Journal of Visualized Experiments

Humanized NOD/SCID/IL2rγnull (hu-NSG) for HIV replication and latency studies

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58255R2
Full Title:	Humanized NOD/SCID/IL2rγnull (hu-NSG) for HIV replication and latency studies
Keywords:	Neonatal NSG mouse; Humanized mice; HSCs; HIV; cART; Latency
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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 **full address** at which this article will be **filmed**.	1500 E Duarte Road, 2002 Fox North, Duarte, CA 91010

1 **TITLE:**

2 **Humanized NOD/SCID/IL2rγ^{null} (hu-NSG) Mouse Model for HIV Replication and Latency**

3 **Studies**

4

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26 **Summary:**

27 This protocol provides a method to establish humanized mice (hu-NSG) via intrahepatic

28 injection of human hematopoietic stem cells into radiation-conditioned neonatal NSG mice. The

29 hu-NSG mouse is susceptible to HIV infection and combinatorial antiretroviral therapy (cART)

30 and serves as a suitable pathophysiological model for HIV replication and latency investigations.

31

32 **Abstract:**

33 Ethical regulations and technical challenges for research in human pathology, immunology, and

34 therapeutic development have placed small animal models in high demand. With a close

35 genetic and behavioral resemblance to humans, small animals such as the mouse are good

36 candidates for human disease models, through which human-like symptoms and responses can

37 be recapitulated. Further, the mouse genetic background can be altered to accommodate

38 diverse demands. The NOD/SCID/IL2rγ^{null} (NSG) mouse is one of the most widely used

39 immunocompromised mouse strains; it allows engraftment with human hematopoietic stem

40 cells and/or human tissues and the subsequent development of a functional human immune

41 system. This is a critical milestone in understanding the prognosis and pathophysiology of

42 human-specific diseases such as HIV/AIDS and aiding the search for a cure. Herein, we report a

43 detailed protocol for generating a humanized NSG mouse model (hu-NSG) by hematopoietic

44 stem cell transplantation into a radiation-conditioned neonatal NSG mouse. The hu-NSG mouse

45 model shows multi-lineage development of transplanted human stem cells and susceptibility to
46 HIV-1 viral infection. It also recapitulates key biological characteristics in response to
47 combinatorial antiretroviral therapy (cART).
48
49 **INTRODUCTION:**
50 Because establishing suitable animal models for human diseases is key to finding a cure,
51 appropriate animals models have long been pursued and improved over time. Multiple strains
52 of immunocompromised murine models have been developed that permit the engraftment of
53 human cells and/or tissues and the subsequent execution of humanized functions[1,2]. Such
54 humanized mouse models are critical for investigations of human-specific diseases[3–5].
55
56 Acquired immune deficiency syndrome (AIDS) resulting from infection with human
57 immunodeficiency virus (HIV) is one example. Prior to the establishment of humanized mouse
58 models, ethical and technical limitations confined HIV/AIDS preclinical animal studies to non-
59 human primates[3]. However, the high expenses and requirements for specialized care for such
60 animal hinder HIV/AIDS studies in typical academic settings. HIV primarily infects human CD4+
61 T-cells and impacts the development and immune responses of other human immune cells such
62 as B-cells, macrophages, and dendritic cells[6]; therefore, small animal models transplanted with
63 functional human immune systems are in high demand.
64
65 A breakthrough came in 1988, when CB17-*scid* mice with a *Prkdc^scid* mutation were developed
66 and showed successful engraftment of the human immune system[1]. The *Prkdc^scid* mutation
67 results in defective T- and B-cell functions and an ablated adaptive immune system in mice,
68 thereby enabling the engraftment of human peripheral blood mononuclear cells (PBMCs),
69 hematopoietic stem cells (HSCs), and fetal hematopoietic tissues[7,8]. Nonetheless, low levels of
70 engraftment are frequently observed in this model; possible causes are 1) residual innate
71 immune activity modulated via natural killer (NK)-cells and 2) the late-stage development of
72 mouse T- and B-cells (leakiness)[5]. The subsequent development of the non-obese diabetic
73 (NOD)-*scid* mouse model achieved dramatic down-regulation of NK-cell activity; thus, it is able
74 to support a higher level and more sustainable engraftment of human immune system
75 components[9]. To further suppress or impede development of innate immunity, mouse models
76 bearing truncation or total knockout of the interleukin-2 receptor γ-chain (Il2rg) in the (NOD)-
77 *scid* background were established. Il2rg, also known as common cytokine-receptor γ-chain, is an
78 indispensable component of various cytokine receptors[10–13]. Strains such as NOD.Cg-
79 *Prkdc^scidIl2rg^tm1Wji* (NSG) and NODShi.Cg-*Prkdc^scidIl2rg^tm1Sug* (NOG) present robust disruption of
80 mouse cytokine signaling and complete ablation of NK-cell development, in addition to severe
81 impairment of adaptive immunity[14–16].
82
83 Three humanized mouse models bearing a *scid* mutation and Il2rg knockout are frequently
84 employed in HIV/AIDS research: the BLT (Bone marrow/Liver/Thymus) model, the PBL
85 (Peripheral Blood Leukocyte) model, and the SRC (SCID Repopulating Cell) model[3]. The BLT
86 model is created via surgical transplantation of human fetal liver and thymus under the mouse
87 kidney capsule accompanied with intravenous injection of fetal liver HSCs[3,17,18]. The BLT mouse
88 model offers high engraftment efficacy, development of human hematopoietic cells in all

89   lineages, and establishment of a strong human immune system; additionally, T-cells are
90   educated in a human autologous thymus and exhibit HLA-restricted immune responses[4,5,17,19].
91   However, the requirement for surgical procedures remains the major drawback of the BLT
92   model. The PBL mouse model is established by intravenous injection with human peripheral
93   lymphoid cells. The PBL model offers convenience and yields successful T-cell engraftment, but
94   its application is limited due to insufficient B-cell and myeloid cell engraftment, low
95   engraftment levels overall, and the onset of severe graft-versus-host disease (GVHD)[3,20]. The
96   SRC mouse model is established through injection of human HSCs into newborn or young adult
97   SCID mice. It exhibits average engraftment efficiency above 25% (assessed as peripheral blood
98   CD45 percentage) and supports the multiple-lineage development of injected HSCs and the
99   elaboration of an innate human immune system. However, the limitation of the SRC model is
100   that the T-cell response is mouse H2-restricted instead of human HLA-restricted[14,21].

