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Stimulation of the Liver X Receptor Pathway Inhibits HIV-1 Replication via Induction of ATP-Binding Cassette Transporter A1

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ABSTRACT

Cholesterol plays an important role in the HIV life cycle, and infectivity of cholesterol-depleted HIV virions is significantly impaired. Recently, we demonstrated that HIV-1, via its protein Nef, inhibits the activity of the major cellular cholesterol transporter ATP binding cassette transporter A1 (ABCA1), suggesting that the virus may use this mechanism to get access to cellular cholesterol. In this study, we investigated the effect on HIV infection of a synthetic liver X receptor (LXR) ligand, *N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide (TO-901317), which is a potent stimulator of ABCA1 expression. We demonstrate that TO-901317 restores cholesterol efflux from HIV-infected T lymphocytes and macrophages. TO-901317 potently suppressed HIV-1 replication in both cell types and inhibited HIV-1 replication in ex vivo cultured lymphoid tissue and in RAG-hu mice infected in vivo. This anti-HIV activity was dependent on ABCA1, because the effect of the drug was significantly reduced in ABCA1-defective T cells from a patient with Tangier disease, and RNA interference-mediated inhibition of ABCA1 expression eliminated the effect of TO-901317 on HIV-1 replication. TO-901317-mediated inhibition of HIV replication was due to reduced virus production and reduced infectivity of produced virions. The infectivity defect was in part due to reduced fusion activity of the virions, which was directly linked to reduced viral cholesterol. These results describe a novel approach to inhibiting HIV infection by stimulating ABCA1 expression.

HIV-1 requires cholesterol for its assembly and budding and for infection of target cells. HIV-1 budding from the host cell occurs at the cholesterol-enriched membrane microdomains, "lipid rafts," resulting in the high cholesterol/phospholipid molar ratio (>1.0) of the viral envelope (Brügger et al., 2006). The physical nature and functions of lipid rafts are a subject of intensive debates and controversies (Lingwood and Simons, 2010), but the emerging view is that sphingolipid-cholesterol rafts generate membrane heterogeneity that serves to functionalize, focus, and coordinate the bioactivity of membrane constituents. Recent comprehensive analysis of HIV-1 lipid content revealed its general similarity to that of the plasma membrane, but several lipid components of lipid rafts, in particular cholesterol, ceramide, and raft-associated ganglioside GM3, were enriched in the virions (Chan et al., 2008). This affinity for rafts is determined by the Pr55(Gag) precursor, which specifically associates with phosphatidylinositol-(4,5)-bisphosphate enriched in these membrane domains (Saad et al., 2006). As a result, cholesterol depletion markedly and specifically reduces HIV-1 particle production

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ABBREVIATIONS: ABCA1, ATP-binding cassette transporter A1; ApoA-I, apolipoprotein AI; FACS, fluorescence-activated cell sorting; GFP, green fluorescence protein; LXR, liver X receptor; MDM, monocyte-derived macrophages; PHA, phytohemagglutinin; TO-901317, *N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; HEK, human embryonic kidney; AU, arbitrary units; AZT, 3′-azido-2′,3′-dideoxythymidine; RT, reverse transcriptase; GW3965, (3-{3-[(2-chloro-3-trifluoromethyl-benzyl)-(2,2-diphenyl-ethyl)-amino]-propoxy}-phenyl)-acetic acid; RNAi, RNA interference.

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(Ono and Freed, 2001) and reduces infectivity of the virions produced by such cells (Zheng et al., 2001).

Notably, this reduction of infectivity correlates with the amount of virion-associated cholesterol. Indeed, treatment of HIV virions with cholesterol-sequestering drugs, such as β-cyclodextrin, renders the virus incompetent for cell entry (Guyader et al., 2002). Analysis of replication of this cholesterol-poor virus identified a mild inhibition of viral attachment to target cells and a major defect at the fusion step of infection (Guyader et al., 2002).

Dependence of HIV replication on cholesterol suggests that the virus might activate mechanisms that ensure adequate delivery of cholesterol to nascent virions and virion assembly sites. One such mechanism involves Nef, which has been shown to up-regulate enzymes involved in cholesterol biosynthesis (van' t Wout et al., 2005). In addition, we have reported previously that Nef inhibits the activity of the ATP-binding cassette transporter A1 (ABCA1) in HIV-infected macrophages, thus leading to the suppression of cholesterol efflux and accumulation of intracellular cholesterol (Mujawar et al., 2006). This finding suggested that inhibition of ABCA1 may be an important viral mechanism to ensure cholesterol delivery to viral assembly sites and that stimulation of ABCA1 activity may impair viral replication.

The current study was set up to determine whether the observed relationship between HIV and cholesterol efflux can be exploited as a new approach to anti-HIV therapy. To stimulate ABCA1, we used liver X receptor (LXR) agonists. LXRs (LXRα and LXRβ) are ligand-activated transcription factors that control cellular cholesterol and fatty acid homeostasis and have been established to exert atheroprotective effects in mouse models. Specifically, activation of LXRs by agonists such as *N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide (TO-901317) induces the expression of genes involved in cellular cholesterol trafficking, including Niemann Pick type C1 and 2 proteins (Rigamonti et al., 2005), and efflux, including ABCA1, ABCG1, and apolipoprotein E (Rader, 2007). It has been suggested that LXR may also regulate ABCA1 at a protein level by binding to ABCA1 and functionally inactivating it; ABCA1 is released when LXR binds to an agonist (Hozoji et al., 2008). The development of LXR agonists into therapeutics was complicated by the fact that activation of LXR also activates sterol regulatory element-binding protein-1c (Repa et al., 2000), which leads to enhanced lipogenesis in the liver, resulting in hypertriglyceridemia and hepatic steatosis (fatty liver). This problem, however, was resolved when the first tissue-specific LXR agonist, (3-{3-[(2-chloro-3-trifluoromethyl-benzyl)-(2,2-diphenyl-ethyl)-amino]-propoxy}-phenyl)-acetic acid (GW3965), was described (Miao et al., 2004). Another steroidal LXR agonist, *N*,*N*-dimethyl-3β-hydroxy-cholenamide, exerted potent anti-atherogenic activity in apolipoprotein E-deficient mice without increasing hepatic triglyceride levels and liver steatosis (Kratzer et al., 2009).

We demonstrate that HIV-1 infection impairs cholesterol efflux from $CD4^+$ T lymphocytes, and stimulation of ABCA1 expression via the LXR nuclear receptors reverses this effect. Importantly, LXR agonists exerted a potent ABCA1-dependent inhibitory effect on HIV-1 replication in both macrophages and T cells, and systemic administration of the LXR agonist effectively suppressed HIV replication in a mouse model of HIV infection. These results suggest that stimulation of ABCA1 expression may be used as a novel anti-HIV therapeutic strategy.

Materials and Methods

Reagents. LXR agonist TO-901317 was purchased from Sigma-Aldrich (St. Louis, MO). GW3965 was synthesized by following the procedure described by Collins et al. (2002). The anti-p24 monoclonal antibody used for FACS analysis was KC57-FITC (Coulter Clone; Beckman Coulter, Fullerton, CA) and for Western blotting, AG3.0 (National Institutes of Health AIDS Research and Reference Reagents Program, Bethesda, MD); HIV-1 JR-CSF Nef monoclonal antibody (6.2) and antiserum to HIV-1 gp120 were obtained from the National Institutes of Health AIDS Research and Reference Reagents Program.

Monocyte-Derived Macrophage and $CD4^+$ T-Cell Cultures, Infection, and Drug Treatment. Monocyte-derived macrophages (MDMs) were prepared from peripheral blood mononuclear cells of normal donors using adherence to plastic and differentiated in the presence of macrophage colony-stimulating factor essentially as described previously (Schmidtmayerova et al., 1997). $CD4^+$ T cell-enriched peripheral blood lymphocytes (labeled as $CD4^+$ T cells) were prepared from nonadherent cells by depleting $CD8^+$ cells using Multiney kit and were activated with PHA (5 μg/ml) for 48 h. Cells were cultured in the presence of interleukin-2 (20 U/ml).

Viruses. The following HIV-1 strains were used in this study: CCR5-tropic virus BaL (Watkins et al., 1990); CCR5-tropic virus ADA isolated from macrophages (Gendelman et al., 1988); CXCR4-tropic virus LAI (Wain-Hobson et al., 1991); and NL4-3 based Nef-positive and Nef-negative constructs (Schindler et al., 2005).

Patient with Tangier Disease. Peripheral blood mononuclear cells were collected from a 53-year-old patient with Tangier disease. The diagnosis was confirmed by showing defective cholesterol efflux to lipid-free ApoA-I from skin fibroblasts.

