TITLE:
Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection

AUTHORS AND AFFILIATIONS:
Anna H.F. Andersen[1,2], Stine S.F. Nielsen[1,2], Rikke Olesen[1,2], Katharina Mack[1], Frederik Dagnæs-Hansen[3], Niels Uldbjerg[1,4], Lars Østergaard[1,2], Ole S. Søgaard[1,2], Paul W. Denton[1,2,5], Martin Tolstrup[1,2]

[1]Department of Clinical Medicine, Aarhus University, Denmark
[2]Department of Infectious Diseases, Aarhus University Hospital, Denmark
[3]Department of Biomedicine, Aarhus University
[4]Department of Gynecology and Obstetrics, Aarhus University Hospital, Denmark
[5]Department of Biology, University of Nebraska at Omaha, USA

Correspondence author: Anna H.F. Andersen (ahfa@clin.au.dk)

Email addresses of co-authors:
Stine S.F. Nielsen (stsoni@rm.dk)
Rikke Olesen (rikkol@rm.dk)
Katharina Mack (katharinamack@yahoo.com)
Frederik Dagnæs-Hansen (fdh@biomed.au.dk)
Niels Uldbjerg (uldbjerg@clin.au.dk)
Lars Østergaard (lars.ostergaard@clin.au.dk)
Ole S. Søgaard (ole.schmeltz.sogaard@clin.au.dk)
Paul W. Denton (pdenton@unomaha.edu)
Martin Tolstrup (mtol@clin.au.dk)

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KEYWORDS:
Immunology and infection, NOG mouse, Humanized mice, HIV, cART, CCR5, Stem cells, ddPCR

SUMMARY:
We have developed a protocol for the generation and evaluation a humanized and HIV-infected NOG mouse model, based on stem cell transplant, intravaginal HIV exposure and ddPCR RNA quantification.

ABSTRACT:
Humanized mice provide a sophisticated platform to study human immunodeficiency virus (HIV) virology and to test antiviral drugs. This protocol describes the establishment of a human immune system in adult NOG mice. Here, we explain all practical steps from isolation of cord-blood derived human CD34+ cells and their subsequent intravenous transplantation into the mice, to the manipulation of the model through HIV infection, combination antiretroviral therapy (cART) and blood sampling. Approximately 75,000 hCD34+ are injected intravenously into the mice and the level of human chimerism, also known as humanization, in the peripheral blood is estimated longitudinally for months by flow cytometry. 75,000 hCD34+ cells yield 20-

50% human CD45+ cells in the peripheral blood. The mice are susceptible to intravaginal infection with HIV and blood can be sampled once weekly for analysis, and twice monthly for extended periods. This protocol describes an assay for quantification of plasma viral load using droplet digital PCR (ddPCR). We show how the mice can be effectively treated with a standard-of-care cART regimen in the diet. The delivery of cART in the form of regular mouse chow is a significant refinement of the experimental model. This model can be used for preclinical analysis of both systemic and topical pre-exposure prophylaxis compounds as well as for testing of novel treatment and HIV cure strategies.

INTRODUCTION:

Human immunodeficiency virus (HIV) is a chronic infection with more than 37 million infected individuals worldwide[1]. Combination antiviral therapy (cART) is a life-saving therapy, but a cure is still warranted. Thus, there is a need for animal models that mirror the human immune system and its responses in order to facilitate continued research in HIV. Multiple types of humanized mice have been developed by transplanting human cells into severely immunodeficient mice, that are capable of supporting cell and tissue engraftment[2]. Such humanized mice are susceptible to HIV infection and provide an important alternative to nonhuman primate SIV models, as they are cheaper and simpler than nonhuman primates. Humanized mice have facilitated research in HIV viral transmission, pathogenesis, prevention, and treatment[3–11].

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We present a flexible humanized model system for HIV research developed by transplanting cord-blood derived human stem cells into mice of the NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac (NOG) background. Besides being of non-fetal origin, the practical bioengineering of these mice is less technically demanding compared to the microsurgical procedures involved in the transplant of e.g. the blood-liver-thymus (BLT) construct.

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We show how to establish HIV infection through intravaginal transmission, and how to monitor the plasma viral load with a sensitive droplet digital PCR (ddPCR)-based setup. Subsequently we describe the establishment of standard cART given as part of the daily mouse diet. The aim of these combined methods is to reduce stress to the animals and facilitate large-scale experiments where limited time can be spent handling each animal[12].

