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Senior Review Editor
Alisha DSouza, Ph.D.

Dear Dr. DSouza,

We very much appreciate the time and effort you have put into your comments. Based on the comments and suggestions, we have made careful modifications to the original manuscript, and related figures. Changes are made in red color fonts, and text for filming is highlighted in yellow color. The responses, and explanations related to editorial comments are uploaded as a separate document by name "Revised MS with comments' response".
We believe that the manuscript has been greatly improved and hope it has reached JoVE journal's standard.
Thank you once again for your time and interest. We look forward to hearing from you.

Sincerely,

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TITLE:

Establishment of the Dual Humanized TK-NOG Mouse Model for HIV-associated Liver Pathogenesis

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KEYWORDS:

Dual humanized mice, albumin, hepatocytes, humanized liver, human immune system, human immunodeficiency virus-1 (HIV-1), hematopoietic stem cells, hematopoietic progenitor cells

SUMMARY:

This protocol provides a reliable method to establish humanized mice with both human immune system and liver cells. Dual reconstituted immunodeficient mice achieved *via* intrasplenic injection of human hepatocytes and $CD34^+$ hematopoietic stem cells are susceptible to human immunodeficiency virus-1 infection and recapitulate liver damage as observed in HIV-infected patients.

ABSTRACT:

Despite the increased life expectancy of patients infected with human immunodeficiency virus-1 (HIV-1), liver disease has emerged as a common cause of their morbidity. The liver immunopathology caused by HIV-1 remains elusive. Small xenograft animal models with human hepatocytes and human immune system can recapitulate the human biology of the disease's pathogenesis. Herein, a protocol is described to establish a dual humanized mouse model through human hepatocytes and $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) transplantation, to study liver immunopathology as observed in HIV-infected patients. To achieve dual reconstitution, male TK-NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(Alb-TK)7-2/ShiJic) mice are intraperitoneally injected with ganciclovir (GCV) doses to eliminate mouse transgenic liver cells, and with treosulfan for nonmyeloablative conditioning, both of which facilitate human

hepatocyte (HEP) engraftment and human immune system (HIS) development. Human albumin (ALB) levels are evaluated for liver engraftment, and the presence of human immune cells in blood detected by flow cytometry confirms the establishment of human immune system. The model developed using the protocol described here resembles multiple components of liver damage from HIV-1 infection. Its establishment could prove to be essential for studies of hepatitis virus co-infection and for the evaluation of antiviral and antiretroviral drugs.

INTRODUCTION:

Since the advent of antiretroviral therapy, there has been a substantial decrease in deaths related to HIV-1 monoinfection. However, liver disease has emerged as a common cause of morbidity in HIV-infected patients[1,2]. Coinfections of hepatitis viruses with HIV-1 infection are more common, accounting for 10% - 30% of HIV-infected persons in the United States[3-5].

The host-specificity of HIV-1 and hepatitis viruses limits the utility of small animal models to study human-specific infectious diseases or to investigate multiple aspects of HIV-1-associated liver pathogenesis. Immunodeficient mice that permit the engraftment of human cells and/or tissues (termed humanized mouse models) are acceptable animal models for preclinical studies[6-8]. Since the introduction of humanized mice in the early 2000s, multiple preclinical studies of cholestatic human liver toxicity, human-specific pathogens, including HIV-1 and HIV-associated neurocognitive disorders, Epstein Barr virus, hepatitis, and other infectious diseases, have been investigated in these mice[6,9-11]. Multiple mouse models for $CD34^+$ HSPCs and/or human hepatocyte transplantation have long been developed and have improved over time to study the disease pathogenesis of Hepatitis B virus (HBV)-associated liver disease[12-14]. Several models for HSPC and human hepatocyte (HEP) transplantation are based on strains, known as NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac)[8,13], NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ*)[15], Balb/C-Rag2$^{-/-}$ γc$^{-/-}$ (Rag2$^{tm1.1Flv}$ Il2rg$^{tm1.1Flv}$/J)[12], and fah$^{-/-}$ NOD rag1$^{-/-}$il2rγnull mouse[16]. However, each model has its own advantages and limitations; for example, AFC8 dual humanized mice for HEPs and human stem cells (HSCs) on a Balb/C-Rag2$^{-/-}$ γc$^{-/-}$ background enables the successful engraftment of immune cells and HSCs, but there is an absence of an antigen-specific T- and B-cell response in this model[12]. The major concerns in reconstituting double humanized mice include suboptimal engraftment, a lack of suitable models to support different tissues, mismatched conditions, immune rejection, or graft-*versus*-host disease (GVHD), and technical difficulties, such as risky manipulations with newborns and high mortality rates due to metabolic abnormalities[13].

Although humanized mice have been used for HIV research for many years[17-19], the use of humanized mice to study liver damage caused by HIV-1 has been limited[20]. We previously reported the establishment of a dual humanized TK-NOG mouse model and its application in HIV-associated liver disease[8]. This model shows the robust engraftment of liver and immune cells and recapitulates HIV infection pathogenesis. This discussion presents a detailed protocol, including the most critical steps in the transplantation of human hepatocytes. A description of the HSPCs required for a successful engraftment of HEPs and the establishment of a functional immune system in TK-NOG mice is also presented. The use of these mice to study HIV-associated liver immunopathogenesis is detailed. TK-NOG male mice carrying a liver-specific herpes simplex virus type 1 thymidine kinase (HSV-TK) transgene are used. Mouse liver cells expressing this transgene

can easily be ablated after a brief exposure to a nontoxic dose of GCV. Transplanted human liver cells are stably maintained within the mouse liver without exogenous drugs[21]. The mice are also preconditioned with nonmyeloablative doses of treosulfan to create a niche in the mouse bone marrow for human cells[8]. Immunodeficient TK-NOG mice are intrasplenically injected with HEPs and multipotent HSPCs. The mice are then regularly monitored for blood and liver reconstitution by blood immunophenotyping and measurements of serum human-albumin levels, respectively. Mice with a successful reconstitution of more than 15% for both human immune cells and HEPs are intraperitoneally injected with HIV-1. The effect of HIV on the liver can be assessed as early as 4 - 5 weeks postinfection. It is critical to note that, because HIV-1 is used, all necessary precautions must be taken while handling the virus and injecting it into mice.

