Stimulation of Liver X Receptor Has Potent Anti-HIV Effects in a Humanized Mouse Model of HIV Infection

Ali Ramezani, Larisa Dubrovsky, Tatiana Pushkarsky, Dmitri Sviridov, Sara Karandish, Dominic S. Raj, Michael L. Fitzgerald, and Michael Bukrinsky

George Washington University School of Medicine and Health Sciences, Washington, DC (A.R., L.D., T.P., S.K., D.S.R., M.B.); Baker International Diabetes Institute, Heart and Diabetes Institute, Melbourne, Victoria, Australia (D.S.); and Harvard Medical School, Lipid Metabolism Unit, Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts (M.L.F.)

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ABSTRACT

Previous studies demonstrated that liver X receptor (LXR) agonists inhibit human immunodeficiency virus (HIV) replication by upregulating cholesterol transporter ATP-binding cassette A1 (ABCA1), suppressing HIV production, and reducing infectivity of produced virions. In this study, we extended these observations by analyzing the effect of the LXR agonist T0901317 [*N*-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-*N*-(2,2,2-trifluoroethyl)benzenesulfonamide] on the ongoing HIV infection and investigating the possibility of using LXR agonist for pre-exposure prophylaxis of HIV infection in a humanized mouse model. Pre-exposure of monocyte-derived macrophages to T0901317 reduced susceptibility of these cells to HIV infection *in vitro*. This protective effect lasted for up to 4 days after treatment termination and correlated with upregulated expression of ABCA1, reduced abundance of

lipid rafts, and reduced fusion of the cells with HIV. Pre-exposure of peripheral blood leukocytes to T0901317 provided only a short-term protection against HIV infection. Treatment of HIV-exposed humanized mice with LXR agonist starting 2 weeks postinfection substantially reduced viral load. When eight humanized mice were pretreated with LXR agonist prior to HIV infection, five animals were protected from infection, two had viral load at the limit of detection, and one had viral load significantly reduced relative to mock-treated controls. T0901317 pretreatment also reduced HIV-induced dyslipidemia in infected mice. In conclusion, these results reveal a novel link between LXR stimulation and cell resistance to HIV infection and suggest that LXR agonists may be good candidates for development as anti-HIV agents, in particular for pre-exposure prophylaxis of HIV infection.

Introduction

Despite the availability of effective anti-human immunodeficiency virus (HIV) treatments, the HIV epidemic continues and 2.3 million new infections were reported worldwide in 2012 (Beyrer and Abdool Karim, 2013). Because an effective HIV vaccine is still unavailable, preventive measures are the best option to curb the incidence of new infections. Pre-exposure prophylaxis (PrEP), which treats uninfected subjects with oral or topical antiretroviral medications to protect against HIV acquisition, is a promising HIV prevention strategy. This approach was suggested about 10 years ago (Youle and Wainberg, 2003), and has gained support from simian/HIV (SHIV) challenge studies in macaques, which indicated high

levels of protection conferred by daily oral dosing of tenofovir (Garcia-Lerma et al., 2008). Moreover, studies of infants born to HIV-infected mothers demonstrated that postnatal administration of antiretrovirals to the baby can substantially reduce the risk of HIV infection in the newborn (Chasela et al., 2010). However, PrEP with available antiretroviral drugs encounters an inherent problem with adverse effects and potential transmission of drug-resistant HIV strains [reviewed in (Baeten and Celum, 2013)].

As shown in a number of published reports, HIV-1 replication is critically dependent on cellular cholesterol [reviewed in (Waheed and Freed, 2009)], as cholesterol depletion in infected cells markedly and specifically reduces HIV-1 particle production (Ono and Freed, 2001) and infectivity of the virions produced by the cholesterol-depleted cells (Zheng et al., 2001). An important component of the viral strategy aimed at securing sufficient amounts of cholesterol in HIV-infected cells is Nef-mediated impairment of the function of ABCA1 cholesterol transporter responsible for cholesterol efflux (Mujawar et al., 2006). Reversion of this impairment by drugs stimulating

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ABBREVIATIONS: AM, acetoxymethyl ester; AZ876, 2-*tert*-butyl-5-phenyl-4-[(4-piperidin-1-ylphenyl)amino]isothiazol-3(2*H*)-one 1,1-dioxide; CCF2, coumarin cephalosporin fluorescein; CTB, cholera toxin subunit B; HIV, human immunodeficiency virus; hu, humanized; IMB-170, 2-(phenoxyacetic acid-2-yl)-3-(benzo[d]-[1,3]dioxol-5-yl)-2,3-dihydroquinazolin-4(1*H*)-one; lysoPC, lysophosphatidylcholine; LXR, liver X receptor; MDM, monocyte-derived macrophage; PBL, peripheral blood leukocyte; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PrEP, pre-exposure prophylaxis; T0901317, *N*-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-*N*-(2,2,2-trifluoroethyl)benzenesulfonamide.

