Journal of Visualized Experiments

Chronic, Acute, and Reactivated HIV Infection in Humanized Immunodeficient Mouse Models

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60315R2
Full Title:	Chronic, Acute, and Reactivated HIV Infection in Humanized Immunodeficient Mouse Models
Keywords:	NSG, CD34+, PBMC, Intrahepatic and Intraperitoneal injections, retro-orbital bleeding, HIV.
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Baltimore, Maryland, USA

TITLE:

Chronic, Acute, and Reactivated HIV Infection in Humanized Immunodeficient Mouse Models

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KEYWORDS:

$CD34^+$, PBMC, intrahepatic injections, intraperitoneal injections, retroorbital bleeding, HIV

SUMMARY:

Described here are three experimental approaches for studying the dynamics of HIV infection in humanized mice. The first permits the study of chronic infection events, whereas the two latter allows for the study of acute events after primary infection or viral reactivation.

ABSTRACT:

Humanized NOD/SCID/IL-2 receptor γ-chain^{null} mice recapitulate some features of human immunity, which can be exploited in basic and pre-clinical research on infectious diseases. Described here are three models of humanized immunodeficient mice for studying the dynamics of HIV infection. The first is based on the intrahepatic injection of $CD34^+$ hematopoietic stem cells in newborn mice, which allows for the reconstitution of several blood and lymphoid tissue-confined cells, followed by infection with a reference HIV strain. This model allows monitoring for up to 36 weeks post-infection and is hence called the chronic model. The second and third models are referred to as the acute and reactivation models, in which peripheral blood mononuclear cells are intraperitoneally injected in adult mice. In the acute model, cells from a healthy donor are engrafted through the intraperitoneal route, followed by infection with a reference HIV strain. Finally, in the reactivation model, cells from an HIV-infected donor under antiretroviral therapy are engrafted via the intraperitoneal route.

In this case, a drug-free environment in the mouse allows for virus reactivation and an increase in viral load. The protocols provided here describe the conventional experimental approach for humanized, immunodeficient mouse models of HIV infection.

INTRODUCTION:

The humanized NOD/SCID/interleukin (IL)-2 receptor γ-chain^{null} (hereafter referred to as huNS γ-chain^{null}) mouse model has been widely used for studying the pathogenesis of infections, autoimmunity, and cancer, as well as for pre-clinical studies of drugs and human cell-based therapies[1,2]. These mice are based on a non-obese diabetic (NOD) background, with the *scid* mutation and targeted mutation at the IL-2 receptor γ-chain locus (common γ-chain for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21), which induce a severe impairment in the development of mouse T-, B-, and natural killer (NK) cells[1]. Thus, they support the engraftment of human tissue, human CD34$^+$ hematopoietic stem cells (HSCs), and human peripheral blood mononuclear cells (PBMCs)[3–5]. In addition, transgenic expression of human hematopoietic factors, such as stem cell factor (SCF), granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-3 promotes the engraftment of human myeloid populations[6–8].

For HIV studies, several huNS γ-chain^{null} mouse models have been described, which differ in the mouse strain, type of human cells used, type of tissues for the engraftment, and origin of cells (i.e., healthy *vs*. HIV-infected donor)[9,10]. The original strain, however, is widely used due to the high levels of human cells engraftment and viral replication following infection with a reference HIV strain[11–13]. Similar immunodeficient mouse strains with transgenic expression of human hematopoietic factors (e.g., NOG-EXL or NSG-SGM3) or with implants of human liver and thymus tissues (bone marrow-liver-thymus [BLT] mice) are useful for evaluating the role of myeloid populations in the anti-HIV immune response, effects of HIV on these tissues, and their participation as viral reservoirs[14,15]. Furthermore, some strains with transgenic expression of human leukocyte antigen (HLA) molecules, as well as BLT mice, can be used for studying the T-cell response to HIV infection[16,17].

In general, in these mice, humanization depends on the cellular origin, delivery route (intraperitoneal, intrahepatic, intravenous, intracardiac) and mouse age at the time of engraftment[18–20]. Regarding the cell origin, human CD34$^+$ HSC derived from cord blood, fetal liver, or mobilized peripheral blood can be injected in newborn or young mice[3,21]. In addition, adult γ-chain^{null} mice can be humanized by the injection of PBMC (here, referred to as hu-PBL-NS γ-chain^{null} mice), allowing the temporal circulation of these cells in the blood, secondary lymphoid organs, and inflamed tissues[22–24].

Described here is a detailed protocol for the establishment of huNS γ-chain^{null} mouse models for the study of HIV infection. The first is the chronic model, in which human CD34$^+$ HSCs derived from cord blood from a healthy donor are injected in newborn mice, followed by infection with a reference HIV strain after 14 weeks of human immune system reconstitution. This model allows monitoring of mice for up to ~36 weeks after infection. The second model is

an acute model, in which PBMCs derived from a healthy donor are injected in adult NS γ-chain^{null} mice, followed by infection with a reference HIV strain after 3 weeks of human T-cell expansion in the mouse. Finally, the third model is the reactivation model, in which PBMCs derived from an HIV-infected donor under suppressive antiretroviral therapy (ART) are injected in adult NS γ-chain^{null} mice. In this case, a drug-free environment allows for viral reactivation and increase in the viral load. The two latter models allow monitoring for up to ~9 weeks after engraftment.

Overall, these three models are useful for virological studies, pre-clinical studies of novel drugs, and evaluation of HIV infection effects on the global immune response. It is also important to consider that use of HIV-infected humanized mice requires review and approval by the Institutional Biosafety Committee (IBC) as well as by the Institutional Animal Care and Use Committee (IACUC) before any experiment. This ensures that the study follows all internal and external institutional regulations for the use of hazardous biological material and humane handling of experimental animals.

PROTOCOL:

In this work, all animal care and procedures were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine (protocol numbers 1018017 and 1018018).

1. Human CD34$^+$ HSC engraftment of newborn mice

1.1. Always use disposable personal protection equipment (PPE), including sterile scrubs, gloves, dedicated shoes, shoe covers, mask, goggles, hair/beard bonnet, and sterile lab coats.

1.2. Re-suspend 1 x 10^6 of frozen CD34$^+$ HSCs (see **Table of Materials**) in 10 mL of RPMI 1640 media 10% FBS under a certified biosafety cabinet and maintain sterile conditions.

1.3. Use 10 µL of the suspension to count (to assure the presence of the expected number of cells) and check cell viability by trypan blue exclusion staining in a hemocytometer.

1.4. Centrifuge at 400 *x g* for 15 min at room temperature (RT). Discard the supernatant and resuspend in cold 1x PBS to the required concentration (1.4 x 10^5 of CD34$^+$ HSCs in 50 µL). Keep the cells on ice until injection.