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In humans, a *CCR5*$^{\Delta32/wt}$ or *CCR5*$^{\Delta32/\Delta32}$ genotype causes reduced susceptibility to HIV infection with transmitter/founder viruses[13], and some precautions must be taken when bioengineering humanized mice with stem cells, with the purpose of HIV studies. This is especially true in our region because naturally occurring variants in the *CCR5* gene, particularly Δ32 deletions, are more prevalent in Scandinavian and Baltic native populations compared to rest of the world[14, 15]. Thus, our protocol includes an easy, high-throughput assay for screening donor hematopoietic stem cells for CCR5 variants prior to transplantation.

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For the intravaginal exposure herein we chose the transmitter/founder R5 virus RHPA4259, isolated from a woman in an early stage of infection who was infected intravaginally[16]. We exposed the mice to a viral dose that was sufficient to yield successful transmission in the

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majority of mice, but below a 100% transmission rate. Choosing such a dose enables a sufficient dynamic range in transmission rate such that antiviral effects of a drug candidate can result in protected animals in HIV prevention experiments and decreased viral load for treatment studies.

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STATEMENTS OF ETHICS

All cord bloods were obtained in strict accordance following local approved protocols, including informed consent of anonymous donation by the parents. All animal experiments were approved and performed in strict accordance with Danish national regulations under the license 2017-15-0201-01312.

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PROTOCOL:

A list of all reagents and catalogue numbers can be found at the end of this protocol along with a detailed description of relevant PCR programs and primer sequences.

CAUTION: handle HIV exposed mice and blood with extreme caution. Decontaminate all surfaces and liquids that have been in contact with HIV with a confirmed HIV-disinfectant (Table of Materials and Reagents).

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1. **Isolation of human CD34+ stem cells**

   1.1. Collect cord blood samples in EDTA-coated blood-collection tubes. (After planned caesarean sections or vaginal births and according to local ethical approvals)

   1.2. Isolate PMBCs from cord blood by density-gradient separation, according to manufacturer's protocol.

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   1.3. Isolate CD34+ cells from the PBMC population by first pre-enriching with antibodies against common markers for mature cells that which induces crosslinking of cells of undesired lineages with red blood cells. This is followed by CD34+ cell enrichment using magnetic beads, according to manufacturer's protocol.

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   1.3.1. Determine live cell count by standard trypane blue exclusion; Briefly, resuspend 10 µL of cell suspension in 90 µL of trypane blue. Add 10 µL of this solution to a hemacytometer and count non-blue cells, according to manufacturer's protocol.

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   1.3.2. Viably cryopreserve CD34+ cells in 1mL 10% DMSO in fetal bovine serum (FBS) until day of mouse transplantation.

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   1.3.3. Viably cryopreserve a small fraction of both isolated (CD34+) and flow-through cells (CD34neg) separately for assessing CD34+ stem cell purity (Approximately 30,000 cells of each sample). (Alternative: Test purity on freshly enriched cells: Step 2 below.)

   1.3.4. Freeze a fraction of non-pelleted flow-through (CD34neg) for determination of *CCR5*Δ32 status. (Cells can be frozen directly without conditioned freezing solution, but note that the presence of red blood cells in the pellet can inhibit the subsequent PCR if the flow-through is pelleted.)

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2. Assessing CD34+ stem cell purity via flow cytometry

2.1. Thaw the isolated cells (CD34+) and flow-through cells (CD34-). Wash cells by resuspending cells from each vial in 9 mL of room temperature (RT) FACS buffer [2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS)].

2.2. Centrifuge for 5 min at 300 x g, at RT to pellet cells.

2.3. Pour off supernatant, resuspend cells in remaining liquid and transfer to FACS tubes, repeat washing step with 3 mL of FACS buffer. After completion of second centrifugation, pour off supernatant and resuspend cells in remaining liquid.

2.4. Add 5 µL of Fc Receptor blocking solution (Table of Materials and Reagents) and leave for 10 min at RT (Do not wash off Fc Receptor blocking solution).

2.5. Add mix containing pre-determined volumes of antibodies against human CD3 (clone SK7) BUV395, CD34 (clone AC136) FITC and CD45 (clone 2D1) APC (Table 1). Leave cells for 30 minutes at RT in the dark. (Fluorophores must be chosen based on parameters that can be assessed with the available flow cytometers without requiring compensation matrix)

2.6. Wash cells by addition of 3 mL of FACS buffer.

2.7. Centrifuge for 5 min at 300 x g, RT to pellet cells.

2.8. Pour off supernatant and resuspend cells in remaining liquid.

2.8.1. Repeat this washing step twice to ensure all non-bound antibodies have been removed)

2.9. Record samples on flow cytometer (Table of Materials and Reagents) and perform data analysis with appropriate software. (Gating strategy presented in Figure 1A-F)

3. Genetic screening for *CCR5*Δ32 variants in cord bloods.

3.1. Incubate 1.25 µL of non-pelleted flow-through with 11.25 µL PCR mix containing: 200 µM of dNTP mix, 0.01 U/µL high fidelity DNA polymerase, forward and reverse primers detailed in Table 2.

