Blocking type I interferon signaling enhances T cell recovery and reduces HIV-1 reservoirs

Liang Cheng, Jianping Ma, Jingyun Li, Dan Li, Guangming Li, Feng Li, Qing Zhang, Haisheng Yu, Fumihiko Yasui, Chaobaihui Ye, Li-Chung Tsao, Zhiyuan Hu, Lishan Su, and Liguo Zhang

1Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA. 2Key Laboratory of Infection and Immunity, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China. 3State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University, Chengdu, Sichuan, China. 4Cancer Institute, Xuzhou Medical University, Xuzhou, Jiangsu, China. 5Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA.

Despite the efficient suppression of HIV-1 replication that can be achieved with combined antiretroviral therapy (cART), low levels of type I interferon (IFN-I) signaling persist in some individuals. This sustained signaling may impede immune recovery and foster viral persistence. Here we report studies using a monoclonal antibody to block IFN-α/β receptor (IFNAR) signaling in humanized mice (hu-mice) that were persistently infected with HIV-1. We discovered that effective cART restored the number of human immune cells in HIV-1–infected hu-mice but did not rescue their immune hyperactivation and dysfunction. IFNAR blockade fully reversed HIV-1–induced immune hyperactivation and rescued anti–HIV-1 immune responses in T cells from HIV-1–infected hu-mice. Finally, we found that IFNAR blockade in the presence of cART reduced the size of HIV-1 reservoirs in lymphoid tissues and delayed HIV-1 rebound after cART cessation in the HIV-1–infected hu-mice. We conclude that low levels of IFN-I signaling contribute to HIV-1–associated immune dysfunction and foster HIV-1 persistence in cART-treated hosts. Our results suggest that blocking IFNAR may provide a potential strategy to enhance immune recovery and reduce HIV-1 reservoirs in individuals with sustained elevations in IFN-I signaling during suppressive cART.

Introduction

Type I interferons (IFN-I) are critical for controlling virus infections (1, 2), but they also contribute to impaired host immunity and virus persistence (3, 4). The precise role of IFN-I during chronic HIV-1 infection remains unclear (5, 6). HIV-1 infection induces widespread expression of IFN-I and IFN-stimulated genes (ISGs) (7, 8). It has been reported that IFN-I can suppress HIV-1 replication in vitro (5), and the major anti–HIV-1 restriction factors are encoded by ISGs (5). In addition, IFN-I has been shown to inhibit early HIV-1 infection in humanized mice (hu-mice) (9) and SIV infection in rhesus macaques in vivo (10). These observations suggest that a robust IFN-I response helps to control or limit initial HIV-1 and SIV infection.

IFN-I has also been implicated in the immunopathogenesis of AIDS during chronic HIV-1 infection (5, 6). Studies using nonhuman primate models have demonstrated that sustained IFN-I signaling is associated with pathogenic SIV infection (11–14). IFN-I is induced during the acute phase of SIV infection in both pathogenic (rhesus macaques or pigtail macaques) and nonpathogenic hosts (African green monkeys or sooty mangabeys). However, compared with the nonpathogenic natural SIV infection, pathogenic SIV infection leads to AIDS development, associated with sustained IFN-I signaling (11–14). Furthermore, studies in HIV-1–infected patients indicate that expression of IFN-I and ISGs is correlated with a higher level of viral load, enhanced hyperimmune activation, and faster disease progression (8, 15–17). Using the mouse model of lymphocytic choriomeningitis virus persistent infection, it is reported that blocking of IFN-I signaling by IFNAR antibody can reverse immune suppression, restore lymphoid architecture, and accelerate clearance of the virus (3, 4).