PROTOCOL:

This protocol has been approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB) at the University of Nebraska Medical Center.

NOTE: Obtain approval from the local IACUC and IRB before performing experiments on animal or human samples.

1. Processing of Umbilical Cord Blood and the Isolation of Human HSPCs

1.1. Perform all steps of the protocol under sterile conditions in laminar flow cabinets.

1.2. Collect human umbilical cord blood (CB) in heparinized tubes and store the CB samples at room temperature before processing.

1.3. Make a CB volume up to 35 mL by adding phosphate-buffered saline (PBS) and mix well. Layer the sample on top of the lymphocytes separation medium (LSM) as illustrated in **Figure 1** and centrifuge the LSM with the layered CB at 400 x *g* for 35 min at 4 °C with no brakes.

NOTE: Dilute the blood carefully and gently to avoid mixing at the interface.

1.4. Remove the top LSM and plasma layer carefully and transfer the white buffy coat interface to a new tube using a transfer pipette.

1.5. Resuspend the buffy coat in 30 - 40 mL of ice-cold buffer (PBS + 0.5% bovine serum albumin [BSA] + 2 mM ethylenediaminetetraacetic acid [EDTA]). Using a pipette, combine 20 µL of the cell suspension with 20 µL of 0.4% trypan blue and pipette 10 µL of the mixture into the outer opening of either of the two chambers of a counting slide and insert the slide in an automated cell counter to count the cells.

NOTE: Use ice-cold buffer in all steps, as it helps keep the cells viable.

1.6. Centrifuge the cells at 300 x *g* for 10 min at 4 °C and aspirate the supernatant carefully. Then, add 300 µL of ice-cold buffer.

1.7. Add 100 µL of human Fc receptor blocking reagent and 100 µL of monoclonal mouse anti-human CD34 antibody-conjugated microbeads for up to 1 x 10^8 cells (see the **Table of Materials**). Incubate for 30 min at 4 °C, add 10 mL of ice-cold buffer to wash the cells, and centrifuge at 300 x *g* for 10 min at 4 °C.

NOTE: Scale this up according to the cell number if more than 1 x 10^8 cells are present.

1.8. Carefully remove the supernatant, resuspend the pellet in 500 µL of buffer, and proceed with the magnetic separation step to enrich HSPCs.

1.9. Place a positive selection LS column (see the **Table of Materials**) in the magnetic-activated cell sorting field and pass it through with 3 mL of buffer.

1.10. Load the sample to the LS column that can entrap microbeads bound to human CD34+ in samples and allow it to flow under the influence of gravity into the collection tube.

1.11. Wash the column 3x with buffer and collect the elute in the same collection tube of the $CD34^-$ fraction of cells.

1.12. Plunge the column with 5 mL of buffer to elute the $CD34^+$ cells into a new collection tube. Repeat the procedure to achieve a purity of >90%.

1.13. Count the eluted $CD34^+$ cells using trypan blue dye in a hemocytometer. After counting, centrifuge the $CD34^+$ cells at 300 x *g* for 5 min and discard the supernatant.

1.14. Resuspend the $CD34^+$ cells in 25 µL of PBS for an injection to be used immediately in transplantation, or cryopreserve the cells at a concentration of 1-2 million/mL in freezing medium (Roswell Park Memorial Institute medium [RPMI 1640 medium] + 50% fetal bovine serum (FBS) + 10% dimethyl sulfoxide [DMSO]) for further use in transplantation.

NOTE: Always recount viable cells before using them in transplantation.

1.15. To check the purity of the $CD34^+$ elute, take 50 µL of the suspension and incubate it with 10 µL of PE-conjugated anti-human CD34 antibody for 30 min at 4 °C. After the antibody incubation, wash the stained cells with PBS, resuspend them in 100 µL of PBS, and then, proceed to perform flow cytometry. Add an additional tube of cells with no antibody to design the gate in the flow cytometer.

1.16. After acquisition, analyze the data by selecting the region of interest on a forward scatter (FSC) and side scatter (SSC) plot, followed by gating for single cells on FSC-area and FSC-height plots. Gate CD34-positive cells on single cells in the PE channel and SSC-area plot.

2. Preparation of Human Hepatocytes for Transplantation

2.1. Remove the cryopreserved hepatocytes from the liquid nitrogen, quickly submerge the vial in the water bath, and thaw for approximately 90 - 120 s.

2.2. Remove the vial cap and pour the thawed hepatocytes into the 50 mL conical tube of the warmed thawing medium.

2.3. Suspend the cells by rocking the 50 mL tube by hand for a few seconds.

NOTE: Do not vortex the tube.

2.4. Pellet the cells at 100 x *g* for 8 min at room temperature. Wash the pelleted cells in PBS with 0.1% BSA and pool them with either fresh or thawed HSPCs (ratio 10:1) in PBS in a final volume of 80 µL/mouse.

3. Animal Handling, Screening, Genotyping, and Treatment for Human HSPC and Hepatocyte Transplantation

3.1. Animal handling

3.1.1. As a result of severe immunodeficiency, breed, house, and handle TK-NOG mice under aseptic conditions.

3.1.2. Always wear a lab coat, gloves, shoe covers, and a face mask to prevent infection with potentially pathogenic microorganisms.

