Supporting Information

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SI Materials and Methods

Cell Culture. Cell toxicity with liposome treatment was determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)-based cell proliferation assay (CellTiter 96; Promega). Cells were grown in media with liposomes at various concentrations for 4 d before the addition of the MTS agent, at which point color was left to develop for 1–2 h, and plates were read at OD = 405 nm.

Additional Lipids for Liposome Preparations. 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18:1 PE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18:1 PC), 1,2-diarachidonoyl-*sn*-glycero-3-phosphoethanolamine (20:4 PE), and 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine (20:4 PC) were purchased from Avanti Polar Lipids. Cholesteryl hemisuccinate (CH) was purchased from Sigma-Aldrich.

Quantification of Sphingomyelinase Activity. Cells and supernatant containing isolated viral particles were lysed in PBS/1% Triton X-100 and a mixture of protease inhibitors (Sigma). Cell lysates were clarified by centrifugation. Sphingomyelinase activity was determined in each sample using the Amplex Red assay kit (Invitrogen), according to the manufacturer's instructions. The values obtained were normalized using the total protein content, as measured by either the bicinchoninic acid (BCA) (Pierce) or Bradford (Bio-Rad) assay systems.

Hepatitis C Virus Cell Culture Replication and Protein Expression Assays. Jc1-infected Huh7.5 cells were >90% infected as determined by hepatitis C virus (HCV) core immunofluorescence of cells, at which point treatment was started. RNA copy numbers were quantified from within treated cells over a period of 3 d. At each time point, cells were harvested, and HCV RNA was purified using a QIAGEN viral RNA purification kit and quantified by real-time PCR. Viral protein expression was determined by Western blot analysis. At treatment day 3, cellular lysates were prepared to a final protein concentration of 1 mg/mL in 1× PBS/1% Triton X-100. SDS/PAGE was performed by loading 50 μg of protein per well in 4–12% Bis-Tris NuPAGE gels (Invitrogen) and separated using the NuPAGE Mes SDS buffer following the manufacturer's recommendations. Protein was transferred to nitrocellulose membranes, and immunoblotting was carried out using the WesternBreeze chemiluminescent kit (Invitrogen) following the manufacturer's protocol. The mouse HCV core antibody was purchased from Affinity Bioreagents. The antibody against HCV NS5a was a kind gift from Charles Rice (Rockefeller University, New York). The HCV NS5b antibody was a kind gift from Volker Lohmann (University of Heidelberg, Heidelberg). The HCV E1 antibody was a kind gift from Steven Foung (Stanford University, Stanford, CA). The mouse anti-actin antibody was purchased from Abcam.

HIV Replication and Protein Expression Assays. Pooled peripheral blood mononuclear cells (PBMC) were infected with HIV-1 LAI at 50% tissue culture infectious dose = 100 per 4×10^5 cells for 16 h. Cells then were washed three times in medium before further culturing in fresh medium with IL-2 for 4 d. The treatment was started, and cells were harvested every 24 h for 3 d and lysed before quantification of HIV p24 protein from within-cell lysates by p24 capture ELISA as previously described (1). Viral protein expression was determined as described above after harvesting treated infected cells at day 3. All anti-HIV antibodies were obtained from the National Institute for Biological Standards and Control.

Hepatitis B Virus Replication and Protein Expression Assays. Hepatitis B virus (HBV)-infected HepG2.2.2.15 cells were treated at the concentrations indicated for 4 d. Encapsidated viral DNA was purified by phenol-chloroform extraction, as described previously (2), and real-time PCR was performed using the SensiMix Plus Kit (Quantance) and HBV-specific primers. To monitor viral protein expression, cells were lysed in PBS containing 1% Triton X-100 and a mixture of protease inhibitors (Sigma). Cell lysates were clarified by centrifugation. Total protein content was measured by the BCA procedure (Pierce). Equal amounts of proteins (50 μg per sample) were analyzed by 12% SDS/PAGE under nonreducing conditions, and viral envelope proteins were visualized using anti-S monoclonal antibodies (Fitzgerald) and the ECL kit (GE Healthcare).