Administration of exogenous IFN-α can lower HIV-1 burden in HIV-1–infected patients but fail to show a significant benefit in HIV-1 disease progression (6). Interestingly, recent studies report that the administration of IFN-α in HIV-1–monoinfected patients or patients coinfected with HIV-1 and hepatitis C virus (HCV) results in reduction of cell–associated viral RNA and DNA in the blood (18–21). However, other studies in HIV-1–infected patients indicate that persistent expression of ISGs is correlated with higher viral load, enhanced hyperimmune activation, and faster disease progression (8, 15–17). In addition, administration of IFN-α to patients also leads to a decrease in CD4 T cell count (18, 21) and enhanced CD8 T cell activation (22) in the blood. Moreover, despite efficient suppression of HIV-1 replication with combined antiretroviral therapy (cART), abnormally elevated IFN-I signaling persists in some patients even under extensive cART (23, 24), which may impede the reversion of hyperimmune activation and immune recovery in those immune nonresponder patients (25).
suggest that blocking IFNAR will provide a novel strategy to enhance immune recovery and to reduce HIV-1 reservoirs in those patients with sustained IFN-I signaling during suppressive cART.

Results

cART efficiently suppresses HIV-1 replication but fails to clear HIV-1 reservoirs in hu-mice, correlated with low levels of ISG expression. To functionally define the role of IFN-I in HIV-1 persistent infection.
and pathogenesis, we used humanized mice with a functional human immune system (hu-mice) for modeling HIV-1 infection and immunopathogenesis (26, 27). We and others have previously reported that persistent HIV-1 infection in hu-mice led to induction of IFN-I signaling, CD4 T cell depletion, aberrant immune activation, and expression of the exhaustion marker PD-1 on T cells (27–29). As in human patients, cART can efficiently inhibit HIV-1 replication in hu-mice (30, 31). We found that plasma viremia decreased to undetectable levels (<400 genome copies/ml) in all HIV-infected hu-mice within 3 weeks after cART treatment (Figure 1A). HIV-1 replication in lymphoid organs was also effectively inhibited by cART (Figure 1B). However, as observed in some patients (23, 24), cART failed to completely reduce ISG expression in HIV-1-infected mice to the level of uninfected hu-mice (Figure 1C). HIV-1 reservoirs, measured by cell-associated HIV-1 DNA and RNA (Figure 1D), and cells with infectious HIV-1 (Figure 1E), were still detectable in lymphoid organs of cART-treated hu-mice. As in cART-treated patients, HIV-1 reservoirs persisted stably and virus rebounded rapidly after cART cessation (Figure 1F).

IFNAR blockade during cART-suppressed HIV-1 infection reverses aberrant immune activation. It is reported that abnormally elevated levels of IFN-I signaling and ISG expression persist in some patients even under extensive cART (23, 24), which may impede immune recovery and foster viral persistence (6, 25). We hypothesized that IFNAR blockade in the presence of cART would reverse hyperimmune or inflammatory activation and facilitate recovery of functional anti–HIV-1 adaptive immune responses, thereby enabling control of cART-resistant HIV-1 reservoirs. To block IFN-I signaling in hu-mice, we developed a monoclonal antibody (mAb) against human IFN-α/β receptor 1 (α-IFNAR1) that specifically binds to human IFNAR1 (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI90745DS1) and inhibits human IFN-I activity (Supplemental Figure 1, B and C). The α-IFNAR1 mAb neither bound to mouse IFNAR1 (Supplemental Figure 2A) nor blocked mouse IFN-I activity in mouse cells (Supplemental Figure 2B). Furthermore, we showed that the α-IFNAR1 mAb effectively blocked ISG induction in vivo in response to the TLR7/8 agonist R848 (resiquimod) in hu-mice (Supplemental Figure 3). Treatment with α-IFNAR1 mAb alone affected neither the percentage nor the number of human leukocyte subsets (Supplemental Figure 4, A and B), and administration of α-IFNAR1 mAb did not affect the expression of ISGs in splenocytes in hu-mice (Supplemental Figure 4C).