102   The SRC mouse model is considered a facile and reliable model for preclinical HIV/AIDS small
103   animal studies, exemplified by the consistent engraftment of a human immune system and
104   successful hematopoietic development. We previously reported the establishment of a NSG Hu-
105   SRC-SCID (hu-NSG) mouse model and described its application in HIV replication and latency
106   studies[22–24]. This hu-NSG mouse model exhibits high levels of bone marrow homing,
107   susceptibility to HIV infection, and recapitulation of HIV infection and pathogenesis.
108   Additionally, the hu-NSG mouse model responds appropriately to combinatorial antiretroviral
109   therapy (cART) and recapitulates plasma viral rebound upon cART withdrawal, confirming the
110   establishment of an HIV latency reservoir[25–27]. This HIV latency reservoir is further
111   substantiated by the production of replication-competent HIV viruses *ex vivo* induced by human
112   resting CD4+ T-cells isolated from infected and cART-treated hu-NSG mice.

114   Herein, we describe the detailed protocol for establishment of the hu-NSG mouse model from
115   neonatal NSG mice, including procedures related to HIV infection and cART treatment for
116   latency development. We expect this protocol to offer a new set of approaches in HIV animal
117   studies regarding HIV virology, latency, and treatment.

119   **PROTOCOL:**

121   All animal care and procedures have been performed according to protocols reviewed and
122   approved by the City of Hope Institutional Animal Care and Use Committee (IACUC) held by the
123   principal investigator of this study (Dr. John Rossi, IACUC #12034). Human fetal liver tissue was
124   obtained from Advanced Bioscience Resources (Alameda, CA), a nonprofit organization, in
125   accordance with federal and state regulations. The vendor has its own Institutional Review
126   Board (IRB) and is compliant with human subject protection requirements. Human PBMCs are
127   isolated from discarded peripheral blood specimens from anonymous healthy donors from City
128   of Hope Blood Donor Center (Duarte, CA), with no identification regarding age, race, gender, or
129   ethnicity. IRB#/REF#: 97071/075546

131   **1.   General Aseptic Practice**

133   1.1.   Perform tissue culture experiments in designated laminar flow cabinets.

135   1.2.   Keep tissue culture medium and supplements under sterile conditions and filter using a
136   0.22-µm filtration unit prior to use.

138   1.3.   Preserve prepared human HSCs in sterile tubes and keep on ice until injection.

140   1.4.   As a result of severe immune deficiency, handle NSG mice aseptically. Wear a disposable
141   surgery gown, hair cap, face mask, shoe covers, and gloves to prevent contamination. Sanitize
142   the bench surface, irradiation holder, and induction chamber for anesthesia with chlorine
143   dioxide based sterilant (*e.g.*, Clidox) before and after experiments.

145   1.5.   Apply petroleum-based eye ointment after retro-orbital bleeding to prevent dry eyes.

147   1.6.   Change to antibiotic-containing diet to prevent infection due to retro-orbital bleeding.

149   **2.**   **Handling HIV Virus, Infected Rodents, and Virus-containing Blood/Tissue Samples**

151   **CAUTION:** HIV is a class 3 human pathogen; the handling rules must be followed exactly.

153   2.1.   Handle HIV virus-containing samples in a designated BSL2+/3 laboratory space. Lock
154   virus-containing reagents securely in a secondary container during transportation. Disinfect the
155   outer surface of all containers by thorough wiping with 10% bleach and isopropanol prior to
156   transportation.

158   2.2.   Keep all virus-containing wastes in designated waste containers with 2-3 layers of
159   biohazardous waste bags. Prior to waste disposal, disinfect by generously spraying the waste
160   with iodine/ethoxylated nonylphenol solution (*e.g.,* Wescodyne) and autoclave.

162   2.3.   Wear personal protective equipment: hair cap, face cover, anti-splash face shield, shoe
163   covers, disposable surgery gown, and double-layer gloves.

165   2.4.   Handle infected rodents with extreme caution. Conduct injections and blood collections
166   while mice are under anesthesia (Steps 5.1—5.2, 6.3—6.5, 7.3, and 8.1—8.2).

168   **3.**   **Isolation of Hematopoietic Stem Cells**

170   3.1.   Prepare collagenase/dispase solution for tissue digestion.

172   3.1.1.   Dissolve collagenase/dispase powder to make stock solution at a concentration of 100
173   mg/mL, and store collagenase/dispase stock solution in 150 µL aliquots at -20 °C.

175   3.1.2.   For working collagenase solution, dilute 150 µL of collagenase/dispase stock with 15 mL
176   of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin.

177

Note: The final concentration of collagenase/dispase for tissue digestion is 1 mg/mL.

3.2.   With a sterile razor blade, cut fetal liver tissue (16–24 weeks gestation) into small pieces (approximately 2-3 mm^3 in size) followed by digestion with collagenase/dispase solution (1 mg/mL) for 30 minutes at room temperature. Adjust the volume of digestion solution so that all tissue pieces are fully submerged. Use the back end of the syringe plunger to gently grind the tissue sample to facilitate digestion.

3.3.   Pass the digestion mixture through a 70-μm sterile nylon mesh filter to acquire a single cell suspension.

3.4.   Enrich for CD34+ HSCs using a MACS system per manufacturer's instruction.

3.5.   Count the viable percentage of enriched CD34+ HSCs using a hemacytometer[28] and proceed to intrahepatic injection into neonatal NSG mice.

3.6.   Freeze the remaining enriched CD34+ HSCs in freezing media (*e.g.*, CryoStor CS2) and preserve cells in a liquid nitrogen tank. Upon future use, recount the viable percentage of thawed CD34+ HSCs prior to injection. With the use of freezing media, post-thaw viability is between 80 to 90%.

4.   Intrahepatic Injection of Human CD34+ HSCs in Neonatal NSG Mice

Note: Neonatal NSG mice 2-3 days from birth are most suitable for this procedure, as they are strong enough to endure irradiation at half-lethal dose, while young enough to have totally impaired immune systems. No anesthesia is required for the HSC injection.

4.1.   Place the whole litter of neonatal NSG mice (typically 5-10 mice, both genders) in a sterile pie-shaped irradiation cage and expose to a total dose of 200-250 cGy gamma-ray radiation from a Caesium-137 radiation source. Ensure that radiation dose is within the range, as significant weight loss or even death can be observed when NSG mice are exposed to high doses of irradiation.[12]

Note: Radiation is a health hazard, personal protection against radiation should be taken.

4.2.   Following irradiation, inject each neonatal NSG mouse with 5 x 10^5 viable human CD34+ HSCs using a syringe/needle setup (**Figure 1**). Directly inject cells into the liver.