ABCA1 expression, such as liver X receptor (LXR) agonists, inhibits HIV replication by reducing viral production and infectivity (Morrow et al., 2010). Importantly, the mechanism of anti-HIV activity of LXR agonists involves reduction of lipid rafts, the sites of HIV assembly (Cui et al., 2012), and reduction of cholesterol in the viral membrane required for effective fusion (Morrow et al., 2010), making it very difficult for the virus to acquire resistance to these agents. In this study, we tested whether LXR agonists can be used to treat and prevent HIV infection in humanized mice.

Materials and Methods

Generation of Humanized Nonobese Diabetic Severe Combined Immunodeficiency- γ Mice. Humanized NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (The Jackson Laboratory, Bar Harbor, ME) were prepared by engraftment with human cord blood CD34⁺ hematopoietic stem/progenitor cells (Lonza, Walkersville, MD), as previously described (Shultz et al., 2010). Briefly, newborn (within 2 days of birth) recipients received 150 cGy total body irradiation using a ¹³⁷Cs-source irradiator, followed by intrahepatic injection of 0.5×10^6 human CD34⁺ cells. Mice were screened for human cell engraftment at 10–12 weeks postreconstitution. Engrafted mice were further characterized by flow cytometry using monoclonal antibodies against human CD4, human CD8, and human CD14 (eBioscience, San Diego, CA). All animal studies were approved by the George Washington University School of Medicine and Health Sciences Institutional Animal Care and Use Committee (protocol A271).

HIV-1 Infection and Measurement of Viral Loads. Mice with $\geq 20\%$ human cell engraftment levels were used in these experiments. Each study group had equal numbers of male and female animals. They were infected with HIV-1 by intraperitoneal injection of CCR5 tropic strain ADA (1.0×10^5 IU) at ≥ 12 weeks after engraftment. Mice were observed daily, and blood samples were drawn biweekly (2, 4, 6, and 8 weeks after infection) to assess plasma viremia. HIV-1 viral load in plasma of infected mice was measured using COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (Roche Molecular Diagnostics, Pleasanton, CA).

T0901317 Treatment Schedule. The animals were treated with 10 mg/kg synthetic LXR agonist T0901317 (*N*-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-*N*-(2,2,2-trifluoroethyl)benzenesulfonamide, purchased from Sigma-Aldrich, St. Louis, MO) administered intraperitoneally every second day. A group of control humanized (hu)-mice ($n = 4$) received only the solvent [15% Cremophor/phosphate-buffered saline (PBS); Sigma-Aldrich] without the T0901317. Two treatment regimens were tested. In the first group ($n = 8$), treatment was initiated 1 week before the infection and stopped on the day of infection. In the second group ($n = 8$), we started treating mice 2 weeks after infection and continued treatment of 6 weeks. Although the number of animals was limited, it was consistent with numbers used in studies by other groups (Veselinovic et al., 2014; Stoddart et al., 2015) and provided sufficient power for statistical substantiation. At the end of the experiment, mice were euthanized and blood was collected for further analysis.

Triglyceride Analysis. Triglycerides in the plasma were measured using a colorimetric assay from Wako Diagnostics (Richmond, VA) following manufacturer's instructions.

Mass Spectrometry Lipidomic Profiling. To profile the lipid species of the mouse plasma, an electrospray ionization–tandem mass spectrometry approach was used, as reported previously (Fitzgerald et al., 2007; Zuo et al., 2008). In brief, 50 μ l plasma was extracted with 2.5 ml chloroform/methanol/distilled water (1:1:0.5, v/v) and two 0.5-ml chloroform extractions. Combined organic phases were washed with 0.5 ml KCl (1 M, 1 volume) and 0.5 ml dH₂O (2 volumes) and dried with N₂ gas. Samples were resuspended in 1 ml chloroform, and phospholipid scans were performed, as described (Fitzgerald et al.,

2007; Zuo et al., 2008). Mass of extracted lipids was determined by drying 10 μ l extracted lipids and measuring the resulting mass in an electro-microbalance (Cubis; Sartorius, Bohemia, NY). The amounts of the analyte lipids are indicated in units of normalized mass spectral signal, with 1 U representing the amount of lipid producing the same amount of signal as 1 nmol internal standard. These amounts are then expressed as nanomole lipid per milligram total plasma lipid.