3.1.1. Adjust volume with nuclease-free H2O to approximately 12.5 µL for each PCR.

3.2. Amplify genomic fragments with the PCR cycling program detailed in Table 3.

3.3. Separate PCR products on a 2% agarose gel[13].

3.3.1. PCR products from the wild type alleles and the Δ32 alleles yield PCR fragments of 196 base pairs and 164 base pairs bands respectively, making them easily distinguishable by gel electrophoresis[13]. (Figure 1G)

4. Intravenous stem cell transplant (when possible having one person preparing cells in the laboratory and one person preparing the animal mice and workspace for transplants is an efficient approach)

4.1. In animal facility: 4-6 hours before planned transplantation of stem cells, female NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicTac (NOG) mice (Taconic) of 6-7 weeks of age, should be irradiated with 0.75 Gy with a Cs^{137} source (the best preconditioning dose may vary based on mouse age, source of radiation etc.). This process conditions the animals for successful engraftment with human stem cells.

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4.2. In animal facility: Prepare flow bench workspace and all reagents before bringing mice or cells into the workspace.

4.2.1. Place sterile blue pad to cover working surface of the flow bench. Prepare sterile gauze and sharps container.

4.2.2. Place a heating lamp disinfected with 70% ethanol in the flow bench with an empty sterile mouse cage underneath the heat.

4.3. In laboratory: Thaw isolated CD34+ cells and dilute them in 9 mL of 37 °C plain RPMI.

4.4. In laboratory: Centrifuge cells at 350 x g for 5 minutes at RT, discard supernatant by aspiration and resuspend pellet in 1 mL of plain RPMI at 37 °C.

4.5. In laboratory: Determine cell count by trypan blue exclusion, and adjust volume to 200 µL per mouse. (Make extra to take into account possible loss due to subsequent handling steps)

4.5.1. Plan to transplant 75,000 CD34+ cells in 200 µL into each mouse.

4.5.2. The cells can be kept at 4 °C for during transport to the animal facility before the transplant. (Avoid keeping the cells on ice, to reduce aggregation/clumping)

4.6. In animal facility: Bring cage with mice into the flow bench and transfer mice to the cage under the heating lamp to dilate vessels. Leave one end of the cage away from the heat source so that mice can move away from the heat upon becoming warm. Mice that have moved to the end of the cage away from the heat source are sufficiently warmed for a successful tail vein injection.

4.7. In animal facility: Load 1 mL pre-lubricated syringe to above the 800 µL mark with suspended CD34+ cells. (Using a lubricated 1 mL syringe will dramatically ease the intravenous injection and increase the precision of this technique.)

4.8. In animal facility: Attach 30-gauge 13 mm needle and prepare needle and syringe for injection. This order of operation allows for the syringe to be loaded more quickly while protecting the integrity of the cells to be transplanted given the possible damage that can occur during rapid aspiration of cells through such a small gauge needle. Fill the needle hub with liquid by pressing the plunger and remove liquid down to the 800 µL mark of the needle (800 µL is an appropriate volume for 1 cage that houses 4 animals)

4.9. In animal facility: Place a heated mouse (Step 4.6) in a restrainer used for giving IV injections. Carefully inject 200 µL of cell suspension into the tail vein of the mouse. Spend 2 seconds performing the plunge and keep the needle inserted for approximately 2 seconds after completion of injection. (This ensures cells have migrated adequately far from the injection site prior to removal of the needle.)

4.10. In animal facility: As necessary, wipe the mouse tail with sterile gauze to remove any visible blood. Put the mouse back into their non-heated home cage.

4.11. In animal facility: Repeat injection procedure with the remaining mice. It is not necessary to change the needle between the different mice unless the needle becomes dull (possible after 8-12 attempted tail-vein injections.)

5. Blood collection and processing for analysis

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5.1. 3-5 month after human stem cell transplantation, human cell engraftment in the peripheral blood can be evaluated via flow cytometry.

5.2. Draw blood samples from the mice using local IACUC-approved techniques.

5.3. Collect a maximum of 70-100 µL of total blood into sterile PCR-approved microcentrifuge tubes containing 10 µL 0.5 M pH 8.0 EDTA to avoid coagulation of blood.