1.5. Place pups (NS γ-chain^{null} pups of both genders from 1–4 days old) into a sterile 100 mm^2 Petri dish along with a small amount of bedding material from the breeder cage. Additionally, rub clean bedding between hands to further mask any foreign odors on the recipient pups.

NOTE: This minimizes foreign odors being impregnated onto the pups, thereby improving the chances of mothers accepting their pups back into cages after the procedure.

1.6. Put the Petri dish into a clean transport cage and place the cage in a carboy transport cage to protect pups from direct light and noise. Cover the transport cage with a cover pad to avoid exposure of animals to the environment while in transit to the irradiation room.

1.7. Irradiate pups with 100 cGy whole body irradiation (WBI) by exposure to a 137 Cs source. Clean the interior of the irradiator with disinfectant solution, place mice in the irradiator, and turn on the turntable so that all pups are irradiated homogeneously. Close the irradiator and press the power switch to initiate the irradiation process. Wait until the irradiation time is completed and immediately remove the mice.

NOTE: Since irradiation does not generate stress in the mice, previous anesthesia is not required.

1.8. 2–4 h after the irradiation procedure, place pups in a biosafety cabinet inside a chilled sterile Petri dish covered with sterile gauze on ice, until anesthetized (~5–10 min). Sufficient anesthesia is achieved when gross movements cease.

1.9. Load the syringe with an attached needle (29 G, 0.5”) with 50 µL of the cell suspension and 1.4 x 10^5 of $CD34^+$ HSCs under the certified biosafety cabinet.

1.10. For the engraftment via hepatic injection, restrain pups with the thumb and index fingers. To minimize the mouse restraint (~30–45 s), let one investigator hold the pup and administer the injection, and have the second investigator load the syringe with the HSC suspension. Clean the injection site with 70% alcohol and deliver 50 µL of cells directly into the liver. Use a shallow needle angle when injecting to avoid completely piercing the liver. As a control, inject mice with 50 µL of 1x PBS into the liver.

1.11. Place the pups on a pre-warmed sterile Petri dish covered with sterile gauze for 1–5 min to allow recovery. Pre-warm the dish using an infrared warming pad for rodents at 20 °C to ensure that pups will not be over-warmed.

1.12. Immediately before returning the pups to their parents, apply a small amount of menthol- and eucalyptus-based ointment, using the thumb and index fingers, to the snout of both parents to avoid cannibalism or rejection of the pups.

1.13. Check cages every day, looking for any signs of graft-versus-host disease (GVHD) in the pups such as dry skin, no feeding, rash, and alopecia. Euthanize the animals if any of these signs are observed.

1.14. Wean the pups at 3 weeks of age and house them in different cages. Verify engraftment in the peripheral blood by flow cytometry at 14 weeks of age.

NOTE: The success rate of engraftment is between 80%–100%.

2. Human PBMC engraftment of juvenile mice

2.1. For the acute and reactivation models, inject 6–8 week-old NS γ-chain^{null} mice intraperitoneally with human PBMCs derived from a healthy donor or HIV-infected patient who was under ART, respectively. In both models, include mice injected with PBMCs from a healthy donor without HIV infection (sham) as controls.

2.2. Layer 15 mL of whole blood in 5 mL of sterile density gradient medium into a 50 mL conical tube.

2.3. Centrifuge at 400 *x g* for 30 min at RT, without brakes, to avoid the buffy coat from becoming mixed with the density gradient medium.

2.4. Carefully collect the fraction of mononuclear cells (between the density gradient medium and supernatant) and transfer the buffy coat to a 15 mL centrifuge tube containing 10 mL of 1x PBS. Centrifuge at 300 *x g* for 10 min at RT.

2.5. Discard the supernatant and remove the remaining red blood cells by lysing with 5 mL of ACK buffer added to the pelleted cells. Incubate for 4 min at RT.

2.6. Centrifuge at 300 *x g* for 10 min at RT. Discard the supernatant and resuspend in 10 mL of 1x PBS or RPMI 1640 medium.

2.7. Use 10 µL of the cell suspension to count the cells and check viability by trypan blue exclusion staining in a hemocytometer. Typically, 1–2 x 10^6 cells are obtained for each 1 mL of blood, with more than 95% viability.

2.8. Centrifuge at 300 *x g* for 10 min at RT. Discard the supernatant and adjust the cell number to the required concentration (in this case, 3.5 x 10^6 cells in 200 µL of 1x PBS).

2.9. Load the syringe with an attached needle (28 G, 0.5”) with 3.5 x 10^6 of PBMCs in 200 µL of 1x PBS under a certified biosafety cabinet.

2.10. Remove the mouse from the cage and hold it by the tail so that it can grip the mesh, applying gentle traction backward. Then, place the index and thumb fingers on the shoulders of the animal, grabbing the loose skin of the neck and using the middle finger to stabilize its back.

2.11. Slide the mouse head back so that its back is above the head. This allows the viscera in the abdominal cavity to be displaced backward and reduces the risk of puncturing the internal organs during the injection.

2.12. Clean the injection site with 70% alcohol.

2.13. Penetrate the syringe used in step 2.9 through the abdominal wall and aspirate before

injecting the cells, if any material is aspirated, remove the syringe and discard it. Otherwise, inject the cells slowly in the intraperitoneal cavity, remove the syringe and discard it. Inject 1x PBS to control mice.

2.14. Return the animal to its cage.

2.15. Verify engraftment in peripheral blood by flow cytometry at 3 weeks post-injection.

3. Post-engraftment care

3.1. Identify mice by ear tagging.

3.2. Observe the mice used in these experiments closely 2x per day after each procedure for clinical signs of distress.

3.3. After human cells transplantation, monitor mice for GVHD. For monitoring GVHD symptoms in newborn, juvenile, and adult mice, evaluate animals for the appearance of skin disease (i.e., color, dryness, rash, alopecia), in addition to body weight measure. Evaluate animals that show these signs by a veterinarian to consider early euthanasia.

4. HIV infection procedure and sham infection procedure

NOTE: For the chronic and acute models, mice are infected with the HIV BaL reference strain at week 14 and week 3 post-engraftment, respectively. Injections with HIV is administered intraperitoneally into the lower abdominal quadrants.

4.1. Perform the loading process of the virus/PBS into syringes, using a 28 G 0.5" needle, in BSL2 cabinets following ABSL2 procedures. The total amount of virus injected is 15,000 median tissue culture infectious dose ($TCID_{50}$) in 200 μL of sterile RPMI 1640.

4.2. Remove the mouse from the cage and hold it by its tail so that it can grip the mesh, applying gentle traction backward. Then, place the index finger and thumb on the shoulders of the animal, grabbing the loose skin of the neck and using the middle finger to stabilize the back.

4.3. Slide the mouse head back so that its back is above its head. This allows the viscera in the abdominal cavity to be displaced backward and reduces the risk of puncturing internal organs during injection.

