Figure 3A). Some of them may be due to incomplete posttranslational modification of overexpressed proteins. Furthermore, Nef may be cleaved by cellular proteases; in addition, some nef alleles contain internal ATG triplets that may lead to the expression of truncated proteins missing the N-terminus [39].

In HeLa-ABCA1 cells, all Nef constructs inhibited cholesterol efflux, although with different efficiency (Figure 3B). Notably, suppression of cholesterol efflux did not correlate with the expression levels of the respective Nef proteins. For example, the SIVmac239 nef protein was much less effective in suppressing cholesterol efflux than the SIVagm SAB1 Nef protein, although it was expressed with higher efficiency. To demonstrate the activity of SIV Nef in the context of whole-virus infection, we measured cholesterol efflux from terminally differentiated macrophages infected in vitro with Nef-positive or Nef-deficient SIVmac239MER (a macrophage-tropic SIV strain) [40]. Importantly, both SIV variants replicated to similar levels, according to reverse-transcriptase activity (Figure 3C), and no visible cytotoxicity (such as detached cells) was observed in infected cultures, consistent with the notion that macrophages are resistant to the cytotoxic effects of SIV [41] and HIV-1 [42] infection. This experiment demonstrated that, similar to HIV-1, SIV suppressed ABCA1-mediated cholesterol efflux to apoA-1 from infected macrophages in a Nef-dependent fashion (Figure 3C).

Having established that SIV Nef and HIV-1 Nef have a similar effect on ABCA1 cholesterol efflux, we tested whether soluble extracellular Nef has the same ABCA1-repressing activity. We first introduced recombinant myristoylated HIV-1 Nef to the

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**Table 1. High-Density Lipoprotein (HDL) Analysis Performed on Serum Samples from Rhesus Macaques**

<table>
<thead>
<tr>
<th>HDL particle</th>
<th>ND (n = 7)</th>
<th>AD (n = 7)</th>
<th>AD SIV (n = 8)</th>
<th>ANOVA</th>
<th>Posttest comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-β-1a</td>
<td>20.72</td>
<td>31.10</td>
<td>37.08</td>
<td>.003a</td>
<td>ND vs AD</td>
</tr>
<tr>
<td>Pre-β-1b</td>
<td>6.98</td>
<td>7.89</td>
<td>15.75</td>
<td>.009c</td>
<td>AD vs AD SIV</td>
</tr>
<tr>
<td>VL-α</td>
<td>17.74</td>
<td>6.65</td>
<td>8.20</td>
<td>.015a</td>
<td>.05 (1)b</td>
</tr>
<tr>
<td>α-1</td>
<td>17.37</td>
<td>9.29</td>
<td>7.07</td>
<td>.001a</td>
<td>.05 (1)b</td>
</tr>
<tr>
<td>α-2</td>
<td>10.97</td>
<td>11.69</td>
<td>10.11</td>
<td>.489a</td>
<td>.05 (1)b</td>
</tr>
<tr>
<td>α-3</td>
<td>8.75</td>
<td>13.51</td>
<td>8.92</td>
<td>.002a</td>
<td>.05 (1)b</td>
</tr>
<tr>
<td>α-4</td>
<td>6.89</td>
<td>8.96</td>
<td>5.18</td>
<td>.012a</td>
<td>.05 (1)b</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean values ± standard deviations, in milligrams per deciliter. Serum samples were obtained from rhesus macaques during receipt of a normal diet (ND), after being switched to 6 months of an atherogenic diet (AD), and then 2 months after simian immunodeficiency virus (SIV) mac239 inoculation during receipt of an atherogenic diet (AD SIV). ANOVA, analysis of variance.

a One-way ANOVA.
b Tukey posttest comparison.
c Kruskal-Wallis 1-way ANOVA on ranks.
d Dunn method.
extracellular milieu of cultured human monocyte-derived macrophages and observed a dose-dependent suppression of cholesterol efflux (Figure 4A). Next, we tested whether extracellular Nef could repress ABCA1 activity in hepatocytes. Using HepG2 cells, we demonstrated a suppression of cholesterol efflux by 70% at a Nef concentration of 10 ng/mL and by >90% at 50 ng/mL (Figure 4B). These concentrations of Nef are similar to those observed in the blood of HIV-infected patients [26] and SIV-infected macaques, respectively. Interestingly, nonmyristoylated Nef did not impair cholesterol efflux (Figure 4A and 4B), despite there being a similar association of both Nef variants with HepG2 cells (Figure 4C). The requirement of Nef myristoylation for cholesterol efflux down-regulation resembles that of endogenously expressed Nef [8].

