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Latent HIV-1 Infection of Resting CD4⁺ T Cells in the Humanized Rag2^{-/-} γ_c ^{-/-} Mouse

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Persistent human immunodeficiency virus type 1 (HIV-1) infection of resting CD4⁺ T cells, unaffected by antiretroviral therapy (ART), provides a long-lived reservoir of HIV infection. Therapies that target this viral reservoir are needed to eradicate HIV-1 infection. A small-animal model that recapitulates HIV-1 latency in resting CD4⁺ T cells may accelerate drug discovery and allow the rational design of nonhuman primate (NHP) or human studies. We report that in humanized Rag2^{-/-} γ_c ^{-/-} (hu-Rag2^{-/-} γ_c ^{-/-}) mice, as in humans, resting CD4⁺ T cell infection (RCI) can be quantitated in pooled samples of circulating cells and tissue reservoirs (e.g., lymph node, spleen, bone marrow) following HIV-1 infection with the CCR5-tropic JR-CSF strain and suppression of viremia by ART. Replication-competent virus was recovered from pooled resting CD4⁺ T cells in 7 of 16 mice, with a median frequency of 8 (range, 2 to 12) infected cells per million T cells, demonstrating that HIV-1 infection can persist despite ART in the resting CD4⁺ T cell reservoir of hu-Rag2^{-/-} γ_c ^{-/-} mice. This model will allow rapid preliminary assessments of novel eradication approaches and combinatorial strategies that may be challenging to perform in the NHP model or in humans, as well as a rigorous analysis of the effect of these interventions in specific anatomical compartments.

Human immunodeficiency virus type 1 (HIV-1) infection persists despite years of antiretroviral therapy (ART) (16, 18, 28). Proviral latency is established early in infection, even in patients who are treated with ART within the first weeks of infection (13). Latently infected memory CD4⁺ T cells constitute the major reservoir of viral persistence in patients on ART (13, 18, 20, 28, 33) and can replenish systemic infection following interruption of therapy (15). Eliminating HIV-1 latency in this important reservoir is critical to the pursuit of successful eradication strategies.

HIV-1 infection also may persist in a variety of anatomical compartments, such as the central nervous system (CNS), a pharmacologically “privileged” site where the blood-brain barrier limits the penetration of some antiretrovirals and may provide a sanctuary for viral persistence (23). The gut-associated lymphoid tissue (GALT), a site where drug metabolism is poorly understood, has also been suggested to be a source of persistent infection during ART (17). Bailey and colleagues found that viral genomes represented in low-level, persistent viremia despite ART were sometimes different than those found in resting CD4⁺ T cells (5), but Anderson et al. found a concordance of circulating and resting cell viral isolates (1). Primitive hematopoietic cells were shown to resist HIV-1 infection (37), but recent studies claim that HIV-1 infection of multipotent progenitor cells could be a potential source of persistent infection by CXCR4-tropic viruses (11). These findings highlight the need for systems in which a comprehensive analysis of all possible cells and reservoirs that may harbor persistent HIV can be examined. Such studies are difficult to conduct in humans and may be better addressed in animal models of HIV-1 latency.

Currently, the macaque nonhuman primate (NHP) model of simian immunodeficiency virus (SIV) infection on ART is the only animal model available to study HIV-1 latency and persistence (19, 32). Although HIV-1 is closely related to SIV, unique accessory proteins and sequence variation within homologous proteins of this lentivirus may subtly alter the pathogenesis of persistent infection (36). While the macaque NHP model of SIV is important for the study of HIV persistence, given the limited re-

sources available for the study of macaques, progress could be accelerated by a tractable animal model that recapitulates resting CD4⁺ T cell infection. Such a model will allow a rigorous evaluation of preclinical strategies to eradicate HIV-1 infection in tissue reservoirs. Human studies are usually slow and difficult and pose some risks to patients who are otherwise clinically stable. A small-animal model of latency would allow additional preclinical studies to be performed, helping to focus human trials seeking to purge latent reservoirs.

Persistent HIV-1 infection has been demonstrated in CD4⁺ thymocytes in the SCID-hu (Thy/Liv) mouse model, but these animals possess few resting CD4⁺ T cells in the peripheral blood (PB) and secondary lymphoid tissues (9, 10). A humanized mouse model that carries resting memory CD4⁺ T cell infection in the PB and secondary lymphoid tissues may be better suited for the testing of HIV-1 eradication strategies.

Humanized Rag2^{-/-} γ_c ^{-/-} (hu-Rag2^{-/-} γ_c ^{-/-}) mice, first developed by Traggiai and colleagues, show stable reconstitution of human T, B, natural killer (NK), and dendritic cells in both primary and secondary lymphoid organs (35). These mice are readily infected with HIV-1, resulting in high-level plasma viremia and depletion of CD4⁺ T cells in the PB (4, 7, 12, 38). We and others have demonstrated that plasma viremia can be suppressed below the limit of detection with ART (12, 31). The discontinuation of ART results in viral rebound, suggesting the presence of persistent infection (12). In our current study, we show that intensification of a 3-drug ART regimen with enfuvirtide improved suppression of plasma viremia, prevented the emergence of drug resistance,

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and allowed the recovery of resting CD4⁺ T cells that expressed HIV only after *ex vivo* stimulation. This is the first tractable small-animal model of HIV-1 infection, ART, and latency.