4.4. Clean the mice with a pre-wetted alcohol pad in the lower left/right quadrant of the abdomen. Inject 15,000 $TCID_{50}$ of the HIV BaL virus contained in 200 μL of sterile RPMI 1640.

4.5. After injection, return the mouse to its home cage.

5. Blood collection by retroorbital puncture

NOTE: Retroorbital bleeding allows for the fast collection of blood, thereby reducing the overall collection time and increasing the stability of human lymphocyte markers. Use EDTA tubes to collect mice blood.

5.1. In the chronic model, at 14 weeks post-HSC injection, collect the blood via retroorbital vein. In the acute and reactivation models, perform this procedure at 3 weeks post-PBMC injection.

5.2. Anesthetize the animals using isoflurane inhalation prior to blood collection in a biosafety hood class B2 that is ducted externally.

5.3. Dispense the Isoflurane into cotton pads under a wire mesh in a clear 1 L jar in a biosafety cabinet vented outside of the building. The use of the mesh ensures that the animals do not contact the isoflurane-soaked pad, which can cause skin irritation and potential overdosing since isoflurane is also absorbed through the skin. Also, put a soft paper towel between the mesh and animal to avoid limbs injuries.

5.4. Once the jar is saturated with isoflurane (approximately 1 min after adding it), introduce the animal and observe the respiratory rate, which will increase then decrease. Check for the clinical indication of a deep plane of anesthesia, which includes the lack of a righting reflex (upon tipping jar gently) and lack of gross movements. Start the bleeding procedure as soon as the animal is completely relaxed and lacking the toe pinch reflex.

NOTE: Since Isoflurane evaporates, dispense more drug if no signs of anesthesia are observed.

5.5. For the retroorbital bleeding, press the mouse external jugular vein caudal to the mandible with the thumb, and with the same hand, gently elevate the upper eyelid with the index finger.

5.6. Insert a hematocrit tube into the medial canthus of the eye and direct it in a ventrolateral direction until the blood starts fluxing.

5.7. Collect at least 100 μL of blood. Once the desired volume of blood is obtained (a volume no more than the 1% of the body weight of the animal), discontinue the external jugular pressure and remove the hematocrit tube.

5.8. Assure that the hemostasis is complete by applying the direct pressure on the eye using a sterile gauze for a minimum of 30 s.

5.9. Apply tetracaine drops in the eye. Monitor the animal until it has completely recovered from anesthesia and place it back in the cage.

NOTE: The 100 μL of collected blood is used for the evaluation of the level of engraftment of human $CD45^+$ and other blood cell populations, as well as for the evaluation of plasma viral load.

6. Screening of engraftment level and flow cytometry analysis

6.1. Follow a conventional flow cytometry staining protocol for whole blood, which includes the incubation of fluorochrome-labeled anti-human antibodies (for suggested flow panel, see **Table of Materials**), followed by the lysis of red blood cells and washing steps[13,15].

NOTE: For the screening of the level of engraftment, include an anti-human CD45 antibody. For the comparison, an anti-mouse CD45 antibody may also be used. Include compensation controls as well as a human blood sample stained with the same antibody mix, unstained mouse and human blood samples, and non-humanized control to test cross-reactivity of the reagents. After staining, there is always some background signal; however, all positive signals are clearly distinguished from negative and cross-reactive controls.

6.2. In an appropriate flow cytometer, acquire at least 10,000 events on the lymphocyte gate (FSC-A *vs.* SSC-A). For flow cytometry analysis, after duplicate exclusion, determine the percentage of human $CD45^+$ cells as well as other cell populations of interest.

7. Evaluation of plasma viral load

7.1. Evaluate the viral load in HIV infected animals 1x per week after infection.

7.2. After the retroorbital bleeding (approximately 100 μL), obtain plasma by collecting the supernatant after centrifugation of the anticoagulated blood at 3,500 *x g* for 3 min in a microcentrifuge. The pellet is used for blood cell phenotyping.

7.3. Use a commercial viral RNA extraction kit (see **Table of Materials**) to obtain RNA from 40 μL of plasma.

7.4. Convert RNA into cDNA using the first strain synthesis mix (see **Table of Materials**) and HIV gag primer SK431.

7.5. Perform quantitative real-time PCR using HIV Gag primers SK38/SK39 and fluorescent green dyes (e.g., SYBR green) as described in previous studies[13,25].

8. Administration of antiretroviral therapy

8.1. Administer oral ART at least 3 weeks after infection, when high viral load is observed, in the chronic, acute, and reactivation models.

8.2. Calculate the doses of tenofovir disoproxil fumarate (TDF), emtricitabine (FTC), and raltegravir (RAL), according to *Km* values of 37 and 3 for humans and mice, respectively[26]. Typically, the human-equivalent doses of TDF, FTC, and RAL are 61.7 mg/kg/day, 40.7 mg/kg/day, and 164 mg/kg/day, respectively.

8.3. For administration in drinking water, crush drug tablets and add the respective amount in the water bottle, ensuring that each mouse in the cage acquires its daily dose. Since the drug powder may form sediment in the bottle, periodically shake the water bottle to achieve homogeneous suspension.

8.4. Change the water bottle every week with freshly dissolved drugs.

8.5. Collect the blood via retroorbital vein every week or every 2 weeks after ART initiation to evaluate the changes in viral load and CD4:CD8 ratio.

9. Mouse euthanasia, collection of secondary lymphoid organs, and isolation of mononuclear cells

9.1. Euthanasia is performed in the three humanized mouse models, periodically along infection time, or at the end of the experiment.

9.2. Perform the euthanasia of adult mice by CO_2 asphyxiation, followed by the cervical dislocation. For asphyxiation, use a non-precharged chamber, dispense CO_2 from a commercial cylinder with fixed pressure regulator and in line restrictor controlling the gas flow within 20%-30% of the chamber volume/minute to comply with 2013 AVMA guidelines.

9.3. Maintain the CO_2 flow for >60 s monitoring respiratory arrest (which may take up to 5 min), followed by the cervical dislocation to assure euthanasia.

9.4. Euthanize neonates <7 days old by a physical method (i.e., using sharp scissors).

9.5. Visualize axillary, mediastinal, and mesenteric lymph nodes (which are typically observed) and extract them with tweezers and sharp scissors. Also extract the spleen, located in the upper left side of the peritoneal cavity.

9.6. Deposit the lymphoid tissues in 1.5 mL centrifuge tubes containing sterile RPMI 1640 medium.

9.7. Immediately process the lymphoid tissues in a 70 μm pore-size nylon cell strainer, collecting the cells in a 50 mL tube. Do not aspirate the tissues.

