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A long-acting formulation of the integrase inhibitor raltegravir protects humanized BLT mice from repeated high-dose vaginal HIV challenges

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Objectives: Pre-exposure prophylaxis (PrEP) using antiretroviral drugs (ARVs) has been shown to reduce HIV transmission in people at high risk of HIV infection. Adherence to PrEP strongly correlates with the level of HIV protection. Long-acting injectable ARVs provide sustained systemic drug exposures over many weeks and can improve adherence due to infrequent parenteral administration. Here, we evaluated a new long-acting formulation of raltegravir for prevention of vaginal HIV transmission.

Methods: Long-acting raltegravir was administered subcutaneously to BALB/c, NSG (NOD–scid–gamma) and humanized BLT (bone marrow–liver–thymus) mice and rhesus macaques. Raltegravir concentration in peripheral blood and tissue was analysed. Suppression of HIV replication was assessed in infected BLT mice. Two high-dose HIV vaginal challenges were used to evaluate protection from HIV transmission in BLT mice.

Results: Two weeks after a single subcutaneous injection of long-acting raltegravir in BLT mice (7.5 mg) and rhesus macaques (160 mg), the plasma concentration of raltegravir was comparable to 400 mg orally, twice daily in humans. Serum collected from mice 3 weeks post-administration of long-acting raltegravir efficiently blocked HIV infection of TZM-bl indicator cells *in vitro*. Administration of long-acting raltegravir suppressed viral RNA in plasma and cervico-vaginal fluids of infected BLT mice, demonstrating penetration of active raltegravir into the female reproductive tract. Using transmitted/founder HIV we observed that BLT mice administered a single subcutaneous dose of long-acting raltegravir were protected from two high-dose HIV vaginal challenges 1 week and 4 weeks after drug administration.

Conclusions: These preclinical results demonstrated the efficacy of long-acting raltegravir in preventing vaginal HIV transmission.

Introduction

Despite significant advances in treatment of HIV infection that remarkably prolong the lifespan and greatly improve the quality of life of people living with HIV/AIDS, new cases continue to occur in both developed and developing countries.[1] The prevention of HIV infection remains a critical public health priority. Use of antiretroviral drugs (ARVs) for prevention of sexual transmission of HIV in high-risk populations [pre-exposure prophylaxis (PrEP)] has been validated in multiple clinical trials.[2–5] Clinical trials showed that daily use of tenofovir disoproxil fumarate or Truvada (combination of tenofovir disoproxil fumarate with emtricitabine) reduces the risk of HIV-1 infection by 44%–75%; however, only 50%–81% of participants in these clinical trials had consistently detectable tenofovir in blood samples.[6] Efficacy of PrEP for participants with detectable plasma tenofovir increased to 74%–92%, suggesting that high drug regimen adherence is necessary to effectively prevent sexual HIV transmission by PrEP.[7,8] Long-acting, parenteral ARV formulations that would require less frequent dosing may represent a viable alternative for HIV prevention. Two ARVs formulated as long-acting injectable crystalline nanosuspensions have progressed to clinical trials.

Cabotegravir (GSK-744) is a potent integrase strand transfer inhibitor formulated as a long-acting parenteral nanosuspension at a concentration of 200 mg/mL. Monthly or quarterly intramuscular injections of cabotegravir in humans maintained plasma drug concentrations 4-fold higher than the protein-adjusted 90% inhibitory concentration (PA-IC_{90}).[9] Protection from rectal and vaginal SHIV transmission was also observed in macaques treated with cabotegravir and plasma drug concentrations appeared similar to those observed in humans.[10–12]

Long-acting rilpivirine is an NNRTI formulated as a nanosuspension with a concentration of 300 mg/mL. A single intramuscular injection of long-acting rilpivirine provides sustained release of rilpivirine into plasma over 3 months in dogs, 2 months in rats[13,14] and 3 weeks in mice.[14] In humans, a single intramuscular administration of long-acting rilpivirine leads to substantial levels of rilpivirine in plasma, cervico-vaginal fluid and vaginal tissue for 84 days.[15] Recently, we evaluated long-acting rilpivirine for protection from vaginal transmission of transmitted/founder HIVs, using a preclinical *in vivo* model of vaginal HIV transmission, humanized BLT (bone marrow–liver–thymus) mice.[16–20] Our results demonstrated that a single intramuscular injection of 15 mg of long-acting rilpivirine offers significant protection from two consecutive high-dose HIV-1 challenges 1 and 4 weeks after drug administration.[21]

One highly desirable pharmacokinetic property for selection of PrEP agents targeting sexual HIV transmission is their ability to rapidly distribute to genital and rectal tissues, where the initial rounds of viral replication occur.[8] ARVs differ greatly in their capability to penetrate mucosal tissues or secretions.[22–24] Raltegravir (Isentress) is an HIV integrase strand transfer inhibitor. Several studies have shown good penetration and distribution of raltegravir in the female genital tract and cervico-vaginal fluid.[25,26] Similarly, raltegravir penetration in the seminal compartment was found to be higher (although somewhat variable) when compared with blood.[22,27,28] Macaques orally treated with 50 mg/kg raltegravir suspension in PBS also showed a high and sustained concentration of raltegravir in vaginal and rectal secretions.[29] These studies suggest that, in addition to good tolerability, effective antiretroviral activity and ability to inhibit viral replication by binding tightly to pre-integration complex, raltegravir may hold promise as an HIV prophylactic agent for its accumulation in mucosal tissue. This is further supported by the fact that in the RAG-hu (humanized BALB/c-Rag2$^{-/-}\gamma c^{-/-}$) mouse model daily oral administration of raltegravir was able to protect against a single vaginal HIV-1 challenge.[30]

Here, we present the *in vivo* and *in vitro* evaluation of a new, long-acting formulation of raltegravir for HIV-1 treatment during acute infection and for PrEP of vaginal transmission. Our results demonstrate excellent pharmacokinetic properties in non-human primates (NHPs) and robust antiretroviral activity and long-term protection from repeated vaginal HIV challenges in humanized BLT mice.

Methods

Long-acting raltegravir preparation, administration and plasma and tissue level analysis

Suspension of long-acting raltegravir was prepared by reconstitution of milled γ-irradiated raltegravir with sterile vehicle containing 5% polyethylene glycol 3350, 0.2% polysorbate 80 and 5% mannitol in water. The final drug concentration was 50 mg/mL.

All animal experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee guidelines of the University of North Carolina for mice and Merck Research Laboratories, Merck & Co., Inc. for mice and NHPs.

A 30 mg/kg dose was administered to rhesus macaques in two injections per animal (15 mg/kg each injection, 300 μL/kg long-acting raltegravir formulation) subcutaneously between the shoulder blades. One subcutaneous injection of 30 mg/kg (600 μL/kg formulation) and/or 300 mg/kg (6 mL/kg formulation) long-acting raltegravir was administered into the lumbar part of the back of BALB/c (n=3), NSG (n=10) and BLT (n=13) mice.

Plasma was isolated from peripheral blood samples collected in EDTA-treated tubes from mouse retro-orbital venous sinus or the femoral vein of macaques and stored at −80°C until analysis.