MATERIALS AND METHODS

Ethics statement. All animal work was approved by the University of North Carolina Institutional Animal Care and Use Committee (IACUC). Human fetal liver was obtained from Advance Bioscience Resources (Alameda, CA), a nonprofit organization, in accordance with federal and state regulations.

Generation and maintenance of hu-Rag2^{-/-} γ_c ^{-/-} mice. hu-Rag2^{-/-} γ_c ^{-/-} mice were created by transplanting human fetal liver-derived CD34⁺ cells into the livers of newborn conditioned Rag^{-/-} γ_c ^{-/-} mice as described previously (35, 38), with minor modifications. Briefly, human fetal liver at 16 to 24 weeks of gestation was treated with 1 mg/ml collagenase D and 10 μ g/ml DNase I (Roche, Indianapolis, IN) for 1 h at 37°C and filtered through a 70- μ m cell strainer (BD, Franklin Lakes, NJ) to make a single-cell suspension. Mononuclear cells were enriched on Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ), and human CD34⁺ cells were purified to more than 95% purity using a CD34 selection kit (Stemcell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Cells were cultured overnight in RPMI 1640 medium containing 20% fetal bovine serum (FBS), 1 μ g/ml interleukin 3 (IL-3), 1 μ g/ml IL-6, and 2 μ g/ml stem cell factor (R&D Systems, Minneapolis, MN). The next day, 0.5 \times 10⁶ to 1 \times 10⁶ human CD34⁺ cells were injected intrahepatically into newborn mice (1 to 3 days old) previously irradiated with 300 rad (X-Rad 320; Precision X-Ray, North Branford, CT). Ten to 12 weeks posttransplantation, the level of human leukocytes in the peripheral blood (PB) was determined by flow cytometry. All mice were maintained in microisolator cages on racks with HEPA-filtered air blown into each cage.

Flow cytometric analysis of human leukocytes in PB and tissue. PB samples were collected by tail vein bleeding and lysed with ACK lysis buffer (Invitrogen, Carlsbad, CA). Mononuclear cells from bone marrow (BM), thymus, spleen, lymph node (LN), the female reproductive tract (FRT), lung, and liver were collected as described previously (25). Cells were incubated with the appropriate antibodies in 1 \times phosphate-buffered saline (PBS) containing 2% FBS plus 0.02% sodium azide, washed, and fixed in 200 μ l PBS with 2% formaldehyde overnight prior to flow cytometry. Cells were analyzed using a CyAn ADP flow cytometer and Summit 4.3 software (Beckman Coulter Inc., Miami, FL). Human leukocytes (CD45⁺ cells) were analyzed for surface expression of CD3, CD4, CD8, CD45RO, CD45RA, CD62L, CD27, HLA-DR, CD25, and CD69. Unstained cells or cells stained with an appropriate isotype control were used to set markers defining positive reactivity. The following anti-human monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-conjugated CD8, CD69 (Invitrogen, Carlsbad, CA), CD4, and CD27 (BD Pharmingen, San Diego, CA); phycoerythrin (PE)-conjugated CD4 and CD25 (Invitrogen, Carlsbad, CA); peridinin-chlorophyll protein (PerCP)-conjugated CD3 and HLA-DR (BD Pharmingen, San Diego, CA); phycoerythrin cyanine 5.5 (PE-Cy5.5)-conjugated CD45RA (BD Pharmingen, San Diego, CA); allophycocyanin (APC)-conjugated CD45 (Invitrogen, Carlsbad, CA); CD62L and CD45RO (BD Pharmingen, San Diego, CA); and allophycocyanin cyanin Cy7 (APC-Cy7)-conjugated CD45 and CD11b (BD Pharmingen, San Diego, CA). Pacific blue-conjugated anti-mouse CD45 was obtained from BioLegend (San Diego, CA).