5.   Engraftment Validation Through Retro-Orbital Bleeding and Flow Cytometry Analysis

5.1.   At 10–12 weeks post-HSC injection, anesthetize mice using an isoflurane/oxygen inhalation apparatus. Adjust the oxygen flow to maintain isoflurane percentage at 4–5%.

Anesthesia takes 3-5 minutes, depending on individual mouse. Properly anesthetized mice show a slow and deep breathing pattern, and no reaction to toe-pinching stimulation.

5.2.   Collect 50–100 µL of peripheral blood from each mouse through retro-orbital sampling[28]. Store blood samples in K2EDTA or heparinized blood collection tubes to prevent coagulation.

Note: Blood collection tubes are required to have anticoagulant coating so that mouse PBMCs can be isolated from whole blood for flow cytometry analysis. EDTA is known to inhibit enzymatic reactions such as PCR and qRT-PCR; thus, if downstream enzymatic reactions are required, use a heparinized blood collection apparatus.

5.3.   Centrifuge blood samples at 2,000 x g for 20 minutes at 4 °C to pellet blood cells.

5.3.1.   Transfer the plasma supernatants to new microcentrifuge tubes after centrifugation and store at -80 °C if cytokine analysis is required.

5.3.2.   Lyse the red blood cells (RBCs) within the blood cell pellet at room temperature for 10 minutes using Red Blood Cell Lysis Buffer (**Table of Materials**).

Note: If plasma samples are not required, directly lyse the whole blood with lysis solution without centrifugation.

5.4.   Pellet remaining blood cells after RBC lysis at 300 x g for 5 minutes at 4 °C; aspirate the supernatant. Wash the cell pellets with 0.01% BSA containing DPBS, centrifuge at 300 x g for 5 minutes at 4 °C and aspirate supernatant. Repeat the washing step one more time.

5.5.   Block cells with 100 µL of 1x blocking cocktail (**Table 3** for recipe) for 20 minutes at 4 °C.

5.6.   After blocking, directly add each antibody solution into the cell suspension at a concentration of 2 µL/10^6 cells and incubate at 4 °C for 30 minutes. Antibodies for engraftment validation are: anti-human CD45 (leukocytes, RRID: AB_2732068), anti-human CD3 (T-cells, RRID: AB_396896), anti-human CD4 (helper T-cells, RRID: AB_397037), anti-human CD8 (cytotoxic T-cells, RRID: AB_2722501), anti-human CD14 (monocytes, RRID: AB_10373536), and anti-human CD19 (B-cells, RRID: AB_10373382). For suggested flow panel, please see **Table 4** and **Table of Materials** for catalog numbers, RRIDs and lot numbers.

Note: Antibody staining in blocking solution eliminates non-specific binding of the anti-human antibodies toward mouse surface markers, as several surface markers share homology between human and mouse.

5.7.   Isolate human PBMCs from healthy donors and prepare single-stained flow cytometry compensation controls using purified human PBMCs. Alternatively, use fluorescence-labeled microspheres as compensation controls[29].

5.8.   Analyze the peripheral blood samples using a flow cytometer and quantify the percentage of human CD45+ cells, human CD3+ cells, human CD14+ cells, and human CD19+ cells from total peripheral blood cells.

5.8.1.   For downstream HIV infection and latency studies, calculate the percentage of CD4+ T-cells and CD8+ T-cells within the CD3+ cells.

Note: Typically, a CD4:CD8 ratio between 1.5 and 2.5 is observed prior to HIV infection[30].

6.   Analysis of HIV Infection of hu-NSG Mice and Plasma Viral Load Using qRT-PCR

6.1.   Propagate HIV BaL viral stock in human PBMCs from healthy donors, harvest at day 10 post-infection, and titrate using p24 ELISA kit[31].

6.2.   Select hu-NSG mice with more than 20% human CD45+ cells in the peripheral blood for HIV infection.

6.3.   Anesthetize hu-NSG mice using an isoflurane/oxygen inhalation apparatus (Step 5.1).

6.4.   After confirming animal is anesthetized, inject HIV BaL virus at a dose of 200 ng of p24 per mouse through intraperitoneal route.

Note: Be extremely cautious with the needle, do not reuse needles, and discard immediately after use into designated sharp container. Disinfect the area of injection after virus administration.

6.5.   Three weeks post-infection, anesthetize hu-NSG mice and collect peripheral blood through retro-orbital bleeding using heparinized capillary tubes and collection tubes.

6.6.   Separate plasma and blood cells by centrifugation at 2,000 x g for 20 minutes.

6.7.   Lyse RBCs. Block and stain remaining blood cells with antibodies for flow cytometry analysis (Section 5.3–5.8).

Note: Flow cytometry analysis should focus on comparing CD4:CD8 ratio before and after infection. Significant decrease in CD4+ cell count is expected, as the CD4 antigen serves as a co-receptor for viral entry.

6.8.   Use plasma samples to analyze the HIV viral load using qRT-PCR. Isolate viral RNA from plasma samples using a viral RNA mini kit. Perform qRT-PCR with HIV-1 LTR-specific primers and probe sets (sequence details in **Table 5**), using manufacturer's protocol.

7.   Oral Administration of cART and Validation of Viral Suppression (optional)

Note: This step is optional for investigations regarding HIV prognosis, virology, or infection-related pathophysiology during the acute infection phase. cART treatment of HIV-infected hu-NSG is used to recapitulate HIV latency among human patients receiving cART. Successfully HIV-infected hu-NSG mice are given cART for 4 weeks. The cART regimen consists of tenofovir disoproxil fumarate (TDF; 300 mg/capsule), emtricitabine (FTC; 200 mg/capsule), and raltegravir (RAL; 400 mg/capsule). The dose of cART for treating HIV-infected hu-NSG mice is adjusted according to body surface area (**Table 1, Equation 1, Table 2**)[32].

7.1.   Calculate the amount of each medication required for each cage during one week of treatment, taking into consideration the volume of the water bottle, the number of mice in each cage, and an average daily water intake of 4 mL per mouse.

Note: The key point in this step is to ensure that each mouse acquires its daily dose within 4 mL of drinking water.

7.2.   Grind all three medications with adjusted doses into fine powder and dissolve the powder mixture in sweetened water (*e.g.,* Medidrop Suralose). Change the water bottle every week with freshly dissolved cART cocktail supplied in sweetened water.

Note: The drug powder may not dissolve immediately, shake vigorously to achieve a homogeneous suspension before returning the bottle to the holding cage.

7.3.   Collect peripheral blood samples via retro-orbital bleeding every two weeks and analyze both the CD4:CD8 ratio using flow cytometry (Section 5.3–5.8) and the plasma viral load using qRT-PCR (Section 6.6).