We then analyzed the effect of IFNAR blockade on T cell activation and functions in the presence of cART treatment in HIV-1-infected hu-mice. We treated HIV-1-infected hu-mice that were fully cART-suppressed with α-IFNAR1 mAb for 3 weeks from 7 to 10 weeks postinfection (Figure 2A). As in some cART-treated human patients (23, 24), cART failed to completely suppress expression of ISGs (Figure 1C and Figure 2B). In contrast, IFNAR blockade efficiently suppressed HIV-induced ISG expression in cART-treated hu-mice (Figure 2B and Supplemental Figure 4A). HIV-1 persistent infection in hu-mice also induced T cell depletion, hyperimmune activation, and proliferation as indicated by the expression of the activation marker CD38/HLA-DR and the proliferation marker Ki67 (Figure 2, C–E). Although cART alone significantly rescued the number of human T cells and total human leukocytes (Figure 2C and Supplemental Figure 5), it only slightly decreased the expression level of CD38/HLA-DR (Figure 2, D and E) and Ki67 on T cells (Figure 2, F and G). Both CD8 and CD4 T cells from cART-treated hu-mice still expressed significantly higher levels of activation (Figure 2, D and E) and proliferation (Figure 2, F and G) markers in comparison with uninfected hu-mice. Interestingly, IFNAR blockade significantly reversed aberrant CD8 T cell activation and proliferation in the presence of cART (Figure 2, D–G).

IFNAR blockade reverses the exhaustion phenotype of human T cells and restores anti–HIV-1 T cell function. Despite successful viral inhibition by cART, T cells from patients with poor immune reconstitution sustained higher PD-1 expression (32). We therefore investigated whether IFNAR blockade could reverse expression of PD-1 and other exhaustion markers and rescue HIV-1-specific T cell function in the presence of cART. HIV-1 persistent infection in hu-mice induced both PD-1 and TIM-3 expression on CD8 T cells (Figure 3, A and B). We found that cART alone failed to significantly reduce the expression of PD-1 and TIM-3 (Figure 3, A and B). Interestingly, IFNAR blockade combined with cART completely reduced expression of PD-1 and TIM-3 on CD8 T cells (Figure 3, A and B). Whole transcriptome sequencing of purified human CD8 T cells revealed that cART plus IFNAR blockade also significantly reduced the expression of other T cell exhaustion markers including CD160, TIGIT (T cell immunoreceptor with Ig and ITIM domains), and BATF (basic leucine transcription factor, ATF-like) (Figure 3C and ref. 33).

We further determined the function of HIV-1–specific T cells after IFNAR blockade. When stimulated with HIV-1 Gag peptide pools ex vivo, both CD8 and CD4 T cells from hu-mice with cART and IFNAR blockade (but not cART alone) produced significantly higher levels of IFN-γ and IL-2 (Figure 3, D and E, and Supplemental Figure 6), indicating that IFNAR blockade also rescued the function of HIV-1–specific T cell responses. Taken together, these results indicate that, in the presence of cART, IFNAR blockade can reverse aberrant immune activation and T cell exhaustion and rescue anti–HIV-1 immune responses.