The concentrations of raltegravir were determined by tandem LC–MS assays following a protein precipitation step. Aliquots of plasma (50 μL) were precipitated by addition of 150 μL of acetonitrile containing 0.1% formic acid and the internal standard followed by centrifugation at 4000 rpm for 10 min. An aliquot of the supernatant was combined 1:1 with water containing 0.1% formic acid prior to analysis. Tandem LC–MS analysis was performed on a Thermo Transcend LX2 system with an HTS PAL CTC autosampler interfaced to an API-5000 mass spectrometer utilizing the turbo ionspray interface (Life Technologies, Carlsbad, CA, USA). Separation of raltegravir was achieved on an Aquity XSelect HSS T3 column (50×2.1 mm, 2.5 μm) using a mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.75 mL/min. The chromatography was run following initial equilibration using a step gradient as follows: after sample injection, solvent B was maintained at 20% for 0.25 min before it was increased linearly to 98% of solvent B over a 1.5 min period. The fraction of solvent B was maintained for 0.42 min, then returned to the initial conditions and kept for an additional 0.83 min. The total run time was 3 min. Quantification was done by monitoring the transition of m/z 445.2 → m/z 109.1 for raltegravir and m/z 281.3 → m/z 193.1 for imipramine (the internal standard). The method was linear across a concentration range of 2–5000 nM.

Homogenates of tissues [liver, lung, female reproductive tract (FRT) and spleen] from non-infected NSG mice (n=5) were prepared in 3 volumes of deionized water (4 for liver samples) using a Spex Geno/Grinder tissue homogenizer and analysed using the methods described above with calibration curves prepared in the appropriate tissue homogenates. Concentrations of raltegravir after administration of the long-acting formulation were analysed in NSG mice as well as in BLT mice. Similar pharmacokinetic profiles in peripheral blood were noted in both NSG and BLT mice (see Figure 2a and b). Given the higher cost of BLT mice, we used NSG mice for the detailed pharmacokinetic analysis and tissue analysis of drug levels.

TZM-bl cell culture and in vitro HIV-1 inhibition by the long-acting raltegravir formulation

TZM-bl cells were obtained from Dr John Kappes and procured through the NIH AIDS Research and Reference Reagent Program. TZM-bl cells were maintained in DMEM containing 10% FBS, 25 mM HEPES, 500 units/mL penicillin and 500 μg/mL streptomycin (TZM-bl medium) and cultured at 37°C and 5% CO_2. TZM-bl cells were plated in 96-well plates at a density of 1×10^5 cells per well in TZM-bl medium the day before infection. The next day, the medium was removed and serum from long-acting raltegravir-treated or control animals diluted 1:100 or 1:20 in TZM-bl medium was added (100 μL per well). Cells were incubated for 30 min and an additional 100 μL of TZM-bl medium containing 20 μg/mL DEAE–dextran and HIV-1$_{JR\text{-}CSF}$ was added. Final concentration of virus was 3×10^3 tissue culture infectious units (TCIU) per well. Approximately 48 h later, the medium was removed and the luciferase substrate ONE-Glo reagent (Promega, Madison, WI, USA) supplemented with 0.01% Triton X-100 was added to inactivate virus and to allow the measurement of luciferase activity. Average values from five replicates of cells incubated in TZM-bl medium containing serum of treated mice were normalized to the luciferase activity of cells incubated with TZM-bl medium containing serum from untreated mice.

Generation and quantification of HIV

HIV viral stocks were generated by transfecting 293T cells with proviral DNA (pTHRO and pCH040 plasmids obtained from Dr John Kappes via the AIDS Research and Reagent Repository Program, Division of AIDS, NIAID, NIH) using Lipofectamine (Invitrogen). Two days post-transfection, the supernatant was harvested and subsequently concentrated by ultracentrifuging through 20% sucrose solution at 30000 rpm with a Beckman SW-41 Ti rotor for 70 min at 4°C. The titre of the virus was determined by infecting TZM-bl cells. TZM-bl cells were plated in 12-well plates at a density of 1×10^5 cells per well in TZM-bl medium. The next day, the medium was removed and serial dilutions of HIV were made in TZM-bl medium containing 20 μg/mL DEAE–dextran. Approximately 48 h later, the medium was removed and the cells were fixed and stained for β-galactosidase activity. The number of TCIU was calculated.

Generation of humanized BLT mice and intravaginal exposure to HIV-1

BLT mice were generated as described previously.[16,18,19,31–33] Briefly, a 1–2 mm piece of human liver tissue was sandwiched between two pieces of autologous thymus tissue (Advanced Bioscience Resources, Alameda, CA, USA) under the kidney capsule of sublethally irradiated (0.250 Sv) 6- to 8 week-old NOD.Cg- Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (NSG; The Jackson Laboratory, Bar Harbor, ME, USA). Following implantation, mice were transplanted intravenously with haematopoietic CD34+ stem cells isolated from autologous human foetal liver tissue. Human immune cell reconstitution was monitored by flow cytometric analysis of peripheral blood every 2 weeks, as previously described.[16,18,31,33] At the end of the experiments, mice were euthanized by exposure to an excess of tribromoethanol. Mice were maintained at the Division of Laboratory Animal Medicine, University of North Carolina at Chapel Hill (UNC-CH) in accordance with protocols approved by the Institutional Animal Care and Use Committee.

To test the HIV suppression ability of a long-acting raltegravir formulation, female BLT mice ($n=7$) were anaesthetized with sodium pentobarbital and intravaginally challenged with transmission/founder viruses (HIV$_{CHO40}$ 3.5×10^5 TCIU or HIV$_{THRO}$ 3.5×10^5 TCIU and HIV$_{RHPA}$ 3.1×10^5 TCIU). Four weeks later infected mice were treated subcutaneously in the lumbar part of the back with (300 mg/kg) 7.5 mg of long-acting raltegravir formulation. Mice were monitored for the presence of viral RNA and drug level in plasma of peripheral blood and cervico-vaginal lavage (CVL) until virus rebound. For HIV-1 protection, female BLT mice ($n=6$) received a single injection of 300 mg/kg long-acting raltegravir subcutaneously into the lumbar part of the back. One week later, mice were anaesthetized with sodium pentobarbital and intravaginally challenged with transmission/founder viruses (HIV$_{CHO40}$ 3.5×10^5 TCIU or HIV$_{THRO}$ 3.5×10^5 TCIU). Three weeks later, uninfected mice were challenged vaginally with different transmission/founder HIV. Specifically, mice receiving HIV$_{THRO}$ in the first challenge received HIV$_{CHO40}$ in the second challenge and mice receiving with HIV$_{CHO40}$ in the first challenge received HIV$_{THRO}$ in the second challenge. Mice were monitored for the presence of viral RNA and drug level in plasma of peripheral blood for 6 weeks.

Analysis of HIV-1 infection in humanized BLT mice

Infection of BLT mice with HIV-1 was monitored in peripheral blood or cervico-vaginal secretions by determining levels of viral RNA in plasma or CVL by isolation of RNA with the RNeasy Mini Kit (Qiagen) followed by one-step real-time RT–PCR assay, using primers 5′-CATGTTTTCAGCATTATCAGAAGGA-3′, 5′-TGCTTGATGTCCCCCCACT-3′ and the MGB probe carboxyfluorescein (FAM)-5′-CCACCCCACAAGATTTAAACACCATGCTAA-Q (non-fluorescent quencher)-3′ (Applied Biosystems). The percentage of human CD4+ T cells in peripheral blood of BLT mice before and after challenge was determined by flow cytometry with respective antibodies: hCD45-APC, hCD3-FITC, hCD4-PE and hCD8-PerCP (eBioscience). Flow cytometry data were collected using a BD FACSCanto cytometer and analysed using FlowJo software. In the experiment evaluating the ability of long-acting raltegravir to protect against HIV transmission, we defined protection as the absence of viral RNA in the plasma of mice at each timepoint tested as well as the absence of viral DNA in the tissues analysed at necropsy. The presence of viral DNA in tissues and peripheral blood collected from BLT mice at the end of the experiment was determined by real-time PCR analysis of DNA extracted from 5×10^4–4×10^6 cells from harvested tissue (spleen, lymph nodes, bone marrow, liver, lung and FRT) or from 15 μL of peripheral blood cells. DNA was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). Real-time PCR for viral DNA was performed using the same primers and probe combination as listed above. As a control for the presence of human cells as well as for the normalization of viral DNA, real-time PCR was also performed for human γ-globin.