Note: Typically after 4 weeks of treatment, the plasma viral load decreases to below the detection limit and a typical CD4:CD8 ratio is restored.

8.   Validation of Viral Rebound upon cART Withdrawal (optional)

Note: This step is critical in validating the latency model, as viral rebound upon cART withdrawal provides direct evidence of a latency reservoir. It is also recommended to serve as a control experiment for therapeutic investigations on HIV rebound suppressants.

8.1.   Plan for cART withdrawal after plasma viral loads from peripheral blood samples are below the detection limit and the CD4:CD8 ratio is restored to a range between 1.5 and 2.5.

8.2.   Collect peripheral blood samples through retro-orbital bleeding every 2 weeks after cART withdrawal. Closely monitor the change in plasma viral loads (Section 6.6) as well as the CD4:CD8 ratio (Section 5.3–5.8).

REPRESENTATIVE RESULTS

Flow cytometry analysis is frequently performed to validate the purity of isolated HSCs, evaluate engraftment levels, profile immune responses to viral infection, and survey cART efficacy. A typical antibody panel contains 4–6 individual fluorescently labeled antibodies; thus, a flow cytometer with multiple lasers and a wide selection of filters is crucial for achieving accurate results.

For initial engraftment validation, the human CD45+ cell count can range from 20% to 80%, and subsets of human leukocytes should appear as discrete populations on the flow dot-plot (**Figure 2**). The ratio of CD4:CD8 stays between 1.5 and 2.5 for a healthy individual; significant CD4+ depletion is typically observed upon viral infection, yielding a lower ratio of CD4:CD8; and restoration of the healthy ratio is observed upon cART treatment (**Figure 3**).

qRT-PCR gives a detection limit of 40 RNA copies/mL of plasma; proper dilutions are required prior to the experiment. Plasma viral loads detected using qRT-PCR throughout the course of the infection and cART regimen can be plotted and used to evaluate the efficiency of infection and cART (**Figure 4**).

Figure 1. Syringe/needle setup used for intrahepatic injection.
The custom-made Hamilton 80508 syringe/needle setup includes a 30-gauge, 51-mm-long needle with a beveled edge and an attached 50-µL glass syringe. Maximum injection volume in this procedure is 25 µL.

Figure 2. Flow cytometry data represent successful engraftment and the developments of lymphoid and myeloid cells in the peripheral blood.
Successfully prepared peripheral blood samples should have discrete population separations, and a well-engrafted hu-NSG mouse should have T-cell, B-cell and monocyte positive populations presented in the peripheral blood. A CD4:CD8 chart is recommended for ratio calculation. **a)** Successful engraftment showed more than 25% CD45+ human leukocytes in the peripheral blood; discrete population of **b)** B-cells, **c)** monocytes, **d)** T-cells among human CD45+ leukocyte; **e)** CD4+ helper T-cells and **f)** CD8+ cytotoxic T-cells are well separated, and **g)** yields a ratio between 1.5 to 2.5 in this uninfected hu-NSG.

Figure 3. CD4+ cell count changes throughout the course of viral infection, cART and cART withdraw.
As infection progresses, the CD4:CD8 ratio decreases from 1.5-2.5 to >1.0, CD4:CD8 ratio has been served as a clinical parameter in evaluating HIV prognosis as well as treatment efficacies. **a)** Representative flow data indicating the change in CD4:CD8 ratios during the experimental course; **b)** Comparison trend chart indicating the effectiveness of cART, which can be identified as restored percentage of CD4+ T cells. Detection of CD4+ T cell count by flow cytometry. N: number of tested mice = 6; Error bars: means ± SEM. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$, ns: no significant different. Two-way ANOVA analysis is employed.

Figure 4. Changes of serum viral RNA copy numbers throughout the course of viral infection, cART and cART withdraw

396 Detection of plasma viremia in the HIV-infected hu-NSG mice by qRT-PCR. The shaded area
397 indicates the time period during which the mice received cART (from Day 28 to Day 70 as
398 shown). The limit of detection (indicated by the dashed line) of the PCR assay is (~110-160 RNA
399 copies/mL) in 50 to 80 µL of plasma obtained through the tail vein. Star (*) indicates viral RNA
400 not detected in cART treated animals at Day 56. Serum viral RNA copy number analyzed from
401 peripheral blood samples serves as direct evidence concerning the degree of viral infection. It
402 should be in the agreement with the CD4 flow analysis. N: number of tested mice = 6; Error
403 bars: means ± SEM. * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001, ns: no significant
404 different. Two-way ANOVA analysis is employed.
405
406 **Equation 1. Dose translation based on body surface area (BSA).**
407 Equation for translating human dose to dosing against small experimental animals. The K_m
408 factor can be found in **Table 2**.
409
410 **Table 1. Daily dose of individual medication in cART regimen**
411
412 **Table 2. Weight, BSA and K_m factor chart for dose conversion**
413
414 **Table 3. Blocking cocktail for isolated blood cells**
415
416 **Table 4. Suggested multi-color flow panel**
417
418 **Table 5. HIV-1 LTR Primers and probe**
419
420 **DISCUSSION**
421 Immunocompromised mice engrafted with human cells/tissue present human-like physiological
422 characteristics and are a tremendous value for pathology, pathophysiology, and immunology
423 studies concerning human-specific diseases. Among multiple strains of immunocompromised
424 mice, the NOD.Cg-*Prkdc^scid^Il2rg^tm1Wji^* (NSG) model is the most immunodeficient due to its lack of
425 both innate and adaptive immunity, as well as ablated mouse-specific cytokine signaling[3, 12, 19].
426 Therefore, NSG mice have been extensively utilized in the humanization process, and it is well
427 established that human cells repopulate in the murine peripheral blood, lymphoid and myeloid
428 tissues, and that the mice exhibit appropriate human immune responses to infectious
429 stimulations such as HIV[27, 33].
430
431 Due to the host restriction of HIV, preclinical animal studies of HIV virology and infection
432 prognosis were previously limited to non-human primates infected with simian
433 immunodeficiency virus (SIV, the non-human primate version of HIV)[3]. Scientific advancements
434 in stem cell research and the generation of NSG mice have opened up the possibility of
435 conducting HIV research on small murine animals with lower cost and faster experimental
436 turnover. Currently, NSG humanization can be achieved through transplantation of 1) fetal
437 tissues and human HSCs (BLT model), 2) human PBMCs (PBL model), and 3) human HSCs (SRC
438 model). The BLT model offers the highest engraftment efficiency as well as comprehensive
439 development of both lymphoid and myeloid functions[34, 35], but is technically demanding. The