IFNAR blockade during cART reduces cART-resistant HIV-1 reservoirs. The administration of cART cannot achieve HIV-1 eradication, and virus rebounds quickly after cART discontinuation, owing to the persistence of HIV-1 reservoirs during cART (34, 35). It was demonstrated that adaptive immune response contributes to the control of cART-resistant reservoirs (36, 37). Thus the improvement of anti–HIV-1 adaptive immune response by therapeutic vaccine or by other immune modifiers has been proposed as an immunological strategy for HIV cure (38, 39). We postulated that the reversal of immune hyperactivation and the induction of elevated anti–HIV-1 T cell response by blocking of IFNAR might reduce the size of the cART-resistant HIV-1 reservoir and control HIV-1 rebound after cART cessation. We treated HIV-1–infected hu-mice that were fully cART-suppressed with α-IFNAR1 mAb for 3 weeks from 7 to 10 weeks postinfection (Figure 4A). Interestingly, IFNAR blockade, in the presence of cART, led to low blips of HIV-1 replication, which returned to undetectable levels after α-IFNAR1 mAb treatment was stopped (Figure 4A). Thus, IFNAR blockade induced activation of HIV-1...
reservoirs. We speculated that the increase of anti-HIV-1 T cell immune responses by IFNAR blockade would eliminate or control the HIV-1–expressing cells and finally reduce HIV-1 reservoirs. We next analyzed the HIV-1 reservoir size in lymphoid organs 2 weeks after IFNAR blockade. We measured cell-associated HIV-1 DNA and RNA by PCR, and replication-competent HIV-1 by the quantitative virus outgrowth assay. We found that IFNAR blockade reduced cell-associated HIV-1 DNA 14-fold in the spleen and 4.4-fold in the bone marrow (Figure 4B). Cell-associated HIV-1 RNA was also reduced in both spleen (17.7-fold) and bone marrow (4.4-fold) (Figure 4C). More importantly and consistently, IFNAR blockade significantly reduced the size of replication-competent HIV-1 reservoirs measured by quantitative virus outgrowth assay (Figure 4D).
IFNAR blockade during cART suppresses HIV-1 infection reverses the exhaustion phenotype of CD8 T cells and restores anti–HIV-1 T cell function. Hu-mice were treated as in Figure 2. (A) Representative dot plots show percentage PD-1+ and TIM-3+ of CD8 T cells from spleens. (B) Summarized data show percentage PD-1+ and TIM-3+ of CD8 T cells from spleens. (C) RNA sequencing was performed with purified CD8 T cells from spleens. Shown is expression of CD160, TIGIT, and BATF in CD8 T cells from mock-treated (n = 2), HIV-1+cART+mlgG2a-treated (n = 3), and HIV-1+cART+α-IFNAR1-treated (n = 3) hu-mice. Transcripts per kilobase million (TPM) indicates the relative abundance of transcripts. Unpaired, 2-tailed Student’s t test was performed to compare between groups (C). (D and E) Splenocytes were stimulated ex vivo with HIV-1 Gag peptide pools for 8 hours (Brefeldin A added at 3 hours) followed by intracellular cytokine staining. Representative dot plots (D) and summarized data (E) show percentages of IFN-γ- and IL-2-producing CD8 T cells. Shown are combined data from 2 independent experiments (A, B, D, and E) with mean values ± SEM (mock, n = 7; HIV-1, n = 7; HIV-1+cART+mlgG2a, n = 8; HIV-1+cART+α-IFNAR1, n = 8). *P < 0.05, **P < 0.01, ***P < 0.001, by 1-way ANOVA and Bonferroni’s post hoc test.
activation and exhaustion. This model thus partially recapitulates the phenotype of those immune nonresponder patients. We showed that blocking IFN-I signaling by using our newly developed anti-IFNAR1 mAb completely inhibited ISG expression, reversed T cell immune activation and exhaustion, and rescued anti–HIV-1 T cell function. Our results agree with recent findings showing that persistent IFN-I signaling plays a detrimental role during chronic lymphocytic choriomeningitis virus infection and that blockade of IFN-I signaling by IFNAR antibody could reverse T cell exhaustion and enhance antiviral immune response (3, 4). The IFNAR blocking antibody will thus facilitate novel therapeutic development aimed at those difficult-to-treat HIV-1–infected patients with sustained IFN-I signaling during cART (23, 24, 40).

HIV-1 reservoirs are refractory to antiretroviral therapies (ART) and remain the major barrier to curing HIV-1 (34, 35). We report here that IFNAR blockade transiently increased HIV-1 RNA in the blood (viral load “blipping”) during cART, indicating that IFN-I signaling contributed to the low replication or latency of the HIV-1 reservoirs. Multiple mechanisms may lead to the reduction of HIV-1 reservoir size after IFNAR blockade during cART. The rescued immune response could target the HIV-1 reservoirs with elevated gene expression and kill the reservoir cells. Other factors, including HIV-1–induced death of reservoir cells and reduced general T cell activation and proliferation, after IFNAR blockade may also contribute to the reduction of HIV-1 reservoir size. The underlying mechanism of reservoir reduction by IFNAR blockade will be further elucidated in the future. Therefore, blocking IFN-I signaling in cART-treated subjects may provide a novel therapeutic approach for HIV-1 cure (38).

