

Accumulation of Neutral Lipids in Liver and Aorta of Nef-Transgenic Mice

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Abstract

HIV-infected individuals are at high risk of developing atherosclerosis and cardiovascular disease, in part, due to HIV-induced impairment of cholesterol metabolism. *In vitro* studies demonstrated that HIV-1 protein Nef inhibits activity of ABCA1, the main cellular cholesterol transporter, leading to cholesterol accumulation in macrophages and conversion of these cells into foam cells, characteristic for atherosclerosis. However, the mechanisms of Nef-mediated effects on cholesterol metabolism *in vivo* are not well characterized. In this study, we generated Nef-transgenic mice and evaluated the accumulation of neutral lipids in liver and aorta of these animals. *Nef* expression was low in all transgenic mice, with some mice carrying the *Nef* transgene, but not expressing the *Nef* RNA. Using Oil Red O staining, we demonstrated increased levels of neutral lipids in liver and aorta of mice expressing *Nef* relative to transgenic animals, with no detectable *Nef* expression or control wild-type mice. These results provide direct evidence that Nef promotes cholesterol deposition in tissues.

Keywords: cholesterol efflux, Nef, HIV-1, lipid droplets

COMBINED ANTIRETROVIRAL THERAPY (cART) has dramatically changed the clinical course of HIV infection, converting it from an acute frequently fatal disease to a chronic condition. Consequently, primary clinical concerns in HIV-infected subjects have shifted from AIDS and AIDS-related conditions to HIV-associated comorbidities. Atherosclerosis and cardiovascular disease (CVD) are particularly prominent, with HIV-infected subjects being at increased^{1,2} and earlier³ risk for developing atherosclerosis and dying from CVD.

Various factors may contribute to HIV-associated atherosclerosis, including persistent inflammation and immune activation due to bacterial translocation through damaged mucosa⁴ and side effects of anti-HIV medications.⁵ In addition, HIV can directly affect cholesterol metabolism of infected cells by inhibiting activity of the cellular cholesterol transporter, ABCA1.⁶ As a result of such inhibition, cholesterol efflux from macrophages is blocked and cells accumulate

cholesterol, converting into foam cells, which are characteristic cells for atherosclerotic plaques.

Our previous *in vitro* studies demonstrated that Nef is the HIV-1 protein responsible for downregulation of ABCA1.^{6,7} However, the only *in vivo* evidence for the effects of Nef on cholesterol metabolism are studies where recombinant Nef was injected into mice.⁸ These studies demonstrated an enhanced development of atherosclerosis in a mouse model of this disease (*apoe*^{-/-} mice on high fat diet), and in wild-type C57BL/6 mice fed high-fat/high-cholesterol diet, Nef induced accumulation of lipid-laden macrophages in adventitia of aorta. These findings are consistent with the role of Nef in impairment of cholesterol efflux.⁶

In this study, we aimed to establish whether endogenously produced Nef causes accumulation of cholesterol in tissues *in vivo*, a key event in the proposed function of Nef in pathogenesis of HIV-induced atherosclerosis.⁸ To characterize the *in vivo* effects of Nef on lipid metabolism,

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we used *Nef*-transgenic mice. The model where *Nef* is expressed in all cells was selected because previous studies demonstrated that *Nef* released from infected cells induces ABCA1 downregulation in uninfected cells of SIV-infected macaques, and recombinant *Nef* inhibits cholesterol efflux in treated cells similar to the inhibition observed in HIV-infected cells.⁹ It has also been shown that *Nef* mRNA can be packaged in exosomes and induce *Nef* expression in uninfected cells.¹⁰

Transgenic mice were generated on C57BL/6 background using genetic construct with chicken actin gene promoter and cytomegalovirus enhancer, and removable STOP-cassette^{11,12} containing LoxP-flanked chloramphenicol acetyltransferase gene in front of *Nef*-IRES-EGFP bicistronic cassette expressing consensus *Nef* sequence.¹³ Transgenic construct was tested *in vitro* in HEK293 cells cotransfected with plasmids containing *Nef* cassette and PGK-Cre. Several transgenic founders were bred with *PGK-cre* deleter mice, and all progenies showed low levels of *Nef* expression. Specifically, *Nef* protein was undetectable in plasma and tissues (spleen, aorta, liver) from these animals by IHC, Western blotting, or ELISA [the threshold of the ELISA assay (Immunodiagnosics #108) was 10 pg/mL], but *Nef*-specific RNA could be detected by reverse transcription-polymerase chain reaction (RT-PCR) in some mice carrying the *Nef* transgene.