RNAi Experiments. Monocyte-derived macrophages were transfected with ABCA1 siRNA(h) or control siRNA-A (150 pmol/10^6 cells) (Santa Cruz Biotechnology, Santa Cruz, CA). Transfection was performed using Metafectene (Biontex, San Diego, CA) by following the manufacturer's protocol. Twenty-four hours after the first transfection, cells were infected with HIV-1 ADA, and after another 24 h, the second round of siRNA transfection was performed to achieve significant down-regulation of ABCA1, and cells were cultured in the presence or absence of TO-901317 (0.5 μM). Viral replication was analyzed by p24 ELISA performed on days 4 and 10 after infection.

Cytotoxicity Assays. Cytotoxicity was analyzed by MTT assay (Sigma-Aldrich) or Apoptosis Detection Kit (BioVision, Mountain View, CA).

Determination of Viral Cholesterol Content. HIV-1 virions were treated with 4 mg/ml subtilisin (Sigma-Aldrich) for 4 h at 37°C. Subtilisin digestion was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 5 μg/ml. Virions were recovered by subsequent ultracentrifugation (35,000 rpm, 2 h, 4°C) through a 30% sucrose cushion. Viral pellets were solubilized in lysis buffer (0.1% Triton X-100, 0.05% SDS, 5 mM CHAPS, 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl) and normalized according to p24 content. Viral pellets were then analyzed for cholesterol content using the Amplex Red Cholesterol Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Media from uninfected drug- or antibody-treated macrophages were prepared in the same way as the virions and were used as a control in the cholesterol assay to account for any contaminating cholesterol that was not virion-associated.

In some experiments, viral cholesterol was analyzed by mass spectrometry using protocol described by Raith et al. (2005). In brief, samples were lyophilized, and lipids were extracted with *N*-hexane/isopropanol mixture (3:2) according to Hara and Radin (1978). The

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extracts were dried, dissolved in 100 μl of water/acetonitrile (1:1) mixture, and used for analysis by liquid chromatography/tandem mass spectrometry. A Jupiter C5 5 μm (2 × 250 mm; Phenomenex, Torrance, CA) column was used for separation. The flow rate was 0.4 ml/min, and the column was held at 50°C. The separation was conducted on an Agilent 1100 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA) interfaced to an Esquire 3000 (Bruker Daltonics, Billerica MA) ion-trap mass spectrometer equipped with an atmospheric pressure chemical ionization ion source. The mass spectrometer was set up to monitor ion current corresponding to a transition of 369 ($[M\text{-}H_2O+H]^+$) →161 Da at retention time window centered at 17.6 min. The ionization parameters were as follows: nebulizer pressure, 50 psi; dry gas flow, 4 l/min; desolvation capillary temperature, 350°C; atmospheric pressure chemical ionization temperature, 450°C.

Reconstitution of Viral Cholesterol. To restore viral cholesterol, a modified procedure described by Campbell et al. (2002) was used. Virus was collected from infected TO-901317-treated or untreated $CD4^+$ T lymphocytes by ultracentrifugation as described above. Concentrated virus was incubated in 0.5 mM 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) complexed with 1 mM cholesterol (Sigma-Aldrich) for 1 h at 37°C. The virus was subsequently purified from cyclodextrin and cholesterol by ultracentrifugation, and p24 content and free cholesterol content was measured by ELISA and Amplex Red Cholesterol Kit (Molecular Probes), respectively.

Assays for Viral Infectivity. HIV-1 virions were normalized according to RT activity. Infectivity was measured using R5/X4/R3 GHOST cells or TZM-bl cells (AIDS Research and Reference Reagent Program). Thirty-six hours after infection, the cells were trypsinized and fixed in 4% formaldehyde. Fixed GHOST cells were analyzed for GFP fluorescence on FACScalibur (BD Biosciences, San Jose, CA); TZM-bl cells were analyzed for luciferase activity.

Fusion Assay. Fluorescence resonance energy transfer-based HIV-1 virion fusion assay was performed essentially as described previously (Cavrois et al., 2002).

Cholesterol Efflux. Cholesterol efflux was measured as described previously (Mujawar et al., 2006).

Infection of Ex Vivo Cultured Lymphoid Tissue. Human tonsils, removed during routine tonsillectomy, were received within 5 h of excision. Tissue was examined by experienced personnel, and all of the parts of the tonsils that had blood clots or looked abnormal based on color and/or microscopic structure were removed before experiments. Tonsils were dissected into 27 to 54 (the number previously shown to bring the variation in the cell number between sets from the same tissue to statistical insignificance) 2- to 3-mm^3 blocks and cultured as described previously (Glushakova et al., 1999). Experiment was repeated with tissue from three different donors. To minimize the influence of donor-dependent variability on the results, experimental and control sets always used donor-matched tissues.

To measure the cytotoxicity of TO-901317, uninfected tissue blocks were treated or not with 0.5 μM TO-901317 for 12 days. Single-cell suspensions were prepared from tissue blocks by digestion with a 1% solution of collagenase IV (Invitrogen) for 30 min, followed by a mechanical dispersion with a pestle. Dead cells were identified using the LIVE/DEAD Fixable Blue Dead Cell Stain kit (Invitrogen).

Animal Studies. Eleven neonate Rag2(−/−)γ_c(−/−) mice (1 day after birth) were sublethally irradiated with 3.5 Gy (0.6 min) using the Cesium-137 irradiator. Five hours after irradiation, the mice were injected intrahepatically with 10^6 $CD34^+$ progenitor cells purchased from Lonza Walkersville (Walkersville, MD). Sixteen weeks after transplantation, animals were infected by intraperitoneal injection of HIV-1 ADA strain (~10^6 $TCID_{50}$/ml; $TCID_{50}$ is the median tissue culture infectious dose). Five mice were treated every 3 days with TO-901317 (10 mg/kg) injected intraperitoneally starting from the first day of infection for 2 weeks after infection, three were injected with AZT (10 mg/kg), and three control mice received vehicle alone. Two weeks after infection, blood was collected, and viral RNA in the plasma was analyzed using a quantitative polymerase chain reaction kit (Roche Diagnostics, Indianapolis, IN).

Results

Pharmacological Reversion of Reverse Cholesterol Transport Impairment in HIV-1-Infected Macrophages and T Lymphocytes. Our previous study of HIV effects on ABCA1-mediated cholesterol efflux was performed with monocyte-derived macrophages (Mujawar et al., 2006). Here, we examined ABCA1-mediated cholesterol efflux from T cells infected or not with HIV-1. Results in Fig. 1A show that cholesterol efflux from HIV-infected T cells to ApoA-I acceptor, a process controlled by ABCA1 (Yvan-Charvet et al., 2010), was significantly reduced compared with efflux from uninfected cells ($p < 0.05$). Similar to results with HIV-infected macrophages (Mujawar et al., 2006), this effect was dependent on Nef, because no significant impairment of cholesterol efflux was observed in T cells infected with Nef-deficient HIV-1. Moreover, pharmacological stimulation of the LXR by a synthetic agonist TO-901317, which up-regulates ABCA1 expression and cholesterol efflux in macrophages (Tall, 2008) (Fig. 1C), increased cholesterol efflux from both HIV-infected and uninfected T cells (Fig. 1B). Of note, the difference in cholesterol efflux between unstimulated cells in Fig. 1, A and B, was probably due to donor-dependent variability, because these experiments were performed with cells from different donors. Interestingly, the difference in cholesterol efflux between HIV-infected and uninfected cells stimulated with TO-901317 (Fig. 1B) was not statistically significant ($p = 0.06$), suggesting that LXR agonist-stimulated cholesterol efflux overwhelms the suppressive capacity of HIV-1. Importantly, consistent with our previous findings in macrophages (Mujawar et al., 2006), ABCA1 in T cells was down-regulated by HIV-1 infection (Fig. 1D). Taken together, these results indicate that the stimulatory effect of TO-901317 on ABCA1 expression in T cells can overcome the suppressive effect of HIV-1 infection.