HIV-1 infection and ART in hu-Rag2^{-/-} γ_c ^{-/-} mice. hu-Rag2^{-/-} γ_c ^{-/-} mice with stable human leukocyte reconstitution (with a 10% or higher percentage of human CD45⁺ T cells in at least two determinations, 2 weeks apart) were infected with the CCR5-tropic HIV-1 clone JR-CSF (25 ng p24/mouse) by retro-orbital injection. Plasma viremia was assayed with the use of the Abbott RealTime reverse transcription-PCR (RT-PCR) automated m2000 system (Abbott Molecular Inc., Des Plaines, IL). Infected mice were treated daily with intraperitoneal injections of combined

emtricitabine (FTC), tenofovir (TFV), and L-870812 (60, 60, and 20 mg/kg of body weight, respectively, in a volume of 150 μ l) reconstituted in PBS, pH 11. Mice also received daily subcutaneous injections of enfuvirtide (100 mg/kg of body weight) reconstituted in water. Mice were bled by tail nicking, and the plasma viral loads and PB cell populations were assayed periodically. Due to a limited volume of plasma available at pre-terminal bleeds (50 to 100 μ l), samples were expanded by dilution for assay in the Abbott system. The limit of detection in this assay is 40 copies/ml or less, but due to dilution, the limit of detection of our assays was 500 to 800 copies/ml, depending on the available sample volume. Mice with suppressed plasma viral loads below this level of detection for at least 2 weeks were used in terminal experiments to determine the frequency of resting CD4⁺ T cell infection (RCI), and plasma viremia was measured in a large bleed volume at the time of sacrifice.

Purification of resting CD4⁺ T cells from hu-Rag2^{-/-} γ_c ^{-/-} mice. Human leukocytes from spleen, BM, liver, lung, FRT, and PB were enriched on 40 to 70% Percoll gradients by centrifugation (Percoll plus; GE Healthcare, Piscataway, NJ). As the thymus and LN already contain high percentages of human leukocytes, these tissues were not subjected to Percoll enrichment to minimize cell loss. Cells were pooled from all tissues and resuspended at 5 million cells/ml in separation medium (Ca²⁺- and Mg²⁺-free PBS containing 2% FBS), and human resting CD4⁺ T cells were enriched using a mouse/human enrichment kit (Stemcell Technologies, Vancouver, Canada), with modifications. Briefly, cells were incubated with a mouse/human enrichment cocktail (35 μ l/ml) supplemented with anti-mouse biotinylated CD31 and CD105 antibodies (0.5 μ g/ml each; eBioscience Inc., San Diego, CA), washed once with separation medium, and then incubated for 15 min with anti-biotin tetrameric antibody complex (TAC). Additionally, a custom TAC-conjugated human antibody cocktail (CD8, CD14, CD16, CD19, CD56, glycophorin A, CD41, HLA-DR, and CD25) (Stemcell Technologies, Vancouver, Canada) was added at this step to enrich human resting CD4⁺ T cells. Following incubation with magnetic colloids, cells were subjected to column chromatography to purify the human resting CD4⁺ T cell population by negative selection.

Viral outgrowth assay and determination of the frequency of RCI. Purified cells were cultured in RPMI 1640 medium containing 20% FBS, 15 nM efavirenz, and 1 μ M raltegravir at high densities (3 to 5 million cells/ml) for 2 to 3 days in U-bottom, 96-well culture plates. The presence of active viral replication in the culture supernatant was determined by p24 assay before phytohemagglutinin (PHA) stimulation. Cells were washed and plated at 10,000 to 100,000 cells/well in 12-well culture plates and maximally activated for 2 days with 1 μ g/ml PHA, 100 units/ml IL-2, and a 10-fold excess of irradiated peripheral blood mononuclear cells (PBMCs) from an HIV-seronegative donor. Control cultures received only 20 units/ml of IL-2. Cultures were fed twice with CD8-depleted, PHA-stimulated PBMCs. The culture supernatant was removed every 3 to 4 days and replaced with an equivalent volume of fresh medium containing 20 units/ml IL-2. We scored cultures as positive if p24 was detectable at 15 days following stimulation and confirmed on day 19. RCI frequency was estimated by a maximum likelihood method and is expressed as the number of infectious units per million resting CD4⁺ T cells (IUPM) (2).

RESULTS

Resting CD4⁺ T cells constitute the predominant cell population in the lymphoid tissue of hu-Rag2^{-/-} γ_c ^{-/-} mice. Secondary lymphoid tissues are the sites where the majority of lymphocytes reside in humans. They are also the sites of antigen presentation and lymphocyte activation and therefore a critical venue for HIV-1 replication and establishment of HIV-1 latency (29). We, therefore, enumerated human resting CD4⁺ T cells in several secondary lymphoid tissues, including LN, spleen, and BM, in hu-Rag2^{-/-} γ_c ^{-/-} mice with stable human-cell engraftment in PB (>10% of the engrafted cells in PB were human CD45⁺ cells) at 12 to 14 weeks posttransplantation. We observed