Preincubation of HCV Cell Culture with Free Cholesterol. The RNA of HCV cell culture (HCVcc) particles secreted from untreated and treated Huh7.5 cells following a 4-d incubation period was quantified by real-time PCR as previously described. Samples were normalized by dilution in complete DMEM/10% FBS to the lowest concentration. HCVcc particles were incubated with free cholesterol (Sigma) for 1 h at 37 °C as previously described (3) and were used to infect naïve Huh7.5 cells for HCV core protein immunofluorescence infectivity assays.

1. Pollock S, Dwek RA, Burton DR, Zitzmann N (2008) N-Butyldeoxynojirimycin is a broadly effective anti-HIV therapy significantly enhanced by targeted liposome delivery. *AIDS* 22:1961–1969.
2. Lazar C, et al. (2007) Treatment of hepatitis B virus-infected cells with alpha-glucosidase inhibitors results in production of virions with altered molecular composition and infectivity. *Antiviral Res* 76:30–37.
3. Aizaki H, et al. (2008) Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J Virol* 82:5715–5724.

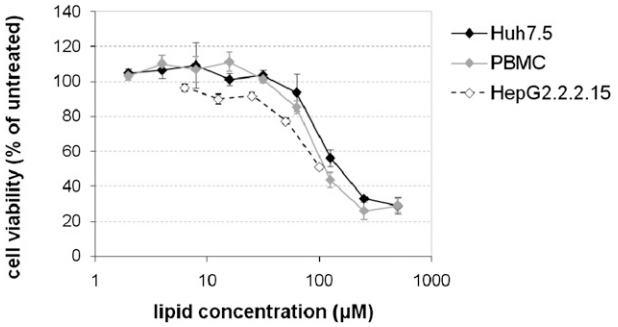

Fig. S1. Toxicity of 22:6 polyunsaturated endoplasmic reticulum (ER) liposomes (PERLs) in Huh7.5 cells, PBMCs, and HepG2.2.2.15 cells. Viability of Jc1-infected Huh7.5 cells, HIV-1–infected PBMCs, and HepG2.2.2.15 cells following 4-d incubation with PERLs encapsulating 1× 150PBS. Final lipid concentrations in the medium ranged from 0 to 500 μM. Cells were assayed after the incubation using an MTS-based assay to calculate decreases in cell viability in relation to the mock-treated control sample. Data represent the mean values from three independent experiments performed in triplicate.