Stimulation of Cholesterol Efflux Causes a Reversible Reduction of HIV Cholesterol Content and Infectivity and Inhibits Virus Production. We have shown previously that stimulation of cholesterol efflux in HIV-infected macrophages reduces virus-associated cholesterol and limits viral infectivity (Mujawar et al., 2006). To test whether the same mechanism operates in T cells, we measured virion-associated free cholesterol of a virus produced from untreated and TO-901317-treated $CD4^+$ T lymphocytes. Viral cholesterol was reduced by ~50% (when adjusted to p24 content, which was similar between the viruses produced from treated and untreated cells) (Fig. 2D) in the virus produced from TO-901317-treated T cells compared with the virus collected from untreated cells (Fig. 2A). This depletion was fully reversible, because viral cholesterol content could be replenished by incubation with cholesterol-saturated cyclodextrin (Fig. 2A). To determine whether cholesterol depletion is the main cause of the infectivity defect of the virus produced by TO-901317-treated cells, we compared infectivity of the virions before and after restoration of the viral cholesterol content using GHOST indicator cells (human osteosarcoma cells expressing CD4, CXCR4, CCR5, and GFP under control of HIV LTR). As shown in Fig. 2B, infectivity of the virus produced from TO-901317-treated $CD4^+$ T cells was

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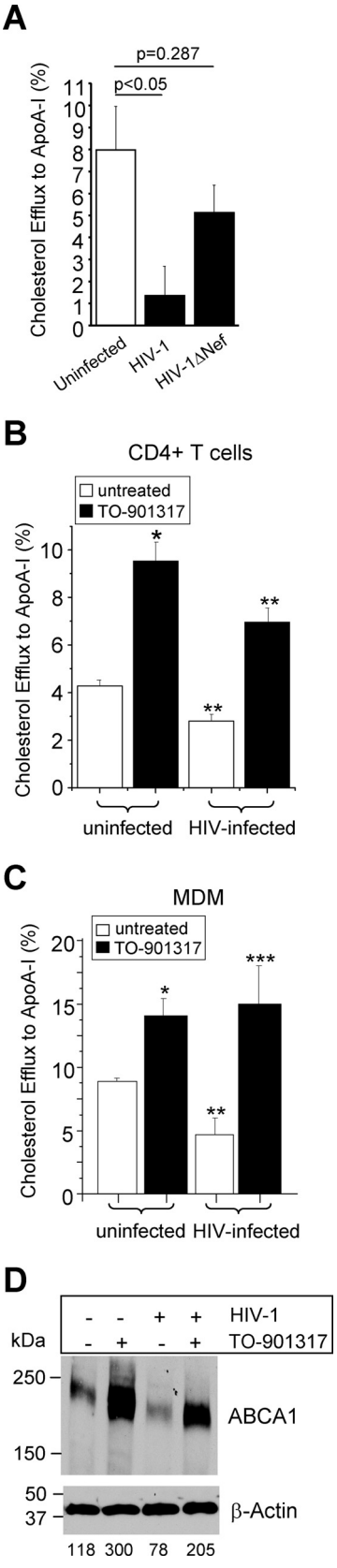

Fig. 1. LXR agonist TO-901317 reverses HIV-mediated impairment of cholesterol efflux. A, triplicate PHA-activated CD4$^+$ T-cell cultures were infected (or mock-infected) with NL4-3 pBRIeG-nef+ (HIV-1) or NL4-3 pBR43IeG-nef- (HIV-1ΔNef) (Schindler et al., 2005), and 11 days after infection, cholesterol efflux to ApoA-I was measured. The data are mean ± S.E. p Values calculated using Student's t test are shown above the bars. B, triplicate CD4$^+$ T-cell cultures were infected (or mock-infected) with HIV-1 LAI, and 7 days after infection, they were treated or not with 5 μM LXR agonist TO-901317. After additional culture for 5 days in the presence or absence of the drug, cholesterol efflux to ApoA-I was analyzed. The data are mean ± S.E. of three independent experiments with cells from different donors. *, $p < 0.003$ versus untreated uninfected cells; **, $p < 0.02$ versus untreated uninfected cells, calculated using Student's t test. C, triplicate MDM cultures were infected (or mock-infected) with HIV-1 ADA and 7 days after infection were treated or not with 0.5 μM TO-901317, an LXR agonist. After additional culture for 10 days in the presence of the drug, cholesterol efflux to ApoA-I was analyzed. The data are mean ± S.E., and results are presented for a representative experiment of three performed. *, $p < 0.05$ versus untreated uninfected cells; **, $p < 0.05$ versus untreated uninfected cells; ***, $p < 0.001$ versus untreated HIV-infected cells, calculated using Student's t test. D, Western blot analysis of ABCA1 and β-actin (loading control) in CD4$^+$ T cells used in A. Multiple glycosylation sites on ABCA1 result in smearing of the band. Results of densitometry of the bands using ImageJ program are shown in arbitrary units at the bottom.

restored after cholesterol replenishment. Some residual defect in infectivity of this virus is probably due to interaction between overexpressed ABCA1 and Nef (Mujawar et al., 2006), resulting in inactivation of enhancing activity of Nef on HIV-1 infectivity (see below). This result indicates that HIV infectivity is highly sensitive to the amount of virus-associated cholesterol, and cholesterol depletion is one of the main reasons for reduced infectivity of the virus produced by TO-901317-treated cells. Consistent with this result, analysis of the viral proteins in TO-901317-treated cells did not reveal any decrease in protein expression (there was an increase in p55 protein), indicating normal (or even enhanced) viral protein synthesis (Fig. 2C). Analysis of virion proteins demonstrated a slight decrease of Nef but otherwise normal protein content (Fig. 2D). Moreover, cholesterol-depleted virus exhibited normal p24/gp120 ratio, suggesting normal viral protein packaging and maturation.

Given that viral lipid content mimics that of lipid rafts (Brügger et al., 2006) and that raft cholesterol depletion interferes with HIV-1 particle production (Ono and Freed, 2001), our finding that TO-901317 reduces virion-associated cholesterol suggested that the drug may also inhibit viral egress from infected T cells. To test this possibility, we infected CD4$^+$ T cells with high input of Nef-positive or Nef-negative HIV-1 (multiplicity of infection ~10), allowed the infection to proceed for 3 days, and then treated the cells with reverse-transcriptase inhibitor AZT (to block the spread of infection) and TO-901317. Viral production was assayed 2 days later by measuring extracellular p24 (by ELISA) and was adjusted to the percentage of infected cells (measured by FACS analysis of cell-associated p24). Interestingly, consistent with higher Gag expression in TO-901317-treated cells (Fig. 2C), the percentage of p24-positive drug-treated cells was also higher than p24-positive uninfected cells (41.8 versus 23.9% for cells infected with the Nef-positive HIV-1, and 31.3 versus 19.9% for cells infected with Nef-negative HIV-1). Nevertheless, as shown in Fig. 2E, a significant reduction in the levels of virus production was observed in TO-901317-treated cultures, both for Nef-positive and Nef-negative HIV constructs. This result supports the suggestion that TO-901317 inhibits virus release from infected cells. Production of Nef-positive virus from TO-901317-untreated cells was higher than of Nef-deficient construct, but this difference did not reach statistical significance. Taken together, these results demonstrate that pharmacological stimulation of

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LXR in HIV-infected T cells leads to reduced production of progeny HIV and produced virus has reduced cholesterol content and reduced infectivity.

ABCA1 Expression Reduces Viral Cholesterol and Infectivity. Because LXR agonists are potent stimulators of ABCA1 expression, we tested the effect of ABCA1 on viral cholesterol and infectivity. Cholesterol content of Nef-positive virus was higher than that of Nef-deficient HIV-1, but this difference was not significant (Fig. 3A). ABCA1 expression significantly reduced viral cholesterol in both Nef-positive and Nef-negative HIV-1 (Fig. 3A).

Infectivity of produced viruses was analyzed using TZM-bl indicator cells expressing luciferase under control of a Tat-responsive promoter. For the Nef-negative virus, expression of ABCA1 reduced the cholesterol level by approximately 50% (Fig. 3A), leading to approximately 50% reduction of infectivity (Fig. 3B). However, Nef-positive virus collected from ABCA1-negative cells was noticeably more infectious than Nef-negative HIV-1, and the difference in infectivity was much greater than could be expected from differences in cholesterol levels between these viruses. This result is consistent with previously demonstrated cholesterol-independent beneficial effects of Nef on HIV infectivity, such as facilitation of intracytoplasmic transport and evasion of proteasomal degradation (Qi and Aiken, 2008). Infectivity of Nef-positive virus collected from ABCA1-expressing cells was greatly reduced compared with infectivity of Nef-positive virus collected from ABCA1-negative cells and was not significantly different from infectivity of Nef-negative virus (Fig. 3B). This reduction in infectivity (approximately 5-fold) was greater than what would be expected from a reduction in viral cholesterol (2-fold) and much greater than ABCA1-specific reduction of infectivity of Nef-deficient virus, pointing to a possibility that over-