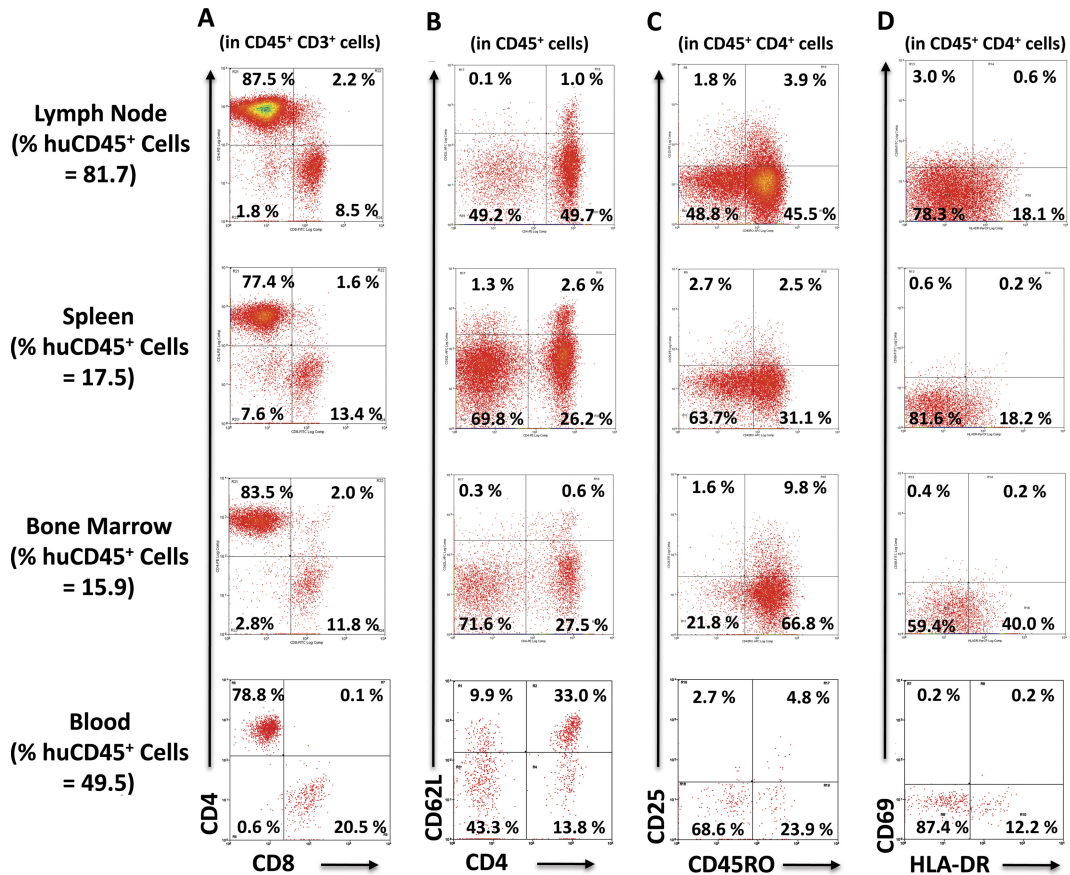


FIG 1 Characterization of human CD4⁺ (huCD4⁺) T cell reconstitution in lymphoid tissues and peripheral blood (PB). Cells from mesenteric lymph nodes, bone marrow, and spleen were harvested at 14 weeks posttransplantation and characterized by flow cytometric analysis. Live cells were discriminated based on forward and side scatter and gated on human CD45⁺/mouse CD45⁻ cells to quantitate human-cell reconstitution in each tissue (shown in parentheses). (A) Human CD45⁺ cells were gated on CD3⁺ cells to analyze CD4/CD8 T cells. (B and C) CD4⁺ T cells were characterized as naïve or memory cells based on expression of CD62L or the CD45RO antigen, respectively. (C and D) The resting state of CD4⁺ cells was determined by the lack of activation markers CD25, CD69, and HLA-DR. Data are representative of results from 5 uninfected animals.

the presence of several mesenteric and cervical LNs in these humanized mice. Axillary, brachial, and superficial inguinal LNs were also present, but infrequent. LNs were highly reconstituted with human cells; 70% (median; range, 56 to 82%) of cells present in the LNs of four mice were human CD45⁺ cells. Forty to 60% of the engrafted human cells were CD4⁺ T cells, and more than 48% uniformly expressed CD45RO but lacked CD62L, suggesting that they were memory cells (Fig. 1). Furthermore, greater than 75% of CD4⁺ T cells lacked early (CD25, CD69) and late (HLA-DR) activation markers, suggesting that they were resting cells. Spleen and BM were also significant sources of engrafted human cells (range, 50 to 72% of total human cells engrafted). As in the LNs, the majority of human cells in the spleen and BM were resting memory CD4⁺ T cells (Fig. 1). Human CD45⁺ T cells were also recovered from liver, lung, and the female reproductive tract (FRT), but they constituted less than 5% of the total human cells in the mice. Very rare human CD4⁺ T cells were found in the gut-associated lymphoid tissue (GALT) of this humanized mouse model. Therefore, it appears that LN, spleen, and BM are the major sources of resting memory CD4⁺ T cells in the lymphoid tissue of hu-Rag2^{-/-} γ_c ^{-/-} mice.

In contrast to lymphoid tissue, PB contained more naïve CD4⁺

T cells than memory cells (Fig. 1). The majority of CD4⁺ T cells in PB also lacked the activation markers CD69, CD25, and HLA-DR.