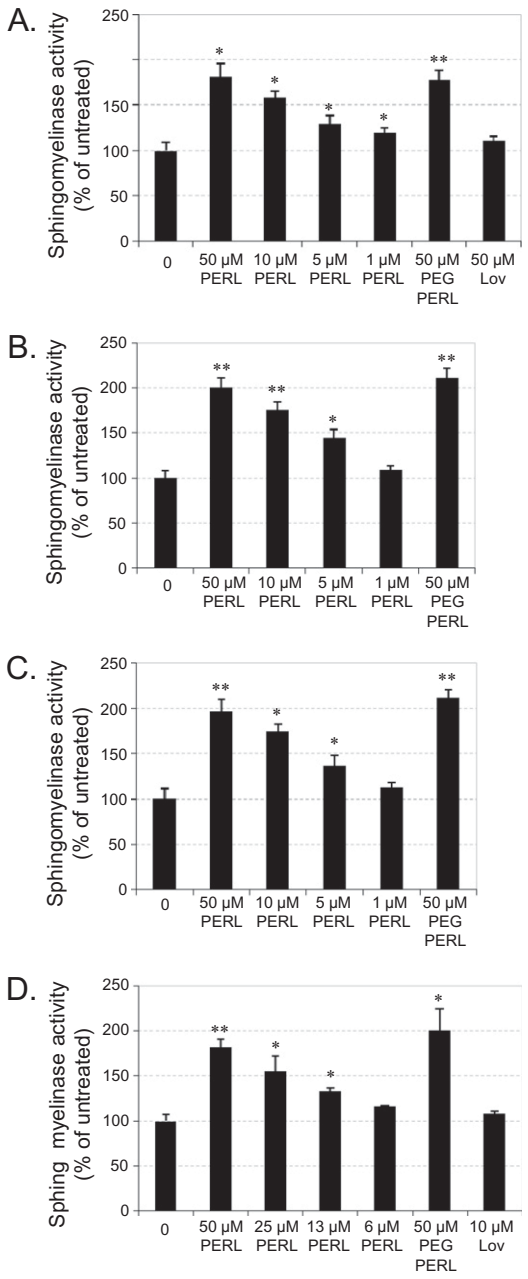

Fig. S2. PERL-treated cells increase sphingomyelinase activity following a 4-d treatment period. Enzymatic activity was quantified by kinetic spectrofluorometric assays on cells, and results were normalized to total protein levels within the same samples. Cells assayed include (*A*) Huh7.5 cells, (*B*) PBMCs, (*C*) $CD4^+$ cells purified from pooled PBMCs, and (*D*) HepG2.2.2.15 cells. PEGylated PERLs (PEG) are PERL liposomes in which 3% of the lipids have been replaced with a PEG-PE 2000 lipid for increased in vivo stability. Lovastatin (Lov) is used as a control for both Huh7.5 and HepG2.2.2.15 cells. Data represent the mean and SD from three independent experiments performed in triplicate and are presented as a percentage of the untreated samples. Significant differences from this value are denoted by $*P < 0.05$ and $*(P < 0.001$ (*t* tests).

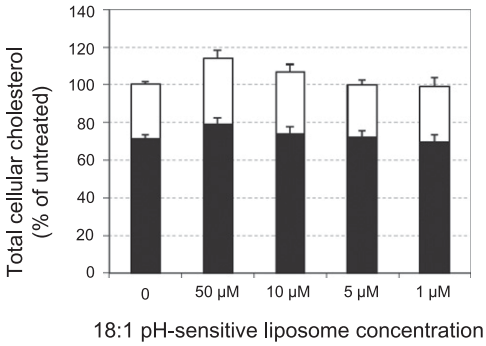

Fig. S3. Cells treated with pH-sensitive liposomes (18:1 PE:CH, 3:2 molar ratio) do not have decreased cholesterol levels following a 4-d treatment period. Free (black) and esterified (white) cholesterol levels were quantified by kinetic spectrofluorometric assays on Huh7.5 cells, and results were normalized to total protein levels within the same samples. Data represent the mean and SD of three independent experiments performed in triplicate and are presented as a percentage of the untreated samples. No significant differences observed in treated and untreated samples were observed.

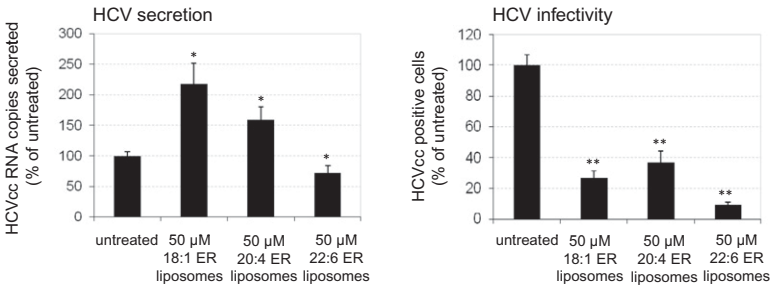

Fig. S4. Jc1 HCVcc secretion and the infectivity of secreted virions with ER liposome treatment using varying lipid saturations. Huh7.5 cells were infected at a multiplicity of infection = 0.5, and the secretion of HCVcc RNA copies was quantified from cellular supernatant following a 4-d incubation period by quantitative PCR. Infectivity of the secreted HCVcc was determined by infection of naïve Huh7.5 cells for 1 h with cell supernatant normalized to HCVcc RNA levels and incubated for 48 h. Cells then were fixed and stained with an anti-HCV core antibody to quantify the number of infected cells and with DAPI to visualize all cells. All results represent the mean and SD of samples from three independent experiments performed in triplicate. Data are presented in relation to the untreated controls (100%), and significant differences from this value are denoted by $*P < 0.05$ and $**P < 0.001$.