Flow Cytometry. Whole blood was collected by tail bleed, and red blood cells were lysed using the red blood cell lysis buffer (Qiagen, Valencia, CA). The white blood cells were immunophenotyped using fluorescein isothiocyanate–conjugated anti-human CD45 and allophycocyanin–conjugated anti-human CD4, CD8, or CD14. Corresponding allophycocyanin- or fluorescein isothiocyanate–conjugated isotype controls were included in each analysis (all antibodies were purchased from eBioscience). Cells were stained with the labeled antibodies and analyzed using a FACSCalibur analyzer (BD Biosciences, San Jose, CA), as described previously (Ramezani et al., 2011).

Cells and Infection. Monocytes were prepared from peripheral blood of uninfected donors by plastic adhesion and differentiated into macrophages by macrophage colony-stimulating factor treatment of 7 days, as previously described (Schmidtayerova et al., 1997). Monocyte-depleted cells [peripheral blood leukocyte (PBLs)] were activated with phytohemagglutinin/interleukin-2 for 3 days. Both cell populations were treated with T0901317 (3 μ M) for 3 days prior to infection. Our previous study (Morrow et al., 2010) demonstrated that this drug concentration effectively inhibits HIV replication while having minimal cytotoxicity. HIV-1 ADA virus (5×10^5 cpm reverse-transcriptase activity per 1×10^6 cells) was added to cells for 3 hours immediately after removal of T0901317, or 1, 2, 3, 4, or 7 days after T0901317 removal. Viral replication was assessed by reverse-transcriptase activity in the supernatant at day 8 after infection in macrophages and day 4 in PBL using previously described procedure (Willey et al., 1988).

Fusion Assay. Fluorescence resonance energy transfer–based HIV-1 virion fusion assay was performed, as previously described (Cavrois et al., 2002). Briefly, monocyte-derived macrophages (MDM) or PBLs were inoculated for 4 hours at 37°C with BlaM-Vpr–carrying virus [200 pg p24/10⁶ cells corresponding to multiplicity of infection = 3 (O'Doherty et al., 2000)] produced by HEK 293T cells cotransfected with AD8 or NL4-3–expressing vectors. Cells were washed, resuspended in β -lactamase loading solution (Invitrogen, Carlsbad, CA) containing 1 μ M coumarin cephalosporin fluorescein (CCF2)–acetoxymethyl (AM) ester, and incubated for 1 hour at room temperature. After washing, cells were resuspended in development medium [2.5 mM probenecid, 10% fetal bovine serum in CO₂-independent medium (Gibco/Life Technologies, Grand Island, NY)], incubated at room temperature for 16 hours in the dark, and fixed in 1.2% paraformaldehyde in fluorescence-activated cell sorting staining buffer for 2 hours at 4°C. 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.

Lipid Raft Staining. Cholera toxin subunit B (CTB) Alexa Fluor 488 conjugate prepared according to the manufacturer's protocol was used for lipid raft staining (Life Technologies, Frederick, MD). A total of 2×10^6 cells was rinsed with cold growth media, one half of the cells were stained in 1 ml CTB-Alexa488 solution (1 μ g/ml in growth media) at 4°C for 30 minutes, and the other half was incubated with 1 ml growth medium without CTB-Alexa488. The cells were washed with cold PBS, fixed with 4% formaldehyde in PBS at 4°C for 15 minutes, and analyzed using FACSCalibur DxP8 Analyzer and FlowJo software (FlowJo, Ashland, OR).

Western Blotting. A total of 2×10^6 cells was washed with PBS and lysed in 100 μ l M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Rockford, IL). A quantity amounting to 40 μ g/well clarified lysate was loaded on 7.5% SDS-PAGE (Bio-Rad Laboratories, Hercules, CA), separated, and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked with

5% blocker in 1× Tris/borate EDTA buffer (Bio-Rad Laboratories) and blotted with rabbit anti-ABCA1 (Novus Biologicals, Littleton, CO) or rabbit anti-glyceraldehyde 3-phosphate dehydrogenase antibody (Sigma-Aldrich), followed by anti-rabbit goat horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). ABCA1 and glyceraldehyde 3-phosphate dehydrogenase proteins were revealed with chemoluminescence substrate (Protein Simple, San Jose, CA) using a *FluorChem E* Digital Darkroom and Alfa View Software (Cell Biosciences, Santa Clara, CA).