To demonstrate that Nef present in the blood of SIV-infected macaques can suppress cholesterol efflux, we compared efflux to apoA-I from human macrophages preincubated with archived serum samples from macaques infected with Nef-positive or Nef-negative SIVmac239. To neutralize Nef, serum samples were also treated with anti-Nef monoclonal antibody (or control IgG), and the immune complexes were either depleted or not depleted before incubation. Serum from a monkey infected with wild-type SIVmac239 (viral load, RNA73.2 \times 10^7 copies/mL) suppressed cholesterol efflux, and this suppression was reversed by treatment with anti-Nef antibody, both when immune complexes were depleted or not depleted (Figure 4D). This result indicates that antibody to Nef neutralizes the suppressive effect that this HIV protein has on cholesterol efflux. Serum samples from an uninfected macaque and a macaque infected with Nef-deficient SIV (RNA copies/mL) did not affect cholesterol efflux, regardless of treatment with anti-Nef antibody. Much lower viral loads in animals infected with Nef-deficient SIV did not allow us to directly control for possible Nef-independent effects on cholesterol efflux. However, reversal of the inhibitory effect by anti-Nef antibody provides strong evidence for the role of Nef. A similar Nef-dependent decrease in cholesterol efflux to ApoA-I by serum samples from SIVmac239-infected animals was observed on HepG2 cells (Fig-
representative experiment is shown of 2 performed with cells from different animals. * For the comparison with cells infected with a multicopy construct of HIV or SIV strains in HeLa cells, tested using anti-AU1 monoclonal antibody. β-Actin expression was used as loading control. B: Cholesterol efflux to apolipoprotein A-I (apoA-I) from HeLa–adenosine triphosphate–binding cassette transporter A1 (ABCA1) cells transfected with AU1-tagged Nef derived from the indicated viral strains. HIV-1 NL4–3 is a well-characterized laboratory clone used as a positive control; HIV-1 Na7 is a primary HIV-1 from a patient with AIDS [34]; HIV-2cbl is from a patient from the Gambia [35]; HIV-2 BEN is from a patient with predominantly neurological defects [36]; SIVmac239 is a well-characterized SIV clone [37]; SIVagm SAB1 Nef was amplified from a green monkey (Chlorocebus sabaeus) [38]; SIVcpz Nok5 nef allele was amplified from plasma RNA derived from a chimpanzee (Noah; subspecies, Pan troglodytes schweinfurthii) infected with the highly divergent SIVcpz-ant strain in Zaire [31]; and SIVcpz GAB2 nef allele was amplified from uncultured peripheral blood mononuclear cell DNA from a chimpanzee (subspecies, Pan troglodytes troglodytes) infected in Gabon [31]. Control cells were transfected with an empty vector. Cholesterol efflux was measured as described elsewhere [8]. Data are mean values ± standard deviations for triplicate wells. A representative experiment is shown of 2 performed. C: Mean cholesterol efflux to apoA-I from rhesus macaque macrophages either mock infected or infected in vitro with Nef-positive or Nef-deleted (ΔNef) SIVmac239MER. Analysis was performed 18 days after infection, when virus replication reached a plateau. Reverse-transcriptase (RT) values are shown beneath the legend; data are mean values ± standard deviations for triplicate wells. A representative experiment is shown of 2 performed with cells from different animals. *P < .05 for the comparison with cells infected with a ΔNef virus (t test; n = 3).