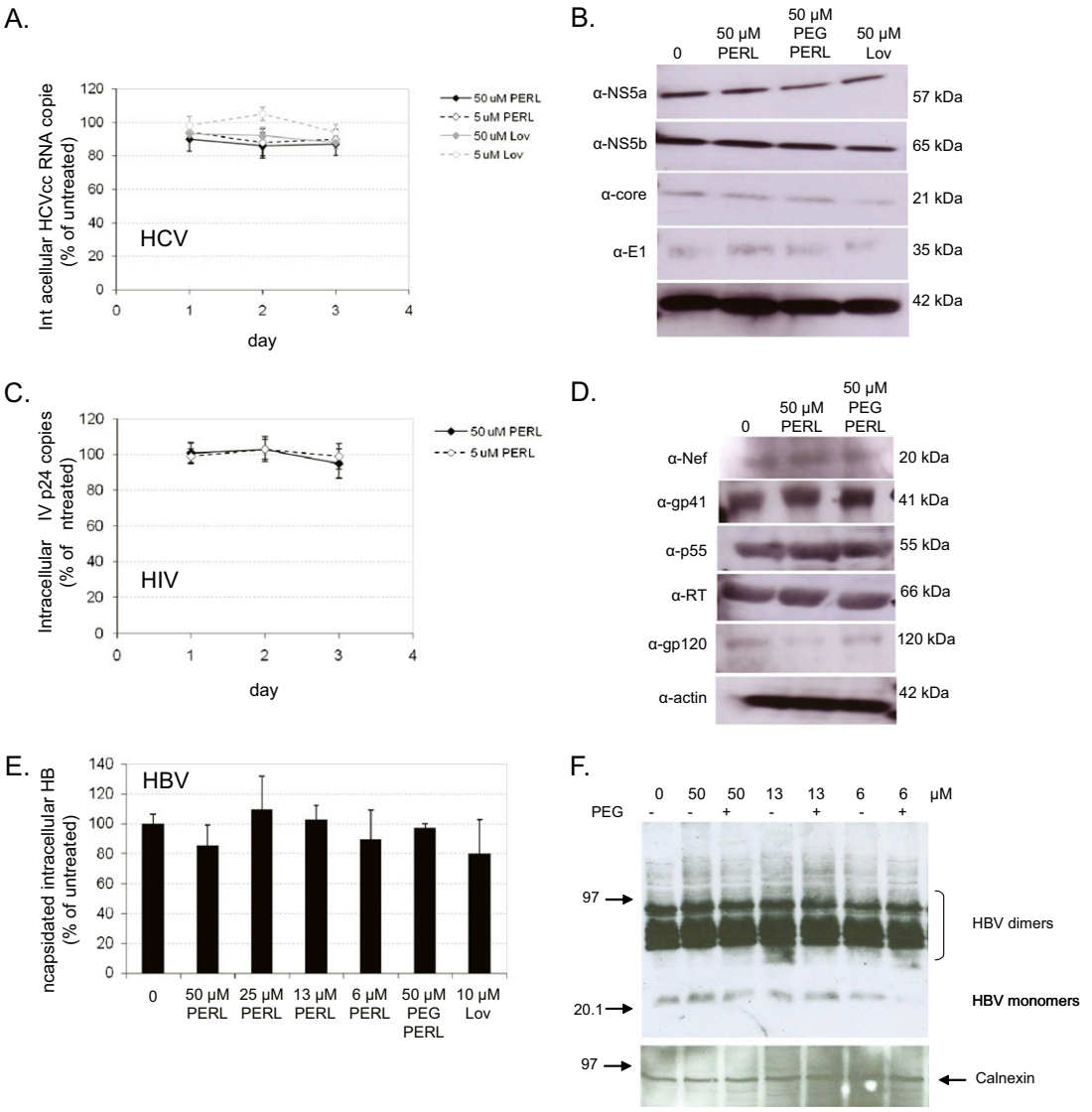

Fig. S5. Treatment with PERLs does not affect the intracellular replication or protein expression of HCV, HIV, or HBV. (*A*) HCVcc RNA within infected Huh7.5 cells was quantified by real-time PCR during treatment with both PERLs and lovastatin at different concentrations over a period of 3 d. (*B*) On day 3 of treatment, HCVcc-infected Huh7.5 cell lysates were separated on SDS/PAGE gels and used for immunoblotting of various HCV proteins to monitor their expression compared with an untreated control. Actin was used as a loading control. (*C*) HIV-infected PBMCs were treated with PERLs at different concentrations, and intracellular p24 levels were quantified over 3 d by capture p24 ELISAs. (*D*) HIV-infected cell lysates from day 3 of treatment with PERLs separated by SDS/PAGE. HIV proteins were detected by Western blot analysis, and treated samples are compared with an untreated control. (*E*) HBV replication assays in which infected HepG2.2.2.15 cells were treated with PERLs or lovastatin for 4 d. Viral replication was monitored by specific real-time PCR on intracellular HBV following purification of encapsidated virus (encapsidation being strictly dependent on the viral polymerase). (*F*) HBV proteins were analyzed using a disulfide-dependent antibody specific for the S domain of the envelope proteins. This antibody also determines whether any major conformational change occurred as a result of treatment. This analysis was performed under nonreducing conditions. Calnexin expression was used as a loading control and run under reducing conditions. All experiments were repeated at least twice, and data are presented in relation to an untreated control.