Identification of transmitted viruses

Viruses replicating in infected animals were identified by direct sequence analysis. Viral RNA was isolated from plasma using QIAamp viral RNA columns (Qiagen) according to the manufacturer's protocol, and cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen) with the primer 5′-GTGGGTACACAGGCATGTGTGG-3′. cDNA was amplified by nested PCR using the Expand High Fidelity PCR System (Roche). PCR primers were designed to anneal in regions with the fewest possible primer mismatches to HIV-1$_{CH040}$, HIV-1$_{RHPA}$ and HIV-1$_{THRO}$ sequences. Primer sequences were as follows: outer forward primer, 5′-TGCATATTGTGAGTCTGTTACTATGTTTACT-3′; reverse primer 5′-CAGGAGCAGATGATACAG-3′; inner forward primer, 5′-GTAGGACCTACACCTGTCAAC-3′; reverse primer 5′-CCTGCAAAGCTAGGTGAATTGC-3′. Amplified viral DNA was sequenced and compared with sequences of transmitted/founder viruses.

Statistical analysis

Statistical differences between treated and control animals in the efficacy of long-acting raltegravir in protecting against vaginal HIV-1 transmission were determined with the log-rank/Mantel–Cox test. Correlation between plasma level of raltegravir and viral RNA in plasma and CVL was evaluated using the non-parametric Spearman correlation coefficient. All statistical analyses were performed using GraphPad Prism software (version 6).

Results

Pharmacokinetic profiles of long-acting raltegravir in peripheral blood of BALB/c mice and NHPs

First, we evaluated the pharmacokinetics of long-acting raltegravir in naive BALB/c mice and NHPs. The plasma profile of a single subcutaneous dose of 30 mg/kg long-acting raltegravir administered to naive BALB/c mice is shown in Figure 1(a). Raltegravir was measureable in plasma at 1 h post-treatment (first timepoint) and diminished below PA-IC$_{90}$ within 4 days. The plasma profile of the same dose of 30 mg/kg long-acting raltegravir in uninfected rhesus monkey administered subcutaneously is shown in Figure 1(b and c). The decline of raltegravir concentrations in plasma of NHPs was slow compared with that observed in BALB/c mice. Notably, mean plasma raltegravir concentration was 3-fold above PA-IC$_{90}$ at 14 days post-administration of long-acting raltegravir in NHPs. Differences seen in plasma profiles of raltegravir between BALB/c mice and rhesus macaques administered the same dose of long-acting raltegravir may reflect interspecies differences in rates of drug release from the administration depot and physiology. Similar interspecies differences in plasma profiles have also been observed for formulations of long-acting rilpivirine.[14]

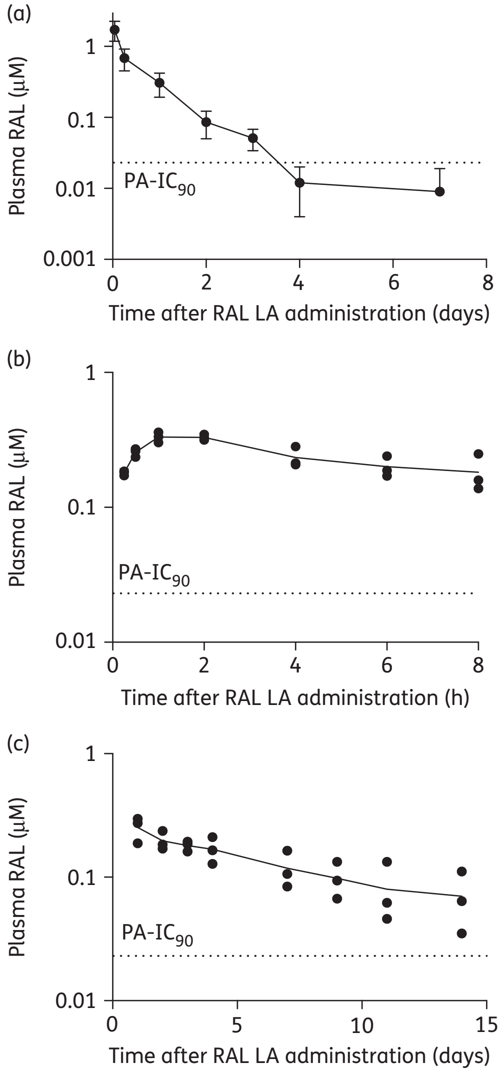

Figure 1. Pharmacokinetic profiles of long-acting raltegravir in BALB/c mice and NHPs. (a) Plasma concentration of raltegravir in BALB/c mice was evaluated at the indicated times after a single subcutaneous injection of 0.75 mg (30 mg/kg) of long-acting raltegravir (1, 6, 24, 48, 72, 96 and 168 h) using tandem LC–MS assays as described in the Methods section. (b and c) Pharmacokinetic profiles of long-acting raltegravir in plasma of rhesus macaques during a period of 8 h (0.25, 0.5, 1, 2, 4, 6 and 8 h) (b) or 14 days (1, 2, 3, 4, 7, 9, 11 and 14 days) (c) after subcutaneous administration of a single dose of 160 mg (30 mg/kg) of long-acting raltegravir. Continuous lines represent mean plasma concentrations and broken lines represent PA-IC$_{90}$ values (0.023 μM). RAL, raltegravir; RAL LA, long-acting raltegravir.

Plasma and tissue concentrations of raltegravir and antiviral activity of serum in NSG and BLT mice treated with long-acting raltegravir

In order to achieve plasma concentrations of raltegravir in mice that will reach levels above PA-IC$_{90}$ for several weeks, we tested a higher dose of long-acting raltegravir. Uninfected immunodeficient mice (NSG) and humanized BLT mice received 300 mg/kg of long-acting raltegravir subcutaneously and drug levels in plasma were monitored for 4 weeks. The higher dose of long-acting raltegravir formulation was well tolerated and no injection site reactions were observed. We also did not observe any signs of overt toxicity, changes in mouse behaviour, movement, water consumption or weight loss. Plasma concentrations were similar in NSG and BLT mice (Figure 2a and b). Interestingly, the higher dose of subcutaneous long-acting raltegravir led to sustained concentrations of plasma raltegravir as mean concentrations were at or above PA-IC$_{90}$ for 4 weeks after the administration. The concentration of raltegravir in liver, lung, spleen and FRT of NSG mice was measured at day 7 ($n=5$) and day 28 ($n=5$) after subcutaneous administration of 300 mg/kg long-acting raltegravir (Figure 2c). At day 7 after administration of long-acting raltegravir, the concentration of drug in most tissues evaluated was at least 10-fold higher than PA-IC$_{90}$. Lung and FRT had raltegravir levels comparable to plasma and the tissue/plasma concentration ratios for lung and FRT were 0.83 (range 0.63–1.20) and 0.93 (range 0.51–2.05), respectively. The concentration of raltegravir in spleen was lower than in plasma; the mean spleen/plasma concentration ratio was 0.43 (range 0.33–0.56), whereas the liver/plasma concentration ratio was much higher (4.89; range 3.51–6.12), as would be anticipated given that the liver is the major organ of elimination for this drug. Twenty-eight days after administration of long-acting raltegravir we were not able to detect raltegravir in most samples. Levels of raltegravir decreased below the limit of quantification (LOQ: lung, spleen 0.04 μM, liver 0.05 μM; FRT 0.016 μM) in most tissues. It should be noted, however, that the LOQ for several tissues was above the PA-IC$_{90}$.