3.1.3. Use sterile gloves and instruments for surgery and handle the animals aseptically throughout the surgery.

3.2. Selecting TK-NOG mice for the experiment

3.2.1. Maintain the TK-NOG strain colony by breeding female TK-NOG mice with male non-TK-NOG littermates and select transgenic offspring by genotyping.

NOTE: Perform genotyping (see step 3.3) to determine the presence or absence of the transgene in newborn male and female mice at the time of weaning.

3.2.2. Select males at 6 - 8 weeks of age for transplantation due to their high sensitivity to the GCV-mediated depletion of HSV-TK transgene-expressing hepatocytes[21].

3.2.3. Ear-tag the mice at the time of weaning or surgery to ease identification. Note down the weight and health status of the animals.

3.3. Genotyping for the presence of the HSV-TK transgene using quantitative real-time polymerase chain reaction

3.3.1. Perform genotyping at the time of weaning (usually at 3 - 4 weeks of age). For genotyping, cut a piece of the mouse ear in a laminar flow biological safety cabinet to maintain sterility and extract genomic DNA by using a genomic DNA isolation kit.

3.3.2. Amplify genomic DNA extracted from tail tissue in a 20 µL reaction mixture to screen for HSV-TK transgene under control of human albumin promoter by adding 1 µL of forward primer 5'-CCATGCACGTCTTTATCCTGG-3', 1 µL of reverse primer 5'-TAAGTTGCAGCAGGGCGTC-3', 0.5 µL of FAM probe 5'-FAM-AATCGCCCGCCGGCTGC-MGB-3', and 10 µL of master mix on a real-time polymerase chain reaction (PCR) instrument[22].

3.3.3. Set the real-time PCR settings as follows: 60 °C for 30 s (preread stage), 95 °C for 10 min (hold stage), 40 cycles of 95 °C for 15 s and 60 °C for 1 min (PCR stage), and 60 °C for 30 s (postread stage).

NOTE: A cycle of threshold (Ct) below 22 is considered positive for HSV-TK transgene.

3.4. Treatment using ganciclovir and treosulfan

3.4.1. Using 27 G needle, inject the TK-NOG mice with intraperitoneal GCV injections (6 mg/kg) 2x a day at day 7 and at day 5 in 100 µL of saline before surgery to deplete the mice's transgenic parenchymal cells (as shown in the experimental strategy in **Figure 2**)[23].

3.4.2. On days 3, 2, and 1 before the surgery, precondition mice with nonmyeloablative intraperitoneal doses of treosulfan (1.5 g/kg/day) in 100 µL of saline, using a 27 G needle.

3.4.3. One day before the surgery, draw two to three drops (~100 µL) of blood from the submandibular vein by pricking it with a 5 mm lancet, and isolate the serum by centrifuging (1,500 x *g* for 10 min at 4 °C) for the alanine aminotransferase (ALT) assay to assess the degree of liver damage.

3.5. Preparation for the surgery

3.5.1. Use clippers to shave the mouse's fur surrounding the incision site (at the left of the peritoneal wall) before surgery.

3.5.2. Adjust the oxygen flow to 1 L/min and the isoflurane flow to 3% - 5% in an induction chamber using a mouse anesthesia machine. Place one mouse at a time in the induction chamber for anesthesia.

3.5.3. Attach the one end of a sterile extension tube (with a holding capacity of 550 µL of suspension; see the **Table of Materials** for specifications) to a 30 G needle and the other end to a 1 mL syringe.

3.5.4. Fill the syringe with the suspension (80 µL/mouse) of pooled HEPs and HSPCs (see section 2), fit the syringe in the notch of a repetitive dispensing pipette, and adjust the dispenser to dispense 10 µL in each press.

3.5.5. Once the mice are anesthetized (usually after 3 - 4 min), switch the isoflurane flow to the nose cone and reduce the isoflurane flow rate to 1% - 3%.

3.6. Intrasplenic transplantation of human HSPCs and hepatocytes in mice

3.6.1. Perform all surgery steps in a laminar flow cabinet under sterile conditions.

3.6.2. Place a clean sterile drape over the working surface and scrub the left side of the body of each mouse with 70% ethanol followed by 10% povidone-iodine, before making an incision.

3.6.3. Make a small incision (~1 - 1.5 cm in length and 5 mm deep) in the skin, muscle, and peritoneum at the left of the peritoneal wall with Vanna's type scissors to enter the peritoneal cavity approximately 5 mm below the lower edge of the rib cage.

3.6.4. Locate the spleen, pull it slightly with forceps to the operating area for easy access, and insert the 30 G needle into the lower pole of the spleen.

3.6.5. Unlock the plunger of the dispensing pipette and dispense 10 µL of the volume at a time, with a limit of 60 - 80 µL per spleen. Retract the needle slowly and clip the spleen with ligating clips using a ligation applier.

3.6.6. Push the spleen back into the body cavity with cotton-tipped applicators wetted with sterile PBS. Close the peritoneum and skin with 6-0 synthetic absorbable sutures.

3.6.7. Use warm water circulating pads to protect the mice from hypothermia after surgery.

3.7. Postoperative care

3.7.1. When the transplanted animal wakens, inject analgesic buprenorphine (0.1 mg/kg) intraperitoneally, 2x a day for a consecutive 3 days.

3.7.2. Observe the animals at least 1x a day until they return to normal physical conditions.

NOTE: Check each animal's body weight, since some mice may lose weight postsurgery. Mice typically regain their original weight in 1 to 2 weeks.

4. Engraftment Validation of the Human Liver by ELISA and the Human Immune System by Flow Cytometry

NOTE: Evaluate the reconstitution of the human liver and immune system monthly, starting 1 month posttransplantation by enzyme-linked immunosorbent assay (ELISA) and flow cytometry, respectively.