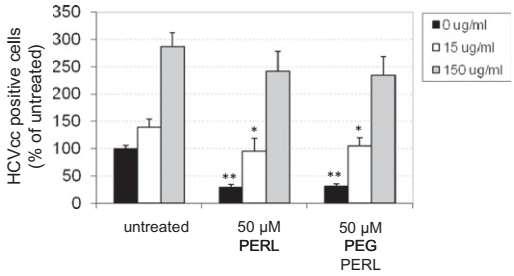

Fig. S6. Infectivity of 22:6 ER liposome-treated, PEGyated 22:6 ER liposome-treated, and untreated Jc1 HCVcc treated with exogenous cholesterol (final concentrations of 15 μg/mL and 150 μg/mL cholesterol). Virus samples were taken from a 4-d incubation in the presence of liposomes, normalized to the lowest concentration of Jc1 RNA (determined by quantitative PCR), and pretreated with cholesterol for 1 h at 37 °C. After the addition of cholesterol, viral samples were used to infect naïve Huh7.5 cells, and the infectivity of these samples was quantified as previously described. Results represent the mean and SD of triplicate samples from three independent experiments. Data are presented in relation to the untreated control (100%), and significant differences between samples treated with similar amounts of free cholesterol are denoted by $*P < 0.05$ or $**P < 0.001$.

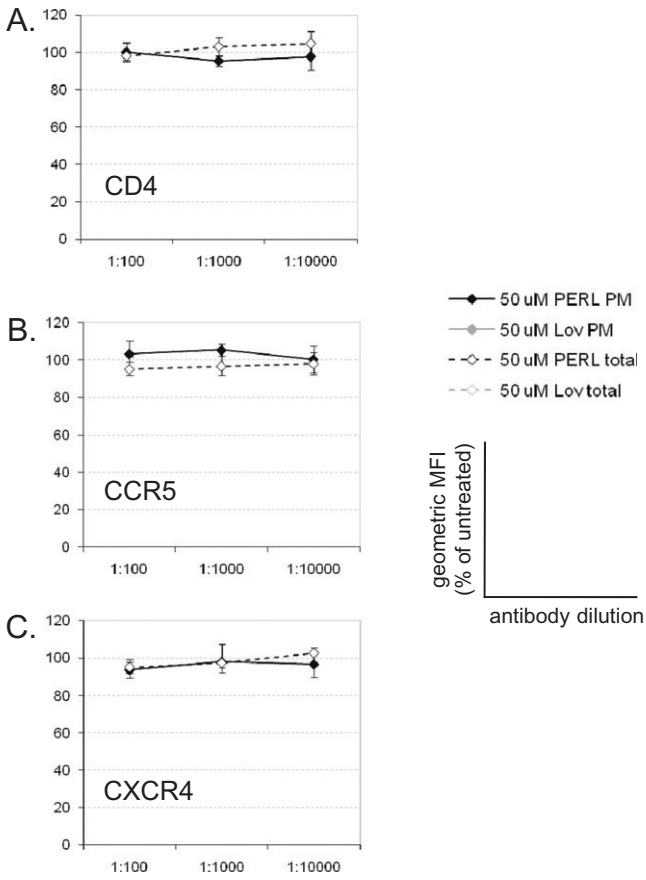

Fig. S7. Pretreatment of $CD4^+$ T cells with PERLs does not affect the localization of HIV receptors CD4, CCR5, and CXCR4. Flow cytometric analysis of uninfected $CD4^+$ T cells purified from PBMCs and treated with 50 μM PERLs for 4 d. Both plasma membrane (PM) and total expression of (*A*) CD4, (*B*) CCR5, and (*C*) CXCR4 were quantified as described in the main text. Flow cytometry data are presented as the geometric mean fluorescent intensities (MFI) of each sample and represent the mean and SD of triplicate samples from two independent experiments. Data are presented in relation to the untreated control (100%).