6. Evaluation of human engraftment via flow cytometry

6.1. Transfer 40-50 µL of blood to FACS tubes

6.2. Add 5 µL of Fc Receptor blocking solution to prevent non-specific binding of antibodies and leave for 10 min at RT.

6.3. Add mouse anti-human antibody mix containing CD4 (clone SK3) BUV 496, CD8 (clone RPA-T8) BV421, CD3 (clone OKT3) FITC, CD19 (clone sj25c1) PE-Cy7, CD45 (clone 2D1) APC (Table 4) and leave to stain in the dark at RT for 30 min. (Fluorophores must be chosen based on parameters that can be assessed with the available flow cytometers without requiring compensation matrix)

6.4. Add 2 mL of appropriate red blood cell lysing buffer to each tube to lyse red blood cells. Use a lysis buffer is one that is specifically formulated for antibody staining prior to red blood cell lysis (one suitable example is given in the Table of Materials and Reagents). Vortex briefly to ensure equal distribution of cells in the lysing solution (important) and leave for 10 min at RT.

6.5. Add 2 mL of FACS buffer to stop lysis reaction.

6.6. Centrifuge for 5 min at 300 x g at RT to pellet cells.

6.7. Pour off supernatant and vortex gently until cells are resuspended.

6.8. Add 3 mL of FACS buffer, centrifuge for 5 min at 300 x g at RT.

6.9. Pour off supernatant and resuspend cells.

6.10. Record samples on appropriate flow cytometer and analyze using appropriate software (Table of Materials and Reagents) (Representative analysis and results are depicted in Figure 2 and Figure 3)

7. Intravaginal HIV exposure

7.1. Virus used for intravaginal exposure of mice can be produced using previously published protocols[17]. Virus is kept at -80 °C and transported between locations while stored on dry ice following local approved protocols. Virus is stored on dry ice until immediate before exposure of the mice. Virus can be diluted into plain RPMI (avoid using RPMI that has antibiotics or serum additives) to achieve the appropriate concentration immediately prior to exposure. (21,400 IUs were used for this IVAG exposure). Once they are generated, keep diluted stock on wet ice throughout procedure (to avoiding freeze-thaw cycles that would occur if diluted virus was placed back on dry ice once thawed.

7.2. Prepare all equipment and flow bench workspace as presented in Figure 4 before bringing mice or virus into the flow bench (similar to step 4.2.)

7.2.1. Place heating lamp focus in the center of the workspace where the mouse will be located during the HIV exposure procedure. The heating

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lamp will ensure no decrease in body temperature of the mice. (Other equipment that controls temperature can also be used, e.g. a heated gel pad or a circulating-warm-water blanket, according to local IACUC regulations[18].)

7.2.2. Bring sterile 20 µL pipette tips and appropriate pipette into the bench. Place a container with liquid disinfectant (Table of Materials and Reagents) in the bench for immediate inactivation of materials and liquids that have been in contact with virus.

7.3. Place a mouse into a chamber supplied with 3% isoflurane gas and enriched with paper towels. This percentage of gas will take the animals into the plane of anesthesia within 2-4 minutes. As with all other materials that immunodeficient mice encounter, the anesthesia apparatuses must be properly disinfected prior to use in this protocol.

7.4. Once anesthetized, transfer the mouse to a sterile blue pad under the heating lamp. Insert the mouse snout into a mask supplying continuous 3% isoflurane gas to maintain anesthesia. Hold the mouse at the base of the tail, stomach facing up, with your hand supporting the mouse back as depicted in Figure 4.

7.5. With a sterile pipette tip, stimulate genital area by gently stroking upwards towards the anus to induce emptying of the rectum, relieving pressure on the vagina.

7.6. Carefully bare vaginal opening by wrapping the mouse tail across your fingers such that the vulva naturally opens, perhaps with the slightest nudging using a sterile pipette tip.

7.7. Change pipette tip and pipette 20 µL of virus a-traumatically into the mouse vagina without creating bubbles. Do not insert the tip deep into the vagina. Rather, with the vulva opened, place the pipette tip at the level of the vaginal opening (avoid going deeper) to eliminate the potential for abrasions during the inoculation process, release the virus and allow gravity to pull the virus into the vagina (Alternative: use a 22G 1.25 mm blunt-end, straight needle, as described in[6])

7.8. Retain the mouse in this position with the vagina facing up for 5 minutes after exposure to avoid gravity-induced leakage of virus suspension.

7.8.1. Carefully place the mouse into the home cage, taking care to place the mouse on its back.

8. Processing of blood samples prior to viral load analysis

8.1. Collect blood as described in step 5 above.

8.2. Centrifuge blood samples for 5 min 500 x g at RT to separate plasma and cells.

8.3. Collect 40 µL plasma for viral load measurement into a new sterile PCR-approved microcentrifuge tubes and store at -80 °C for at least 1 hour until further processing. (It is important to freeze all samples before RNA extraction do avoid the risk of bias from comparing RNA levels in samples that have not been frozen prior to RNA isolation to samples frozen prior to RNA isolation.)