**Figure 3.** Inhibition of simian immunodeficiency virus (SIV) Nef by reverse cholesterol transport in infected cells. A, Expression of AU1-tagged Nef constructs of HIV and SIV strains in HeLa cells, tested using anti-AU1 monoclonal antibody. β-Actin expression was used as loading control. B, Cholesterol efflux to apolipoprotein A-I (apoA-I) from HeLa–adenosine triphosphate–binding cassette transporter A1 (ABCA1) cells transfected with AU1-tagged Nef derived from the indicated viral strains. HIV-1 NL4–3 is a well-characterized laboratory clone used as a positive control; HIV-1 Na7 is a primary HIV-1 from a patient with AIDS [34]; HIV-2cbl is from a patient from the Gambia [35]; HIV-2 BEN is from a patient with predominantly neurological defects [36]; SIVmac239 is a well-characterized SIV clone [37]; SIVagm SAB1 Nef was amplified from a green monkey (Chlorocebus sabaeus) [38]; SIVcpz Nok5 nef allele was amplified from plasma RNA derived from a chimpanzee (Noah; subspecies, Pan troglodytes schweinfurthii) infected with the highly divergent SIVcpz-ant strain in Zaire [31]; and SIVcpz GAB2 nef allele was amplified from uncultured peripheral blood mononuclear cell DNA from a chimpanzee (subspecies, Pan troglodytes troglodytes) infected in Gabon [31]. Control cells were transfected with an empty vector. Cholesterol efflux was measured as described elsewhere [8]. Data are mean values ± standard deviations for triplicate wells. A representative experiment is shown of 2 performed. C, Mean cholesterol efflux to apoA-I from rhesus macaque macrophages either mock infected or infected in vitro with Nef-positive or Nef-deleted (ΔNef) SIVmac239MER. Analysis was performed 18 days after infection, when virus replication reached a plateau. Reverse-transcriptase (RT) values are shown beneath the legend; data are mean values ± standard deviations for triplicate wells. A representative experiment is shown of 2 performed with cells from different animals. *P < .05 for the comparison with cells infected with a ΔNef virus (t test; n = 3).

**DISCUSSION**

Although the association between HIV infection, dyslipidemia, and cardiovascular disease has been demonstrated in a large number of clinical studies [2], the mechanism behind this association remains elusive. The relative contribution of HIV infection itself remains unresolved, because most HIV-infected patients in developed countries receive ART early during disease progression. Elevations in concentrations of LDL-C and triglycerides may be caused by ART, given that elevation of plasma LDL-C and triglyceride levels coincided with initiation of the treatment, depended on the treatment regimen, and subsided with treatment interruption [43, 44]. There is little evidence, however, that the concentration of HDL is negatively affected by ART [45]. Plasma apoA-I concentration positively correlated with CD4+ T cell level [5], suggesting that HIV infection may be the primary cause of changes in HDL levels. A major difficulty in explaining how HIV infection itself could cause impairment of HDL metabolism is that HIV infects CD4+ T cells and macrophages, cells that do not have a major effect on HDL metabolism. Systemic effects of HIV infection were previously attributed mainly to the elevated inflammatory status of HIV-infected patients [46], and no specific mechanisms related to lipid metabolism were proposed.

We previously demonstrated that dyslipidemia and atherosclerosis could be induced in macaques by feeding these animals an atherogenic lipid-rich diet [13]. In the present study, we have demonstrated that an atherogenic diet induces a decrease in the proportion of large, mature HDL particles and an increase in the proportion of small, lipid-poor HDL particles. Infection of atherogenic diet–fed macaques with SIV led to a specific increase in pre–α-1:α-4 and pre–α-1:α-3 ratios, suggesting that SIV infection inhibits conversion of pre–β-1 HDL to α-4 and α-3 particles. This process is dependent on ABCA1 and occurs as a result of reverse cholesterol transport from peripheral tissues as well as the liver [22, 24, 47]. Therefore, we investigated SIV-induced changes in ABCA1 with a focus on the liver, the organ mainly responsible for HDL composition.