Next, we measured the ability of serum from uninfected NSG mice treated with long-acting raltegravir to block *in vitro* infection of TZM-bl cells with HIV-1$_{\text{JR-CSF}}$. For this analysis we administered a single subcutaneous dose (300 mg/kg) of long-acting raltegravir to NSG mice, collected serum at days 1, 4, 13 and 22 post-administration and evaluated for antiviral activity *in vitro*. The inhibitory activity of serum from mice treated with long-acting raltegravir was compared with antiviral activity of serum from naive (control) mice. As shown in Figure 2(d), serum from treated animals collected 2 weeks post-drug administration and diluted 1:20 resulted in ~90% inhibition of HIV-1 infection in TZM-bl cells *in vitro*, and ~45% inhibition when collected 3 weeks after administration of long-acting raltegravir. Serum collected 2 weeks after long-acting raltegravir administration and diluted 1:100 reduced infection by ~35% (Figure 2d). Collectively, these data showed the presence of active raltegravir in plasma of drug-treated mice beyond 3 weeks post-drug administration.

Suppression of HIV-1 replication in vivo by long-acting raltegravir

To establish the effect of long-acting raltegravir on HIV infection *in vivo*, BLT mice were challenged vaginally with three different transmitted/founder viruses (HIV-1$_{\text{THRO}}$, HIV-1$_{\text{CH040}}$ and HIV-1$_{\text{RHPA}}$). Infection, as determined by the presence of HIV RNA in plasma, was established 1–2 weeks after exposure to virus. At 3 weeks post-infection, mice were treated with a single dose of long-acting raltegravir (300 mg/kg) administered subcutaneously and the levels of viral RNA and drug concentration were monitored for an additional 6 weeks or until evidence of viral rebound. Viral rebound was defined as the timepoint after long-acting raltegravir administration when we were able to detect a continuous increase in

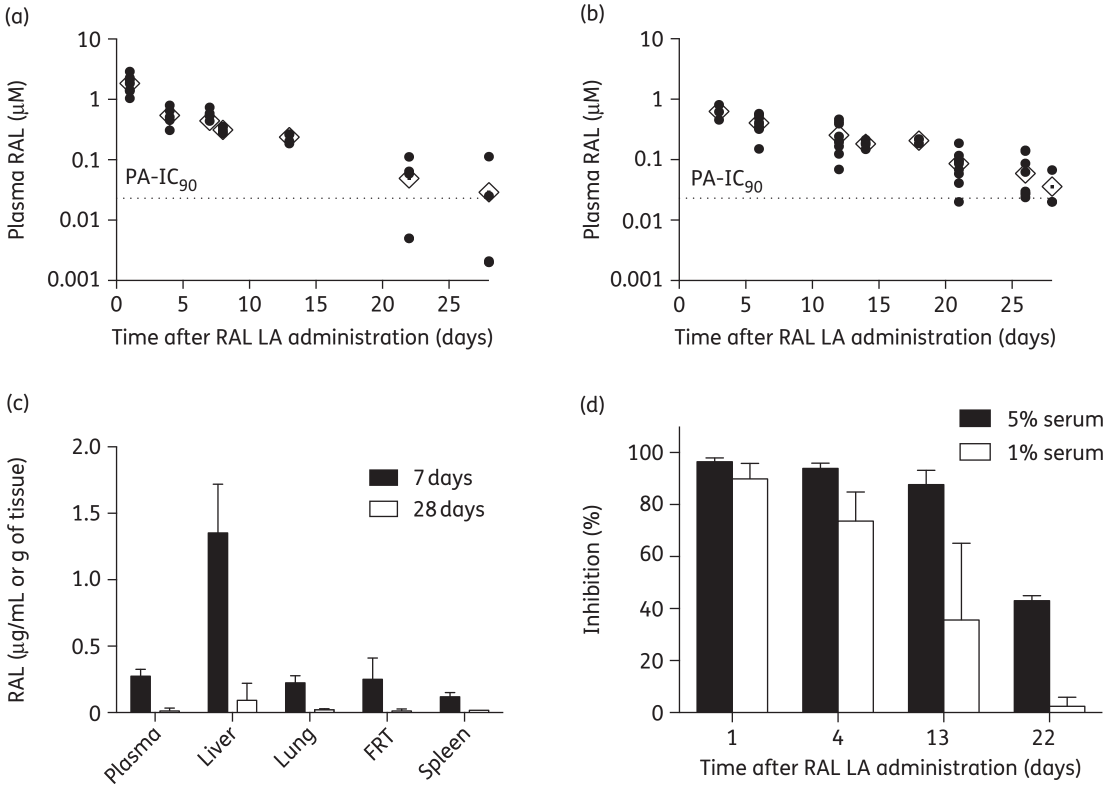

Figure 2. Pharmacokinetic profiles of long-acting raltegravir in NSG and BLT mice and *in vitro* inhibition of HIV-1 infection with serum obtained from mice treated with long-acting raltegravir. (a) Female NSG mice ($n=10$) received 300 mg/kg long-acting raltegravir subcutaneously. The drug concentration in peripheral blood was measured at days 1, 4 and 7 (24, 96 and 168 h) ($n=5$) and at days 1, 8, 13, 22 and 28 (24, 192, 312, 528 and 672 h) ($n=5$) by tandem LC–MS assays following a protein precipitation step as described in the Methods section. The assay had an LOQ of 0.002 μM. (b) Raltegravir concentrations in the plasma of BLT mice (black circles) administered 300 mg/kg of long-acting raltegravir subcutaneously. Diamonds denote the mean plasma concentration at each timepoint. The broken line represents PA-IC_{90}. The LOQ was 0.02 μM. $n=13$. (c) Tissues of NSG mice were collected 7 and 28 days after subcutaneous administration of 300 mg/kg long-acting raltegravir and analysed for raltegravir concentration by HPLC. $n=5$. LOQ: liver, 0.05 μM; lung, 0.04 μM; FRT, 0.016 μM; and spleen, 0.04 μM. (d) The TZM-bl indicator cell line was pre-incubated (30 min) in the presence of 1% and 5% serum from NSG mice treated with 300 mg/kg of long-acting raltegravir that was collected at the indicated days post-administration. Cells were then infected with HIV-$1_{JR\text{-}CSF}$ (still in the presence of mouse serum). Infection of cells was evaluated as a function of luciferase activity produced by the indicator cells (ONE-Glo assay) 48 h after administration of the virus. Data were normalized to the luminescence of cells cultured in serum from untreated mice (0% inhibition). RAL, raltegravir; RAL LA, long-acting raltegravir.