8.4. Adjust the volume of blood back to the original volume by adding 40 µL suspension media (PBS with 2.5% bovine serum albumin (BSA), 50 U/ml penicillin G

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and streptomycin, and 10 U/mL DNase and sterile-filtered at 0.22 µm) and vortex briefly to mix.

8.5. Transfer 15 µL of adjusted blood volume to a new PCR-approved microcentrifuge tube.

8.6. Add 1 mL of 1X RBC lysis solution (Table of Materials and Reagents), vortex and incubate for 10 min at RT.

8.7. Centrifuge 9,600 x g for 1 min at RT to pellet cells.

8.8. Aspirate supernatant and leave only the tiny white cell pellet as red blood cell contamination can inhibit PCR.

8.9. Store pellet at -80 °C for at least 1 hour until further processing.

8.10. Optional: any remaining blood from step 8.4 can be used for flow cytometry analysis, as described above in step 6.

9. DNA extraction using a proteinase K extraction method

9.1 Extract DNA from peripheral cell pellets (generated in step 8.8) using a proteinase K extraction method as described below. This method has been demonstrated to maintain the highest DNA yield from a small volume of blood such as is required for serial blood collections utilized herein[19].

9.2 Add 25µL of proteinase K (20 µg/ml) to 1ml of 0.1M TRIS buffer.

9.3 Vortex proteinase K solution briefly.

9.4 Add 50 µL proteinase K solution to each cell pellet to be digested.

9.5 Mix by pipetting up and down – watch to ensure resuspension of the cell pellet.

9.6 Shake on a thermoshaker (Table of Materials and Reagents) at 400 rpm (depending on instrument) at 56 °C for 1 hour. (Tape tubes down to hold them in place, if necessary.)

9.7 Immediately and in the same thermoshaker, inactivate proteinase K with a temperature shift to 95 °C while shaking continues for an additional 20 min.

9.8 Vortex each sample.

9.9 Place each sample at -80 °C for a minimum of 30 min.

9.10 Thaw, then centrifuge samples at 17,000 x g for 1 min at RT to pellet unwanted cellular fragments.

9.11 Place the DNA-containing supernatant into a new microcentrifuge tube.

9.12 The DNA template is ready for PCR. The DNA templates can be stored at -80 °C.

10. RNA extraction, cDNA synthesis and ddPCR quantification of viral RNA.

10.1. Isolate RNA from thawed mouse plasma with a virus RNA isolation kit following manufacturer's protocol (Table of Materials and Reagents).

10.2. After addition of sample to the column, add an on-column DNase treatment step to ensure removal of all DNA in the plasma sample.

10.2.1. For each sample, 95 µL of RNase-free DNase solution to the column and incubate for 15 min at RT. (mix 2 µL RNAse-free DNase and 98 µL reaction buffer)

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10.3. Store RNA samples at -80 °C for at least 1 hour, before further processing. (It is important to freeze all samples after RNA extraction do avoid the risk of bias when comparing samples that have not been frozen to samples that were frozen)

10.4. Synthesize cDNA using a reverse transcriptase step using reagents as described previously[20]. Important: Add 0.5 µL of an RNase inhibitor to the cDNA reaction to avoid degradation of RNA.

10.4.1. Perform cDNA synthesis with the program detailed in Table 5.

10.5. Store cDNA samples at -80 °C for at least 1 hour. (It is important to freeze all samples after cDNA synthesis do avoid the risk of bias when comparing samples that have not been frozen to samples that were frozen)

10.6. Prepare samples for ddPCR as follows[20]:

10.6.1. Mix 3 µL cDNA sample with 11 µL ddPCR probe mixture (no dUTP)[20], 250 nM minor groove-binding probe and 900 nM of each of the forward and reverse primers as detailed in Table 6.

10.6.2. Adjust total PCR volume to 22 µL with nuclease-free water.

10.7. Emulsify PCR mixes with Droplet Generation Oil for Probes, on a droplet generator according to manufacturer's protocol and described previously[20].

10.8. Run PCR program as detailed in Table 7. Note: The primer/probe sequences and PCR programs displayed here have been specifically designed and optimized for sensitive detection of the HIV strain RHPA4259. Primer and probe sequences can easily be adjusted to detect any other HIV strain of choice.

10.9. Detect droplet fluorescence from samples on a droplet reader, and analyze results with appropriate software, according to manufacturer's protocol.