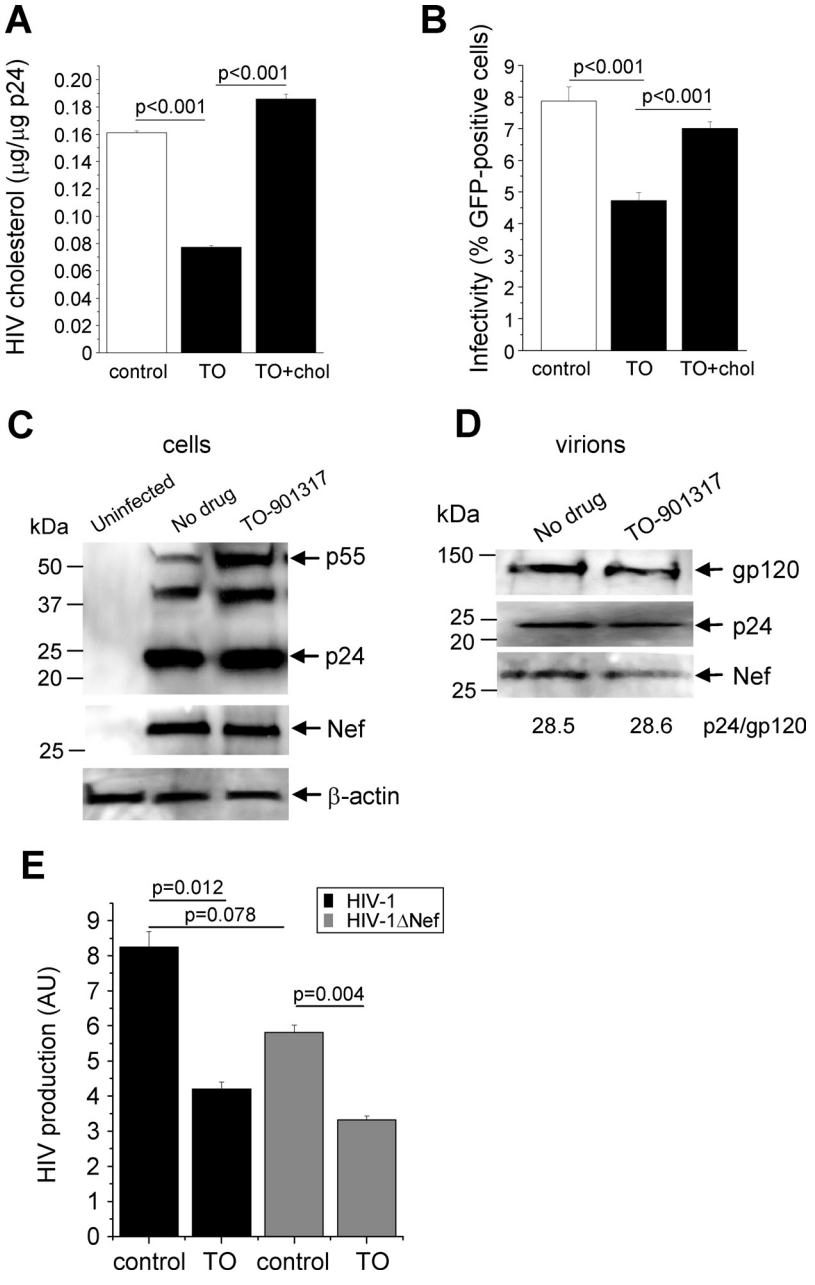

Fig. 2. TO-901317 reduces viral production, cholesterol, and infectivity. A, HIV-1 LAI harvested from untreated (control) or TO-901317-treated (TO) PHA-activated $CD4^+$ T-cell cultures was pelleted via ultracentrifugation and normalized according to p24 assay. An aliquot of TO-treated virus was incubated with cholesterol-saturated cyclodextrin (TO + chol) to restore cholesterol. Virions were analyzed in triplicate for free cholesterol content using Amplex Red Cholesterol Assay. Results are presented as amount of virion cholesterol (in micrograms) normalized to p24 content and are the mean ± S.E. of triplicate determinations. Results are shown for one representative experiment of two performed. *p* Values calculated using Student's *t* test are shown above the bars. B, HIV-1 virions described in A were used to infect indicator GHOST cells. Infected cells (GFP-positive) were detected by flow cytometry. Results are presented as the percentage of GFP-positive gated cells and are the mean ± S.E.M. of triplicate independent determinations. *p* Values calculated using Student's *t* test are shown above the bars. C, Western blot analysis of cell lysates of HIV-infected $CD4^+$ T cells. T cells were infected with HIV-1 LAI and treated with TO-901317 (5 μM) for 5 days (the drug was added 7 days after infection). Molecular weight markers are shown on the left. β-Actin was used as loading control. D, Western blot analysis of viral proteins in pelleted virions harvested from HIV-infected $CD4^+$ T-cell cultures treated as in C. Samples were equalized by p24 content. Molecular weight markers are shown on the left. p24 and gp120 were assayed by ELISA to calculate p24/gp120 ratio. E, PHA-activated $CD4^+$ T lymphocytes were infected with Nef-positive (HIV-1) or Nef-deficient (HIV-1ΔNef) HIV-1 NL4-3 (60 ng p24/10^6 cells, multiplicity of infection ~10). Infection was allowed to proceed for 3 days, and then AZT (3 μM) with or without TO-901317 (5 μM) was added for another 2 days. p24-Positive cells were quantified by flow cytometry, and extracellular p24 was measured by ELISA. Results are presented in arbitrary units (concentration of extracellular p24 normalized to the percentage of infected cells). *p* Values calculated using unpaired Student's *t* test are shown above the bars.

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expression of ABCA1 not only reduces the amount of viral cholesterol but also reduces the activity or availability of Nef. Of note, analysis of virus produced by TO-901317-treated cells (Fig. 2) did not reveal a discrepancy between viral cholesterol content and infectivity, probably because of lower ABCA1 expression in TO-901317-stimulated cells compared with transfected cells. Therefore, the effect of ABCA1 on HIV infectivity may be a combination of reduced viral cholesterol and inhibited Nef activity.

Because the effect of cholesterol depletion is expected to manifest at the step of virus-cell fusion, we used the fluorescence resonance energy transfer-based HIV-1 virion fusion assay (Cavrois et al., 2002) to measure fusion of viruses produced in HEK 293T cells in the presence or absence of ABCA1. Results presented in Fig. 3C (top) show that fusion capacity of virions produced in the presence of ABCA1 was reduced by approximately 50% (from 18.4 to 8.5%), in excellent correlation with the reduction of viral cholesterol (Fig. 3A). Thus, the effect of ABCA1 on the fusion step can be fully accounted for by changes in virus cholesterol content. In summary, ABCA1 affects the infectivity of HIV-1 virions by targeting both cholesterol-dependent (fusion) and cholesterol-independent (Nef-mediated enhancement) components of the process.

LXR Agonists Inhibit HIV-1 Replication In Vitro. We next analyzed the anti-HIV effect of TO-901317 on HIV replication in natural target cells of the virus. TO-901317 exhibited a dose-dependent inhibition of HIV replication in both $CD4^+$ T cells and MDM cultures (Fig. 4, A, left, and B). This effect was not due to cytotoxicity, because cytotoxic effect measured by the MTT assay did not exceed 20% at the highest drug concentrations used in the experiments (Fig. 4C). At the peak dose, TO-901317 inhibited replication of the M-tropic HIV strain ADA in macrophages and of the T-tropic HIV strain LAI in PHA-activated $CD4^+$ T cells by >90%. A similar effect was observed with other HIV variants, such as primary isolates YU-2 and 92US660 (data not shown).

Because both Nef and TO-901317 ultimately target the same factor (ABCA1), it was possible that the sensitivity of Nef-negative and Nef-positive virus to TO-901317 may be different. To test this assumption, we analyzed the effect of TO-901317 on the replication of Nef-deficient virus. This analysis produced a result similar to that observed with Nef-positive HIV-1 (Fig. 4A, right). In fact, suppression of the