plasma viral RNA after suppression (Figure 3a). Rebound virus was sequenced to determine whether mutations associated with drug resistance developed. As shown in Figure 3(b), viral RNA levels declined rapidly after treatment with long-acting raltegravir in all animals and reached a nadir within 2 weeks of treatment. In the case of the animals infected with HIV-1_{THRO}, viraemia was suppressed below the LOQ (800 copies of HIV RNA per mL). Two animals infected with HIV-1_{RHPA} were suppressed below the LOQ and in another we observed a >2 log decrease in viraemia. In the case of HIV-1_{CH040}, viraemia dropped by >1.5–2.5 log but was not fully controlled in any of the animals treated with long-acting raltegravir (Figure 3c). Viral suppression was maintained for an average 11.8 ± 7.7 days (range 1–19 days) and viral rebound was observed 26.4 ± 7.6 days (range 19–39 days) after administration of long-acting raltegravir (Table 1). In order to determine whether drug resistance mutations were acquired, viral RNA from plasma was sequenced. We did not observe any mutations in any of the samples obtained from animals infected with RHPA and CH040 strains. In the case of one THRO-infected mouse, we found a single-nucleotide mutation resulting in an amino acid change at position 268 (integrase domain) from isoleucine to leucine (I268L). This mutation, however, has not been associated with resistance to raltegravir.[34] We also monitored levels of viral RNA in the CVL of some animals. Following administration of long-acting raltegravir, we observed levels of viral RNA in CVL to be suppressed below the LOQ (400 copies/mL) in all animals analysed (Figure 3d). Like in plasma, viral rebound was noted in all animals. Suppression of viral RNA persisted in CVL for an average 12.5 ± 8.3 days (range 1–21 days) and viral rebound appeared on average 29.8 ± 8.8 days (range 21–42 days) after administration of long-acting raltegravir (Table 1). These results show that active raltegravir effectively penetrated the FRT and suppressed viral replication for several weeks. Interestingly, HIV-1_{CH040} replication was suppressed in CVL longer and more efficiently than in plasma (Figure 3c and d). However, these differences were not found to be statistically significant ($P=0.0773$). The concentration of raltegravir in plasma was sustained and remained above or at PA-IC_{90} for 4 weeks after administration of long-acting raltegravir (Figure 3e). To assess whether

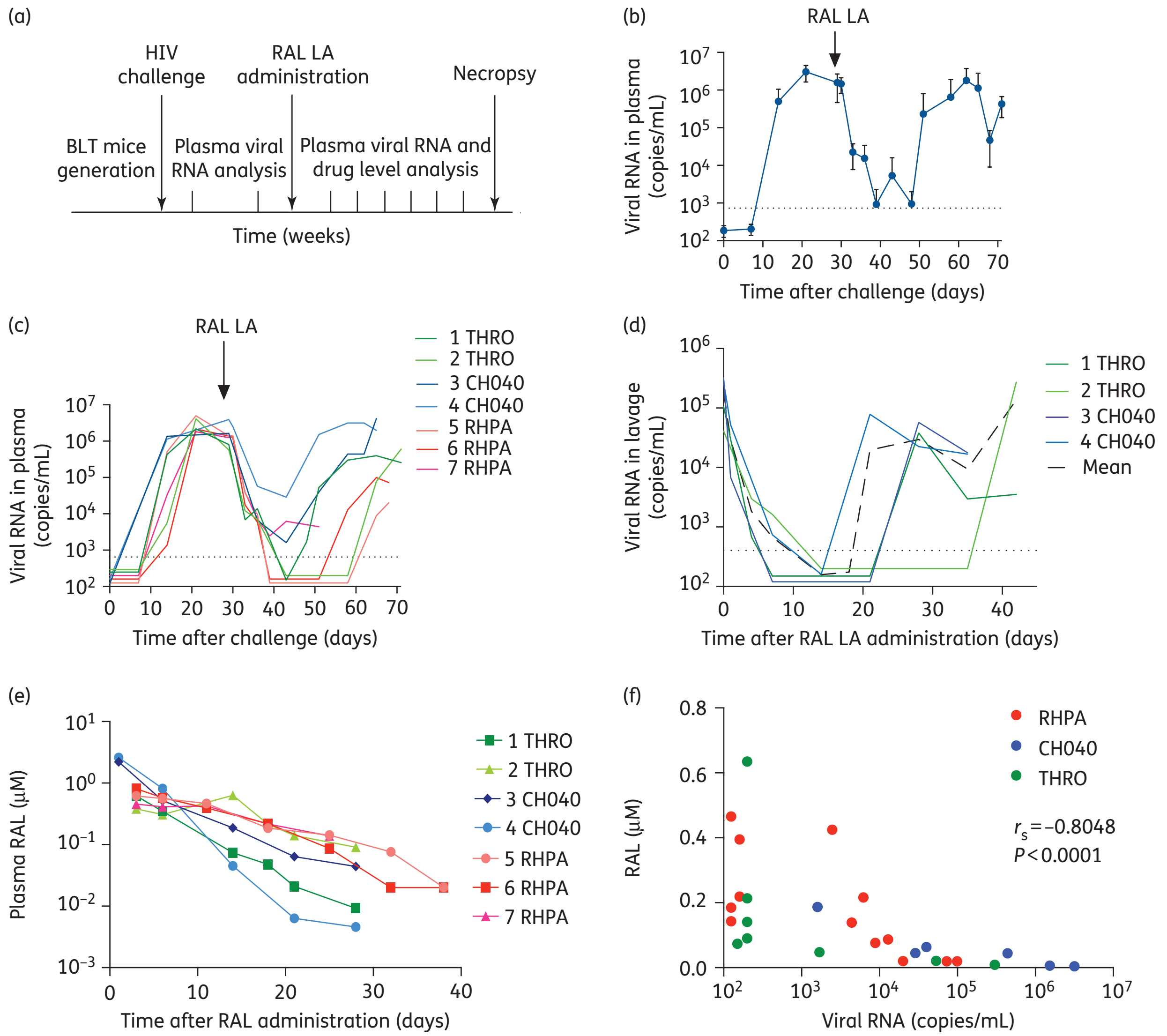

Figure 3. Suppression of HIV-1 replication by long-acting raltegravir in infected humanized BLT mice. (a) Experimental design. BLT mice were inoculated with a transmitted/founder virus (HIV-1_{RHPA}, HIV-1_{CH040} or HIV-1_{THRO}) vaginally. Four weeks later, infected mice were treated once subcutaneously with 300 mg/kg of the long-acting raltegravir formulation; viral load and drug level in plasma were monitored weekly. (b) Mean plasma viral load in BLT mice challenged with different transmitted/founder viruses and treated with long-acting raltegravir 1 month after infection. The broken line indicates the LOQ for the assay (800 copies/mL). $n=7$. (c) Viral load in plasma of individual BLT mice infected with indicated transmitted/founder viruses and treated with long-acting raltegravir 1 month after infection. (d) Viral load in CVL samples from infected BLT mice treated with 300 mg/kg 1 month after viral challenge (the LOQ for this assay was 400 copies/mL lavage fluid). (e) Longitudinal analysis of raltegravir level in plasma of mice treated with long-acting raltegravir and infected with various HIVs. The raltegravir concentration was analysed by tandem LC–MS assays as described in the Methods section. (f) Analysis of correlation between plasma raltegravir concentration and viral RNA in plasma 14–28 days after administration of long-acting raltegravir. Spearman correlation coefficient (r_s) = −0.8048, 95% CI −0.9094 to −0.6044, $P<0.0001$. RAL, raltegravir; RAL LA, long-acting raltegravir.

suppression of HIV correlates with plasma raltegravir concentration, we compared the concentration of raltegravir in plasma with viral RNA in plasma and CVL between 14 and 28 days after long-acting raltegravir administration. As shown in Figure 3(f), raltegravir plasma concentration negatively correlated with plasma viral RNA (Spearman correlation coefficient = −0.8048, 95% CI −0.9094 to −0.6044, $P<0.0001$). However, no significant correlation between raltegravir plasma concentration and CVL viral RNA was found (Spearman correlation coefficient = −0.4179, 95% CI −0.7832 to 0.1618, $P=0.1203$; data not shown).