11. Treatment with ART containing chow

11.1. Mice can be fed with pellets containing a standard cART regiment containing 4800 mg/kg raltegravir (RAL), 720 mg/kg tenofovir disoproxil fumarate (TDF) and 520 mg/kg Emtricitabine (FTC) [21] (Table 8).

11.2. The doses were determined assuming that a mouse weighs 25 g and eats 4 g of chow per day. This corresponds to a daily dose of 768 mg/kg RAL, 2.88 mg/kg TDF and 83 mg/kg FTC[21].

11.3. cART diet was prepared by an external vendor (See Table of Materials and Reagents) from prescription drugs. Other companies could potentially also produce this regimen. cART diet was produced with a red color to easily distinguish it from ordinary mouse chow.

11.3.1. Control chow diet without cART can be produced in a standard brown color for easy distinction.

11.4. For initiation of cART, sterile mouse cages are prepared with the addition of cART-containing chow diet, and then mice are simply transferred from the old cage to the new cage.

11.4.1. Monitor weights of the mice and consumption of cART-containing chow by visual inspection to ensure that the mice are adjusting to the change.

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<#>Run PCR program as detailed in Table 5.

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Table 1: *CCR5Δ32* variant detection PCR primers ¶
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Table 2: CCR5Δ32 variant detection PCR

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REPRESENTATIVE RESULTS:

Gating strategy for analysis of stem cell purity is depicted in Figure 1. Figure 1A-C show the purified CD34+ population and Figure 1D-F the CD34neg flow-through used to illustrate that minimal amount of the CD34+ population is lost in the isolation process. Purity of isolated CD34+ stem cells was between 85-95% with less than 1% T-cell contamination. Figure 1G depicts CCR5 bands from one adult human control donor with the $CCR5^{\Delta32/wt}$ genotype, followed by bands from two $CCR5^{wt/wt}$ and one $CCR5^{\Delta32/wt}$ stem cell donors. The frequency of the genotype $CCR5^{\Delta32/wt}$ in a group of 19 donors was 15.8% (Figure 1H). This is in agreement with larger epidemiological studies[14, 15] reporting the genotype in up to 23.6% of investigated persons in Denmark.

3-5 months after transplantation of human CD34+ stem cells, human levels in peripheral blood was assessed via flow cytometry. The gating strategy is presented in Figure 2A-E. Figure 3A and Figure 3B illustrates the variability between 10 and 16 individual mice receiving stem cells from two different donors. Transplantation of 75,000 hCD34+ cells yielded 20-50% human CD45+ in the peripheral blood. All mice developed human B and T cells, including both CD4- and CD8-positive T cells.

For atraumatic intravaginal exposures, the setup depicted in Figure 4 was used. Mice were anaesthetized in a closed chamber and kept under anesthesia during the exposure. Mice were held vagina facing up for 5 minutes after exposure to ensure virus solution engagement with mucosal surfaces.

Shown in Figure 5A is the 64% HIV transmission success rate observed using this model. Mice were challenged with 21,400 infectious units (IU) of RHPA4259 intravaginally. This dose resulted in 64% of mice becoming HIV infected following vaginal exposure. For comparison, data from two different cohorts of mice, both exposed through intravenous route, are included. As expected, 100% of the mice became HIV+ with similar doses of RHPA and an additional strain, YU2, using this route.

Figure 5B depicts representative results from 3 mice which have been infected with HIV and switched to a diet containing standard cART. Mice were switched back to regular mouse chow after 40 days of cART. In this assay setup, the limit for viral load detection was 725 copies/mL. Viral loads were all below the detection limit after 4 weeks of cART. After cessation of cART, virus rebounded, mirroring clinical data[22]. Mice on cART tolerated the change in diet well as indicated in Figure 5C.

FIGURE AND TABLE LEGENDS:

Figure 1A-H Representative flow cytometry gating strategy for validation of stem cell purity and CCR5 donor variant status.
Panel A-C depicts the gating strategy used for the isolated CD34+ cell population. Doublets and debris are excluded in panel A and B respectively (FSC-A vs FSC-H and FSC-A vs SSC-A). Panel C shows the frequency of CD34+ stem cells and CD3+ T cell contamination. Similarly, the CD34neg

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flow-through gating strategy is presented in Panel D-F. Percentages in gates are calculated as a fraction of the parent population. The results of a *CCR5*$^{\Delta 32/wt}$ PCR analyses are presented in Panel G. Lane 1: DNA from a human *CCR5*$^{\Delta 32/wt}$ donor, lane 2+3: two *CCR5*$^{wt/wt}$ human stem cell donors, lane 4: A *CCR5*$^{\Delta 32/wt}$ human stem cell donor. Frequency of the genotype *CCR5*$^{\Delta 32/wt}$ in our group of 19 stem cell samples is 15.8% (Panel H).