In a recent report, blocking IFN-I signaling with an antagonistic IFN-α2 mutant (IFN-ant) with increased IFNAR2 binding but diminished IFNAR1 binding activity (41) during the acute phase of SIV infection (0–4 weeks postinfection) in rhesus monkeys led to elevated SIV replication and accelerated disease progression (10). Conversely, while preinfection IFN-α2a administration results in decreased SIV transmission, continued IFN-α2a treatment appears to induce IFN-I desensitization and decrease antiviral gene expression, resulting in increased SIV replication and accelerated CD4 T cell loss (10). This study has major differences from our studies in that IFN-I signaling is blocked (or desensitized) only during acute SIV infection. The higher levels of SIV infection probably lead to the accelerated disease progression during the late
stage of infection, in the absence of IFN-I blocking. It is generally believed that persistent IFN signaling during chronic infection can lead to general immune suppression (42). Therefore, IFN-I signaling is beneficial during the acute stage to inhibit or prevent virus infection but becomes harmful during the chronic stage of HIV-1 infection. Blocking IFN-I signaling with either the IFNAR mAb or the antagonistic IFN-α2 mutant protein in rhesus monkeys with persistent SIV infection and cART will be of great interest to further clarify these therapeutic strategies.

Several recent reports have shown that the administration of IFN-α in HIV-1-monoinfected or HIV-1/HCV-coinfected patients leads to reduction of cell-associated viral RNA and DNA in the blood cells of a subset of treated patients (18–21). The study by Livio Azzoni et al. (18) reports that long-term administration of IFN-α during and after ART in HIV-1–infected patients leads to suppression of HIV-1 rebound in approximately 40% of patients, whose PBMC-associated HIV-1 DNA (after 12 weeks with IFN-α only but no ART) is lower in comparison with their PBMCs during ART alone when normalized to their CD4 T cell counts. However, HIV-1 reservoirs (cell-associated DNA) were not significantly changed by IFN-α treatment during ART. The administration of IFN-α may induce the migration of activated CD4 T cells into lymphoid organs and subsequently reduction in the peripheral blood (43); thus the reduction may be due to the redistribution of HIV-1 reservoir cells to lymphoid organs induced by IFN-α. Interestingly, treatment of HIV-1/HCV-coinfected patients with IFN-α/ribavirin appears to lead to a significant reduction of both CD4 and CD8 T cells (18, 20, 21), which is consistent with our previous finding that IFN-I contributes to T cell depletion during chronic HIV-1 infection (28, 44).

In addition, a low level of HIV-1 replication in the presence of cART may also contribute to the HIV-1 reservoir pool (45). High levels of exogenous IFN-I may inhibit the low-level HIV-1 replication as well as enhance anti-HIV immune responses (46). Therefore, IFN-I signaling may play complex roles during the acute and chronic phases of HIV-1 infection, both inhibiting viral replication and fostering viral persistence by inducing immune dysfunction.

It is important to point out, however, that the human immune system in hu-mice is not fully functional as found in immunocompetent hosts (26, 27). The restoration of anti–HIV-1 T cell immune response by IFNAR blockade in hu-mice may not be robust enough to eliminate or fully control the HIV-1 reservoir. Another concern is the xenoreactive graft-versus-host...
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in hu-mice than in primate models or in humans. The findings reported here in hu-mice should accordingly be confirmed with the IFNAR blockade in SIV-infected and cART-treated monkey models before clinical testing in HIV-1 patients.

Methods

Construction of hu-mice. NRG (NOD Rag2−/−γc−/−) mice were obtained from the Jackson Laboratory. All mice were housed and bred in a specific pathogen–free environment. Humanized NRG mice with a functional human immune system were generated by intrahepatic injection of newborn mice with human fetal liver–derived CD34+ hematopoietic progenitor cells as previously reported (28). Humanized BLT (bone marrow/liver/thymus) mice were generated as previously reported (50). Briefly, 6- to 8-week-old NRG mice were sublethally irradiated and anesthetized, and 1-mm³ fragments of human fetal thymus were implanted under the kidney capsule. CD34+ hematopoietic progenitor cells purified from fetal liver of the same donor were injected i.v. within 3 hours. Human immune cell engraftment was detected by flow cytometry 12 weeks after transplantation. All animal studies were approved by the University of North Carolina IACUC (ID 14-100).