A single administration of long-acting raltegravir offers significant protection from two separate high-dose vaginal challenges with transmitted/founder HIVs

The ability of long-acting raltegravir to offer protection against vaginal HIV transmission was evaluated using BLT mice. BLT mice have a full complement of human haematopoietic cells present in the FRT that renders them susceptible to HIV infection after vaginal exposure.[16,20] BLT mice have been validated for the evaluation of a number of PrEP approaches.[16–18,31] The overall

Table 1. Suppression of HIV-1 infection by long-acting raltegravir in BLT mice

Mouse number	hCD45 (%)	hCD4 (%)	Virus	Duration of suppression below LOQ in plasma (days)	Time of viral rebound in plasma (days)[a]	Duration of suppression below LOQ in CVL (days)	Time of viral rebound in CVL (days)[a]
1	53.4	60.4	THRO	1	20	14	28
2	54.3	71.0	THRO	15	37	21	42
3	88.4	82.5	CH040	NA	22	14	28
4	91.5	80.4	CH040	NA	22	1	21
5	73.3	72.2	RHPA	19	36	not analysed	not analysed
6	74.1	70.3	RHPA	12	29	not analysed	not analysed
7	83.6	71.7	RHPA	NA	19	not analysed	not analysed
Mean	74.1	72.6		11.8	26.4	12.5	29.8
SD	15.4	7.3		7.7	7.6	8.3	8.8

NA, not applicable; suppression of infection was above LOQ.

BLT mice with the indicated amount of human CD45+ (hCD45) cells and human CD3+CD4+ (hCD4) cells in peripheral blood were challenged with the indicated transmitted/founder virus. Four weeks after the challenge, infected mice were treated subcutaneously with 300 mg/kg long-acting raltegravir formulation. Suppression of viral RNA level in plasma was monitored weekly until virus rebound. The LOQ was 800 copies/mL in plasma and 400 copies/mL in CVL.

[a] Rebound of infection above LOQ or the lowest level of viral RNA in plasma or CVL is presented in days after long-acting raltegravir administration.

approach to evaluating the effect of long-acting raltegravir on vaginal HIV transmission is depicted in Figure 4(a). BLT mice were treated once with long-acting raltegravir (300 mg/kg) via subcutaneous injection or left untreated (controls). One week later mice were challenged vaginally with a high dose of HIV-1$_{\text{THRO}}$ or HIV-1$_{\text{CH040}}$. The presence of viral RNA and raltegravir in plasma was monitored weekly. Four weeks after long-acting raltegravir administration, mice were exposed to a second high dose of HIV-1. Mice exposed to HIV-1$_{\text{CH040}}$ in the first viral challenge were subsequently exposed to HIV-1$_{\text{THRO}}$ and mice challenged with HIV-1$_{\text{THRO}}$ in the first challenge were subsequently exposed to HIV-1$_{\text{CH040}}$. Monitoring for the presence of viral RNA and raltegravir in plasma was continued as indicated above (Figure 4a). As shown in Figure 4(b), all control mice became infected 2 weeks after HIV challenge as viral RNA was detected in their peripheral blood. In contrast, none of the mice treated with long-acting raltegravir was infected 2 weeks after the first challenge (Figure 4c). However, 1 week after the second challenge, one mouse became infected and viral RNA sequence analysis identified the virus as HIV-1$_{\text{CH040}}$. The mouse (T3) was exposed to HIV-1$_{\text{CH040}}$ during the first challenge and HIV-1$_{\text{THRO}}$ in the second challenge (Table 2), suggesting that HIV-1 transmission occurred during the first challenge 1 week after drug administration. Two weeks after the second challenge, two more mice treated with long-acting raltegravir developed viraemia. Sequence analysis identified the virus in both mice as HIV-1$_{\text{CH040}}$, the virus used for the second challenge. No evidence of plasma viral RNA was noted in any of the other long-acting raltegravir treated mice. Analysis of DNA from tissues of the three uninfected mice treated with long-acting raltegravir revealed the absence of viral sequences in all tissues analysed and confirmed their protection from HIV transmission after two high-dose challenges (Figure 4g). Longitudinal analysis of peripheral blood by flow cytometry confirmed that levels of human CD45+ and human CD3+CD4+ cells were similar and remained stable throughout the course of the experiment in mice treated with long-acting raltegravir. Gradual decreases in the levels of human CD3+CD4+ cells were seen only in the infected mice (Figure 4e and f). In summary, long-acting raltegravir protected five out of six mice after the first challenge and four out of six after the second challenge. These results demonstrate that long-acting raltegravir offered significant protection from a high dose of virus administered 1 week (83%, $P<0.0016$) or 4 weeks (66%, $P=0.0495$) later. In summary, in the presence of long-acting raltegravir only three transmission events were seen after 12 high-dose exposures to transmitted/founder HIV.

Analysis of plasma levels of raltegravir during the course of exposures

Raltegravir concentrations were monitored in plasma during the challenge experiments described above (Figure 4d). At the time of the first virus challenge the lowest concentration of plasma raltegravir was observed in mouse T1. However, this mouse was protected from HIV-1 infection after both challenges. At the time of the second challenge, treated mice, including those that became infected after this second challenge, had similar raltegravir concentrations in plasma, with the exception of mouse T4, which had plasma raltegravir levels 2.5-fold higher than other mice. Because virus first appeared in plasma while there were raltegravir levels significantly higher than its PA-IC$_{90}$, viruses from breakthrough infections were sequenced and analysed for the presence of known drug resistance mutations that might have been acquired during the course of infection. No mutations associated with raltegravir resistance were found in the virus present in any of the mice treated with long-acting raltegravir. These results indicate that long-acting raltegravir offers significant protection from HIV infection but that raltegravir plasma levels do not directly correlate with protection and do not result in the development of drug-resistant viruses.