4.1. Collect blood samples from the submandibular vein using lancets in EDTA tubes, and centrifuge at 1,500 x *g* for 10 min at 4 °C. Isolate serum to check human albumin levels to assess the engraftment efficiency of the mouse liver for transplanted human hepatocytes, using a human albumin ELISA quantitation set (see the **Table of Materials**) by following the manufacturer's instructions.

NOTE: Do not discard the pellet and use the pelleted cells for a flow cytometry analysis to evaluate the human immune system reconstitution.

4.2. Resuspend the cell pellet without serum in 35 µL of FACS buffer (PBS + 2% FBS) and stained with 5 µL of mouse-specific CD45 (concentration 0.5 mg/mL), 5 µL each of human-specific antibodies CD45 (0.1 mg/mL), CD3 (0.2 mg/mL), CD8 (0.1 mg/mL), and CD19 (0.5 mg/mL), and 20 µL of CD4 (0.25 mg/mL) and CD14 (0.25 mg/mL) each for 30 min at 4 °C, to check the development of a functional immune system from $CD34^+$ HSPCs.

NOTE: Consider adding one additional tube of unstained cells to determine the gating of the stained cells.

4.3. After incubation, transfer the stained suspension (~100 µL) in a polystyrene round-bottom flow cytometry tube and use 2 mL of 1x lysis buffer (see the **Table of Materials**) by diluting 1 part of 10x lysis buffer with 9 parts of distilled water and incubating 10 - 15 min to lyse red blood cells.

NOTE: Observe turbidity to evaluate the red blood cell lysis. Once the sample becomes clear, the lysis is complete.

4.4. After the lysis, add 3 mL of the FACS buffer in the tube and centrifuge at 300 x *g* at 4 °C for 5 min to get a pellet. Repeat the washing by adding 3 mL of FACS buffer to the pellet and centrifuge at 300 x *g* at 4 °C for 5 min.

4.5. Fix the cells in freshly made 1% paraformaldehyde (PFA) and acquire stained cells on the flow cytometer, analyzed with flow software.

4.6. For the analysis, select lymphocytes gating on an FSC/SSC plot, followed by single-cell gating on FSC-area/FSC-height.

4.7. Further, gate for human-specific CD45 (hCD45) on the single-cell population and include mouse-specific CD45 (mCD45) for the exclusion of cells of murine origin. Strategize gating of the stained population based on the gating of unstained cells.

4.8. Gate $hCD45^+$ cells to determine the frequency of $CD3^+$ T cells and $CD19^+$ B cells. Gate T cells to determine CD4 and CD8 subsets. To evaluate monocytes, gate on hCD45 to determine $CD14^+$ monocytes.

5. HIV Infection of TK-NOG Mice and Its Effect on the Human Liver and Immune System

5.1. Handle the HIV-1 virus and all infected mice in a designated biosafety level 3 facility.

CAUTION: Autoclave and discard all HIV-infected wastes in double biohazard bags. For safety reasons, wear cut-resistant gloves while handing HIV-infected mice.

5.2. Wear personal protection equipment (PPE), including a disposable coverall gown, shoe covers, a face mask, and double gloves at all times while working with the virus.

5.3. Select mice with a reconstitution of more than 15% of human $CD45^+$ cells (tested in step 4.7) and with a presence of human albumin in the serum for HIV-1 infection (tested in step 4.1).

5.4. Inject the mice with 1 x 10^3 to 1 x 10^4 tissue culture infectious doses 50 ($TCID_{50}$) $HIV\text{-}1_{ADA}$ in a volume of 100 - 200 µL per mouse, intraperitoneally.

5.5. Euthanize the HIV-infected mice 5 weeks postinfection by using isoflurane (with an isoflurane flow rate of >5%).

5.6. After euthanizing the mice, collect blood in EDTA tubes by a cardiac puncture for the isolation of serum, to see the effect of HIV-1 on the liver by evaluating human-specific albumin levels by ELISA (see step 4.1) and blood cells to check for changes in the human immune cells using flow cytometry (see steps 4.2 - 4.8).

NOTE: Assess the peripheral viral load 5 weeks postinfection on a bioanalyzer to confirm if the mice are infected.

5.7. After drawing blood, excise the liver from the euthanized mice.

5.8. For the liver excision, expose the abdominal cavity by making an incision of 1.5 - 2 cm long and 0.5 cm deep in the skin, muscles, and peritoneum, from the xyphoid. Make a cut perpendicular to the spine between the liver and the diaphragm. Lift the liver and sever any membranes attaching it to the stomach and intestines.

5.9. Collect and fix the liver in 4% paraformaldehyde overnight and follow a standard immunohistochemical protocol to evaluate the effect of HIV on CK18$^+$ hepatocytes by using human-specific CK18 antibody[8].

REPRESENTATIVE RESULTS:

The establishment of a dual humanized mouse model with human liver and immune cells can be easily monitored at each step with very simple ELISA and flow cytometry, respectively. Flow cytometry is regularly performed to evaluate the development of a functional immune system and to see the effect of HIV infection on immune cells. In dual humanized mice, the development of functional immune cells can range from 15% to 90% of the lymphocyte gate. Representative subsets of immune cells are shown in dot plots (**Figure 3**). For the evaluation of the engraftment of human hepatocytes, ELISA for human-specific albumin levels is performed monthly on mouse serum. Mice engrafted with both HSPCs and HEPs show human-specific albumin levels ranging from ~7 µg/mL to 377 µg/mL at one month, continuing to grow over the time of observation (6 months) (**Figure 4**). The effect of HIV infection on human immune cells in the blood of dual humanized mice is monitored by flow cytometry and on HEPs in the liver by human-specific albumin ELISA. By 5 weeks, HIV-1 causes a decrease in human albumin levels in the serum, as assessed by ELISA, and there is a depletion of human CK18$^+$ hepatocytes in the liver sections of dual humanized mice, as evaluated by immunohistochemistry (**Figure 5**). A lower ratio of CD4:CD8 is typically observed, by flow cytometry, in the blood and liver of HIV-infected mice, compared to levels noted in the same mouse before infection (**Figure 6**). All reagents and materials important for the protocol are discussed in the **Table of Materials**.