Table 1. IFNAR blockade in the presence of cART delays HIV-1 rebound after cART cessation

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<th>Mouse no.</th>
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Hu-mice were treated as in Figure 5E. APercentage of human CD45+ of total cells in PBMCs. BPercentage of CD3+ from human CD45+ cells. CPercentage of CD4+ from CD3+ cells. DWeeks after cART treatment. ND, not detectable.
**HIV-1 infection of hu-mice.** The R5 tropic strain of HIV-1 (JR-CSF) was generated by transfection of 293T cells with plasmid containing full-length HIV-1 (JR-CSF) genome. Hu-mice with stable human leukocyte reconstitution were anesthetized and infected with HIV-1 (JR-CSF) (10 ng p24 or 3,000 infectious units per mouse) through retro-orbital injection. Hu-mice infected with 293T supernatant were used as mock control groups. Both male and female mice were used for all the experiments.

**Development of anti-IFNAR1 blocking antibody.** The mouse cell line L-929 transfected with the human IFNAR1 (extracellular domain and transmembrane domain) expression plasmid mentioned was used as the immunogen for immunization. For each immunization, the WT BALB/c female mice were injected i.p. with 5,000,000 immunogen cells with 10 μg CpG-1826 as adjuvant. After 5 times immunization, the spleen cells were fused with the mouse myeloma cell line SP2/0. For screening of the clones that could secrete the IFNAR1 binding antibody by flow cytometry, 293T cells transfected with the human IFNAR1 expression plasmid were used. Briefly, the human IFNAR1 expression 293T cell line was firstly incubated with the supernatant of the hybridoma, then incubated with the PE-labeled goat anti-mouse IgG secondary antibody. Then, an IFN-1 reporter 293T cell line stably transfected with a mouse A2 promoter–driven EGFP was used to screen antibody clones that could block the human IFNAR1 signaling.

**In vitro blocking assay.** The IFN-1 reporter 293T cell line or human PBMCs or mouse splenocytes were preincubated with antibodies for 1 hour in 37 celsius, and the human IFN-a2b or mouse IFN-α was added with a final concentration of 5 ng/ml. IFN-1 reporter 293T cells were harvested and EGFP expression was analyzed by flow cytometry 24 hours later. The IFN activity after anti-human IFNAR1 treatment relative to samples with IFN-α2a treatment only was calculated. To detect expression of ISGs in human PBMCs or mouse splenocytes, cells were harvested 4–5 hours later for ISG detection by quantitative real-time PCR. The primers used for the quantitative real-time PCR in the in vitro assay were as follows: human ISG15 (5′-CCGAGATCACCCAGAGAGTCTG-3′ and 5′-TCTGGTCTAGTGGGGCAGCTG-3′), mouse MX2 (5′-CAGAGGCAGCGGAATC -TGAAGCTCTAGCTC-3′ and 5′-TGAAGCTCTAGCTC-3′), human EF1A (5′-ATATGGTTTCTCGCAAGCCCC-3′ and 5′-GTGGGTTGTCAGGTATTAGG-3′), mouse IgG5 (5′-TGGTACAGAAGCTCACAGGAG-3′ and 5′-AGCCAGACCTGTCATGGTT-3′), mouse IRF7 (5′-GCTGGACGTGACCATATGTA-3′ and 5′-AGCGGGCTGGTTGTCATGGTTT-3′), mouse Gapdh (5′-GAAGGGCAACATTGGCTCTG-3′ and 5′-GAGGGGCCATCCAGGTCTTT-3′).

**In vivo IFNAR blocking antibody treatments.** To confirm the in vivo neutralizing activity of α-IFNAR1 mAbs, hu-mice were treated i.p. with α-IFNAR1 mAb or mlgG2a as isotype control 6 hours before R848 treatment. HIV-1-infected, cART-treated mice were treated i.p. with α-IFNAR1 blocking antibodies from 7 to 10 weeks postinfection twice a week. HIV-1–infected, cART-treated mice were treated i.p. with α-IFNAR1 mAbs, hu-mice were treated i.p. with neutralizing activity of human IFNAR1 blocking antibodies from 7 to 10 weeks postinfection twice a week.