All transgenic animals were genetically identical and differed from the wild-type animals only by the presence of the *Nef* transgene. Therefore, variability of *Nef* expression levels in individual transgenic animals was likely due to varying levels of epigenetic silencing of the transgene, although special study would be required to test this assumption. The fact that the *Nef* expression was so low in these transgenic mice was actually advantageous for our purposes, as it approximated the situation in cART-treated subjects where HIV replication is suppressed to undetectable levels. Mice carrying the *Nef* transgene, but not expressing *Nef* RNA, served as an internal control, as they are genetically identical to *Nef*-expressing siblings.

The first set of animals was used for analysis of liver sections. This set included three transgenic (L2, L3, L5) mice (descendants of the same founder) and two wild-type mice, as tested by polymerase chain reaction (PCR) of genomic DNA (Fig. 1A1) using primers amplifying *Nef* fragment that spans base pairs 341-560 (forward primer 5'-CTACCACACA CAAGGCTACTTC-3', reverse primer 5'-GCTGTCAAACC TCCACTCTAAC-3'). *Nef* RNA was detected in two out of three transgenic mice (animals L3 and L5), as revealed by RNA PCR (Fig. 1A2). Staining liver sections with Oil Red O (ORO, stains neutral lipids in red), anti-mouse CD68 (Bio-Rad) mAb followed by goat anti-rat IgG conjugated with Alexa488 (Life Technologies, stains macrophages in green), and DAPI revealed increased amount of ORO-stained lipid droplets in liver sections from *Nef*-expressing mice L3 and L5 (Fig. 1B1). Quantitative analysis of ORO-stained droplets using Volocity software (PerkinElmer Life Sciences) demonstrated a significant increase ($p < .0001$ by one-way ANOVA with *post-hoc* Tukey HSD test) in the size of lipid droplets in *Nef*-expressing mice L3 and L5 relative to the WT mouse or nonexpressing transgenic mouse L2 (Fig. 1B2).

Given the limited number of mice available for this study, we could not statistically compare the three groups, that is,

nontransgenic mice, transgenic mice with no *Nef* expression, and transgenic mice with *Nef* expression. Therefore, we combined nontransgenic (two animals) and transgenic mice with no *Nef* expression (one animal) into one group (*Nef* negative) and compared it to the *Nef*-expressing group (*Nef* positive, two animals). Results presented in Figure 1B3 demonstrate that *Nef*-expressing animals accumulated significantly more lipid droplets in liver than *Nef*-negative mice ($p = .045$ by unpaired Student's *t*-test). Of particular interest, ORO staining did not colocalize with CD68 staining (there were few CD68-positive cells), suggesting that lipids accumulated in other than macrophages cells (e.g., hepatocytes, which also express *Nef* in these mice), and *Nef* did not induce massive macrophage migration into the liver. This conclusion is consistent with only a very low inflammation observed in *Nef*-injected mice.⁸

The second set of mice was used to stain aorta. This set included two transgenic animals (A19 and A20, descendants of the same founder, which was different from the founder of mice used for liver analysis) and three wild-type mice, as confirmed by PCR (Fig. 1C1) using primers amplifying *Nef* fragment spanning base pairs 26-360 (forward primer 5'-TGTGATTGGATGGCCTACTG-3', reverse primer 5'-GTA GCCTGTGTGTGGTAGAT-3'). One of the transgenic animals (A20) did express *Nef*, whereas the other (A19, used as control) did not (Fig. 1C2).