440 PBL model is the most convenient to establish but has a short experimental window upon
441 engraftment resulting from GVHD; additionally it only offers decent peripheral T-cell responses
442 and lacks lymphoid and myeloid development. For these reasons, we employed the SRC model
443 in this protocol, with adaptations to meet the requirements for HIV investigations. The hu-NSG
444 model is completely devoid of immune function and efficient at bone marrow homing upon
445 radiation and transplantation; furthermore, it allows viral challenge and subsequent
446 investigations at a younger age, avoiding the development of age-related pathological problems
447 known to the NOD strain background. Although T-cells in the hu-NSG model are educated in a
448 mouse thymic environment and are only H2-restricted, multiple, if not all, subsets of human
449 immune cells can develop in and colonize mouse lymphoid and myeloid organs. Noticeably, gut-
450 associated lymphoid tissue of the hu-NSG model is also reconstituted with human immune
451 cells; thus, this model could potentially support HIV mucosal transmission, similar to the BLT
452 model[22, 23].

453
454 One of the major obstacles to eradicating HIV is the existence of a latency reservoir among
455 patients treated with suppressive cART[33, 36–39]. Latently infected cells remain quiescent, and
456 contribute to HIV viral rebound upon cART withdrawal. Latency reservoirs are anatomically
457 located in the liver, spleen, brain, and other lymphoid tissues, and are mainly composed of
458 memory CD4+ T-cells[36, 40, 41]. As reported by our group, the hu-NSG model fully supports
459 development of the T-cell lineage, making it suitable for pathological modeling of HIV latency.
460 We showed this model is highly susceptible to viral infection and subsequently to the cART
461 regimen via oral administration. Dramatic viral rebound occurs immediately upon cART
462 withdrawal, which fully supports the existence of a latency reservoir. Latently infected memory
463 CD4+ T-cells can be isolated from the lymphoid organs of HIV-infected hu-NSG during cART, and
464 viral outgrowth assays recapitulate viral rebound *ex vivo*[41]. The hu-NSG model and the HIV
465 infection/cART regimen described in this protocol thus have broad applications in fields related
466 to HIV virology, infection prognosis, latency pathophysiology, and antiretroviral therapeutics
467 development.

468
469 The hu-NSG mouse model in this protocol requires irradiation and intrahepatic injection of HSCs
470 at day 2–3 after birth; therefore, in-house breeding and specific housing conditions are
471 required. Although neonatal HSC transplantation generally yields high engraftment efficiencies,
472 in some cases severe GVHD can occur. In some cases, a significant decrease in peripheral CD45+
473 cell count can be observed upon infection, and in extreme conditions, peripheral blood CD45+
474 depletion can be observed; therefore, blood collection and flow cytometry analysis can be
475 challenging. One of the major limitations of this hu-NSG mouse model lies within the chimeric
476 nature of its T-cells. T-cells in the hu-NSG mouse model are educated within the mouse thymic
477 environment and are H2-restricted instead of being HLA-restricted; therefore, full
478 recapitulation of the dynamic changes of T-cell subsets upon infection is less likely. Further,
479 although the hu-NSG mouse model exhibits good susceptibility to HIV-1 infection, the observed
480 plasma viral load can be several-fold lower than in the BLT model, possibly due to
481 incomprehensive reconstitution of human lymphoid and myeloid functions.

482

In summary, the hu-NSG mouse depicted in this protocol offers an easy and efficient methodology for generating a humanized mouse model for HIV virology and latency studies, as an alternative to the BLT model. Despite its limitations in comprehensive lymphoid and myeloid development, the hu-NSG model has been proven susceptible to infection and responsive to cART treatment or other therapeutics[22–24]. The latency pathology model established according to this protocol recapitulates HIV viral rebound *in vivo* and latently infected memory CD4+ T-cells can be further isolated for additional investigation.

DISCLOSURES

The authors disclose no conflicts of interest.

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Equation 1. Dose translation based on body surface area (BSA)