FIGURE LEGENDS:

Figure 1: Schematic of the enrichment of CD34$^+$ cells from cord blood. (**A**) Cord blood is layered on lymphocytes separation medium (LSM) and centrifuged to isolate buffy coat. (**B**) LS columns are placed on a magnetic stand and rinsed with BSA buffer, followed by adding buffy coat. Cells positive for CD34 are trapped in the columns, and CD34$^-$ cells are eluted in separate tubes. Trapped CD34$^+$ cells in column resins are plunged with a plunger, and the cells are collected in a new tube.

Figure 2: Schematic view of the experimental design for the dual reconstitution of humanized liver and immune system mice, followed by HIV-1 infection. TK-NOG mice are injected with ganciclovir (GCV) at a dose of 6 mg/kg, 2x a day, on day -7 and day -5, followed by a treosulfan injection on days -3, -2 and -1. To screen the mice for the transplantation (Tx), an alanine aminotransferase (ALT) assay is performed one day before the surgery, and mice with ALT levels of >200 and <600 U/L are selected. After transplantation, the mice are checked for a reconstitution of the human immune system by flow cytometry (FACS) and for liver reconstitution by assessing their albumin level using ELISA. The mice are infected with HIV-1 5 weeks before they are sacrificed.

Figure 3: Flow cytometry analysis gating strategy for the human cell distribution of blood. (**A**) First, lymphocytes are gated on whole blood based on FSC-A and SSC-A. (**B**) Single cells are gated

on lymphocytes. (**C**) Human $CD45^+$ leukocytes are gated on single cells using mouse CD45 and human CD45. (**D**) $CD3^+$ T cells and $CD19^+$ B cells are identified on gated $CD45^+$ human leukocytes. (**E**) $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells are identified in gated $CD3^+$ T cells. (**F**) $CD14^+$ monocytes are gated from human $CD45^+$ leukocytes. The results represented here are from one mouse transplanted with dual human hepatocytes and HSPCs.

Figure 4: Albumin concentration is measured by ELISA in the serum of dual humanized mice. The mice are transplanted with both human hepatocytes (HEPs) and $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) (*n* = 11). Serum is collected at different times at 1, 4, and 6 months posttransplantation, and dilutions are made to adjust the unknown sample concentrations in the range of standards. Each symbol represents an individual mouse value. The results represent the median, as well as individual values. * $P < 0.05$, by one-way ANOVA. This figure has been modified from Dagur *et al.*[8].

Figure 5: Effect on HIV-1 on albumin levels in serum and the depletion of $CK18^+$ human hepatocytes in the liver of dual humanized mice. (**A**) Albumin concentrations are monitored in uninfected mice (*n* = 9) transplanted with both human HEPs and HSPCs at 1 and 4 months. The mice are infected (*n* = 10) with HIV at 4 - 5 months posttransplantation and sacrificed 5 weeks postinfection. Each symbol represents an individual mouse value. The results represent the median, as well as individual values. * $P < 0.05$, by one-way ANOVA. This figure has been modified from Dagur *et al.* [8]. (**B**) Five-micron liver sections from uninfected (HEPs + HSPCs, left panel) and HIV-infected TK-NOG mice (HEPs + HSPCs + HIV, right panel) are fixed, paraffin embedded, and stained for anti-human cytokeratin-18 (CK18) antibody. HIV-1 causing a depletion of $CK18^+$ hepatocytes is evidenced by a less occupied area by the $CK18^+$ human cells. The results represented here are from one uninfected and one HIV-infected mouse transplanted with dual human hepatocytes and HSPCs. The scale bars = 100 µm.

Figure 6: Ratio of $CD4^+$ cells to $CD8^+$ T cells in peripheral blood and in the liver of dual reconstituted uninfected and HIV-1-infected mice. For dual reconstituted uninfected mice: closed circle; HEPs + HSPCs; blood *n* = 7; liver *n* = 6. For HIV-1 infected mice: open circles; HEPs + HSPCs + HIV; blood *n* = 10; liver *n* = 11. The results represent the median, as well as individual values. * $P < 0.05$, by one-way ANOVA test between HIV-infected and uninfected mice. This figure has been modified from Dagur *et al.*[8].

DISCUSSION:

The liver is compromised and damaged in HIV-infected patients[24]. Experimental small animal models for studying human liver diseases in the presence of HIV-1 is extremely limited, despite the availability of a few cotransplanted animal models with $CD34^+$ HSPCs and hepatocytes[7,12,25]. In *in vitro* experiments, hepatocytes are shown to have low-level HIV-1 infection[26]. Humanized mice that carry both types of human cells are a desirable model. The liver of mice reconstituted with only human immune system has been shown to be affected by HIV infection under the experimental depletion of human regulatory T cells[20,27]. However, the difference in immune and functional properties of mouse and human hepatocytes may underline the differences in their responses to HIV-1 and immune cells. In this review, a protocol is described to reconstitute both

human immune system and liver and to address HIV-1-associated liver immunopathology, as observed in HIV-1-infected patients. TK-NOG male mice were selected due to their liver-selective high mRNA expression of HSV-TK transgene and the susceptibility of GCV toxicity to mouse transgenic liver[21]. Moreover, they can be maintained for long periods after transplantation without the use of exogenous drugs and do not develop spontaneous systemic diseases[28]. To establish human immune system and liver reconstitution, ablation of the mouse immune system and damage to mouse-specific liver cells are required and achieved using nonmyeloablative doses of treosulfan and GCV, as shown previously in TK-NOG male mice[13,23]. Mice are injected with GCV and treosulfan at the age of 6 - 8 weeks, as the expression of transgene and GCV-induced hepatic injury as assessed by ALT levels are optimal, then, for providing niche-to-transplanted human cells[21]. Mice showing ALT levels of >200 U/L, but <600 U/L, are usually selected for transplantation. Mice showing ALT levels of >600 U/L are at a greater risk of death as human hepatocytes are not able to rescue the damaged mouse liver function.