**Combination antiretroviral therapy.** Food formulated with antiretroviral individual drug was prepared as reported with elevated dose modifications (30). In brief, tablets of emtricitabine and tenofovir disoproxil fumarate (Truvada; Gilead Sciences) and raltegravir (Isentress; Merck) were crushed into fine powder and manufactured with TestDiet 5105 feed (Modified LabDiet 5058 with 0.12% acamoxicillin) into half-inch irradiated pellets. Final concentrations of drugs in the food were 4,800 mg/kg raltegravir, 1,560 mg/kg tenofovir disoproxil, and 1,040 mg/kg emtricitabine. The estimated daily drug doses were 768 mg/kg raltegravir, 250 mg/kg tenofovir disoproxil, and 166 mg/kg emtricitabine.

**Flow cytometry and cell sorting.** For surface staining, single-cell suspensions prepared from peripheral blood, spleen, or mesenteric lymph nodes of hu-mice were treated with surface markers and analyzed on a CyAn ADP flow cytometer (Dako). For intracellular staining, cells were first stained with surface markers, and then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), followed by intracellular staining. FITC–conjugated anti-human HLA-DR (L243), PE-conjugated anti-human CD38 (HIT2), CD303 (SJ25-C1), and LIVE/DEAD Fixable Yellow Dead Cell Stain Kit were purchased from Beckman Coulter. PE-conjugated anti-human caspase-3 (C92-605) was purchased from BD Pharmingen. Pacific Orange–conjugated anti-HIV-1 p24 was purchased from Invitrogen. Data were analyzed using Summit 4.3 software (Dako).

For CD8 T cell sorting, after staining with viability dye and surface markers (anti-hCD45, -mCD45, -hCD3, -hCD4, -hCD8, -hCD11c, -hCD14, -hCD123), CD8 T cells (hCD45+mCD45 hCD3+hCD8+) were sorted on a BD FACSAria II using a 70-mm nozzle and collected into Falcon round-bottom polystyrene tubes containing RPMI 1640/10% FBS. The purity of sorted CD8 T cells was above 99%.

**T cell stimulation and intracellular cytokine staining.** For nonspecific stimulation, splenocytes from hu-mice were stimulated ex vivo with PMA (phorbol 12-myristate 13-acetaet) (50 ng/ml) and ionomycin (1 μM) (Sigma-Aldrich) for 4 hours in the presence of brefeldin A (BioLegend).
For antigen-specific stimulation, splenocytes from hu-mice were stimulated ex vivo with peptide pools (2 μg/ml for each peptide) for HIV-1 GAG protein [PepMix HIV (GAG) Ultra; JPT Innovation Peptide Solutions] for 3 hours without brefeldin A and then 5 hours in the presence of brefeldin A. Cells were then fixed and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), and intracellular staining was then performed.

HIV-1 genomic RNA detection in plasma. HIV-1 RNA was purified from the plasma with the QIAampkit Viral RNA Mini Kit. The RNA was then reverse-transcribed and quantitatively detected by real-time PCR using the TaqMan Fast Virus 1-Step PCR kit (Thermo Fisher Scientific). The primers used for detecting the HIV gag gene were 5′-GGTGCAGACGCTCTGATTAA3′ and 5′-AGCTCCTTGGTGTCTCCAATA-3′. The probe (FAM-AAAATTCGTTAAGGCCAGGGGAAAAGA-QSY7) used for detection was ordered from Applied Biosystems, and the reactions were set up following the manufacturer’s guidelines and were run on the QuantStudio 6 Flex PCR system (Applied Biosystems). The detection limit of the real-time PCR reaction is 4 copies per reaction. Accordingly, due to the relatively small volume of each bleeding in mice (around 50–100 μl total blood), the limit of detection of the assay is 400 copies/ml plasma. We set the copy numbers that were below the detectable limit as 1.