9.8. Wash the cells with 5 mL of RPMI 1640 medium supplemented with 1% FBS to facilitate cells filtering.

9.9. After the tissue disaggregation, centrifuge the cells suspension at 3,500 *x g* for 10 min in a microcentrifuge.

9.10. Discard the supernatant and resuspend the cells with 500 μL of 1x PBS.

9.11. Transfer the cells suspension into a 1.5 mL centrifuge tube containing 500 μL of sterile density gradient medium.

9.12. Centrifuge at 3,500 *x g* for 3 min in a microcentrifuge, without brake, to prevent the buffy coat from mixing with the density gradient medium

9.13. Carefully collect the fraction of mononuclear cells (between the density gradient medium and supernatant) and transfer the buffy coat to a 1.5 mL centrifuge tube containing 500 μL of 1x PBS. Centrifuge at 3,000 *x g* for 3 min.

9.14. Remove the remaining red blood cells by lysing with 500 μL of ACK buffer, incubating for 4 min at RT.

9.15. Centrifuge at 3,000 *x g* for 3 min. Discard the supernatant and resuspend in 1 mL of 1x PBS or medium.

REPRESENTATIVE RESULTS:

As described above, at 14 weeks post-HSC injection (chronic model) or at 3 weeks post-PBMC injection (acute and reactivation models), the mice are bled for screening the level of human cells engraftment by flow cytometry. A representative gating strategy for the evaluation of 1) human $CD45^+$ cells reconstitution and 2) percentage of $CD4^+$ and $CD8^+$ T-cells is shown in **Figure 1A**. Typically, the level of engraftment (percentage of human $CD45^+$ cells) ranges from 10%–80% after $CD34^+$ HSC injection and depends on the route of injection and mouse strain, among other previously described factors (**Figure 1B**). After PBMC injection, the level of engraftment (percentage of human $CD45^+$ or $CD3^+$ cells) ranges from 5%–65%, also with differences between the mouse strains (**Figure 1B**). In addition, some differences between mice injected with PBMC derived from a healthy versus an HIV-infected donor, can be observed (**Figure 1D**). Usually, for HIV infection, levels of engraftment above 5%–10% are enough for active viral replication.

Importantly, a characteristic of hu-PBL-NS γ-chain^{null} mouse models is the development of xenogeneic GVHD within a few weeks after cell engraftment, due to the human T-cell recognition of murine major histocompatibility complex (MHC) molecules [23]. This process is evident, even after 3 weeks post-PBMC injection, by signs such as hair and weight loss (**Figure 2A,B**), as well as by the increased expression of activation markers in T-cells such as HLA-DR and CD38 (**Figure 2C,D**). On the other hand, GVHD is more slowly developed in mice injected with human $CD34^+$ HSC and is directly correlated with the initial level of engraftment.

Following HIV infection, there is a rapid increase in plasma viral load, usually being detectable after 2–3 weeks post-infection, both in the chronic and acute models (**Figure 3A,B**), with similar kinetics in the reactivation model (**Figure 3C**). The increase in viral load coincides with a decrease in the CD4:CD8 ratio (**Figure 3D,E,F**). These changes are not observed in control mice (without HIV infection, **Figure 3**). Of note, in the hu-PBL-NS γ-chain^{null} mouse model, an initial

inversion of the CD4:CD8 ratio can be observed, being reconstituted along monitoring time (**Figure 3E,F**). Finally, if ART is administered to HIV-infected mice, a suppression of the viral load as well as recovery in the CD4:CD8 ratio is expected, reaching levels similar to those in uninfected controls (**Figure 3A,C,D,F**). Typically, after 2–3 weeks of treatment, a decrease in viral load and increase in the CD4:CD8 ratio is observed in the chronic, acute, and reactivation models. If this is not observed, the drug doses and the route of administration needs an evaluation.

FIGURE LEGENDS:

Figure 1: Representative gating strategy for evaluation of engraftment levels of human CD45$^+$ and T-cells. (**A**) Gating strategy used for the screening of the percentage of human CD45$^+$ (huCD45), CD3$^+$, CD4$^+$, and CD8$^+$ T-cells in huNS γ-chain^{null} mice, at week 14 following injection with cord blood CD34$^+$ HSCs. The numbers indicate the percentage of each population. (**B**) Representative levels of engraftment (percentage of huCD45$^+$ cells) in huNS γ-chain^{null} (n = 6) and a similar immunodeficient strain with transgenic expression of IL-3 and GM-CSF (huNOG-EXL, n=6), as reported previously[15]. (**C**) Representative levels of engraftment (percentage of huCD3$^+$ cells) in hu-PBL-NS γ-chain^{null} and hu-PBL-SGM3 mice (NS γ-chain^{null} mice with transgenic expression of SCF, GM-CSF, and IL-3), at week 3 following injection with PBMCs from a healthy donor (acute model, n = 7 and n = 8, respectively). (**D**) Representative levels of engraftment (percentage of huCD3$^+$ cells) in hu-PBL-NSG-SGM3 mice after injection with PBMCs from a healthy or HIV-infected patient who was under ART (reactivation model, n = 10 and n = 12, respectively). In B–D, the line indicates the median, and the p-value of the Mann-Whitney test is shown.

Figure 2: Development of GVHD in hu-PBL-NS γ-chain^{null} mouse model. (**A**) Hair loss in two representative hu-PBL-NSG-SGM3 mice, at week 7 following injection with PBMC from a healthy donor. (**B**) Mouse body weight loss throughout monitoring time normalized to the percentage of starting weight in hu-PBL-NSG-SGM3 mice injected with PBMC from a healthy donor (n = 10) and HIV-infected patient (n = 12). (**C**) Representative expression (at week 7 post-engraftment) of HLA-DR and CD38 in CD4$^+$ and CD8$^+$ T-cells from hu-PBL-NSG-SGM3 mice injected with PBMCs from a healthy donor. The numbers indicate the percentage of each population. (**D**) Representative percentages of CD4$^+$ and CD8$^+$ T-cells that are HLA-DR$^+$ CD38$^+$ in hu-PBL-NSG-SGM3 mice injected with PBMC from a healthy donor. Of note, in cells before the injection into mice, the levels of HLA-DR$^+$ CD38$^+$ CD4$^+$ and CD8$^+$ T-cells were 2.0% and 5.7%, respectively. In B and D, the median and interquartile range is shown.

Figure 3: Representative changes of viral load and CD4:CD8 ratio in huNS γ-chain^{null} mice after HIV infection and after ART introduction. (**A, D**) CD4:CD8 ratio and plasma viral load in huNS γ-chain^{null} mice after infection with HIV BaL (red dots and line, n = 3), which were performed after week 14 of injection with cord blood CD34$^+$ HSCd. Uninfected controls (PBS-injected) were also included (green dots and line, n = 5). (**B, E**) Plasma viral load and CD4:CD8 ratio in hu-PBL-NSG-SGM3 mice after infection with HIV BaL (red dots and line, n = 4), which was performed at week

3 following injection with PBMC from a healthy donor (acute model). Uninfected controls (PBS-injected) were also included (green dots and line, n = 3). (**C and F**) Plasma viral load and CD4:CD8 ratio in hu-PBL-NSG-SGM3 mice injected with PBMCs from an HIV-infected donor (red dots and line, n = 9) or healthy donor (green dots and line, n = 10) (reactivation model). In all cases, the median and interquartile range is shown. In A–C, the dashed line indicates the limit of detection of the assay (150 copies/mL). To samples with undetectable viral load, a value equal to one-half of the limit of detection was assigned. In D–F, the dashed line indicates a CD4:CD8 ratio of 1. In A, C, D, and F, the gray box indicates the time with administration of ART.