$$Human\ Dose\ (mg/kg)$$
$$= Animal\ equivalent\ dose\ (mg/kg) \times \frac{Animal\ K_m\ factor}{Human\ K_m\ factor}$$
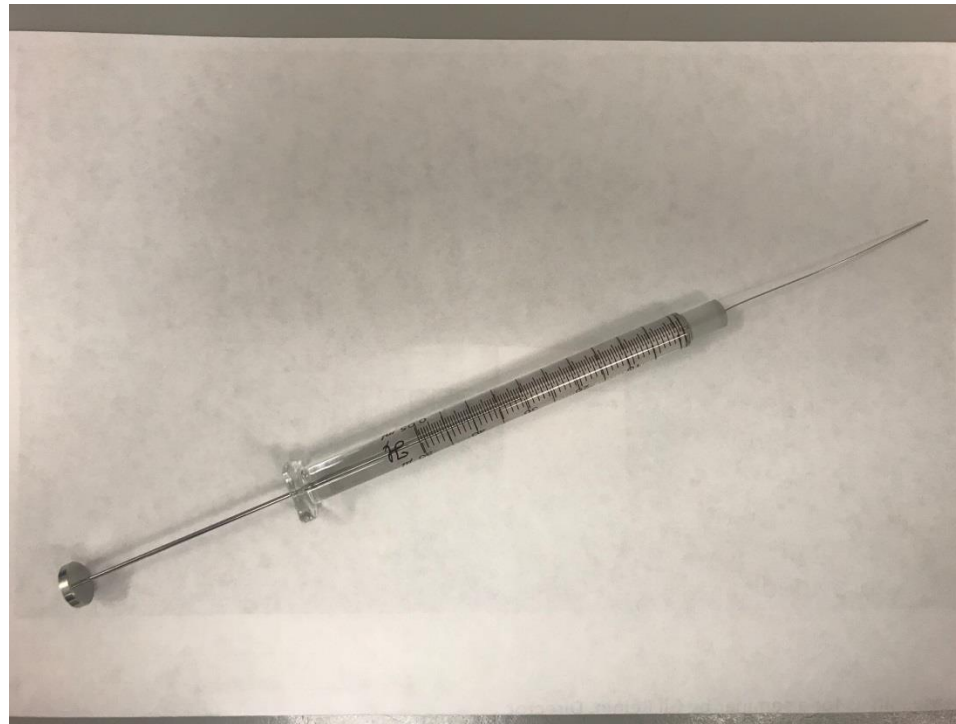
Figure 1.
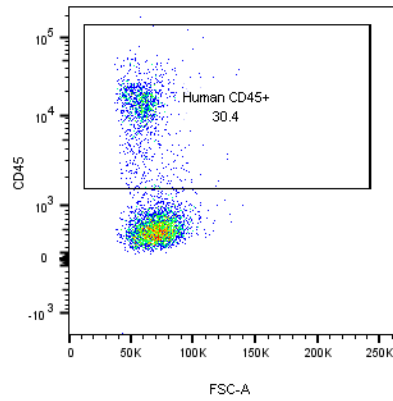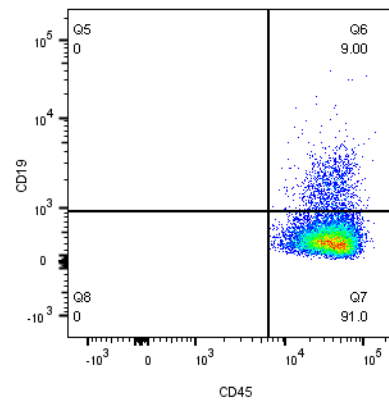
Figure 2
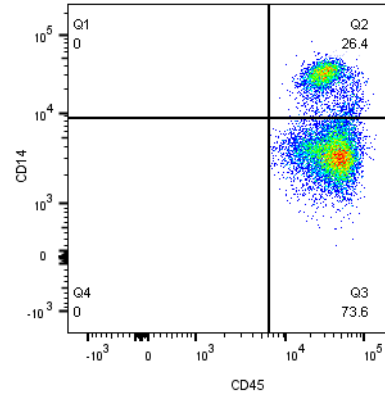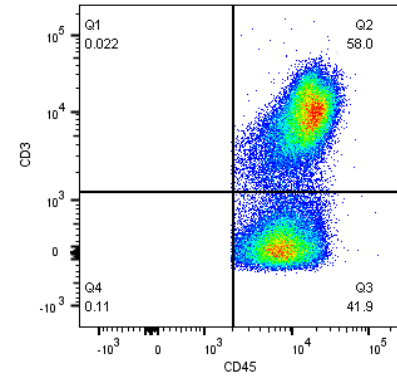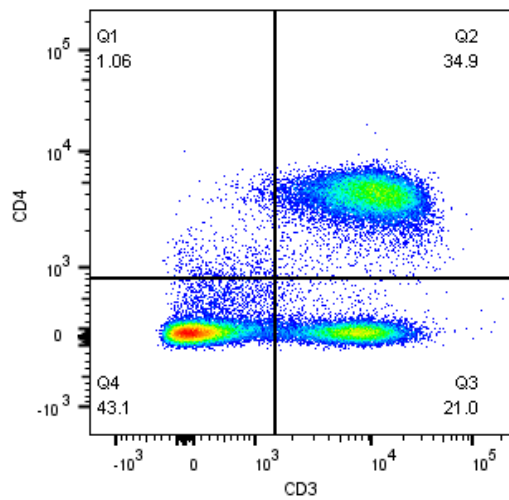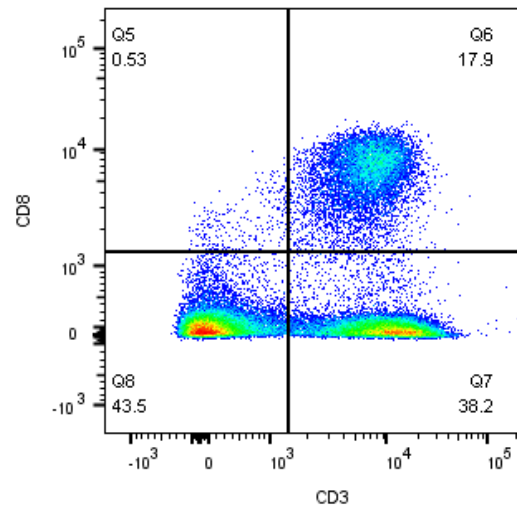
Figure 3