Cell-associated HIV-1 DNA detection. To measure total cell-associated HIV-1 DNA, nucleic acid was extracted from spleen and bone marrow cells using the DNEasy mini kit (Qiagen). HIV-1 DNA was quantified by real-time PCR. DNA from serial dilutions of ACH2 cells, which contain 1 copy of HIV genome in each cell, was used to generate a standard curve.

Cell-associated HIV-1 RNA detection. To measure total cell-associated HIV-1 RNA, nucleic acid was extracted from spleen or bone marrow cells using the RNeasy plus mini kit (Qiagen). HIV-1 RNA was detected as described above. The HIV-1 RNA expression levels were normalized to human CD4 mRNA (5′-GGCTCTTCGCTGCTGAACTTTGC) and CCGCTTCGAGACCTTTGC) controls, and the result was calculated as fold change in gene expression.

Viral outgrowth assay. Viral outgrowth assay was performed as reported (52). Serial dilutions of human cells from splenocytes of hu-mice (1 x 10⁶, 2 x 10⁵, 4 x 10⁴ human cells) were stimulated with phytohemagglutinin (2 μg/ml) and IL-2 (100 U/ml) for 24 hours. MOLT4/CRRS cells were added on day 2 to enhance the survival of the cultured cells as well as to support and facilitate further HIV-1 replication. Culture medium containing IL-2 (NIH AIDS Reagent Program; https://www.aidsreagent.org/) and T cell growth factor (homemade as described in methods) were added to support the growth of HIV-1 infection. Culture medium containing IL-2 (NIH AIDS Reagent Program; https://www.aidsreagent.org/) and T cell growth factor (homemade as described in the standard protocol) was replaced on days 5 and 9. After 7 and 14 days of culture, supernatant from each well was harvested and HIV-1 reverse transcriptase quantitative PCR was performed to score viral outgrowth. Estimated frequencies of cells with replication-competent HIV-1 were calculated using limiting dilution analysis.

RNA sequencing. Purified human CD8 T cells from spleens of hu-mice as described above were used to prepare RNA. The cDNA was prepared using SMART Seq v4 Ultra Low RNA-Seq kit for 48 reactions (Clontech). A Nextera kit was used for library construction, and sequencing was performed on Illumina HiSeq2500v4 with paired-end sequencing for 50 cycles. Sequencing data FASTQ files for samples were processed in Salmon workflow in a Linux server operating system to output gene-level abundance estimates and statistical inference as gene-level raw counts. Those raw counts for samples were input into the edgeR program (Bioconductor) for differential gene expression analysis.

Statistics. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software). Experiments were analyzed by 2-tailed Student’s t test, or by 1-way ANOVA and Bonferroni’s post hoc test or Gehan-Breslow-Wilcoxon test, according to the assumptions of the test, as indicated in the figure legends for each experiment. *P < 0.05, **P < 0.01, ***P < 0.001. All the data with error bars are presented as mean values ± SEM. A p value less than 0.05 was considered significant.

Study approval. Human fetal liver and thymus tissues (gestational age 16–20 weeks) were obtained from elective or medically indicated termination of pregnancy through a nonprofit intermediary working with outpatient clinics (Advanced Bioscience Resources, Alameda, California, USA). Informed consent of the maternal donors was obtained in all cases, under regulation governing the clinic. The project was reviewed by the University of North Carolina at Chapel Hill Office of Human Research Ethics, which has determined that this submission does not constitute human subjects research as defined under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c)(e)(l)]. All animal studies were approved by the University of North Carolina IACUC.

Author contributions
LC, LS, and LZ conceived and designed the study. LC, JM, JL, DL, GL, FL, QZ, HY, FY, CY, LCT, and ZH performed experiments. LC, LS, and LZ analyzed and interpreted the data and prepared the manuscript.

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Address correspondence to: Lishan Su, Room 5208, Masco Hall, 125 Mason Farm Road, Chapel Hill, North Carolina 27514, USA. Phone: 919.966.6654; E-mail: lshu@med.unc.edu. Or to: Liguo Zhang, 15 Datun Road, Chaoyang District, Beijing, China, 100101. Phone: 010.64862568; E-mail: liguozhang@ibp.ac.cn.

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