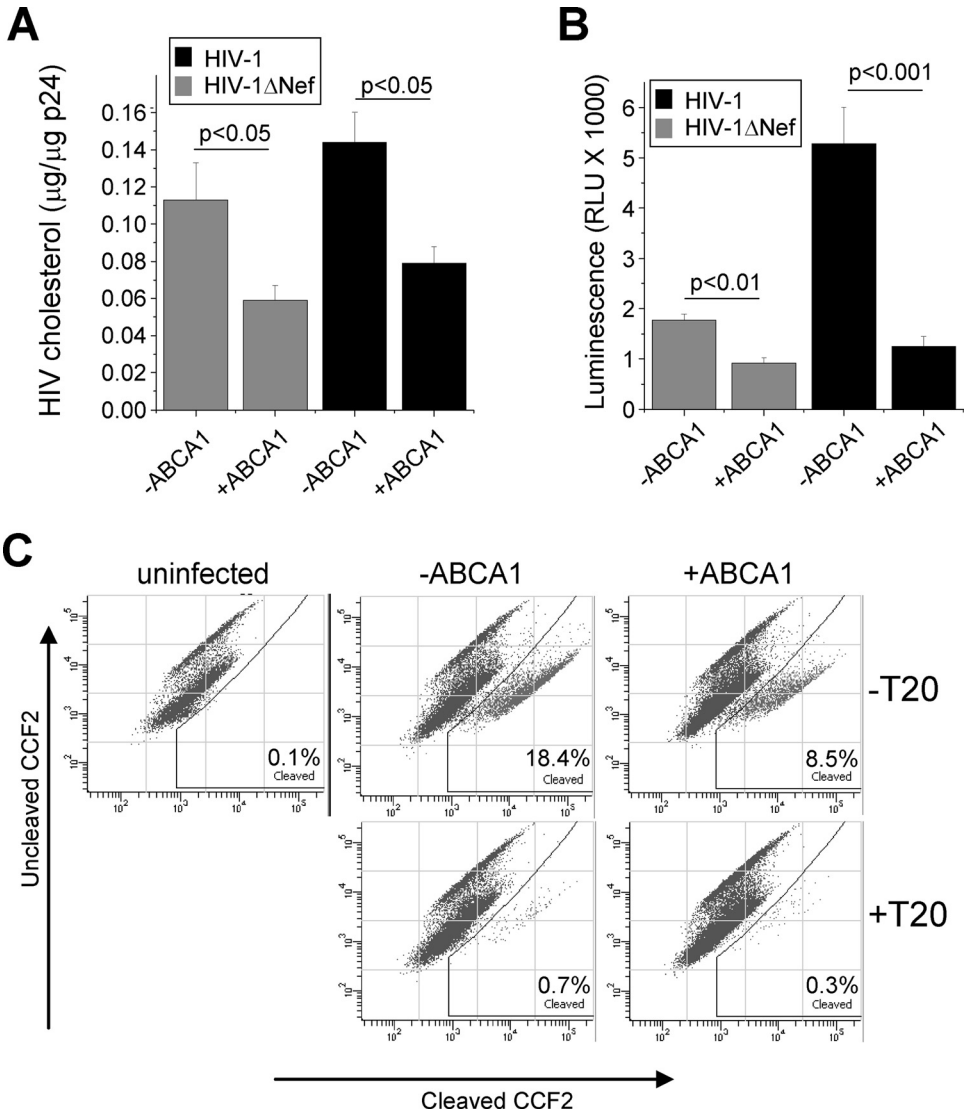

Fig. 3. ABCA1 effects on viral cholesterol and infectivity. A, HEK 293T cells were cotransfected with infectious molecular clones NL4-3 pBR43IeG-nef- (HIV-1ΔNef) or NL4-3 pBRIeG-nef+ (HIV-1) (Schindler et al., 2005) and ABCA1 or control vector. Viruses collected from cells after 48 h were pelleted and analyzed for free cholesterol content by liquid chromatography/tandem mass spectrometry. Results were normalized by p24 content and are presented as mean ± S.E.M. of triplicate determinations. B, Nef-positive (HIV-1) and Nef-negative (HIV-1ΔNef) HIV-1 collected from transfected cells were normalized by RT activity and assayed for infectivity using TZM-bl indicator cells. Results are presented as luminescence (relative luciferase units), and *p* values of triplicate determinations were calculated using Student's *t* test and are shown above the bars. C, HEK 293T cells were cotransfected with HIV-1 NL4-3 molecular clone, BlaM-Vpr, and ABCA1 (or empty vector). Virions were collected by centrifugation, equalized according to RT activity, and used to infect $CD4^+$ T cells loaded with CCF2-AM in the presence or absence of fusion inhibitor T20 (1 μg/ml). Cells were analyzed by flow cytometry, using excitation at 409 nm and measuring emission at 520 nm (uncleaved CCF2) and 450 nm (CCF2 cleaved by BlaM). Percentage of cells with cleaved CCF2 reflects the efficiency of fusion.

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Nef-deficient virus seems to be slightly more potent than that of the wild-type virus (IC_{50} ~0.3 μM for Nef-deficient virus and ~0.5 μM for the wild-type virus).

To demonstrate that observed anti-HIV activity was not an off-target effect of TO-901317 but was related to its LXR agonist activity, we tested the effects of a different LXR agonist, GW3965 (Collins et al., 2002). Similar to TO-901317, GW3965 inhibited HIV-1 replication in MDM and $CD4^+$ T cells in a dose-dependent manner (Fig. 4D). No cytotoxicity of the drug was observed at concentrations used in the experi-

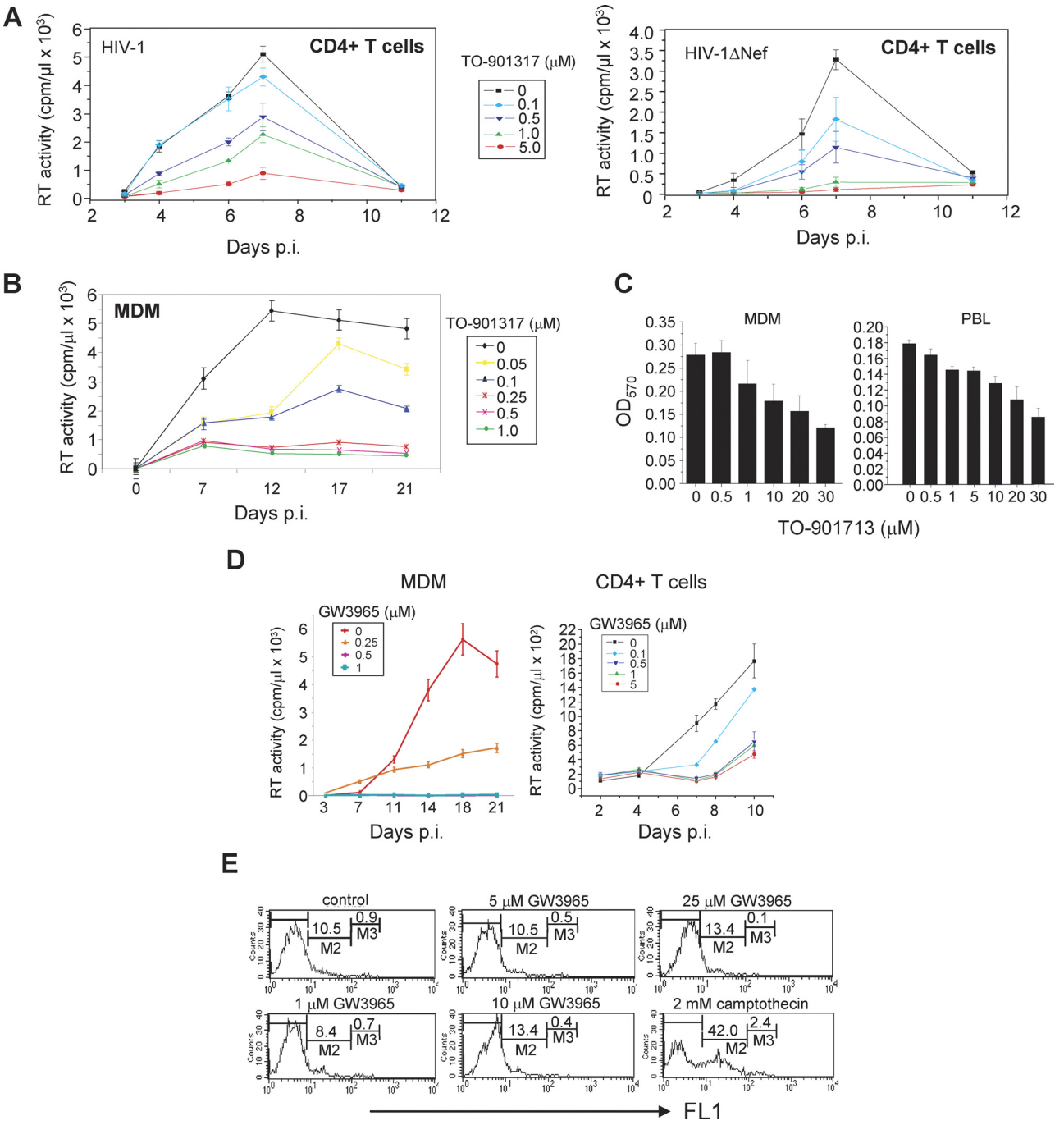

Fig. 4. LXR agonists inhibit HIV-1 replication in vitro. Replication of Nef-positive or Nef-deficient (HIV-1ΔNef) HIV-1 NL4-3 in $CD4^+$ T cells (A) and HIV-1 ADA replication in MDMs (B) in the presence of indicated concentrations of TO-901317 was measured by RT activity in culture supernatant. Drug was added together with the virus and maintained throughout the course of infection. Results are mean ± S.E. of triplicate cultures. Results are shown for a representative experiment of four performed with cells from different donors. C, cytotoxicity of TO-901317 was measured using the MTT assay. Cells were incubated with the drug for 7 days ($CD4^+$ T cells) or 14 days (MDMs). D-HIV-1 ADA replication in MDMs (left) and HIV-1 LAI replication in $CD4^+$ T cells (right) in the presence of indicated concentrations of GW3965 was measured by RT activity in culture supernatant. Drug was added together with the virus and maintained throughout the course of infection. Results are mean ± S.E.M. of triplicate cultures. Results are shown for a representative experiment of two performed with cells from different donors. E, $CD4^+$ T lymphocytes were activated with PHA (5 μg/ml) for 48 h and cultivated for 7 days in the presence of interleukin-2 (20 U/ml) and indicated concentrations of GW3965. Cytotoxicity was analyzed using Annexin V-FITC Apoptosis Detection Kit Plus (BioVision). Apoptotic cells stained with Annexin V-FITC dye are located in M2 gate, and necrotic cells stained with SYTOX Green dye are in M3 gate. The percentage of cells in M2 and M3 gates is shown. As a positive control, cells were treated with 2 mM camptothecin for 2 h. One representative experiment of two performed is shown.