Currently, dual humanization is shown by the transplantation of human $CD34^+$ HSPCs and fetal liver cells; however, the manipulation of newborn animals creates technical problems[13,14]. HSPCs can be derived or isolated from multiple sources, such as fetal liver cells (FLCs), embryonic stem cells (ESCs), and CB. However, ethical issues constrain the use of ESCs and FLCs. CB has no such restriction and is a most useful alternative to obtain HSPCs, as well as being a precious source of primitive hematopoietic stem and progenitor cells that can reconstitute the functional immune system. Cord blood should not be older than one day when used to isolate HSPCs, as the yield of HSPCs is highly affected by age. The purity of the isolated HSPCs needs to be checked before cryopreserving the cells. The cross-contamination of $CD3^+$ T cells is avoided, as it may lead to systemic mouse graft-*versus*-host disease and acute allorejection of HEPs while transplanting with mismatched cells.

Commercially available hepatocytes were used as a source for liver reconstitution[8,13]. Adult hepatocytes are preferred for establishing liver reconstitution due to their increased efficiency in engraftment and sustainability for a long period of time[29].

The presence of human immune system in a mouse model increased ALB levels, as shown previously[30,31]. However, the efficiency of hepatocytes and immune system reconstitution may vary with different sources of donor cells and depend on the recipient mouse. So, each mouse needs to be assessed for engraftment, and the most critical part is to utilize the antibodies or reagent that are human-specific and do not cross-react with mouse cells. The human-specific reagents and antibodies used in the study presented here are detailed in the **Table of Materials**. If antibodies other than provided in the **Table of Materials** are used for the study, be sure to check for the human specificity.

The optimal condition would be the transplantation of syngeneic cells; however, that is technically difficult to achieve. Wherever possible, HSPCs and hepatocytes should be pooled from donors with partially matched human leukocyte class-1 antigens (like HLA-A2).

To screen mice for HIV studies, blood is drawn at multiple time points to determine the optimal immune and liver reconstitution; flow cytometry and ELISA are preferred as they can be performed with only a little amount of blood. Blood cells and serum from the same sample could be used for flow cytometry and ELISA, respectively. It is important to make proper dilutions of serum at each time point (1,000 - 40,000 range) to evaluate ALB levels so that the unknown concentrations can be brought within the range of standard concentrations (kit range: 6.25 - 400 ng/mL).

Proinflammatory cytokines in response to HIV-1 infection in the presence of human immune system can also be useful in addressing the interaction of hepatocytes and immune cells. The model is useful for showing the immunopathogenesis of HIV-1-induced liver disease, given that it recapitulates liver damage in the same manner as in humans, evidenced by a low ratio of CD4:CD8, a decrease in ALB levels, human hepatocyte death, and liver immune activation. The model also has some limitations, such as a low level of cytotoxic T cells activity and impaired immunoglobulin class switching. Due to the presence of both human immune system and liver, the model presented here is promising for studi coinfections of HIV-1 and hepatitis viruses, chronic hepatitis infection (to clarify the mechanisms of the anti-hepatitis immune response), and as a cirrhosis model.

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DISCLOSURES:

The authors have nothing to disclose.

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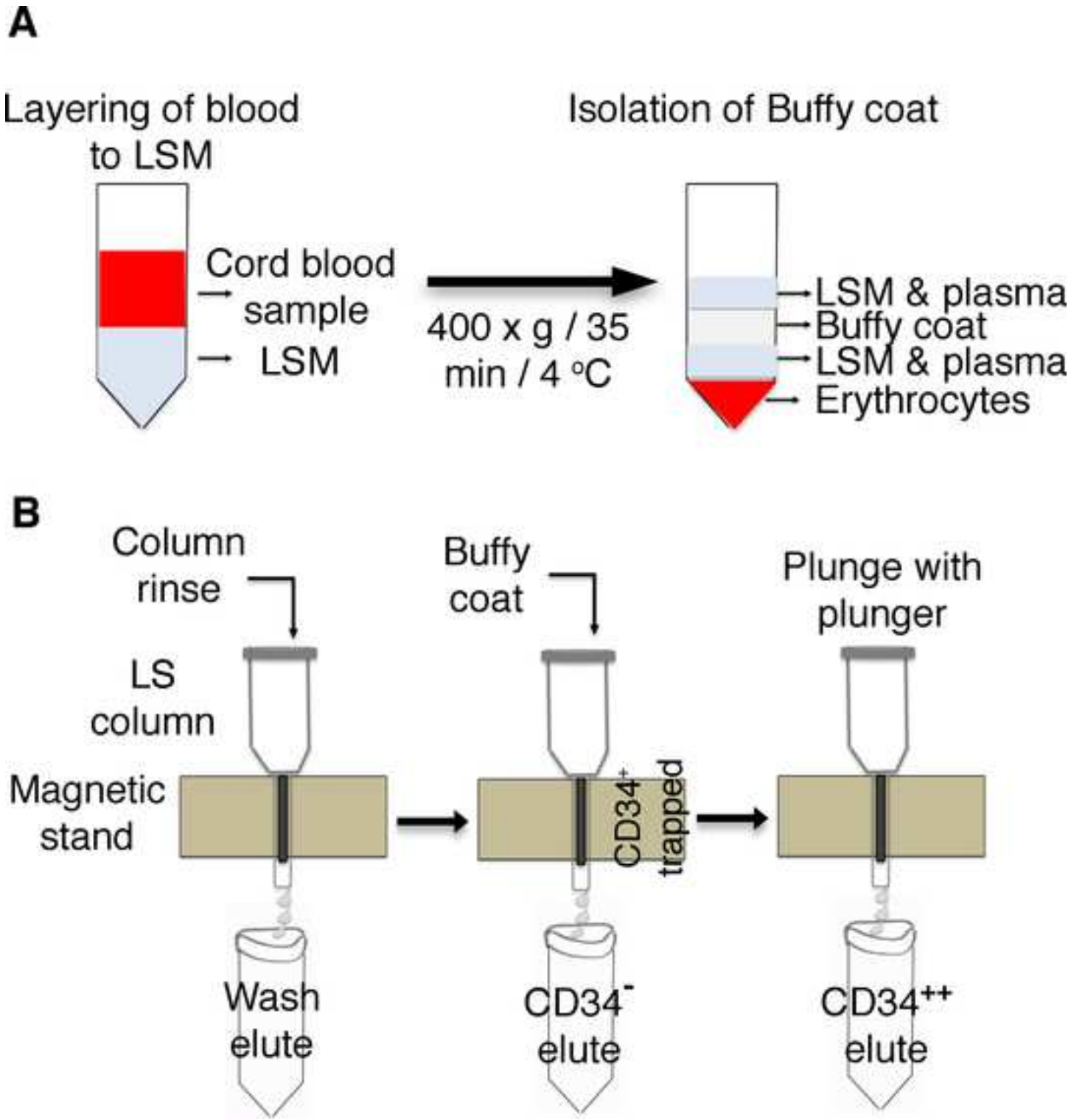