DISCUSSION:

Important advances have been achieved in the development of immunodeficient mouse strains for humanization, with a number of different options that can be used according to the research interest[1]. Provided here is a general protocol for the humanization of NS γ-chain^{null} mice and genetically similar strains to be employed in three different models for studying HIV infection. In the first experimental approach, irradiated newborn mice are injected with human CD34$^+$ HSCs, which can be derived from cord blood, fetal liver, or mobilized peripheral blood[3,21]. Appropriate irradiation of NS γ-chain^{null} mice is a critical step, as it eliminates the mouse bone marrow and other progenitor cells, allowing efficient reconstitution of human cell populations. However, some reports have evidenced reconstitution of human cells in different mouse strains, without irradiation[27]. In this regard, proper doses of irradiation must be provided, since NS γ-chain^{null} mice are radiosensitive, and high γ-irradiation could induce thymic lymphomagenesis[21,28].

Other critical steps and factors that could affect the level of engraftment include the route of injection (intrahepatic, intravenous, intracardiac), mice age, percentage of purity of CD34$^+$ HSCs, and operator expertise[29]. In the second and third approaches based on hu-PBL-NS γ-chain^{null} mouse models, some critical factors include the route of injection (intraperitoneal, intravenous, intrasplenic), mice age, and number of human cells injected, which can influence the final level of engraftment. Regarding this latter factor, several studies have used 5–10 x 10^6 PBMCs for engraftment[22,23,30], whereas the present protocol suggests the use of 3.5 x 10^6 PBMCs. Of note, this number of cells is sufficient for the reconstitution of T-cells and for HIV replication, both in the acute and reactivation models, and also delays the development of GVHD[23]. Nonetheless, investigators should optimize the humanization conditions according to the research objectives. Moreover, it is important to validate the HIV strain used for infection of huNS γ-chain^{null} mice. Here, the R5 tropic HIV-1 BaL strain is used, which yields high levels of viral replication in huNS γ-chain^{null} mice. Other reporter strains, such as those containing luciferase or fluorescent proteins, are also suitable for single-cell analysis of HIV-infected cells[31].

Overall, three major limitations are evidenced in huNS γ-chain^{null} mouse models following engraftment with CD34$^+$ HSC. First, due to the absence of a human thymic environment, T-cells are educated in the context of murine MHC molecules, restraining subsequent antigen-specific stimulation via their T-cell receptors. This issue limits the use of NS γ-chain^{null} mouse models for

studying the HIV-specific T-cell response. Nonetheless, this limitation can be overcome by the use of BLT mice or NS γ-chain^{null} mice with transgenic expression of HLA molecules[16,17]. Second, typically there is poor reconstitution of myeloid populations in NS γ-chain^{null} mouse models, limiting the study of these subsets that have relevance in the context of antigen-presentation and pathogenesis of HIV infection[14,15]. In this case, the use of mouse strains with transgenic expression of hematopoietic factors is recommended[8,15,32].

Third, there is a 1) poor development of lymphoid follicle structures in the secondary lymphoid tissues and 2) lack of tertiary lymphoid tissues, which is related to the low levels of innate immune cells (i.e., dendritic cells in huNS γ-chain^{null} mice) that are critical for the development of follicles[33]. This issue is associated with a poor humoral response in huNS γ-chain^{null} mouse models[34]. Nonetheless, some reports have evidenced the development of follicle-like structures in huNS γ-chain^{null} mice[4], whereas spleen- and lymph node-confined follicular T-cells (expressing the follicle-homing chemokine receptor CXCR5) are detected in huNS γ-chain^{null} mice and related strains[15]). Again, the use of 1) BLT mice or 2) mouse strains with transgenic expression of hematopoietic factors and/or with expression of HLA molecules can improve the reconstitution of myeloid populations, development of organized secondary and tertiary lymphoid structures, and effective T-cell and B-cell responses[8,35,36].

Similar to the limitations of CD34^{+} HSC-humanized NS γ-chain^{null} mouse models, there is a lack of antigen-specific T-cell and humoral responses, absence of myeloid populations, and organized lymphoid structures in hu-PBL-NS γ-chain^{null} mice. In addition, an important limitation of the hu-PBL-NS γ-chain^{null} mouse model (acute and reactivation models of HIV infection) is the short window for monitoring, since these mice develop xenogeneic GVHD[23]. The development of GVHD could also induce undesired phenotypic and functional changes of immune populations, inherent of the pathogenic process[23,37]. Nonetheless, this model has the advantage of being simpler and more accessible, particularly considering that human PBMCs are more easily acquired from healthy or HIV-infected donors[38]. In addition, the injection of primary cells directly from patients is useful for the study of cell- or pathogen-intrinsic conditions of the donor, such as viral drug resistance mutations or donor-specific immune alterations. Of note, for the reactivation model, in vitro assays with HIV reactivation agents can be performed to corroborate the response of PBMCs before injection into mice[39]. Another limitation for some institutions is that this work requires BSL2+ facilities to handle HIV-infected animals due to regulations.

The huNS γ-chain^{null} mouse models have some advantages in comparison with other animal models for studying HIV infection, such as nonhuman primates infected with simian immunodeficiency virus. For instance, huNS γ-chain^{null} mice allow the creation of gene knockout or transgenic strains, which permit the evaluation of specific gene targets. Additionally, the use of primary human cells in huNS γ-chain^{null} mice avoids possible species-specific restrictions, such as the case of interferon-stimulated genes in nonhuman primates, which can influence the antiviral response and course of infection[40]. Thus, the kinetics of infection are highly consistent between huNS γ-chain^{null} mice. Finally, huNS γ-chain^{null} mouse models are less expensive, do not

require complex core facilities, and are more accessible.

In summary, $CD34^+$ HSC-humanized and hu-PBL-NS γ-$chain^{null}$ mouse models offer a variety of possibilities for the study of chronic, acute, and reactivation events in HIV infection. With the recognition and overcoming of the aforementioned limitations of these models, the use of NS γ-$chain^{null}$ mice may be a powerful tool for virological, immunological, and drug pre-clinical studies, as well as for genome editing and cell-based immunotherapies.

ACKNOWLEDGMENTS:

This work was supported by IHV clinical division internal funds to JCZ.