A major finding of this study is that the abundance and function of ABCA1 in the livers of SIV-infected monkeys are decreased despite there being no evidence of SIV infection in this organ. Importantly, we were able to detect Nef in the livers as well as in the plasma of infected macaques. Plasma from infected macaques inhibited cholesterol efflux from hepatocytes in a Nef-dependent fashion. Recent reports have demonstrated...
**Figure 4.** Inhibition of cholesterol efflux by soluble Nef. A. Results of incubation of human monocyte–derived macrophages with the indicated concentrations of myristoylated recombinant Nef (mNef) or nonmyristoylated recombinant Nef (nmNef) derived from the human immunodeficiency virus type 1 SF2 strain. Cholesterol efflux to apolipoprotein A-I (apoA-I) was measured and is presented as the percentage of [3H]-cholesterol in the culture supernatant. Data are mean values ± standard deviations for triplicate wells. B. Results of incubation of HepG2 cells for 48 h with the indicated concentrations of recombinant Nef. Cholesterol efflux to apoA-I was measured as for panel A. Data are mean values ± standard deviations for triplicate wells. C. Western blot analysis of Nef in the lysate and supernatant of HepG2 cells incubated for 48 h with 100 ng/mL mNef or nmNef. Nef in the lysate fractions reflects the amount of cell-associated Nef, and supernatant reflects the amount of Nef added to cells. D. Results of incubation of human monocyte–derived macrophages for 72 h with serum samples from macaques infected with Nef-positive or Nef-negative simian immunodeficiency virus (SIV) mac239 (or from uninfected macaques before SIV inoculation). Serum samples were preincubated with anti-Nef antibody or control immunoglobulin G, and immune complexes were either removed by protein G sepharose (serum samples from uninfected macaques and macaques infected with SIVΔNef, and bars labeled “depleted” for serum samples from SIV-infected macaques) or left with the serum (bars labeled “nondepleted” for serum samples from SIV-infected macaques). Cholesterol efflux to apoA-I was measured as for panel A. Data are mean values ± standard errors of the mean for triplicate determinations. E. Results of incubation of HepG2 cells for 48 h with serum samples from macaques infected with Nef-positive or Nef-negative SIVmac239 (or from uninfected macaques before SIV inoculation) depleted of Nef as described for panel D. Data are mean values ± standard errors of the mean for triplicate determinations. A representative experiment of 2 performed is shown.

That soluble Nef can be taken up by various cells normally not infected by HIV or SIV and affect their function, leading to the impairment of such physiological activities as immunoglobulin class switching by B cells [27] or hematopoiesis by bone marrow CD34+ progenitor cells [48]. Our study suggests that soluble Nef may be responsible for the inhibition of ABCA1 activity in the liver and the suppression of cholesterol efflux, which in turn leads to the inhibition of HDL production and remodeling in SIV-infected macaques. Nef may also alter production by hepatocytes of other factors regulating HDL metabolism, such as cholesteryl ester transfer protein (CETP). CETP mediates the exchange of triglyceride for cholesteryl ester between apoB-containing lipoproteins and HDL. Our recent study suggested that the elevated levels of CETP observed in HIV-infected patients may contribute to hypoalphalipoproteinemia [5], providing another potential link between Nef and HDL.

Our results suggest a 2-pronged atherogenic mechanism of SIV and HIV infection. By impairing cholesterol efflux from infected macrophages, SIV (or HIV) blocks cholesterol release from these cells, thus promoting their conversion into foam cells. At the same time, Nef released from infected cells can affect cholesterol efflux from uninfected peripheral cells, including macrophages and hepatocytes. This effect of Nef would lead to changes in HDL metabolism and contribute to hypoalphalipoproteinemia and the alterations in HDL profiles...
observed in SIV-infected macaques and HIV-infected patients [49, 50]. Our findings indicate that lentiviral infection is associated with fundamental alterations in lipid metabolism that, together with detrimental changes induced by high-fat, high-cholesterol diets and highly active ART, may lead to an increased risk of cardiovascular disease.

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