CD11b⁺ myeloid cells were detected in several lymphoid tissues at frequencies ranging from 1.4 to 7.6% in four mice. However, less than 0.2% of the total human cells were CD14⁺ in BM and LN, and only 0.5% of these cells were observed in the spleen, suggesting that macrophages are a minor population in this humanized mouse model at 12 to 14 weeks posttransplantation. We therefore conclude that resting CD4⁺ T cells constitute the predominant cell population in the lymphoid and peripheral tissue of hu-Rag2^{-/-} γ_c ^{-/-} mice; these cells are critical for the establishment and maintenance of persistent HIV-1 infection in humans.

Suppression of HIV-1 plasma viremia with ART. Infection of hu-Rag2^{-/-} γ_c ^{-/-} mice with CCR5-tropic HIV-1 JR-CSF resulted in productive HIV-1 replication in all mice at 12 to 14 days postinfection. We have previously reported that 3-drug ART comprised of the HIV nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) FTC and tenofovir (TFV) and the HIV integrase strand transfer inhibitor (InSTI) L-870812 at doses of 60, 50, and 20 mg/kg/day, respectively, suppresses HIV-1 plasma viremia below the limit of detection after 7 to 9 weeks of treatment (12). However, one mouse out of six in this earlier study failed therapy

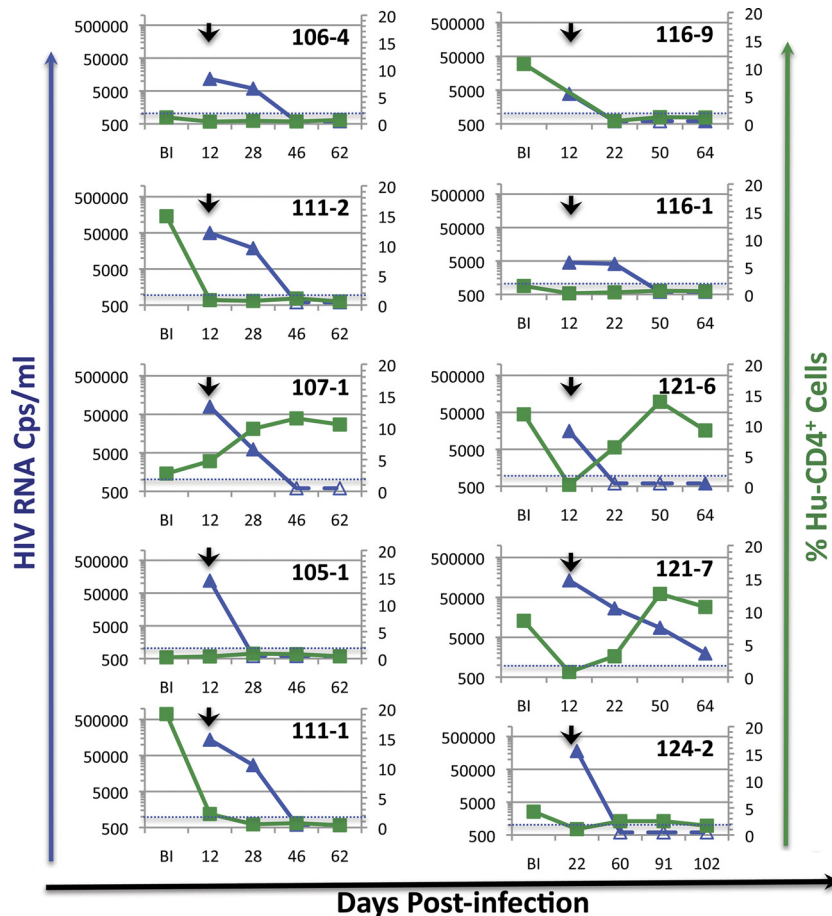


FIG 2 Suppression of HIV-1 plasma viremia with ART in hu-Rag2^{-/-} γ_c ^{-/-} mice. Mice were infected with JR-CSF (25 ng p24/mouse) and treated with the nucleoside/nucleotide reverse transcriptase inhibitors emtricitabine (FTC) and tenofovir (TFV), the integrase strand transfer inhibitor L-870812, and the fusion inhibitor enfuvirtide (60, 60, 20, and 100 mg/kg/daily, respectively). BI, time before infection. The arrows indicate the initiation of ART, the blue lines and triangles show plasma viremia, while the green lines and squares show the percentages of all PBMCs that are human CD3⁺ CD4⁺ T cells. The blue dotted lines show the limit of detection (<800 copies/ml) of plasma viremia in 50 to 100 μ l of samples obtained through the tail vein. The filled markers below the dotted lines indicate detection of plasma viremia in higher sample volumes at necropsy (limit of detection, 40 copies/ml).

due to the emergence of drug resistance mutations selected by tenofovir disoproxil fumarate (TDF) (K65R) and INSTIs (M154I). In our current study, ART was intensified with a fourth drug, enfuvirtide, a fusion inhibitor. The 4-drug ART regimen more rapidly suppressed plasma viremia, and viremia was below the limit of assay detection (500 to 800 copies/ml) (see Materials and Methods) in 15 out of 16 mice within 5 weeks, 2 to 4 weeks more rapidly than previously observed with a 3-drug ART regimen (Fig. 2). Although HIV was subsequently undetectable in most animals, occasional surges of viremia similar to the “blips” reported clinically were observed in a few mice (27). One animal (121-7) had detectable viremia after 64 days of treatment. Nevertheless, even in this animal, we observed a gradual and continuous decline in plasma viremia.