Statistical Analysis. Data were expressed as the mean value $\pm$ S.D., unless indicated otherwise. Significance of the differences between data groups was determined by two-tail Student *t* test analysis. *P* values below 0.05 were considered significant.

Results

In Vitro Studies. Previously, we demonstrated that treatment of HIV-infected cells with LXR agonist T0901317 significantly suppresses viral replication via the mechanism involving upregulation of ABCA1 expression, reduced cellular and viral cholesterol content, and resultant suppression of HIV production and infectivity (Morrow et al., 2010). In those experiments, the drug was added to cells at the time of infection and was present thereafter. To determine whether T0901317-treated cells acquire and sustain a virus-resistant phenotype, we pretreated monocyte-derived macrophages and monocyte-depleted PBLs with T0901317, washed out the drug, and infected the cells at different time points after removal of the drug. Viral replication was assessed on day 8 after infection of MDM and day 4 after infection of PBLs, and is presented in Fig. 1 for three experiments with cells from different donors as percent inhibition relative to mock-treated cultures (to accommodate differences in viral replication between cells from different donors, which prevent statistically valid analysis). In T0901317-pretreated MDM, HIV replication was substantially lower than in mock-treated cells, and partial resistance to infection was sustained for 4 days after T0901317 removal (Fig. 1A). In PBLs, a decrease in viral replication was observed only when cells were infected right after removal of T0901317, and resistance was not sustained (Fig. 1B). This result is consistent with the level of ABCA1 in cells pretreated with T0901317: elevated levels of ABCA1 were maintained in macrophages for at least 3 days (Fig. 1C), whereas, in PBLs used in these experiments, the ABCA1 levels were too low for detection by Western blotting (unpublished data).

Because the main mechanism behind the suppressive effect of LXR agonists on HIV replication is reduction of lipid raft abundance (Morrow et al., 2010; Cui et al., 2012), we analyzed lipid raft content in T0901317-pretreated cells at various times after washing away the LXR agonist. As shown in Fig. 1D, the abundance of lipid rafts was decreased in pretreated MDM by half, and this effect was maintained for at least 4 days. Interestingly, lipid raft content increased transiently on day 1 relative to day 0 for both treated and untreated cells. This was due to full change of culture medium on day 0 necessary to remove T0901317 (only half the medium was changed during normal culturing). No effect of T0901317 on lipid raft abundance was detected in PBLs (unpublished data). This result is consistent with analysis of ABCA1 (Fig. 1C), as elevated expression of

ABCA1 leads to disruption of lipid rafts (Landry et al., 2006; Koseki et al., 2007). It also supports the notion that pretreatment with LXR agonist causes sustained changes in cholesterol metabolism that reduce MDM susceptibility to HIV infection.

Given the role of lipid rafts in virus-cell fusion (Lim and Yin, 2006), we further tested the fusion capacity of T0901317-pretreated cells. Results presented in Fig. 1E show that fusion between HIV-1 ADA virions and pretreated macrophages was decreased by about 60% relative to mock-treated cells. Again, this effect was maintained for at least 4 days after removal of the drug, consistent with the effects on ABCA1 and lipid rafts. Taken together, these results demonstrate that pretreatment of macrophages with LXR agonist raises their resistance to infection. This induced resistance lasts for several days in macrophages; in T cells the effect is smaller and is reversed in less than 24 hours.

In Vivo Studies in Humanized Mice. To investigate the effect of LXR agonist on HIV infection in vivo, we used humanized mice as a model of infection. Nonobese diabetic severe combined immunodeficiency- γ (NSG) mice were inoculated intrahepatically with human cord blood-derived CD34⁺ hematopoietic stem cells, and the development of mature blood cells was monitored. Three months after transplantation, all transplanted mice were engrafted with high levels of human cells (Fig. 2A), and the percentages of human CD45, CD4, and CD14 cells were 75.1 ± 17.4 , 16.7 ± 8.2 , and 2.8 ± 2.4 , respectively (Fig. 2B). It should be noted that, in blood samples, CD4 is typically detectable by fluorescence-activated cell sorting analysis only on helper T cells, so observed levels of CD4⁺ cells most likely correspond to CD4⁺ T helper cells.