Discussion

Advances in ARV therapy of HIV-infected individuals and the availability of potent ARVs with known safety have stimulated interest

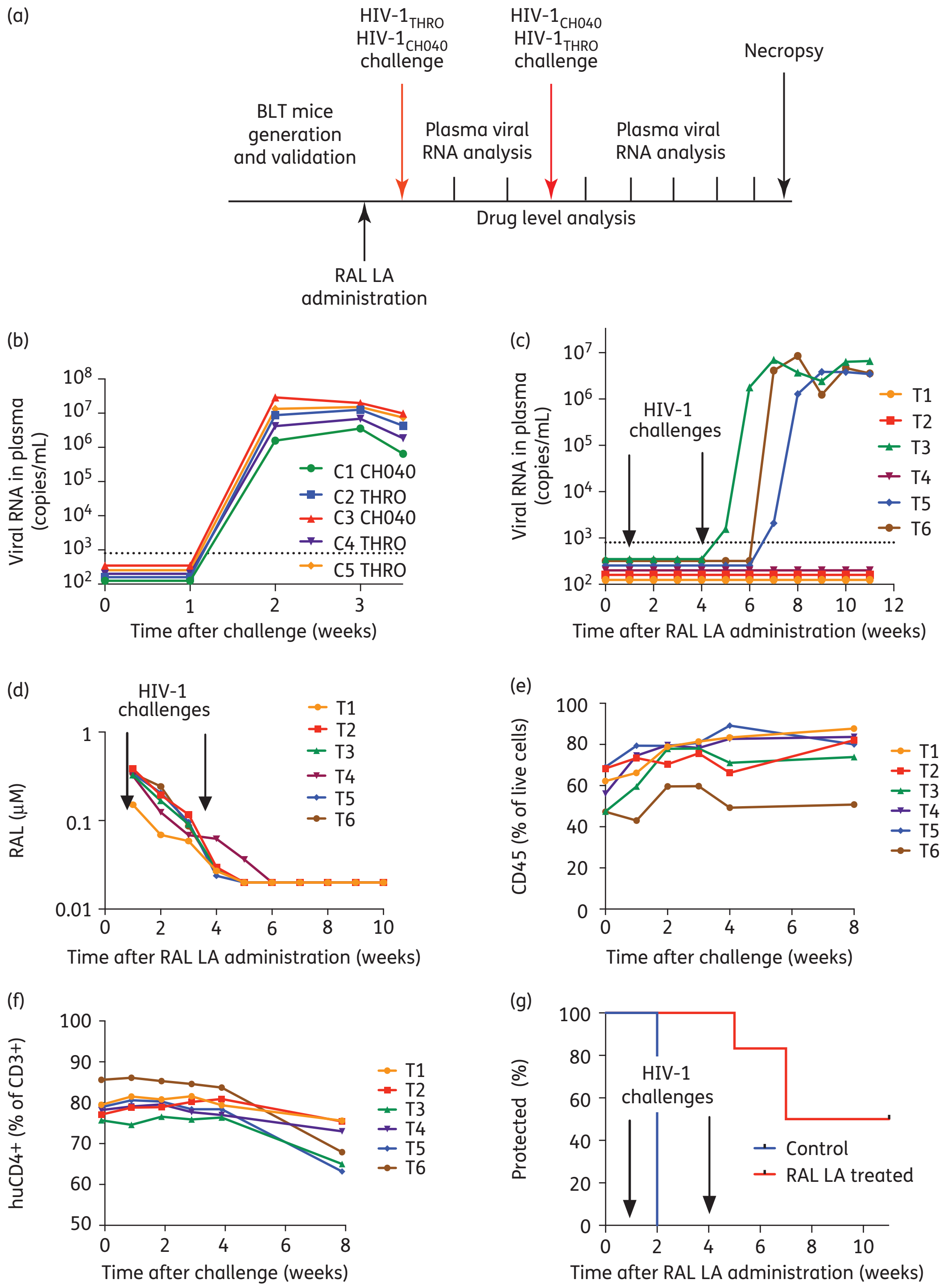

Figure 4. Analysis of long-acting raltegravir protection of BLT mice against two high-dose vaginal challenges with transmitted/founder viruses. (a) Experimental design. BLT mice were challenged vaginally with HIV-1_{CH040} (3.5×10^5 TCID) or HIV-1_{THRO} (3.5×10^5 TCID) transmitted/founder viruses 1 week after subcutaneous administration of 300 mg/kg of long-acting raltegravir. Four weeks after administration of long-acting raltegravir, mice were vaginally challenged again, but this time with a different virus. The mice that were first challenged with HIV-1_{CH040} were challenged with HIV-1_{THRO} and the mice that were first challenged with HIV-1_{THRO} were challenged with HIV-1_{CH040}. (b) Plasma viral load in untreated (control) mice exposed vaginally to HIV-1_{CH040} or HIV-1_{THRO}. The broken line indicates the LOQ. (c) BLT mice ($n=6$) subcutaneously treated with long-acting raltegravir and challenged with HIV-1_{CH040} (T1–T4) or HIV-1_{THRO} (T5, T6) 1 week later. Three weeks after the first challenge (4 weeks after administration of long-acting raltegravir) T1–T4 mice were challenged with HIV-1_{THRO} and mice T5 and T6 were challenged with HIV-1_{CH040}. The viral load in plasma was monitored weekly. The broken line indicates the LOQ. (d) Longitudinal analysis of raltegravir level in plasma of mice treated with long-acting raltegravir (T1–T6) analysed by tandem LC–MS assays as described in the Methods section. (e and f) Human CD45 cell levels in

in their use for HIV prevention. Although the success of several clinical trials using Truvada for PrEP led to its regulatory approval in USA,[35] some of the clinical trials also suggested the importance of adherence to the drug regimen for PrEP efficacy. This was further demonstrated in the VOICE study, in which women from sub-Saharan Africa received oral or topical tenofovir as PrEP.[36] The study failed to demonstrate efficacy, most likely due to the fact that adherence to the drug regimen among participants measured by detectable tenofovir in the plasma or vaginal swab samples was $<$30% and 50%, respectively.[36] Interestingly, in the same geographical region, injectable progestins for contraceptive purposes such as depot-medroxyprogesterone acetate (DMPA) are among the most popular contraceptive methods. This suggests that ARVs formulated as long-acting injectables might have good acceptance and therefore could serve as possible alternatives to oral ARVs for HIV PrEP, with potential for improved adherence.[37]

BLT mice with a functional human immune system have a significant potential to serve as an effective tool for assessing drug efficacy for HIV PrEP, including long-acting ART formulations. Compared with other animal models, important advantages of BLT mice include their human target cells; their susceptibility to infection by highly relevant HIV isolates, including transmitted/founder viruses; the response of these viruses to the same drugs used for human treatment; and the fact that BLT mice can be challenged via relevant routes of transmission. We recently evaluated a long-acting formulation of rilpivirine for prevention of vaginal transmission in this model.[21] Our results and those from others indicated that mice and rats have higher clearance rates of rilpivirine from plasma compared with dogs and humans.[13] Therefore, in order to reach effective levels of plasma rilpivirine to prevent HIV transmission the doses of long-acting rilpivirine administered had to be adjusted for use in BLT mice (15 mg).[21] In the current study, the plasma concentration of raltegravir decreased to the PA-IC_{90} level within 4 days in BALB/c mice dosed with 30 mg/kg long-acting raltegravir subcutaneously. This same dose in macaques resulted in more sustained plasma levels of raltegravir, which were above PA-IC_{90} for 2 weeks after administration. Administration of the higher dose of long-acting raltegravir subcutaneously to BLT or NSG mice resulted in a median raltegravir plasma level of 0.15 μg/mL (range 0.07–0.33) and 0.09 μg/mL (range 0.07–0.12) 7 and 14 days after long-acting raltegravir administration, respectively (Figure 2a and b). In humans, a 400 mg oral dose of raltegravir, administered twice a day, results in a median C_{trough} of 0.09 μg/mL (range 0.002–2.47)[38] or mean C_{trough} of 0.11 μg/mL.[39] Thus, raltegravir plasma concentration in mice 2 weeks after a single administration of 300 mg/kg long-acting raltegravir subcutaneously was within the range of concentration seen in humans administered 400 mg of oral raltegravir twice daily, which makes the higher dose administered to mice relevant for long-acting raltegravir evaluation *in vivo*. Currently, several long-acting ARVs are under development. Those drugs differ in their pharmacokinetic properties and tissue penetration. Future human studies are needed for the selection of an appropriate PrEP agent for targeting sexual HIV transmission. Andrews *et al.*[11] showed the possible limitation of cabotegravir in vaginal protection. Only 10% of drug, compared with plasma, was found in cervical tissues. Low distribution of drug in the FRT may have resulted in the breakthrough events that occurred in two out of eight treated animals. In contrast to cabotegravir, we found comparable distributions of raltegravir in the plasma and FRT of mice treated with long-acting raltegravir. This is consistent with previously reported observations from human studies using oral raltegravir that measured levels of drug in the female genital tract.[25,26] Favourable distribution in mucosal tissue, particularly in the FRT, is a great advantage of long-acting raltegravir.