Aorta sections from these mice were stained with ORO, counterstained with DAPI, and analyzed by fluorescent microscopy. Aortic media from *Nef*-expressing transgenic mouse (A20) were enriched in ORO-stained lipid droplets relative to aorta sections from the wild-type mouse and the *Nef*-transgenic mouse that did not express *Nef* (Fig. 1D1). Accumulation of neutral lipids within aortic elastin fibers is characteristic to early stages in the development of atherosclerotic lesions.¹⁴

The size of ORO-stained droplets within the aorta wall was quantified for 493 vesicles from WT mouse, 635 vesicles from A20, and 238 vesicles from A19 using Volocity software. Lipid droplets from *Nef*-expressing transgenic mouse (A20) were significantly larger ($p < .0001$ by one-way ANOVA with *post-hoc* Tukey HSD test) than vesicles from WT mouse or transgenic mouse with silent *Nef*, A19 (Fig. 1D2). Given that only one *Nef*-expressing and one nonexpressing transgenic mice were available, statistical analysis of the groups could not be done. We therefore performed a 1-sample *t*-test to determine whether the size of the lipid droplets in transgenic mice was different from the size of lipid droplets in nontransgenic group. This analysis revealed that the size of droplets in *Nef*-expressing transgenic mouse (mean size 5.45 μm) was significantly different ($p = .0422$) from the droplet size in three nontransgenic mice (mean size = 4.19 μm , SD = 0.463 μm). In contrast, the droplet size in *Nef*-transgenic mouse not expressing *Nef* (mean size 4.58 μm) was not significantly different from that in nontransgenic mice ($p = .2820$).

Results of this study suggest that *Nef* expression, even at very low levels, promotes accumulation of lipid droplets in liver and aorta, which is characteristic for systemic impairment of cholesterol efflux and reverse cholesterol transport. These findings reproduce the key effect associated with injection of recombinant *Nef*, accumulation of lipids in aorta adventitia.⁶ However, in our transgenic mice, we did not