Previously, we demonstrated that T0901317 significantly reduced HIV replication in hu-mice when treatment was initiated at the time of infection and continued throughout the course of the experiment (Dubrovsky et al., 2012). In this work, we tested the situation more relevant to HIV infection in humans: pretreatment with T0901317 and drug removal prior to infection (a model of PrEP), and post-treatment with the drug, when the drug was added after establishment of infection and continued thereafter (a model of postexposure treatment). Engrafted mice were either pretreated with T0901317 for 1 week and then infected with HIV-1 ADA, or infected with HIV-1 and then treated with T0901317 starting 2 weeks after infection.

Because a characteristic effect of this LXR agonist is an increased synthesis of triglycerides (Grefhorst et al., 2002; Chisholm et al., 2003), we first analyzed triglycerides in a group pretreated with T0901317 prior to infection, HIV-infected mock-treated mice, and mock-infected controls. Figure 3A shows that levels of triglycerides in the blood of T0901317-treated animals were significantly higher than in HIV-infected mock-treated animals 4 and 6 weeks after infection and returned to levels observed in the mock-treated group by week 8. This result reflects the pharmacokinetic properties of T0901317 (Peng et al., 2008) and indicates that the drug's effects are sustained for several weeks after treatment interruption, most likely due to slow drug clearance. Interestingly, triglyceride levels in HIV-infected mock-treated mice were higher than in mock-infected animals, and the difference was highly significant on weeks 4 and 6 after infection. Hypertriglyceridemia is

Fig. 1. Pretreatment with T0901317 induces resistance to HIV-1 infection. (A) MDM were pretreated with T0901317 for 3 days, washed, and infected with R5 HIV-1 strain ADA immediately after washing or at indicated days after drug removal. Control cells were treated with the vehicle (0.1% dimethylsulfoxide). Viral replication was assessed by reverse-transcriptase activity in culture supernatant at day 8 after infection. Results are presented as mean $\pm$ S.D. of three experiments with cells from different donors. Given the differences in viral replication between the experiments, results are presented as percent of inhibition ($\frac{\text{RT activity in control culture} - \text{RT activity in treated culture}}{\text{RT activity in control culture}} \times 100\%$). (B) Monocyte-depleted PBMCs (PBL) were activated with phytohemagglutinin and interleukin-2 for 3 days in the presence of T0901317, washed, and infected with HIV-1 ADA, as in (A). Viral replication was assessed on day 4 after infection and is presented as described in (A). (C) MDM were treated with T0901317 or diluent for 3 days, washed, and analyzed for ABCA1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immediately after washing or at indicated days after drug removal. (D) MDM were treated as in (C), and at indicated time points after drug removal, abundance of lipid rafts was assessed by flow cytometry after interaction with CTB conjugated with Alexa Fluor 488. Upper panel shows representative results with cells from one donor. Bottom panel shows mean $\pm$ S.D. of experiments with five donors, presented as percent inhibition of lipid raft abundance in drug-treated cells. (E) MDM were treated as in (A), and at indicated time points after drug removal were loaded with CCF2-AM and assessed for fusion with HIV-1 AD8-carrying BlaM-Vpr (multiplicity of infection = 3). Percentage of cleaved CCF2 indicates the percentage of cells that fused with the virus. Top panel shows representative results with cells from one donor. Bottom panel shows mean $\pm$ S.D. of experiments with five donors, presented as percent inhibition of virus fusion with drug-treated cells. SSC, side scatter parameter.

a characteristic feature of dyslipidemia in HIV-infected patients (Calvo and Martinez, 2014), and these results are consistent with the notion that HIV-specific dysregulation of lipid metabolism is reproduced in HIV-infected humanized mice (Dubrovsky et al., 2012).

To more broadly characterize the lipid metabolic responses to HIV infection and T0901317 treatment in the human mouse model, we used mass spectrometry to quantitate the glycerophospholipids, ether-glycerophospholipids, and selected lyso derivatives. Many, but not all, of these lipid

Fig. 2. Analysis of human cell engraftment in hu-mice. (A) Peripheral blood cells from humanized mice were analyzed for human CD4 and human CD45 markers. A typical plot is presented. (B) Results of analysis of indicated human markers are presented for all mice used in the experiments.