Despite the dramatic spread of HIV-1, the efficiency of male-to-female intravaginal transmission is surprisingly low, with only about 1 event per 200–10000 coital acts.[40] To model this rare-event situation *in vivo* would be challenging. Instead, high doses of transmitted/founder virus were used for vaginal challenge during long-acting raltegravir evaluation in BLT mice. These doses were several-fold higher than expected in sexual transmission, though it was sufficient to ensure that a single challenge of untreated (control) BLT mice will result in $>$90% HIV transmission. However, as a result of using the high dose of the virus in the evaluation of long-acting AVR for PrEP, the efficacy of the drug could be underestimated, as a higher drug concentration may be needed for prevention of high-dose viral challenge in BLT mice. This possibility is supported by two recent studies in macaques. It was shown that female pigtail macaques treated every 4 weeks with 50 mg/kg of long-acting injectable cabotegravir LA were protected from intravaginal inoculations of lower doses of chimeric simian/human immunodeficiency virus (SHIV, 50 TCIU) twice a week for up to 11 weeks. Using the same dose of SHIV, placebo controls were all infected after a median of 4 (range 2–20) vaginal challenges with the same SHIV.[12] Nevertheless, in a subsequent study only six of eight female rhesus macaques treated with cabotegravir LA (50 mg/kg) monthly were protected against three high-dose SHIV (300 TCIU) challenges and all control animals became infected after the first challenge.[11]

In the long-acting raltegravir protection experiment presented here, all control animals exposed to either HIV-1_{THRO} or HIV-1_{CH040} transmitted/founder viruses were infected within 2 weeks of the viral challenge. One out of six of the BLT mice treated with long-acting raltegravir that were challenged with HIV-1 1 week after drug administration became infected 4 weeks after the challenge. This breakthrough infection cannot be explained by lower plasma concentrations of raltegravir at the time of exposure, as there were no significant differences in plasma level of raltegravir between all the long-acting raltegravir treated mice (protected and unprotected). It is possible that the raltegravir concentration in the FRT in the breakthrough mouse was lower, allowing transmission of HIV. The fact that no correlation was found between plasma raltegravir concentration and viral RNA in CVL during the suppression experiment suggested this possibility. However, this

peripheral blood expressed as a percentage of total live cells (e) and human CD4 levels expressed as a percentage of human CD3-positive cells (f) were analysed by flow cytometry at the indicated times. (g) Kaplan–Meier plots representing the percentage of BLT mice protected against HIV transmission by a single subcutaneous injection of long-acting raltegravir as a function of the number of weeks after first and second challenges until the first viral RNA detection in peripheral blood. Statistical analysis was by the log-rank (Mantel–Cox) test. First and second challenges were analysed separately: first challenge, $P=0.0016$; and second challenge, $P=0.0495$. RAL, raltegravir; RAL LA, long-acting raltegravir.

Table 2. Protection of BLT mice treated with long-acting raltegravir against two high-dose vaginal challenges with HIV-1 transmitted/founder virus

							Presence of viral DNA in tissues					
Mouse code[a]	hCD45 (%)	hCD4 (%)	Treatment	Virus for first challenge	Virus for second challenge	Infecting virus	lymph nodes	spleen	liver	lung	bone marrow	thymic organoid
C1	52.4	83.7	none	CH040	none	CH040	+	+	+	+	+	+
C2	53.4	79.0	none	THRO	none	THRO	+	+	+	+	+	+
C3	62.7	84.5	none	CH040	none	CH040	+	+	+	+	+	+
C4	73.6	79.3	none	THRO	none	THRO	+	+	+	+	+	+
C5	58.4	80.5	none	THRO	none	THRO	+	+	+	+	+	+
T1	62.2	79.5	RAL LA	CH040	THRO	none	−	−	−	−	−	−
T2	68.3	77.1	RAL LA	CH040	THRO	none	−	−	−	−	−	−
T3	47.4	75.7	RAL LA	CH040	THRO	CH040[b]	+	+	+	+	+	+
T4	56.1	78.2	RAL LA	CH040	THRO	none	−	−	−	−	−	−
T5	69.1	78.9	RAL LA	THRO	CH040	CH040[b]	+	+	+	+	+	+
T6	47.3	85.6	RAL LA	THRO	CH040	CH040[b]	+	+	+	+	+	+
Mean	59.2	80.2										
SD	8.8	3.1										

RAL LA, long-acting raltegravir.

BLT mice with the indicated amount of human CD45+ (hCD45) cells and human CD3+CD4+ (hCD4) cells in peripheral blood were treated with 300 mg/kg long-acting raltegravir formulation subcutaneously or left untreated. One week after administration of long-acting raltegravir, mice were inoculated with the indicated HIV-1. Three weeks later mice were inoculated again, but this time with a different type of HIV-1. The presence of viral RNA in plasma was monitored weekly.

Cell-associated DNA was analysed in the indicated tissues: −, negative for viral DNA; +, positive for viral DNA.

[a] C indicates control mice and T indicates treated mice.

[b] Virus in infected mice exposed to two inoculations was identified by sequencing.

could not be directly examined within the constraints of the current experimental design. Specifically, the tissue could not be removed for analysis since it involves terminal surgery and collection of CVL could result in removal of the inoculum. Our hypothesis to explain our breakthrough results and those of Andrews *et al.*[11] is that under these experimental conditions one or a few cells were productively infected. Virus replication was suppressed for several weeks due to the systemic presence of drug. Once drug levels were reduced, replication and spread could take place. Currently, it is not clear why we did not observe the development of drug-resistant viruses. However, this is similar to what was observed in the NHP study.[11] Future studies should consider alternative experimental designs that would allow sampling of the FRT and CVL during the course of the study. While this is clearly possible for NHP studies, it might be challenging for experiments using humanized mice. In summary, we show here that a single dose of long-acting raltegravir results in strong virus suppression during chronic infection. We also show that long-acting raltegravir offers significant protection against vaginal HIV infection in BLT mice after a high-dose challenge administered 1 week and 4 weeks after drug administration. This suggests that long-acting raltegravir is a good candidate for PrEP aimed at preventing vaginal HIV transmission.

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Transparency declarations

M. D. S., R. I. S., J. S., B. J. H. and D. J. H. are employed by Merck & Co., Inc. and have some limited number of shares or options in the company. All other authors: none to declare.

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