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ment by the MTT assay (data not shown) or analysis of apoptosis by flow cytometry (Fig. 4E).

The Inhibitory Effect of LXR Agonist TO-901317 on HIV-1 Replication Is ABCA1-Dependent. Because our previous results suggested that the mechanism of anti-HIV activity of TO-901317 may be ABCA1-dependent, we tested the effect of this drug on HIV-1 replication in T cells obtained from a patient with Tangier disease. Tangier disease is characterized by a genetic defect of ABCA1 resulting in the impairment of cholesterol efflux (Nofer and Remaley, 2005). Consistently, cholesterol efflux to ApoA-I in T cells isolated from a patient with Tangier disease was severely reduced and was not stimulated by TO-901317 (Fig. 5A, left). In contrast to its activity in normal T cells (Fig. 4A, left), TO-901317 delayed but did not reduce HIV replication in cells from the patient with Tangier disease (Fig. 5A, right). This result indicates that functional ABCA1 is required for the anti-HIV effect of the LXR agonist.

Tangier disease is very rare, and samples from patients are not readily available, making it impossible to repeat this analysis on cells from several donors. Therefore, to further prove the dependence of the anti-HIV effect of LXR agonist on ABCA1 expression, we down-regulated ABCA1 in monocyte-derived macrophages using siRNA. MDM transfected with scrambled or ABCA1-directed siRNA were infected with HIV-1 and cultured in the presence or absence of TO-901317 (0.5 μM). The effect of siRNA was verified in mock-infected cells by Western blotting (Fig. 5B), and viral replication was tested on days 4 and 10 by p24 ELISA (Fig. 5C). Quantitative analysis of the blot was performed using ImageJ software (http://rsbweb.nih.gov/ij/) (numbers at the bottom in Fig. 5B). Four days after infection, ABCA1-directed siRNA specifically down-regulated the expression of ABCA1 but not of a related cholesterol transporter ABCG1 (which is also induced by treatment with LXR agonist) in both unstimulated and TO-901317-stimulated cells (Fig. 5B) relative to cells transfected with scrambled siRNA. This effect disappeared on day 10 after infection (results not shown), probably because of degradation of siRNA. TO-901317 increased ABCA1 expression by approximately 4-fold [from 12 to 54 arbitrary units (AU)] in cells transfected with scrambled siRNA, with a corresponding reduction in HIV-1 replication on day 4 (from 390 to 110 pg/ml p24) (Fig. 5C). Consistent with the ABCA1 requirement for the effect of TO-901317, the drug failed to inhibit HIV-1 replication in cells transfected with ABCA1-targeting siRNA (Fig. 5C). Interestingly, despite activity of ABCA1 siRNA, the level of ABCA1 in TO-901317-treated cells was approximately 2-fold higher than in untreated cells transfected with scrambled siRNA (21 versus 12 AU, respectively; Fig. 5B). Lack of virus inhibition in these cells was probably due to the ability of HIV to neutralize this increase in ABCA1 through activity of Nef. Likewise, the decrease of ABCA1 in untreated cells transfected with ABCA1 siRNA versus cells transfected with scrambled siRNA (5 versus 12 AU, respectively; Fig. 5B) did not translate into higher virus production (Fig. 5C). These results suggest a nonlinear relationship between the viral replication and the level of ABCA1.

On day 10 after infection, the anti-HIV effect of TO-901317 in ABCA1 siRNA-transfected cells was detected, but it was diminished relative to cells transfected with scrambled siRNA (50% inhibition in ABCA1 siRNA-transfected cells versus 75% inhibition in scrambled siRNA-transfected cells; Fig. 5C). This result probably reflects gradual degradation of siRNA and loss of its suppressive activity. Consistent with anti-HIV activity of ABCA1, cumulative viral production measured 10 days after infection was substantially higher in cells transfected with ABCA1-targeting siRNA (Fig. 5C).

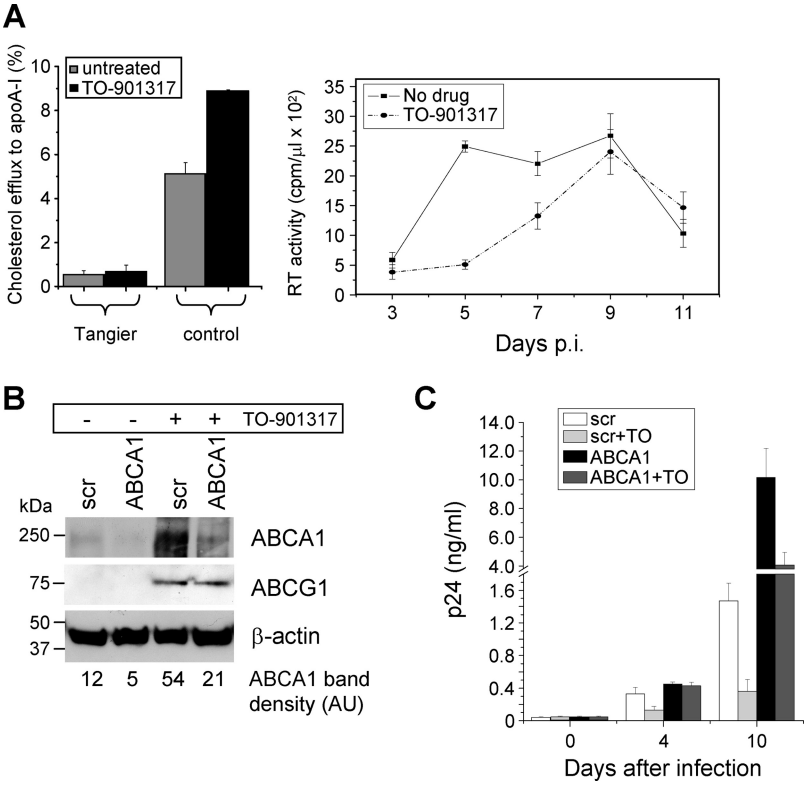

Fig. 5. Anti-HIV activity of TO-901317 requires ABCA1. A, TO-901317 delays but does not inhibit HIV-1 replication in CD4$^+$ T cells from a patient with Tangier disease. Left, cholesterol efflux to ApoA-I was measured with PHA-activated CD4$^+$ T lymphocytes from normal donor (control) or patient with Tangier disease. Cells were treated or not with TO-901317 (5 μM). Results are the mean ± S.E. of triplicate determinations. Right, replication of HIV-1 LAI in TO-901317-treated (5 μM) CD4$^+$ T cells from a patient with Tangier disease. The drug was added at the time of infection (day 0). B and C, suppression of ABCA1 expression by RNAi reduces the anti-HIV effect of TO-901317 in MDM. Monocyte-derived macrophages were transfected with ABCA1-specific siRNA (ABCA1) or scrambled siRNA (scr), infected with HIV-1 (or mock-infected), transfected with siRNA the second time, and cultivated in the presence or absence of TO-901317 (0.5 μM). B, ABCA1, ABCG1, and β-actin (loading control) were analyzed by Western blotting 4 days after mock infection (3 days after the second transfection). C, viral replication was analyzed by p24 ELISA on days 4 and 10 after infection.

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The LXR Agonist TO-901317 Inhibits HIV Replication Ex Vivo and In Vivo. Replication of HIV-1 in vivo occurs predominantly in lymphoid tissue (Embretson et al., 1993; Pantaleo et al., 1993). A system for culturing human tonsils ex vivo (Glushakova et al., 1999) provided an opportunity to test the anti-HIV activity of LXR agonist in the context of intact human lymphoid tissue. Results presented in Fig. 6A demonstrate a potent anti-HIV effect of TO-901317 in this system. At a concentration of 0.5 μM, the drug reduced HIV production in this system by more than 80%. Importantly, this anti-HIV activity was not associated with cytotoxicity of the drug, because flow cytometric analysis of single-cell suspensions prepared from tissue blocks of drug-treated and untreated uninfected lymphohistocultures on day 12 of culture did not reveal differences in the percentage of dead cells (8.3 ± 0.4% in untreated tissue blocks and 8.4 ± 0.5% in blocks treated with TO-901317). Drug-treated tissues also maintained normal CD3/CD19 and CD4/CD8 ratios (data not shown).