Fig. 3. Plasma lipids in HIV-1-infected hu-mice. (A) Humanized mice were pretreated with T0901317 (Pre-TO + HIV) or 15% Cremophor/PBS (HIV) for 7 days and infected with HIV-1 ADA. Triglycerides were analyzed by colorimetric assay on indicated time points (weeks) after infection. Mock-infected animals served as control. (B–D) Mass spectrometry was used to profile plasma lipids in the hu-mice. Shown are results (mean $\pm$ S.E.M.) for PC, lysoPC, and PI. * $P < 0.05$ compared with HIV-infected mock-treated animals.

classes were elevated in the HIV-infected samples, and pretreatment with T0901317 reduced, but did not completely eliminate, these changes (Table 1). Of interest, the lipidomic data available for one animal that remained HIV-positive in the T0901317 pretreatment group had values more similar to those of the HIV-infected untreated group. Both phosphatidylcholine (PC) and its lyso derivative (lysoPC) were elevated by HIV infection, and T0901317 pretreatment significantly blocked this increase (Fig. 3, B and C). Likewise, phosphatidylinositol (PI) was also significantly elevated in the HIV-infected mice, and T0901317 treatment again significantly blocked this HIV-associated elevation (Fig. 3D).

We then measured plasma viral load on weeks 2, 4, 6, and 8 after infection. As shown in Fig. 4A, pretreatment with T0901317 protected five of eight mice from infection. In these animals, viral load remained at or below the level of detection (50 copies/ml) for the whole duration of the experiment. Two of three infected mice were able to control viral replication: viral load at weeks 4–8 after infection was at or below the level of detection. Only one pretreated mouse showed viral replication, but it was significantly lower on weeks 2 and 4 than in untreated animals (Fig. 4A). On week 8, viral replication in the mock-treated group plummeted, most likely due to killing of CD4⁺ T cells (Sun et al., 2007). This result demonstrates

TABLE 1

Lipid analysis in plasma by mass spectrometry

P values were calculated using Student's unpaired two-tailed *t* test. Control group consists of mock-treated uninfected animals; HIV group, HIV-infected mock-treated animals; preTO + HIV group, animals pretreated with T0901317 and then infected with HIV (results for nonrespondent mouse, which was infected and maintained virus load on weeks 6 and 8, is shown in a separate column).

Lipid Class	Mean $\pm$ S.E.M. (nmol/mg Total Lipid)				<i>P</i> Values		
	Control	HIV	preTO + HIV		Control versus HIV	Control versus preTO + HIV	HIV versus preTO + HIV
			All	Nonrespondent			
LysoPC	10 $\pm$ 7.7	49 $\pm$ 8.1	20 $\pm$ 3.8	33.9	0.03 ^a	0.22	0.006 ^a
PC	61 $\pm$ 51	193 $\pm$ 24	115 $\pm$ 18	186	0.04 ^a	0.21	0.031 ^a
EtherPC	10 $\pm$ 8.2	10 $\pm$ 1.2	8 $\pm$ 1.1	11.2	0.97	0.54	0.283
LysoPE	0.02 $\pm$ 0.01	0.09 $\pm$ 0.03	0.05 $\pm$ 0.01	0.11	0.23	0.26	0.229
SM, DSM	7 $\pm$ 5.6	19 $\pm$ 2.7	11 $\pm$ 2.4	19	0.051	0.44	0.080
PE	0.02 $\pm$ 0.02	0.02 $\pm$ 0.003	0.03 $\pm$ 0.01	0.05	0.76	0.48	0.159
PI	0.42 $\pm$ 0.4	8.42 $\pm$ 1.9	2.90 $\pm$ 0.6	5.56	0.01 ^a	0.01 ^a	0.015 ^a
PS	0.04 $\pm$ 0.04	0.06 $\pm$ 0.01	0.09 $\pm$ 0.01	0.09	0.54	0.14	0.102
PA	0.14 $\pm$ 0.13	0.08 $\pm$ 0.02	0.06 $\pm$ 0.01	0.03	0.35	0.12	0.452
Chol Ester	645 $\pm$ 485	1579 $\pm$ 435	649 $\pm$ 97	886	0.22	0.99	0.056

Chol Ester, cholesterol ester; DSM, dihydrosphingomyelin; EtherPC, ether-linked phosphatidylcholine; LysoPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

^aSignificant difference.

Fig. 4. The effects of T0901317 treatment on HIV-1 infection in hu-mice. Hu-mice were either pretreated with T0901317 for 1 week and then infected with HIV-1 (T0901317 pretreatment) or infected with HIV-1 and treated with T0901317 starting at 2 weeks postinfection (T0901317 post-treatment). Mock-treated HIV-infected animals (no treatment) served as control. Viral load was assessed at indicated time points (weeks) postinfection. (A) Data for individual mice in each group are presented, together with mean and S.E.M. (B) Mock-treated and post-treated groups were compared. Statistical significance was determined using the Holm-Sidak method, with $\alpha = 5.000\%$.

a potent activity of T0901317 as a prophylactic anti-HIV agent.