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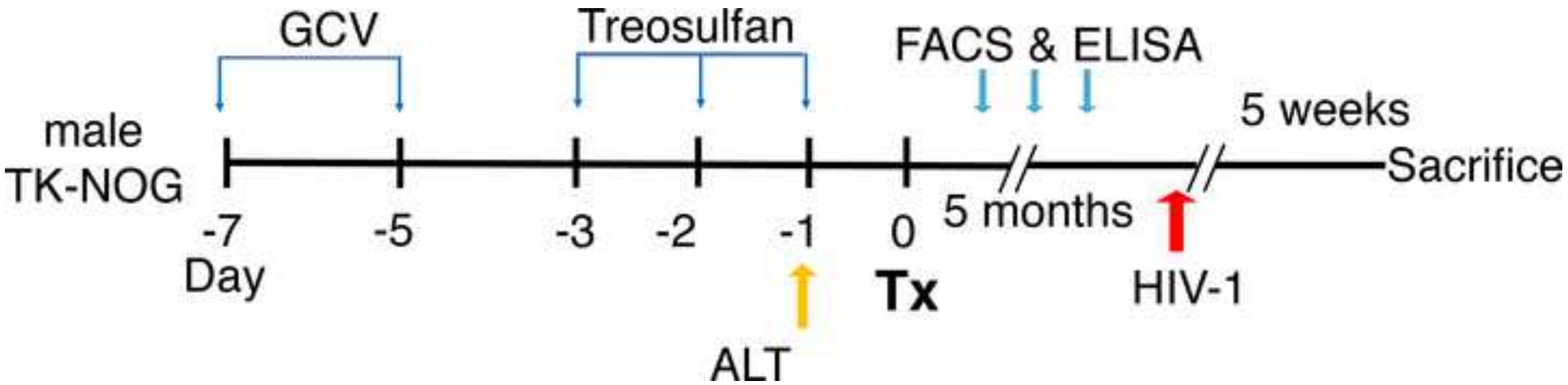

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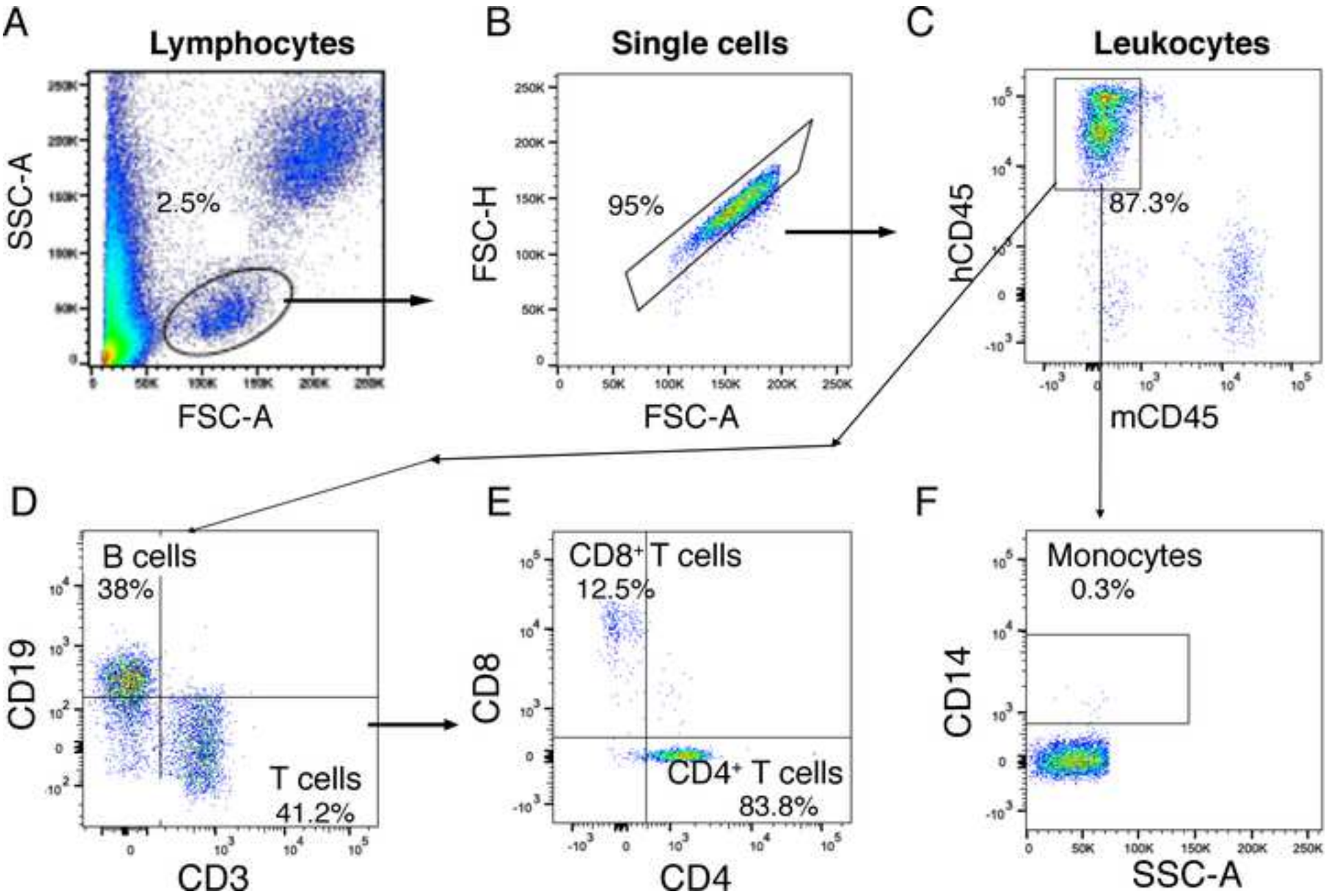