The possibility to humanize the immune system of Rag2(−/−)γc(−/−) knockout mice using $CD34^+$ hematopoietic stem cells (Baenziger et al., 2006) allowed us to test the effect of TO-901317 in an in vivo model. Rag-hu mice infected by intraperitoneal injection of HIV-1 ADA were treated or not with 10 mg/kg TO-901317 or AZT (used as a positive control) administered every 3 days. Two weeks after infection, blood was collected from the mice, and the number of viral RNA copies in the plasma was analyzed. As shown in Fig. 6B, treatment with TO-901317 reduced the viral load in infected animals by 1.5 log to a level similar to that in AZT-treated animals. It should be noted that for practical reasons AZT in this study was given every 3 days rather than daily (to mimic TO-901317 treatment). Given the short half-life of AZT (Rossi et al., 2002), such treatment produced incomplete suppression of HIV replication.

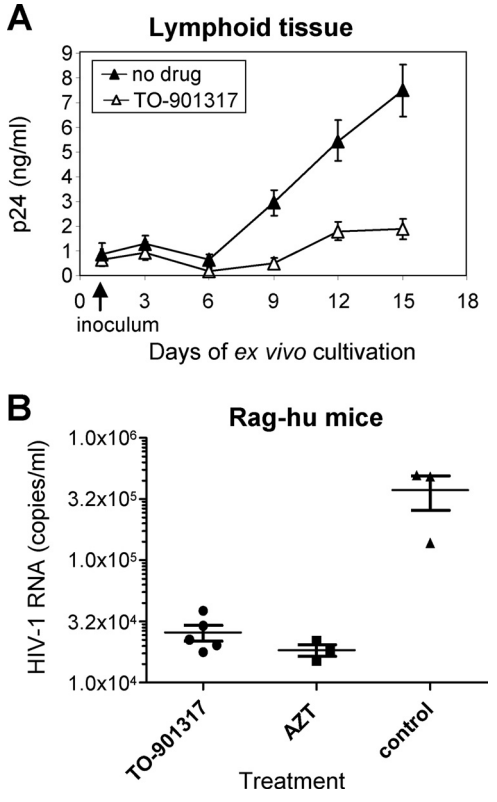

Fig. 6. TO-901317 inhibits HIV-1 replication ex vivo and in vivo. A, ex vivo cultured pieces of tonsil tissue (49 blocks per each treatment) were infected with HIV-1 BaL and cultured in the presence of TO-901317 (0.5 μM). HIV-1 replication was monitored by measuring p24 in culture supernatants pooled from all 49 tissue blocks. Results are mean ± S.E.M., where S.E.M. shows variations of the assay done in triplicate. One representative experiment is shown of three performed with tissues from different donors. B, Rag-hu mice were infected with HIV-1 ADA and treated with TO-901317 (10 mg/kg), AZT (10 mg/kg), or vehicle control injected IP every 3 days. After 2 weeks, mice were sacrificed and viral load in the blood was assayed by Roche quantitative polymerase chain reaction kit.

Discussion

Results presented in this report demonstrate that LXR agonists inhibit HIV-1 replication. This inhibition correlated with the stimulation of cholesterol efflux in HIV-infected cells. This anti-HIV effect of LXR agonists relied on ABCA1 and combined cholesterol-dependent and independent events. Cholesterol-dependent mechanisms include diminished virus production and reduced fusogenic ability of virions because of a reduction of virion-associated cholesterol. ABCA1 has additional suppressive effect on Nef-positive virus, probably because of its binding to Nef (Mujawar et al., 2006) and inhibiting the postentry functions of Nef in HIV infectivity, such as facilitation of intracytoplasmic transport (Qi and Aiken, 2008). The anti-HIV activity of ABCA1 explains the benefit that the virus gains by specifically targeting ABCA1 via Nef (Mujawar et al., 2006).

Nef and ABCA1 interact with each other and exist in a state of a dynamic balance: under normal infection conditions, Nef inhibits ABCA1 activity and reduces cholesterol efflux; however, upon induction of ABCA1 expression by agents such as LXR agonists, ABCA1 stimulates cholesterol efflux and also inhibits Nef activity. Inhibition of ABCA1 seems to be the main mechanism by which Nef influences viral cholesterol levels. Indeed, differences in cholesterol levels between Nef-positive and Nef-negative virions in ABCA1-negative cells are small (Fig. 3A). This finding is consistent with recently reported data that Nef does not significantly affect virion levels of phosphoglycerolipids or cholesterol (Brügger et al., 2007).

The Nef-inhibiting activity of ABCA1 requires additional studies. Two possible mechanisms can be considered: ABCA1 may bind Nef and prevent its incorporation into virions, or ABCA1 bound to Nef can incorporate into virions and inhibit the ability of Nef to stimulate postentry steps of replication. Analysis of viral proteins in Fig. 2D shows only a small decrease of Nef, thus favoring the second possible mechanism.

The key role of ABCA1 in the antiviral activity exhibited by the LXR agonist is supported by our finding of a greatly reduced effect of TO-901317 on HIV-1 replication in T cells isolated from a patient with Tangier disease. In these cells, the LXR agonist delayed HIV-1 replication, but the viral production reached the level observed in untreated cells by day 9, when replication was still at the maximal level (Fig. 5A). Delayed replication of HIV-1 in TO-901317-treated T cells from a patient with Tangier disease might be due to residual activity of ABCA1 (Clee et al., 2000), which was not revealed in cholesterol efflux analysis, or to the ability of mutant ABCA1 to bind and inhibit Nef despite inability to support cholesterol efflux. In both cases, the anti-HIV effect

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would disappear when viral replication and Nef production reach high levels. Failure of the LXR agonist to inhibit HIV-1 replication in Tangier cells suggests selective sensitivity of HIV-1 to ABCA1-dependent pathway of cholesterol efflux. This notion is consistent with specific targeting of ABCA1, but not related transporter ABCG1, by Nef (Mujawar et al., 2006). The role of ABCA1 in TO-901317-mediated anti-HIV effect is further underscored by experiments with macrophages where ABCA1 was down-regulated by RNAi. In these cells, the anti-HIV effect of TO-901317 was abolished at an early time point (4 days after infection) and significantly reduced at a later time point. Despite this evidence, contribution of additional factors controlled by LXR to anti-HIV activity of LXR agonist cannot be excluded. For example, ABCG1 is stimulated by LXR agonist (Fig. 5B) and may contribute to cholesterol depletion of virions.

The mechanisms responsible for the observed inhibition of HIV production from TO-901317-treated cells require additional studies. It is interesting that this inhibitory effect occurs on the background of increased Gag expression. One possibility is that depletion of cholesterol disrupts the lipid domains, thus affecting virion assembly. It is also possible that LXR agonists stimulate the expression of tetherin, a cellular innate immunity factor that blocks virion release (Sauter et al., 2010) or impair the ability of Vpu to counteract this factor.

Before this study, cholesterol depletion of HIV virions in vitro has largely been achieved via treatment of mature, cell-free virus using cyclodextrin compounds (Liao et al., 2003). Although such treatment leads to a significant reduction in viral infectivity, cyclodextrin is not suitable for clinical use because of its lack of specificity: it indiscriminately extracts cholesterol from cellular lipid membrane, resulting in high cytotoxicity. Using the LXR agonists, we demonstrate a novel approach to targeting HIV-associated cholesterol. In contrast to the effect of cyclodextrins, LXR agonists do not nonspecifically extract cholesterol, but redirect it to the physiological efflux pathway, thus diverting cholesterol to the efflux pathway at the expense of HIV assembly.

In support of the concept of possible future administration of LXR agonists for the treatment of systemic HIV infection in humans, TO-901317 treatment resulted in a 1.5-log reduction of the viral load in HIV-infected Rag-hu mice. The primary indication for LXR agonists is treatment or management of dyslipidemia and atherosclerosis (Miao et al., 2004). LXR agonists would be highly beneficial to HIV-positive patients who exhibit altered cholesterol profiles, including significantly reduced high-density lipoprotein levels, and are at increased risk of developing atherosclerosis (Mangili et al., 2007). Our previous studies demonstrated that HIV infection impairs cholesterol efflux from macrophages and promotes their conversion into foam cells (Mujawar et al., 2006), suggesting a direct connection between HIV replication and atherosclerosis (Bukrinsky and Sviridov, 2007). Therefore, treatment of systemic HIV infection with LXR agonists could not only inhibit the progression of infection, but could also have the benefit of reversing or inhibiting the development of atherosclerosis linked to HIV infection.

In summary, results presented in this report demonstrate a potent anti-HIV effect of LXR agonists. The drugs affect two critical steps of HIV replication: they inhibit viral production and infectivity of produced virions, the latter by reducing viral cholesterol and fusion activity and by inactivating Nef. Although each of these effects may seem small, together and in the context of spreading infection, they result in potent suppression of HIV replication. This study also emphasizes the critical role of ABCA1 in the regulation of HIV infectivity. Stimulation of ABCA1 expression may thus be an effective approach to limit HIV infectivity and inhibit viral replication.