When treatment with T0901317 was initiated 2 weeks after infection, a significant reduction of HIV replication was observed on weeks 4 and 6 after infection (Fig. 4B). Again, the difference between drug-treated and mock-treated groups disappeared on week 8 when viral load plummeted in the mock-treated group. This result not only confirmed our previous finding (Dubrovsky et al., 2012), but also suggested that LXR agonist can be used as a supplemental treatment of an established HIV infection.

Discussion

This study analyzed the effects of a prototypical LXR agonist T0901317 on HIV replication in humanized mice. We investigated two treatment scenarios, as follows: pretreatment of cells or animals with T0901317 prior to infection (no drug was given after infection), and post-treatment of the animals starting 2 weeks after infection. In the first scenario, the treatment protected five of eight animals from infection, and substantially reduced viral load in three infected animals (to levels at the limit of detection in two). Treatment of infected animals with T0901317 significantly reduced viral load, supporting results published in our previous report (Dubrovsky et al., 2012), in which hu-mice were treated with T0901317 starting at the time of infection.

There are two plausible mechanisms for the protective effect of pretreatment with T0901317 in humanized mice. The first is that the observed effect was due to continued presence of the drug in the bloodstream during the critical period of establishment of HIV infection. This explanation is consistent with sustained increase of triglycerides, which was observed for up to 6 weeks after T0901317 removal (see Fig. 3A). However, viral replication in hu-mice occurs mostly in CD4 T cells (Baenziger et al., 2006; Berges et al., 2006), and the magnitude of viral suppression by T0901317 in PBLs was relatively small (Fig. 1) to explain such a profound effect. This is most likely due to very low levels of ABCA1 expression in T cells. In addition, a small increase of ABCA1 observed in T0901317-stimulated PBLs from some donors (Cui et al., 2012) was very short-lived. Whereas we cannot rule out other possible reasons for the differences between PBLs and MDM

in response to LXR agonist, we believe that different level of ABCA1 expression is the most likely explanation. Indeed, the essential role of ABCA1 in anti-HIV effects of T0901317, both in MDM and CD4⁺ T cells, has been substantiated in previous studies by our and other groups (Morrow et al., 2010; Jiang et al., 2012). In particular, our study (Morrow et al., 2010) demonstrated that CD4⁺ from patients with Tangier disease, who do not express ABCA1, and MDM, in which ABCA1 was downregulated by small interfering RNA, is not sensitive to anti-HIV effects of T0901317. Therefore, low expression of ABCA1 in PBLs is likely to limit the anti-HIV effect of T0901317.

This brings us to a second possible mechanism for T0901317-induced protection from HIV infection: the effect on macrophages. In macrophages, T0901317-induced resistance was much stronger than in PBLs and was long-lasting (up to 7 days). Pretreatment of macrophages with T0901317 induced sustained upregulation of ABCA1, reduced abundance of lipid rafts, and reduced fusion of treated cells with the virus, all of which were associated with suppression of HIV replication (Morrow et al., 2010). Therefore, a significant protective effect of the drug would be expected if infection of macrophages is essential for establishing high viral load. Although Rag-hu mice can be infected with 4 $\times$ virus that does not replicate in macrophages (Baenziger et al., 2006; Berges et al., 2006), and NSG-hu thy/liv mice lacking macrophages can be infected with HIV (Honeycutt et al., 2013), the role of these cells in the HSC-NSG model used in our studies is unknown. Previous studies demonstrated that macrophages are infected in hu-mice (Watanabe et al., 2007; Gorantla et al., 2010), and results presented in this report are consistent with an important contribution of these cells to establishment and persistence of HIV infection in this animal model. Given that anti-HIV activity of LXR agonist is more potent in macrophages than in PBLs, potential utility of LXR agonists as a therapy option for HIV infection in humans would depend on the role of macrophage infection in HIV disease and will ultimately require clinical testing. Available data indicate that infection of macrophages plays important role in comorbidities of HIV infection, including neurocognitive (Rappaport and Volsky, 2015) and cardiovascular disorders (Bukrinsky and Sviridov, 2007).