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Figure 2A-E Flow cytometry gating strategy for validation of human cell engraftment and differentiation
The total mononuclear cell population from humanized mice were analyzed via flow cytometry. The percentage of human CD45+ cells was determined as a fraction of the total recorded events (Panel A). Doublets were subsequently excluded based on FSC-A/FSC-H gating (Panel B). Based on size and granularity the true lymphocyte population was defined (Panel C). Lymphocytes were then characterized as either CD3+ (T cells) or CD19+ (B cells) (Panel D). CD3+ T cells were either CD4+ T cells or CD8+ T cells (Panel E). Percentages in gates were calculated as a fraction of the parent population.

Figure 3A-B Representative humanization levels 4-5 months after stem cell transplantation with cell subtype fractions for 10 and 16 mice generated from two different human donors
The mononuclear cell population (MNC) from 10 (Panel A) and 16 (Panel B) humanized mice were analyzed via flow cytometry and gated as presented in Figure 2. The fraction of human CD45+ cells is presented as %hCD45 (of total MNC), and %B and %T cells as a fraction of hCD45. T cells were subsequently divided into %CD4 and %CD8. Each data point represents one mouse. Data is presented with mean ± S.D.

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Figure 4 Experimental lab bench setup for intravaginal exposure of mice
Experimental setup for HIV exposure of humanized mice through the intravaginal route. Procedure is performed in a flow bench where all reagents and surfaces have been sterilized prior to use.

Figure 5A-C Rate of HIV strain transmission through different exposure routes and efficacy and safety of cART-containing chow in viral suppression
Humanized NOG mice were successfully infected with two different strains of HIV through either the intravaginal or the intravenous route (Panel A) Mice were exposed with 21,400 IUs of RHPA4259 intravaginally, 5157 IUs IV with RHPA4259 or 3000 IUs IV with YU2 (Protocol details regarding IV exposure of humanized mice are not included in this protocol). HIV infections were successfully treated with a cART regimen delivered through mouse chow. Viral load decreased to below detection for all three mice on cART, and rebound reemerged after cessation of cART. The dotted line indicates limit of quantification at 725 copies/mL (Panel B). Mice fed with cART chow had similar weight development as mice housed on non-cART chow during the same time period, indicating no taste-preference or side effects of the cART diet. Weights are presented as fold change compared to start of cART. Each data point represents the mean of three animals ± standard deviation (Panel C).

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Table 1: Antibodies used for determination of stem cell purity

Suggested multicolor flow cytometry panel for evaluation of stem cell purity. Listed are the antibody target, the clone and fluorophore.

Table 2: CCR5Δ32 variant detection PCR primers

Forward and reverse primers used for detection of the 32 bp deletion in the CCR5 gene.

Table 3: CCR5Δ32 variant detection PCR program

PCR cycling program used for amplification of the CCR5 gene.

Table 4: Antibodies used for determination of mouse humanization

Suggested multicolor flow cytometry panel for humanization. Listed are the antibody target, the clone and fluorophore.

Table 5: cDNA amplification program

Program used for amplification of complementary strand DNA to the viral RNA.

Table 6: HIV ddPCR primers

Primers and probes used for ddPCR amplification of viral cDNA.

Table 7: HIV ddPCR program

PCR cycling program used for amplification of viral RNA.

Table 8: Mouse cART chow diet

Mouse chow diet was formulated as previously published[21]. The chow diet was made on a base of standard mouse chow, and after production, the food was γ-irradiated with 25 kGy and double-bagged. The chow was stored at -20 °C until use.

DISCUSSION:

The severely immunocompromised mouse strain, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac (NOG) is extremely well suited for transplantation of human cells and tissues. Both innate and adaptive immune pathways in these mice are compromised. NOG (and NSG) mice harbor a Prkdc^{scid} mutation that results in defective T and B cell function. Furthermore, these mice lack a functional interleukin-2 receptor γ-chain (common gamma chain, IL2rg) which is indispensable in the binding complexes of many key cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Immuno-deficient mice such as the NOG mouse, transplanted with a human immune system are a powerful tool for the study of HIV transmission and immunology. Contributions in these fields made using humanized mice have been extensively reviewed by us and others[2, 23–26]. The use of these mice to study human innate immune responses are also gaining increased attention[27, 28].