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References

Baenziger S, Tussiwand R, Schlaepfer E, Mazzucchelli L, Heikenwalder M, Kurrer MO, Behnke S, Frey J, Oxenius A, Joller H, et al. (2006) Disseminated and sustained HIV infection in CD34$^+$ cord blood cell-transplanted Rag2−/−gamma c−/− mice. *Proc Natl Acad Sci USA* **103:**15951–15956.

Brügger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, and Kräusslich HG (2006) The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci USA* **103:**2641–2646.

Brügger B, Krautkrämer E, Tibroni N, Munte CE, Rauch S, Leibrecht I, Glass B, Breuer S, Geyer M, Kräusslich HG, et al. (2007) Human immunodeficiency virus type 1 Nef protein modulates the lipid composition of virions and host cell membrane microdomains. *Retrovirology* **4:**70.

Bukrinsky M and Sviridov D (2007) HIV and cardiovascular disease: contribution of HIV-infected macrophages to development of atherosclerosis. *PLoS Med* **4:**e43.

Campbell SM, Crowe SM, and Mak J (2002) Virion-associated cholesterol is critical for the maintenance of HIV-1 structure and infectivity. *AIDS* **16:**2253–2261.

Cavrois M, De Noronha C, and Greene WC (2002) A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat Biotechnol* **20:**1151–1154.

Chan R, Uchil PD, Jin J, Shui G, Ott DE, Mothes W, and Wenk MR (2008) Retroviruses human immunodeficiency virus and murine leukemia virus are enriched in phosphoinositides. *J Virol* **82:**11228–11238.

Clee SM, Kastelein JJ, van Dam M, Marcil M, Roomp K, Zwarts KY, Collins JA, Roelants R, Tamasawa N, Stulc T, et al. (2000) Age and residual cholesterol efflux affect HDL cholesterol levels and coronary artery disease in ABCA1 heterozygotes. *J Clin Invest* **106:**1263–1270.

Collins JL, Fivush AM, Watson MA, Galardi CM, Lewis MC, Moore LB, Parks DJ, Wilson JG, Tippin TK, Binz JG, et al. (2002) Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. *J Med Chem* **45:**1963–1966.

Embretson J, Zupancic M, Ribas JL, Burke A, Racz P, Tenner-Racz K, and Haase AT (1993) Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* **362:**359–362.

Gendelman HE, Orenstein JM, Martin MA, Ferrua C, Mitra R, Phipps T, Wahl LA, Lane HC, Fauci AS, and Burke DS (1988) Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. *J Exp Med* **167:**1428–1441.

Glushakova S, Grivel JC, Suryanarayana K, Meylan P, Lifson JD, Desrosiers R, and Margolis L (1999) Nef enhances human immunodeficiency virus replication and responsiveness to interleukin-2 in human lymphoid tissue ex vivo. *J Virol* **73:**3968–3974.

Guyader M, Kiyokawa E, Abrami L, Turelli P, and Trono D (2002) Role for human immunodeficiency virus type 1 membrane cholesterol in viral internalization. *J Virol* **76:**10356–10364.

Hara A and Radin NS (1978) Lipid extraction of tissues with a low-toxicity solvent. *Anal Biochem* **90:**420–426.

Hozoji M, Munehira Y, Ikeda Y, Makishima M, Matsuo M, Kioka N, and Ueda K (2008) Direct interaction of nuclear liver X receptor-beta with ABCA1 modulates cholesterol efflux. *J Biol Chem* **283:**30057–30063.

Kratzer A, Buchebner M, Pfeifer T, Becker TM, Uray G, Miyazaki M, Miyazaki-Anzai S, Ebner B, Chandak PG, Kadam RS, et al. (2009) Synthetic LXR agonist attenuates plaque formation in apoE−/− mice without inducing liver steatosis and hypertriglyceridemia. *J Lipid Res* **50:**312–326.

Liao Z, Graham DR, and Hildreth JE (2003) Lipid rafts and HIV pathogenesis: virion-associated cholesterol is required for fusion and infection of susceptible cells. *AIDS Res Hum Retroviruses* **19:**675–687.

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Lingwood D and Simons K (2010) Lipid rafts as a membrane-organizing principle. *Science* **327:**46–50.
Mangili A, Jacobson DL, Gerrior J, Polak JF, Gorbach SL, and Wanke CA (2007) Metabolic syndrome and subclinical atherosclerosis in patients infected with HIV. *Clin Infect Dis* **44:**1368–1374.
Miao B, Zondlo S, Gibbs S, Cromley D, Hosagrahara VP, Kirchgessner TG, Billheimer J, and Mukherjee R (2004) Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator. *J Lipid Res* **45:**1410–1417.
Mujawar Z, Rose H, Morrow MP, Pushkarsky T, Dubrovsky L, Mukhamedova N, Fu Y, Dart A, Orenstein JM, Bobryshev YV, et al. (2006) Human immunodeficiency virus impairs reverse cholesterol transport from macrophages. *PLoS Biol* **4:**e365.
Nofer JR and Remaley AT (2005) Tangier disease: still more questions than answers. *Cell Mol Life Sci* **62:**2150–2160.
Ono A and Freed EO (2001) Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc Natl Acad Sci USA* **98:**13925–13930.
Pantaleo G, Graziosi C, and Fauci AS (1993) The role of lymphoid organs in the pathogenesis of HIV infection. *Semin Immunol* **5:**157–163.
Qi M and Aiken C (2008) Nef enhances HIV-1 infectivity via association with the virus assembly complex. *Virology* **373:**287–297.
Rader DJ (2007) Liver X receptor and farnesoid X receptor as therapeutic targets. *Am J Cardiol* **100:**n15–n19.
Raith K, Brenner C, Farwanah H, Müller G, Eder K, and Neubert RH (2005) A new LC/APCI-MS method for the determination of cholesterol oxidation products in food. *J Chromatogr A* **1067:**207–211.
Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, and Mangelsdorf DJ (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* **14:**2819–2830.
Rigamonti E, Helin L, Lestavel S, Mutka AL, Lepore M, Fontaine C, Bouhlel MA, Bultel S, Fruchart JC, Ikonen E, et al. (2005) Liver X receptor activation controls intracellular cholesterol trafficking and esterification in human macrophages. *Circ Res* **97:**682–689.
Rossi L, Serafini S, Franchetti P, Casabianca A, Orlandi C, Schiavano GF, Carnevali A, and Magnani M (2002) Inhibition of murine AIDS by a heterodinucleotide of azidothymidine and 9-(R)-2-(phosphonomethoxypropyl)adenine. *J Antimicrob Chemother* **50:**639–647.
Saad JS, Miller J, Tai J, Kim A, Ghanam RH, and Summers MF (2006) Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. *Proc Natl Acad Sci USA* **103:**11364–11369.
Sauter D, Specht A, and Kirchhoff F (2010) Tetherin: holding on and letting go. *Cell* **141:**392–398.
Schindler M, Münch J, and Kirchhoff F (2005) Human immunodeficiency virus type 1 inhibits DNA damage-triggered apoptosis by a Nef-independent mechanism. *J Virol* **79:**5489–5498.
Schmidtmayerova H, Nuovo GJ, and Bukrinsky M (1997) Cell proliferation is not required for productive HIV-1 infection of macrophages. *Virology* **232:**379–384.
Tall AR (2008) Cholesterol efflux pathways and other potential mechanisms involved in the athero-protective effect of high density lipoproteins. *J Intern Med* **263:**256–273.
van 't Wout AB, Swain JV, Schindler M, Rao U, Pathmajeyan MS, Mullins JI, and Kirchhoff F (2005) Nef induces multiple genes involved in cholesterol synthesis and uptake in human immunodeficiency virus type 1-infected T cells. *J Virol* **79:**10053–10058.
Wain-Hobson S, Vartanian JP, Henry M, Chenciner N, Cheynier R, Delassus S, Martins LP, Sala M, Nugeyre MT, and Guétard D (1991) LAV revisited: origins of the early HIV-1 isolates from Institut Pasteur. *Science* **252:**961–965.
Watkins BA, Dorn HH, Kelly WB, Armstrong RC, Potts BJ, Michaels F, Kufta CV, and Dubois-Dalcq M (1990) Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science* **249:**549–553.
Yvan-Charvet L, Wang N, and Tall AR (2010) Role of HDL, ABCA1, and ABCG1 transporters in cholesterol efflux and immune responses. *Arterioscler Thromb Vasc Biol* **30:**139–143.
Zheng YH, Plemenitas A, Linnemann T, Fackler OT, and Peterlin BM (2001) Nef increases infectivity of HIV via lipid rafts. *Curr Biol* **11:**875–879.

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