Consistent with our previous report (Dubrovsky et al., 2012), HIV infection of hu-mice induced changes in lipidomic profile. PC, lysoPC, and PI were all significantly elevated in the HIV-infected mice. In mice pretreated with LXR agonist, the lipidomic profile did not significantly differ from that of control group, consistent with protection of these animals from HIV infection. The only exception was the level of PI, which remained significantly increased in pretreated animals. This finding suggests that protection from infection was not complete, and low levels of infection, undetectable by the assay used, still occurred. This interpretation is supported by the lipidomic profile of the single mouse that acquired HIV infection and maintained detectable virus load on weeks 6 and 8 despite being pretreated with T0901317. Lipids in this mouse, and in particular PC, lysoPC, and PI, were substantially lower than in other mice in this group, and closer to the levels observed in HIV-infected group. These results are not fully consistent with recent lipidomic profiling of plasma from HIV patients with coronary artery disease (Wong et al., 2014). In that study, HIV patients had elevated levels of cholesteryl ester and lysoPC, but reduced levels of PC and PI. However, not all species within each class showed the same association, and a number of individual lipid species were significantly increased in plasmas from HIV-infected subjects (Wong et al., 2014). Elevated levels of lysoPC were also found in the cerebrospinal fluid of simian immunodeficiency virus-infected macaques (Wikoff et al., 2008). Hence, our detection of elevated lipid levels in this mouse model of HIV infection suggests that this may be a general metabolic response to infection. The potential reasons and significance of the increase in lipid levels by HIV infection remain to be determined. Previous studies documented increased triglyceride levels in HIV-infected subjects, both drug-naïve and receiving antiretrovirus treatment (Stein, 2003), and virus-specific mechanisms of this effect included increased lipogenesis (Rasheed et al., 2008) and increased levels of lecithin-cholesterol acyltransferase and cholesteryl ester transfer protein (Rose et al., 2008). These factors may well be responsible for the changes in lipidomic profile observed in this study.

Taken together, results presented in this report demonstrate that LXR stimulation exerts a potent anti-HIV effect on established HIV replication and provides support for considering LXR agonists as candidate drugs for PrEP of HIV infection. The advantage of LXR agonists over current anti-HIV drugs is that the former target a cellular cholesterol pathway rather than a viral enzyme targeted by the latter, thus making it difficult for the virus to acquire resistance to these compounds and rendering PrEP with these agents effective against viruses resistant to antiretrovirals that are currently in use, including tenofovir, which showed promising results in PrEP trials (Martin et al., 2015). LXR agonists are being developed for the treatment of atherosclerosis, as synthetic LXR agonists have been shown to inhibit the progression (Joseph et al., 2002; Terasaka et al., 2003) and even promote the regression (Levin et al., 2005) of atherosclerosis in mouse models. Introduction of LXR agonists into clinical practice was impeded by a significant limitation: LXR activation leads to increased fatty acid synthesis, accumulation of triglycerides, and the development of fatty liver (Grefhorst et al., 2002; Chisholm et al., 2003). A new generation of

LXR agonists that do not induce lipogenic effects, but preserve anti-atherogenic activity, has been described: *N*, *N*-dimethyl-3 β -hydroxy-choleamide (Kratzer et al., 2009), IMB-170 [2-(phenoxyacetic acid-2-yl)-3-(benzo[d]-[1,3]dioxol-5-yl)-2,3-dihydroquinazolin-4(1*H*)-one] (Li et al., 2014), and AZ876 (2-*tert*-butyl-5-phenyl-4-[(4-piperidin-1-ylphenyl)amino]isothiazol-3(2*H*)-one 1,1-dioxide) (Van der Hoorn et al., 2011) are some examples. In this study, T0901317 was used to provide a proof of principle that LXR stimulation can be used to treat and prevent HIV infection. These results provide justification for further development of nonlipogenic LXR agonists as anti-HIV treatment.

Authorship Contributions

Participated in research design: Ramezani, Raj, Bukrinsky.

Conducted experiments: Ramezani, Karandish, Dubrovsky, Pushkarsky, Fitzgerald.

Performed data analysis: Ramezani, Fitzgerald, Dubrovsky, Pushkarsky, Bukrinsky.

Wrote or contributed to the writing of the manuscript: Sviridov, Fitzgerald, Bukrinsky.

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Address correspondence to: Dr. Michael Bukrinsky, George Washington University School of Medicine and Health Sciences, 2300 Eye Street NW, Ross Hall 624, Washington, DC 20037. E-mail: mbukrins@gwu.edu