At the time of necropsy, larger volumes of plasma were collected, allowing the detection of lower levels of viremia in 6 of 8 mice. However, two mice had plasma viremia levels below 40 copies/ml. A viral blip was observed in mouse 127-6 at the time of necropsy. The observation of low but continually declining viremia after 9 weeks or less of ART is consistent with viral dynamics seen in human studies (26).

Suppression of viremia following ART allowed recovery of human CD4⁺ T cells in the PB of several mice, including 107-1, 121-6, 121-7, and 124-2. However, little CD4⁺ T cell recovery was detected in four animals on ART. Overall, these data show that 4-drug ART allows rapid suppression of plasma viremia and some recovery of CD4⁺ T cells in hu-Rag2^{-/-} γ_c ^{-/-} mice, analogous to the experience of ART-treated HIV-1-infected patients.

Quantitation of RCI in ART-suppressed hu-Rag2^{-/-} γ_c ^{-/-} mice. We sought to quantitate the frequency of RCI in ART-suppressed humanized mice in the pooled cells of peripheral blood and other lymphoid tissue, as mentioned in Materials and Methods. We recovered human cells following column purification, with <8% murine contaminants (Fig. 3A and B). More than 99% of human cells were CD3⁺ T cells with no detectable CD19⁺ and CD11b⁺ cells, demonstrating the successful exclusion of B cells, macrophages, and NK cells by column purification (Fig. 3 and data not shown). Virtually all T cells were CD4⁺ cells and lacked the activation markers CD25 and HLA-DR, identifying them as resting cells. We further characterized the resting CD4⁺ T cells based on CD27 and CD45 expression and observed that the vast majority were central memory cells (Fig. 3B). The median