The aim for this manuscript was to supply a comprehensive protocol of mouse and ddPCR procedures to go from a naïve mouse to HIV transmission and treatment data. Our system utilized ddPCR for quantification of viral RNA and DNA. In a ddPCR reaction, the reactants are

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partitioned into up to 20,000 droplets, each containing a single, separate micro PCR reaction. The amplification of a target inside a droplet leads to a positive fluorescent signal for that droplet. Thus, the readout is binary and by applying Poisson statistical analyses, the number of positive reactions can be directly translated to a number of template copies in the original sample. The benefit of ddPCR lies in its ability to directly quantify a target, independent of a standard curve. This is particularly attractive when analyzing RNA samples that are challenging to utilize as PCR standard curves due to their labile nature[29]. Moreover, by analyzing multiple replicas of the same sample and merging the individual data points for the final sample quantification, the binary nature of ddPCR makes it possible to lower the detection limit of template copies per mL of sample[29]. This is especially important in a humanized mouse setting, where only limited sample material is available and high sensitivity is required.

Administration of cART to humanized mice can be done either oral gavage or intraperitoneal injections with solutions of cART[30–32], and as shown recently by formulation into the diet[21]. One of our major aims was the implementation of a cART regimen in the mouse diet to reduce potential stress on the animals due to extra handling steps inherent in other drug delivery methods. The dose of medicine that a mouse will eat can be accurately estimated based on the average daily food intake of mice[33]. Oral delivery through the diet serves as the easiest delivery route with both minimal stress for the animal and minimal workload for the handler. We based our combination of antiviral drugs on previous published studies in humanized mice[21, 30]. Furthermore, our cART strategy is clinically relevant given that the drug combination utilized herein is orally administered by patients around the globe.

Certain limitations are noted regarding the use of NOG mice. Importantly, human T cells in these mice are educated in a mouse thymic environment, as opposed to a human environment. Recent focus is on generating xenorecipient strains that have a favorable environment for the development of robust human immune responses. These new strains include immune-deficient mice which are transgenic for human MHC molecules such as A2. These models enable HLA-restricted antigen T-cell responses, that result in better maturation and effector functions of the adaptive immune system in these mice[34]. Another approach is to replace mouse genes with key human cytokines for IL-3/GM-CSF[35], IL-6[36], IL-15[37], TPO[38], M-CSF and IL-7/TSLP[31]. Such models have gained increased attention for their ability to generate better differentiation of innate cell types. Our protocol will be easily adaptable for the humanization and HIV infection of mice using any such enhanced-genetic background immunodeficient strain.

In summary, the ease and utility of the described approach facilitates research in HIV-related fields *in vivo.* Humanized mice can be a very powerful tool in guiding research towards generating better research hypotheses. Along with the generation of more “human” humanized mice with human transgenes, we believe our standardized protocol will contribute to the streamlining of experimental procedures across different research environments.

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authors would like to thank Professor Florian Klein for developing standard-of-care cART and for guidance.

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DISCLOSURES:

The authors declare no conflicts of interests.

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Suggested multicolour flow cytometry panel for humanization (for use on BD Fortessa X20)
CD4 (clone SK3) BUV 496, CD8 (clone RPA-T8) BV421, CD3 (clone OKT3) FITC, CD19 (clone sj25c1) PE-Cy7, CD45 (clone 2D1) APC

Table 1: *CCR5*Δ32 variant detection PCR primers

CCR5Δ32 detection	Primers
Forward primer	5'CTTCATTACACCTGCAGCT'3
Reverse primer	5'TGAAGATAAGCCTCACAGCC'3

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program

No. of Cycles	1x	45x	1x	∞
Temperature	98°	98°/63°/72°	72°	10°
Time	30 Sec	10sec/30sec/15sec	5min	∞

Table 3: cDNA amplification program

No. of Cycles	1x	1x	∞
Temperature	51°	80°	4°
Time	45 min	15min	∞

Table 4: HIV ddPCR primers

HIV quantification	Primers
Forward primer	5’AGGGCAGCATAGAGCAAAAA’3
Reverse primer	5’CAAAGGAATGGGGGTTCTTT’3
FAM probe	5’ATCCCCACTTCAACAGATGC’3

Table 5. HIV ddPCR program

No. of Cycles	1x	39x	1x	∞
Temperature	95°	95°/54.5°	98°	4°
Time	10min	30 sec / 1 min	10min	∞

Detailed description of mouse cART chow diet
Mouse chow diet contained 4800 mg/kg raltegravir (RAL) (Isentress®, MSD), 720 mg/kg tenofovir disoproxil fumarate (TDF) (Viread®, Gilead) and 520 mg/kg Emtricitabine (FTC) (Emtriva®, Gilead)[16]. The chow was produced by ssniff Spezialdiäten GmbH, Ferd.-Gabr.-Weg 16, DE-59494 Soest. The chow diet was made on a base with PS PicoLab Mouse 20 5058, γ-irradiated with 25 kGy and double-bagged.