a

b
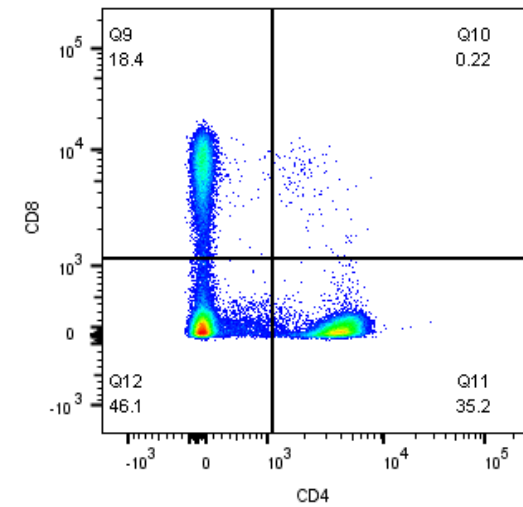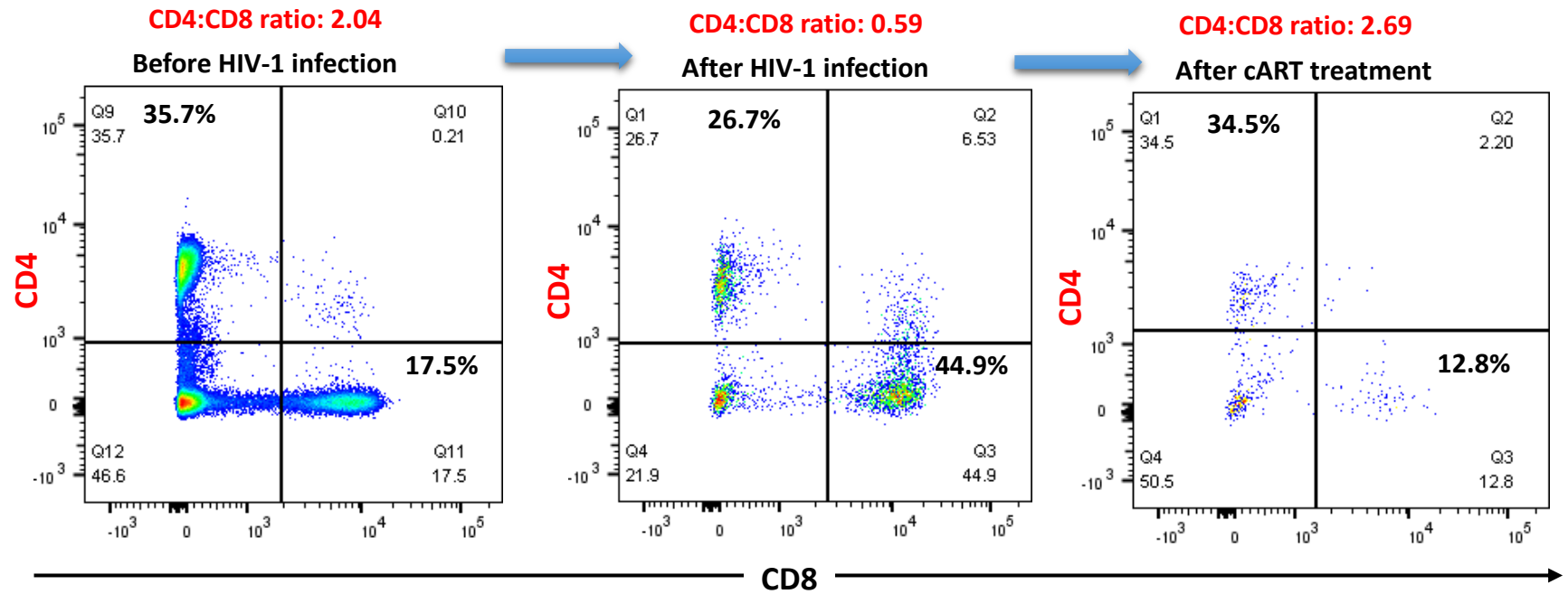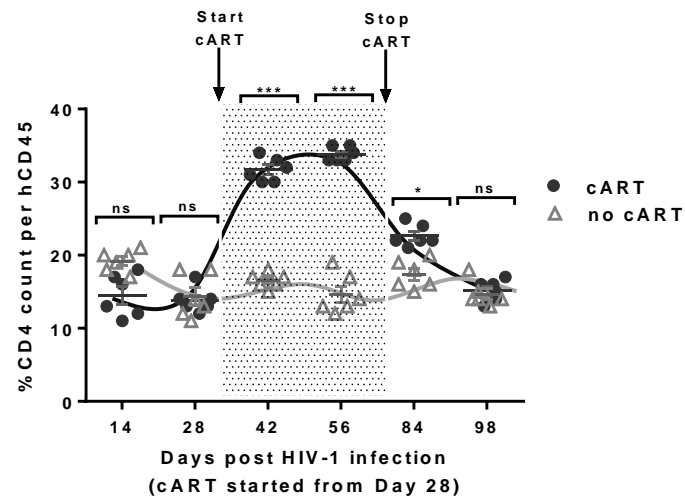
Figure 4
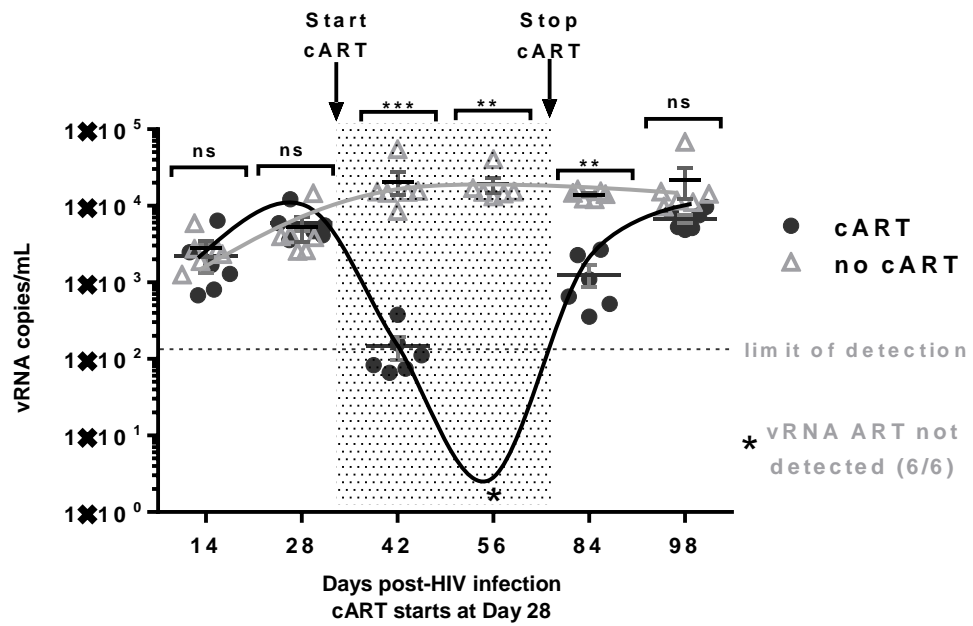
Table 1. Daily dose of individual medication in cART regimen

Medication	Daily Dose (mg)
Tenofovir disoproxil fumarate	300
Emtricitabine	200
Raltegravir	800

Table 2. Weight, BSA and K$_m$ factor chart for dose conversion

Species	Weight (kg)	BSA (m^2)	K$_m$ factor
Human			
Adult	60	1.6	37
Child	20	0.8	25
Mouse	0.02	0.0007	3

Table 3. Blocking cocktail for isolated blood cells

Reagent	Volume (µl)*	Final Concentration
0.01% BSA in PBS	98	
10 mg/ml human IgG	1	100 µg/ml
10 mg/ml mouse IgG	1	100 µg/ml
	100	

* Recipe is for one cell sample blocked in 100 µl blocking cocktail.
Adjust the volume accordingly with sample numbers.

Table 4. Suggested multi-color flow panel

Antibody	Fluorophore	Ex. Max	Em. Max
CD45	BB515	490 nm	515 nm
CD3	PE-Cy7	496 nm	785 nm
CD4	Pacific Blue	401 nm	452 nm
CD8	BUV395	348 nm	395 nm
CD14	APC-Alexa 750	650 nm	774 nm
CD19	PE	496 nm	578 nm

Table 5. HIV-1 LTR Primers and probe

Forward Primer	5'-GCCTCAATAAAGCTTGCCTTG -3'
Reverse Primer	5'-GGCGCCACTGCTAGAGATTTT -3'
Probe*	5'-AAGTAGTGTGTGCCCGTCTGTTAGTGTTGACT -3'