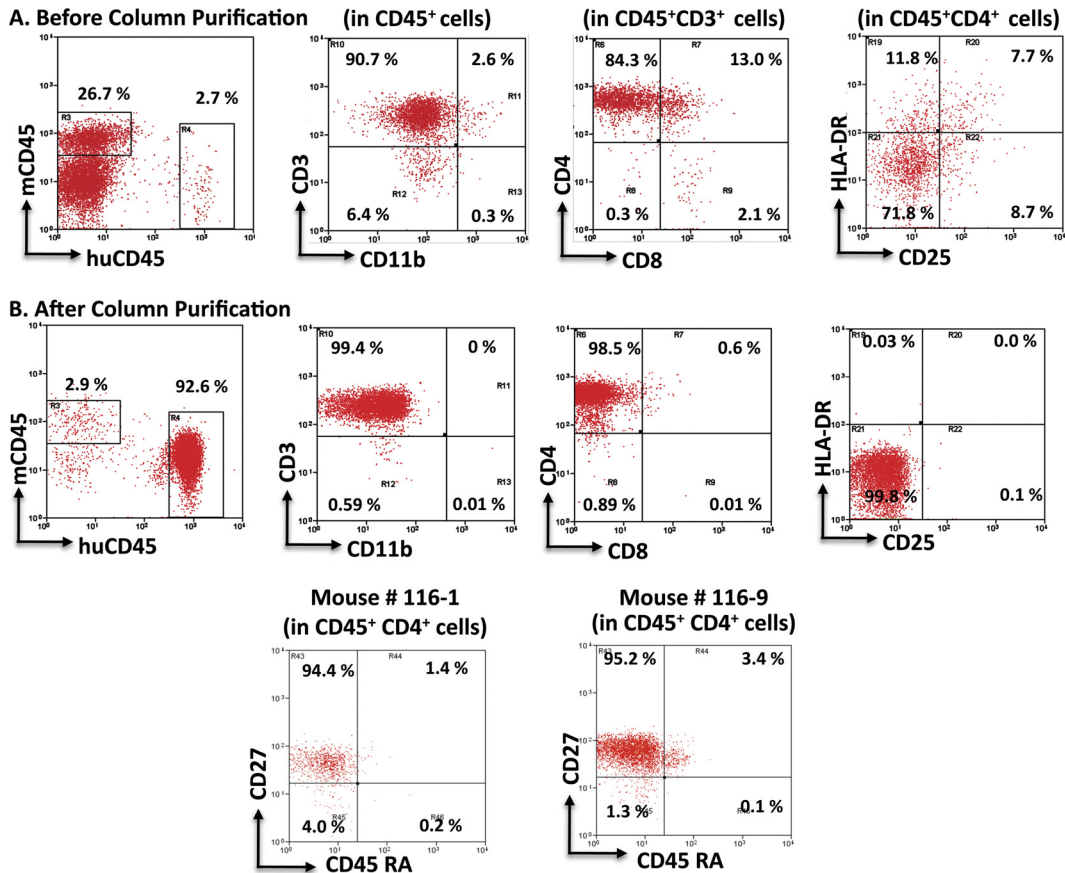


FIG 3 Isolation of resting CD4⁺ T cells from humanized mice. Resting CD4⁺ T cells were isolated from pooled blood and lymphoid tissues of each mouse by negative selection. Cells before (A) and after (B) magnetic bead enrichment are shown. CD4⁺ T cells lacked the activation markers CD25 and HLA-DR and were predominantly central memory cells (CD27⁺ CD45RA⁺). mCD45, mouse CD45⁺ cells.

TABLE 1 Recovery of replication-competent HIV-1 from latently infected resting human CD4⁺ T cells after mitogen activation

Mouse	No. of days on ART before outgrowth assay	No. of cultures that yielded HIV-1 (40,000 cells/culture)/total no. of cultures stimulated with:		Frequency of RCI (IUPM) in cultures stimulated with PHA
		PHA and IL-2	IL-2 only ^a	
111-2	64	2/8	0/4	7
116-1	52	2/7	ND	8
116-9	52	3/9	0/2	10
121-6	52	2/9	0/4	6
127-3	58	1/4	ND	12
124-2	102	1/11 ^b	0/2	2
135-1	102	2/5 ^c	ND	12
111-1	64	0/6	1/4	<7
105-1	90	0/4	0/4	<7
106-4	90	0/4	0/4	<7
107-1	90	0/4	ND	<7
121-7	52	0/8	ND	<3
127-2	58	0/4	ND	<11
127-5	58	0/2	ND	<37
127-6	58	0/2	ND	<37
134-10	102	0/7 ^d	ND	<6

^a ND, not determined.

^b Limiting dilution of 100,000 to 25,000 cells, with a total of 650,000 cells.

^c Limiting dilution of 100,000 to 10,000 cells, with a total of 245,000 cells.

^d Limiting dilution of 40,000 to 15,000 cells, with a total of 230,000 cells.

recovery of resting CD4⁺ T cells following column purification was 450,000 cells, with a range of 110,000 to 800,000 cells.

To validate that the resting CD4⁺ T cells isolated did not express HIV, cells from all of the mice except 105-1, 106-4, 107-1, and 111-1 were cultured for 2 to 3 days without stimulation and immediately tested for HIV-1 expression. No HIV gag p24 antigen was detected in these cultures, suggesting the absence of ongoing viral replication (22). To demonstrate that resting cells contained replication-competent HIV-1, the resting cells were maximally activated with PHA and cocultured with CD8-depleted activated PBMCs. Virus was recovered from resting CD4⁺ T cell cocultures of seven mice following stimulation with PHA (Table 1). Control cocultures performed with cells that were not maximally activated but that were incubated with a low concentration of IL-2 (20 U/ml) sufficient to support cell survival were negative, demonstrating that full activation is usually necessary to disrupt latency and recover replication-competent HIV (14). The outgrowth of HIV from none of the 8 activated cocultures but 1 of the 4 IL-2-supported cocultures (in mouse 111-1) likely reflects a chance event in the context of a low frequency of infected resting CD4 cells, similar to results seen in coculture assays from humans (3).

All mice were treated with ART for 50 to 102 days, and all except mouse 121-7 had no detectable plasma viremia for at least 24 days. The frequency of RCI, when it could be measured, varied in each mouse, ranging from 2 to 12 IUPM, with a median of 8

IUPM. Resting CD4⁺ T cells from the other nine mice yielded no replication-competent virus, but as fewer cells were available in many of these animals, the lack of detection of virus means that the frequency of RCI ranged from less than 3 to less than 37 infected cells per million total cells. If the data for all mice studied are pooled, the estimated RCI frequency is 3.8 infected cells per million.

DISCUSSION

In this study, we treated hu-Rag2^{-/-} γ_c ^{-/-} mice with intensified ART to model the HIV-1 latency in resting CD4⁺ T cells observed in patients. This humanized mouse model supports HIV-1 replication and CD4⁺ T cell depletion after infection with both CCR5- and CXCR4-tropic HIV-1 and displays long-term chronic infection (4, 6, 7, 38). Here, we report that memory CD4⁺ T cells constitute the major cell population in several lymphoid tissues, including the LN, spleen, and BM, 14 to 16 weeks after transplantation. Zhang and colleagues also observed that about 28% of cells were CD45RO⁺ memory CD4⁺ T cells in both HIV-1-infected and uninfected animals (38). We report that the majority of memory CD4⁺ T cells lacked activation markers, such as CD25, CD69, and HLA-DR, suggesting that the lymphoid tissues in this humanized mouse provide the milieu necessary for the maintenance of resting memory CD4⁺ T cells. We speculate that these resting cells might support an RCI within lymphoid tissue similar to that observed in HIV-1-infected patients.