DISCLOSURES:

The authors have nothing to disclose.

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Figure

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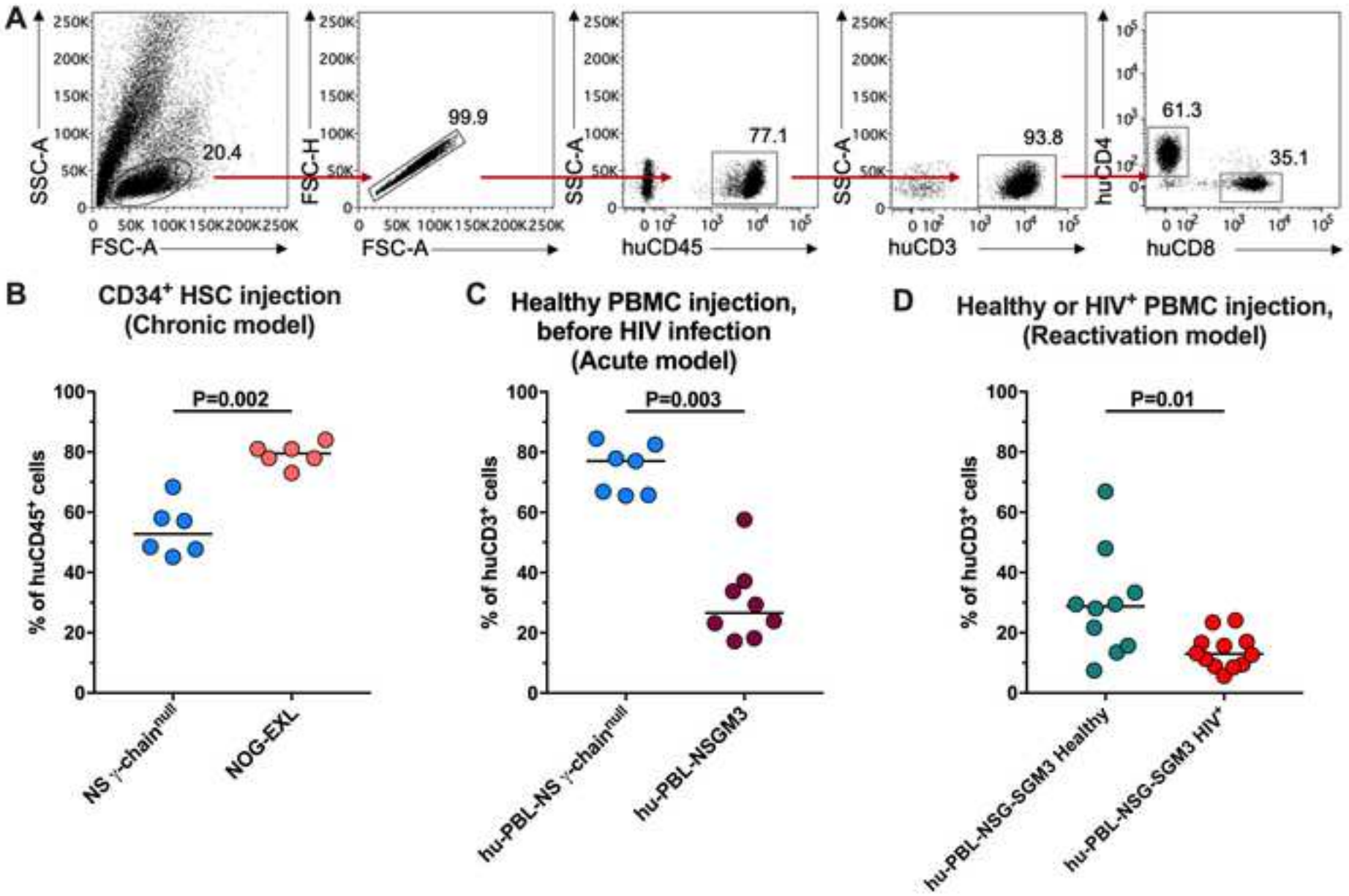