* 5'-FAM, 3'-Black Hole Quenche 1

Name of Material/ Equipment	Company	Catalog Number
CD34 MicroBead Kit, human	MiltenyiBiotec	130-046-703
CryoStor CS2	Stemcell Technologies	07932
NOD.Cg-*Prkdc* ^{scid} *Il2rg* ^{tm1Wji}	The Jackson Laboratory	005557
IsoFlo	Patterson Veterinary	07-806-3204
Clidox disinfectant	Fisher Sicentific	NC9189926
Wescodyne	Fisher Sicentific	19-818-419
Hamilton 80508 syringe/needle	Hamilton	80508
Blood collection tube (K2EDTA)	BD Bioscience	367843
Blood collection tube (Heparin)	BD Bioscience	365965
Capillary tube (Heparinized)	Fisher Sicentific	22-362574
Red Blood Cell Lysis Buffer	Sigma Aldrich	11814389001
QIAamp Viral RNA mini kit	Qiagen	52906
TaqMan Fast VIrus 1-step Master Mix	Thermofisher	4444434
HIV-1 P24 ELISA (5 Plate kit)	PerkinElmer	NEK050B001KT
IgG from human serum	Sigma Aldrich	I4506-100MG
IgG from mouse serum	Sigma Aldrich	I5381-10MG
BB515 Mouse Anti-Human CD45 (clone HI30)	BD Biosciences	564586
PE-Cy7 Mouse Anti-Human CD3 (Clone SK7)	BD Biosciences	557851
Pacific Blue Mouse Anti-Human CD4 (Clone RPA-T4)	BD Biosciences	558116
BUV395 Mouse Anti-Human CD8 (Clone RPA-T8)	BD Biosciences	563795
APC-Alexa Fluor 750 Mouse Anti-Human CD14 (TuK4)	ThermoFisher	MHCD1427
PE Mouse Anti-Human CD19 (SJ25-C1)	ThermoFisher	MHCD1904

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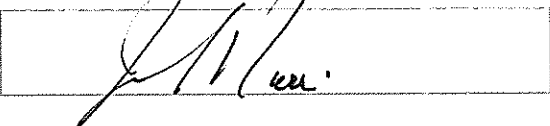
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We sincerely thank reviewer #1 for the detailed review comments. We have adjusted the manuscript accordingly. Following are the answers in regards to the concerns and questions raised by the reviewer:

Reviewer 1

Major Concerns:

1) The manuscript was difficult to read. Major editing for clarity is needed. For example, the second authors affiliation is written "Coty" of Hope, not "City". I had to re-read sentences several times to figure out exactly what was meant. Also, a lot of very subjective or "jargon" terminology was used throughout. I would avoid use of the words "decent," "good," and "properly." What is "decent" engraftment? >5%? >50%? Again, very subjective. You can say one model engrafts better than another, but not that one model has "good" engraftment. Also, please use a single verb tense within a sentence.

We have gone through detailed proof-reading, typos and wording issues have been addressed.

2) Please define SRC in the text (SCID repopulating cell) and PBL (peripheral blood leukocyte). You define BLT but not the other two abbreviations.

We have defined the other two humanized mice models accordingly.

3) In lines 51-52, you make the statement that HIV infects B cells (not true) and DCs (rarely). You could make the statement instead that CD4+ T cells are the major target for HIV infection but other cell types can also be infected, such as myeloid cells.

We have changed the content to "HIV primarily infects human CD4+ T cells, impacts the developments and immune responses in other immune cells such as B cells, macrophages, and dendritic cells, therefore small animal models transplanted with functional human immune system are highly demanded.."

4) In reference to line 233, the CD4 receptor serves as the primary cellular receptor for HIV entry, not as a cofactor.

We have changed the context to "Significant decrease in CD4+ cell count in expected as CD4 antigen serves as coreceptor for viral entry.", since CD4 binding does not necessarily lead to viral entry, depending on the tropism of the HIV viruses, coreceptor CCR5 or CXCR4 is required for infection of CD4+ cells.

5) How many weeks post-engraftment is the mouse shown in Figure 1?

Figure 1 shows the peripheral blood profile at week 10 post-engraftment. We have added the information in the figure legend.

6) In Figure 2, why are there so few total events in the middle and right panels in comparison to the left panel? Did engraftment levels drop? Also, you state that cART started on Day 28, but the shaded box starts around Day 31. Also, if you want to discuss a restoration of CD4 levels, should also show the pre-HIV levels in Figure 2b.

Thank reviewer #1 for the comments. Since HIV only targets human cells in the presented mice model, as the infection progresses, the CD45 level as well as progenitor immune cell levels in peripheral blood drops with the same volume of blood analyzed.

The shadow box is arranged so that the blood analysis on Day 28 is presented as prior to cART and the shadow box does not cover the data points for the Day 28 analysis. The box is just representing the stage of cART treatment in the model development. Further, it has been clearly stated in the manuscript that cART starts right after blood analysis.

Figure 2a dot-plots have indicated the CD4 percentage prior to the infection, during infection and after cART, which sufficiently identifies the cART responsiveness in the reported mouse model. Figure 2b is to further compare the cART group with non-cART group, showing both the cART response and the latency development in the reported model.

Minor Concerns:
1) In lines 54-57, I would make it clear that the deficiencies are in murine cell development.
Context has been changed accordingly
2) The word "researches" is not typically used (found in abstract and conclusion) as "research" is already plural.
Context has been changed accordingly
3) For protocol, if anticoagulants are being used, then plasma not serum is isolated from blood samples. "Serum" should be replaced with "plasma" throughout (including all text and figure legends).
Context has been changed accordingly
4) In Figure 3, should read vRNA not vRNa. Also, why are the undetectable viral loads from day 56 shown so far below the LOD when the undetectable points from day 42 are shown just below the line? Also, the corresponding figure legend is worded oddly.
Context has been changed accordingly. Day 42 shows viral loads with 2 weeks of cART treatment, the analyzed results is at or slightly above the LOD, Day 56 is 4 weeks into cART treatment, as typically observed in patient, the viral loads is undetectable. In our qPCR data no trace appears within the amplification cycles, hence the viral load has a value of 0.

Reviewer #2:
Manuscript Summary:
The manuscript describe a protocol for reconstitution of humanized NSG mice with human CD34+ HSC cells and utilization of the model for HIV research.

Major Concerns:
Not applicable

Minor Concerns:
Though manuscript is very well written, additional informations could also be included to increase the reproducibility of the protocol by others.
Line 75: Remove the musical word.
Line 142: Please mention the collagenase concentration and incubation time.
Line 150: For the freezing of HSCs, include the name or composition of freezing medium. It would also be useful for the readers if the viability of cells are also mentioned after thawing.
Line 156: What is irradiator source and nature of radiation, gamma rays or x-rays?
Line 146: Correct the name of Miltenyl biotech to miltenyi biotec.
Line 194: Please mention the catalog number of the antibodies, as different antibodies may have different working concentrations, also it would assist in making multicolor panel for immunophenotyping.

Line 218: Please provide the catalog number of p24 ELISA kit used.

Line 235: Provide the sequence of HIV-1 LTR specific primer and probe.

We thank the reviewer for the detailed review and the positive feedback. All the minor concerns have been addressed accordingly.