To mimic RCI during ART in humans, HIV-1-infected mice were treated with a 4-drug ART regimen. In the macaque SIV model, 4-drug ART was also used to rapidly suppress viremia (19). The 4-drug ART regimen more reliably suppressed viremia than the 3-drug ART regimen previously reported (12). The new regimen suppressed viremia to below the level of detection in 2 to 5 weeks of treatment. Furthermore, suppression of viremia not only prevented further decline in CD4⁺ T cells but also resulted in an increased CD4⁺ T cell percentage in the PB of several mice. Zhang and colleagues observed an increase in the number of CD4⁺ T cells in the PB during low-level viral infection without ART, indicating the presence of a responsive and functional human immune system in this humanized mouse model (38).

We were able to purify human cells from hu-Rag2^{-/-} γ_c ^{-/-} mice by negative selection, and more than 98% of the human cells were resting CD4⁺ T cells. The activation of resting CD4⁺ T cells isolated from pooled tissue and cocultured with CD8-depleted PBMCs allowed recovery of replication-competent virus in several suppressed mice. Resting cells were cultured for 2 to 3 days in the presence of raltegravir and efavirenz before viral outgrowth to facilitate the degradation or circularization of the labile preintegration complex and to exclude the possibility that preintegration latency might contribute to the recovery of virus (30). No CD11b⁺ cells were detected following column purification, and therefore, macrophages and NK cells could not contribute to the recovery of persistent HIV. In culture, about 7% of the total cells were of murine origin, but these cells are refractory to HIV-1 replication due to several restrictions at the entry, transcription, and assembly stages of the viral life cycle (8, 21, 24). Together, these results suggest that latently infected resting CD4⁺ T cells were indeed the source of persistent infection in this mouse model.

The median frequency of RCI was 8 (range, 2 to 12) infected cells per million (IUPM) in seven mice treated with ART for 52 to

102 days. Resting CD4⁺ T cells from the other nine mice yielded no replication-competent virus on mitogen activation, but as fewer cells were available in many of these animals, the lack of detection of virus means that the frequency of RCI ranged from less than 3 to less than 37 infected cells per million cells. The RCI frequencies in these two cohorts therefore overlap and are similar to that seen in humans (33) and most similar to the RCI frequency of patients treated for a few months after acute HIV infection (34). If the data for all mice studied are pooled, the estimated RCI is 3.8 infected cells per million cells. Cultures supplemented with IL-2 (20 ng/ml) but not treated with mitogen yielded no viral outgrowth in any case except for a single culture from mouse 111-1. This likely reflects a chance event in the context of a low frequency of infected resting CD4⁺ cells. We have similarly observed that suboptimal concentrations of IL-2 can sometimes induce infrequent viral production in resting CD4⁺ cells from aviremic patients (14).

It has been well established that most of the proviral DNA integrated in the genome of a host on ART has intrinsic defects and that only 1% of HIV-1 DNA-positive CD4⁺ T cells can be induced to high-level HIV-1 gene expression after cellular activation (22). Limited numbers of human T cells are available in humanized mice to yield the purified resting CD4⁺ T cells needed to perform viral outgrowth assays. Therefore, we elected to focus on direct measurements of the frequency of resting CD4⁺ T cell infection and did not expend valuable cells to make surrogate measurements. This has allowed us to make direct comparisons of resting CD4⁺ T cell infections in humanized mice on suppressive ART with those in patients on ART and will ultimately allow precise testing of the effect of novel antilateness reagents.

In the SIV-infected macaque model of viral latency, a high RCI frequency in the PB was observed in suppressed animals at 64 and 99 days after ART, but it declined to 1.1 infected cells per million after 150 days of treatment (19). The number of IUPM in the lymphoid tissue was slightly higher than that in the PB of the monkey model, with 1.8 and 1.4 IUPM observed in spleen and gut LNs, respectively (19). It is likely that treatment of infected mice with ART for a shorter time (56 to 90 days), as well as the predominance of resting CD4⁺ T cells in the lymphoid tissue, might have contributed to a higher frequency of RCI.

In summary, this study validates hu-Rag2^{-/-} γ_c ^{-/-} mice as a model of persistent HIV-1 infection after suppressive ART. Moreover, our results suggest that HIV-1 infection persists in central memory CD4⁺ T cells, a reservoir of known importance for the eradication of HIV-1 infection in humans. Since these mice are highly reconstituted with memory CD4⁺ T cells, this model may be amenable to rigorous testing of novel therapeutic approaches before their application to HIV-1-infected patients. We have begun such studies. Our study thus provides a foundation for further optimization, such as improvements in the recovery of resting cells or in the pooling of cells from identical mice within a cohort, and points the way for studies of models of eradication of HIV-1 infection.

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