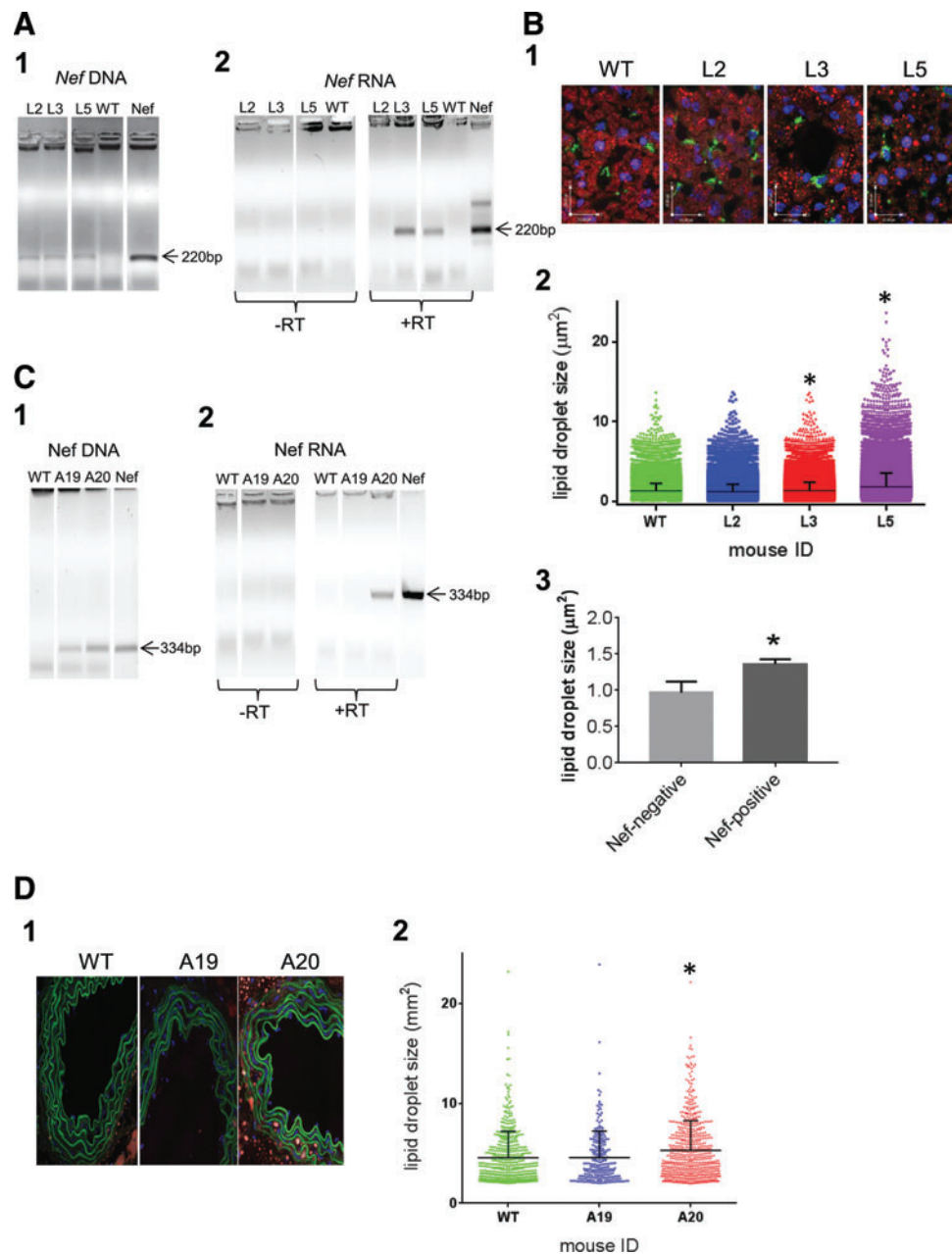


FIG. 1. Analysis of neutral lipids in tissue sections from Nef-transgenic mice. **(A)** Total DNA (**A1**) and RNA (**A2**) were extracted from liver sections of transgenic (L2, L3, L5) and one of the wild-type mice and analyzed by PCR (**1** and *left panels* in **2**) or reverse transcription-polymerase chain reaction (RT-PCR) (*right panels* in **2**). **(B1)** Liver sections were stained with ORO (stains neutral lipids in red), anti-CD68 (stains macrophages in green), and DAPI (stains nuclei in blue) and observed on fluorescent microscope. **(B2)** Sizes of 66,274 ORO-stained vesicles in the liver sections from WT mouse, 22,400 vesicles from L2, 63,503 vesicles from L3, and 39,450 vesicles from L5 transgenic animals were analyzed using Volocity software and are presented as mean \pm SEM. $*p < .0001$ relative to the WT and L2 mice, calculated by one-way ANOVA with *post-hoc* Tukey HSD test. **(B3)** Nef-negative group combined two wild-type mice and one transgenic mouse not expressing Nef (L2), Nef-positive group was represented by L3 and L5 mice. Bars show mean \pm SEM. $*p = .045$, unpaired Student's two-tailed *t*-test. **(C)** Total DNA (**C1**) and RNA (**C2**) were extracted from aorta sections of transgenic (A19, A20) and wild-type mice and analyzed by PCR (**C1** and *left panels* in **C2**) or RT-PCR (*right panels* in **2**). **(D1)** Aorta sections were stained with ORO (stains neutral lipids in red) and DAPI (stains nuclei in blue) and observed on fluorescent microscope. Green staining of elastin was due to autofluorescence. **(D2)** Sizes of the ORO-stained vesicles within aorta wall were analyzed using Volocity software and are presented as mean \pm SEM. $*p < .0001$ relative to the WT and A19 mice, calculated by one-way ANOVA with *post-hoc* Tukey HSD test. PCR, polymerase chain reaction; SEM, standard error of the mean. Color images available online at www.liebertpub.com/aid

observe weight gain, which was prominent in Nef-injected mice. This difference may be due to low *Nef* expression in transgenic animals, or/and to the fact that mice injected with Nef were fed cholesterol/fat-rich diet, whereas transgenic mice were on normal chow. Such interpretation is consistent with the model, whereby Nef and HIV infection provide an additional risk factor that potentiates the proatherogenic effect of other major risk factors, such as unhealthy diet, high cholesterol, smoking, and diabetes.

The small number of transgenic mice available for this study is a clear limitation, which needs to be addressed in the future. Future studies would also be required to determine the chain of molecular events behind the Nef-induced accumulation of lipid droplets. Nef is known to impair the function of the ABCA1 cholesterol transporter,⁶ but may also, through its association with calnexin,⁷ affect other proteins involved in lipid synthesis or transport. Nef interaction with Acyl-CoA thioesterase 8 (ACOT8)^{15,16} may also influence fatty acid metabolism.¹⁷ Findings described in this report provide the rationale for mechanistic studies into the role of Nef in pathogenesis of atherosclerosis and metabolic diseases in HIV-infected subjects.

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Author Disclosure Statement

No competing financial interests exist.

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