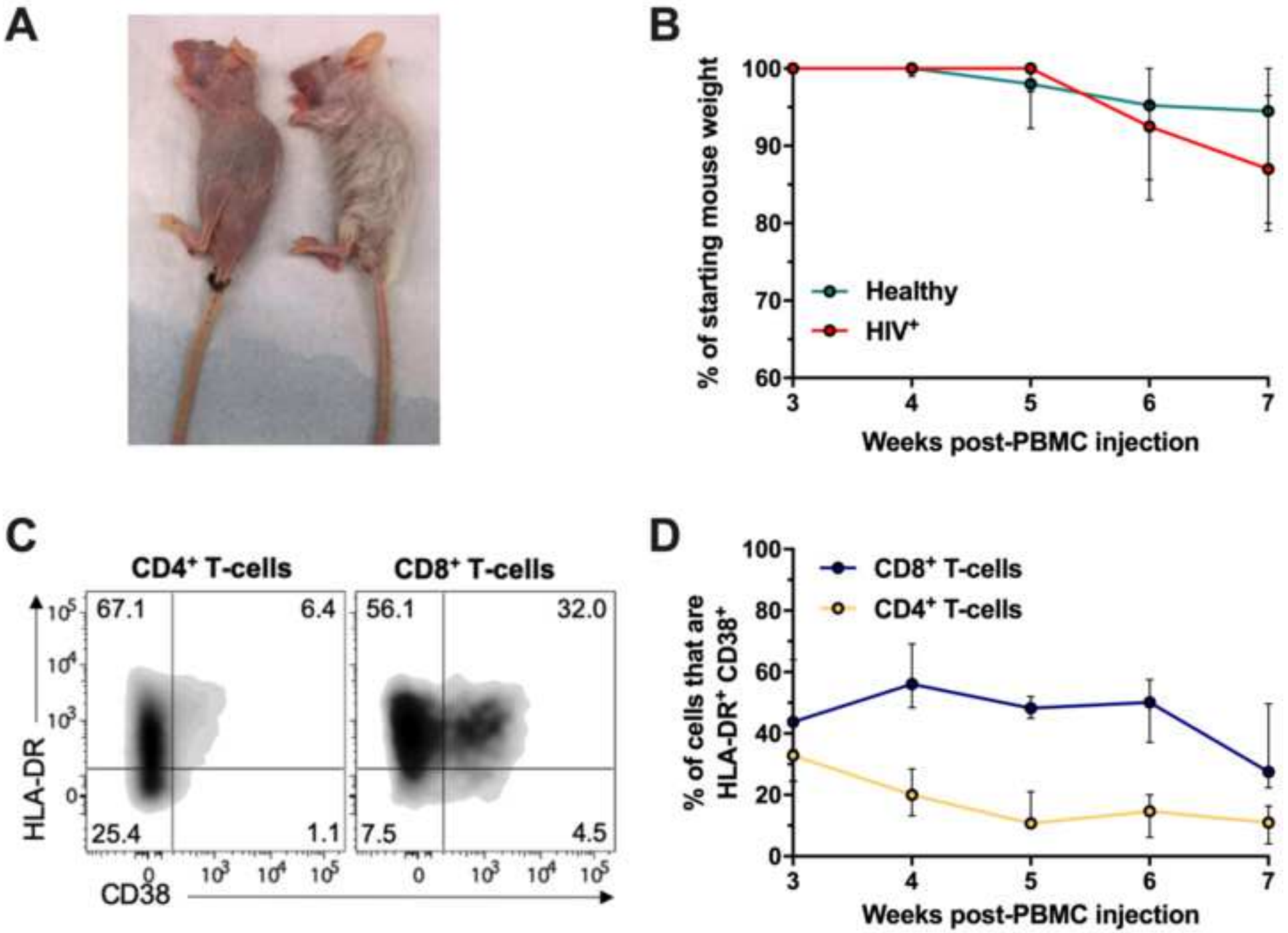

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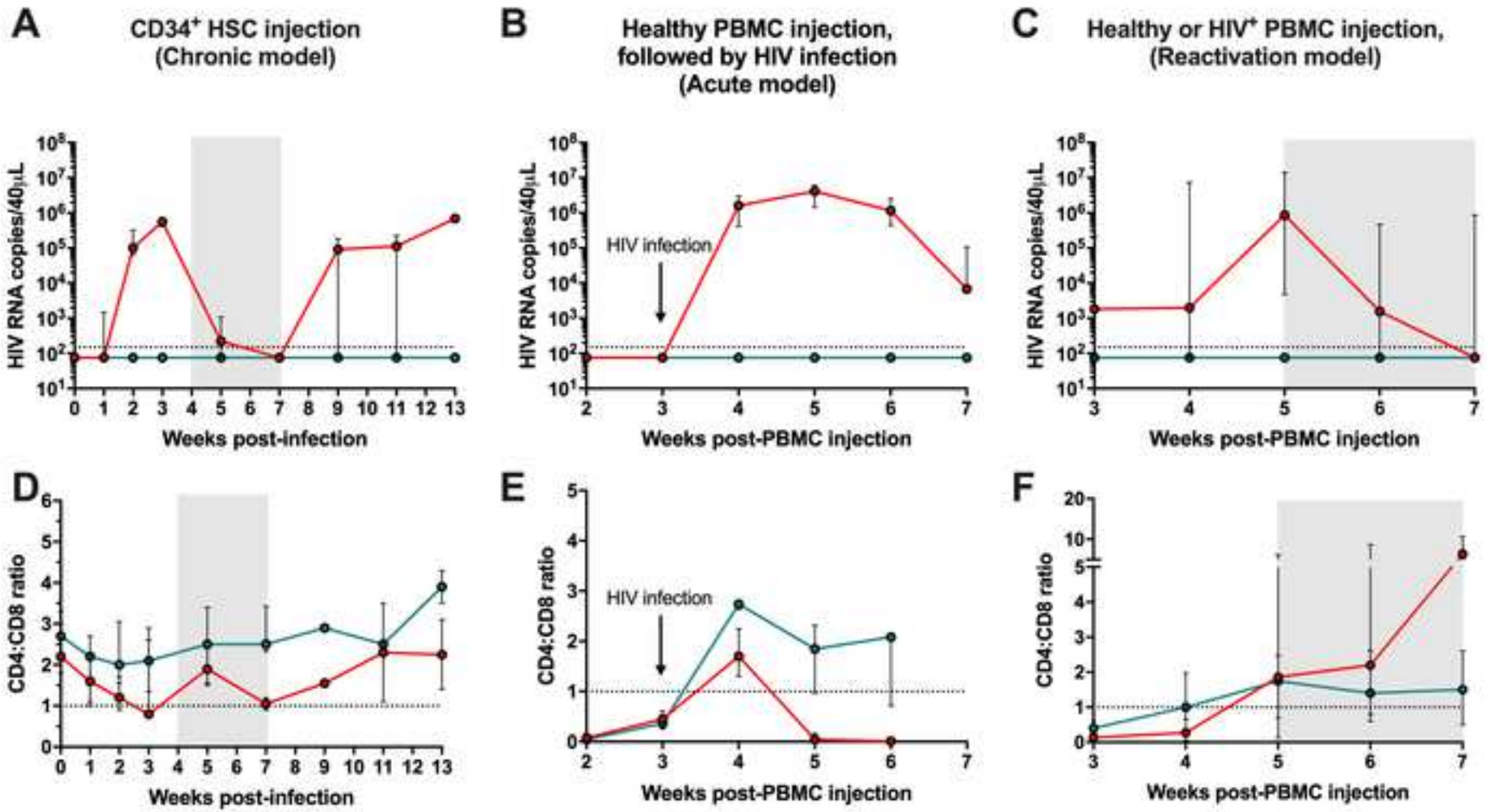

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5 ml Serologial pipetes	stellar sceintific	VL-4090-0005
50 ml conical tubes	Stellar scientific	T50-600
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Surface desinfectant
As many vials available from a single donor

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5 ml polystyrene and round bottom
Prepared in our uQUANT core facility
HIV positive and negative volunteers
Temporary therapeutic warming pad for small animals
Antiretroviral medication to treat human immunodeficiency virus (HIV)-Integrase inhibitor
Equipment belonging to university of Maryland

Time pregnant females for CD34 engraftment and Juveniles for PBMCs engraftment

cDNA synthesis

Antiretroviral medication to treat human immunodeficiency virus (HIV)-Nicleoside analog-transcriptase inhibitor

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A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

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RESPONSE TO REVIEWERS
Humanized NSG mouse models of HIV infection: chronic, acute, and reactivation models
JoVE60315

The authors would like to acknowledge the editor and reviewers for taking the time to correct this manuscript. The following are our answers to your suggestions.

Kind regards,

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: As suggested, we thoroughly revised the manuscript and corrected any errors.

2. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Vicks Vaporub, Truvada, Gilead Sciences, raltegravir, Isentress, Merck, etc

Response: As suggested, we changed the commercial terms as follows:
-Lines 141-142: “Vick Vaporub” was changed for “an ointment based on menthol and eucalyptus”).
-Lines 314-315: Truvada, Gilead Sciences, Isentress and Merck were deleted from the manuscript.

3. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 110-114, 135-139.

Response: As suggested, we edited the section 1.3 in lines 105-109 and section 1.8 in lines 130-134.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

Response: As suggested, we changed all the text for an imperative tense. For instance, see:
-Line 294: “an anti-human CD45 antibody should be included” was changed for “include an anti-human CD45 antibody”.
-Line 340: “Neonates <7 days old should be euthanized by a physical method” was changed for “Euthanize neonates <7 days old by a physical method, such as sharp scissors”.
-Line 377: “mice should be bled for” was changed for “bleed the mice for screening the level”.

5. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

Response: As suggested, we removed discussion from the protocol.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.

Response: As suggested, we edited the sections in the protocol and removed large paragraphs.

7. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Response: As suggested, we added more details to the protocol. Lines 141, 144-145, 160-178, 207, 297, 305-306, 309-310.

8. 1.3: Age, strain sex of the pups used?

Response: The strain, sex and age of pups were added in line 105.

9. 1.5: why is the irradiation performed? Is it ok to perform before anesthesia?

Response: Irradiation should be performed to eliminate mouse bone morrow and other progenitor cells, which improves the engraftment of human cells (discussed in lines 460-462). Since irradiation does not generate stress in the mice, previous anesthesia is not required. This statement was added in line 121.

10. 1.7: How do you obtain the same? How do you calculate the cell number?

Response: Lines from 160 to 180, briefly describe the steps for PBMC isolation and counting.

11. Lines 153-158, 181-187: Please convert to numbered action step in imperative tense.

Response: As suggested, we converted this paragraph in section 2.1 (line 155-158) and section 3.1-3.3 (line 207-209).

12. 4.1: What is the virus used? MOI of infection? What volume? Please include all details.
Response: Information about the virus strain was included in lines 219 and 236. Dose (in $TCID_{50}$), volume and inoculation route are in 4.4 lines 235-237.

13. 5.1, 9.1: Please make substeps and make it an action step in imperative tense. We cannot have paragraph of texts in the protocol section.

Response: As suggested, we divided these paragraphs in three sections (5.1-5.3, lines 247-257; and 9.1-9.3, line 332-340).

14. 6, 7: Please make numbered action steps in imperative tense.

Response: As suggested, we divided these paragraphs in action steps. Lines 282-297.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: The essential steps of the protocol for the video are from section 1 to 4 (lines 96-239).

16. Please do not highlight euthanasia steps.

Response: As suggested, these steps are not highlighted.

17. Please include non-humanized control to test for the cross reactivity of the reagents.

Response: As suggested, we included non-humanized controls in line 296-297.

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: This is not a modification of our previous figure. The first box was generated using old data and the following two boxes were generated with totally new data.

Therefore, the legend of figure 1 was modify as follows: “As reported previously by our group”. Lines 416-417.

19. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique.
c) Any limitations of the technique.
d) The significance with respect to existing methods.
e) Any future applications of the technique.

Response: As suggested, we explicitly covered these issues in the discussion. For example, critical steps are detailed in line 460-462, 465-466, and 469. Limitations are described in lines 480-511. Modifications and alternatives for technique limitations are described in line 486, 489-490, and 500-504. Some advantages of the models in comparison with nonhuman primates are described in line 523-530. Finally, the applications of the technique are summarized in line 532-536.

20. Please sort the materials table in alphabetical order.

Response: As suggested, the table of materials was sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript content seems highly relevant and contemporal to the current experimental techniques in murine models. The models can be used to study HIV-1 associated preclinical research. Robust reconstitution of immune cells in animal models is a highly useful technique in developing disease models which the authors have described in their experimental approach.

Major Concerns:

a. **The authors refer to the use of human-specific reagents (e.g., antibodies to detect human cell engraftment by FACS) but there is no mention of the testing of non-humanized mice to confirm that the reagents are truly human-specific. The authors should state if they have carried out those control experiments, and if any cross-reactivity was detected by FACS.**

Response: As suggested, we included non-humanized controls to test the reactivity of human-specific reagents. Line 297.

b. The discussion should mention if the animal models show comparative advantage over other established animal models? and/or leading to better results.

Response: As suggested, we included some advantages of the huNSG mouse models in comparison with nonhuman primates in line 523-530.

Minor Concerns:
a. Description of protocols should be directive instead of narrative. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. e.g sections 3, 4.1, 4.4, 5.1 etc.

Response: As suggested, we changed all the text for an imperative tense. For instance:
-Line 294: "an anti-human CD45 antibody should be included" was changed for "include an anti-human CD45 antibody".
-Line 340: "Neonates <7 days old should be euthanized by a physical method" was changed for "Euthanize neonates <7 days old by a physical method such as sharp scissors".
-Line 377: "mice should be bled for" was changed for "bleed the mice for screening the level".

b. Section. Lines 214-229 and 249 - 263: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the discussion about the protocol to the 'Discussion.'

Response: As suggested, we removed discussion from the protocol.

Reviewer #2:

Manuscript Summary:

This protocol presents the detailed procedure for generation of humanized mouse models for HIV acute and chronic (Latent) HIV infection and reactivation. This JOVE will be very useful to the researchers in the field of HIV infection, genome editing, drug discovery and immune therapy.

Major Concerns:

1. This procedure required BSL2+ (at least) for IBC and IACUC in most institutes. This should be included or discussed. Why only BSL2 is proposed? Due to this requirement, the application of this JOVE will be limited.

Response: By regulation HIV virus should be handled at least in BSL2. While infected animals should be handled in BSL-2+ facilities. We mention IBC and IACUC requirements in lines 367-371 and 520-521.

2. For the reactivation model, there were no descriptions or discussions about whether the selected HIV latent PBMCs are responsive to HIV reactivation agents. This responsive efficacy should be validated before doing the PBMC i.v. injection.

Response: The rationale of the reactivation model is that in a drug-free and inflammatory/immune activation environment such as GVHD in the mouse, latent HIV will reactivate, leading to increase in viral load. This scenario is similar to an analytical treatment interruption in HIV-infected patients. Based on this context, the evaluation of HIV reactivation agents is not suggested in our protocol. Nonetheless, we added a discussion on this issue in line 518-520.

3. Selection of HIV reference strain for the protocol may need a discussion. The benefits of using HIV-1 reporter strains (Luciferase or EGFP, etc) may be also necessary to be discussed.

Response: As suggested, we included a discussion on this issue in line 478-481.

Minor Concerns:
Careful proofreading is needed. For example, "mice" model sounds wrong.

Response: As suggested, we thoroughly revised the manuscript and corrected any errors.

RESPONSE TO EDITORIAL COMMENTS
Humanized NSG mouse models of HIV infection: chronic, acute, and reactivation models
JoVE60315_R1

Dear editor,

Thank you for working with us to improve this manuscript. The following are the answers to your comments.

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Response: As indicated, the manuscript format is retained.

2. Please address all the specific minor comments marked in the manuscript.

Response: As suggested, all the minor comments were addressed in the manuscript.

3. Once done please proofread the manuscript well for any grammar or spelling issues.

Response: The manuscript was proofread for any errors.

4. Once done please also ensure that the highlight is no more than 2.75 pages including headings and spacings.

Response: The highlights include 2.75 pages (from steps 1 to 3).

Kind regards,

Juan Carlos Zapata