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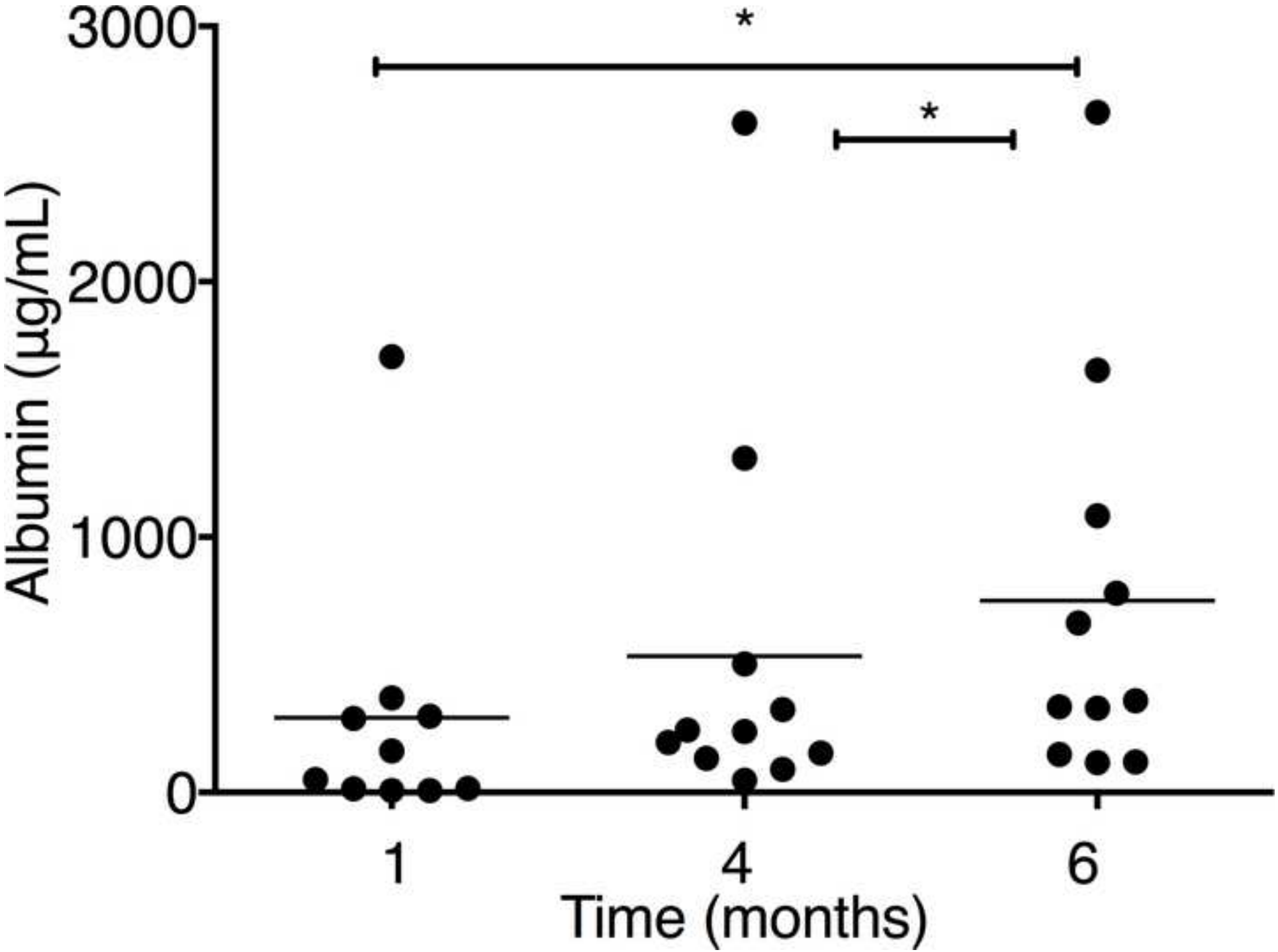

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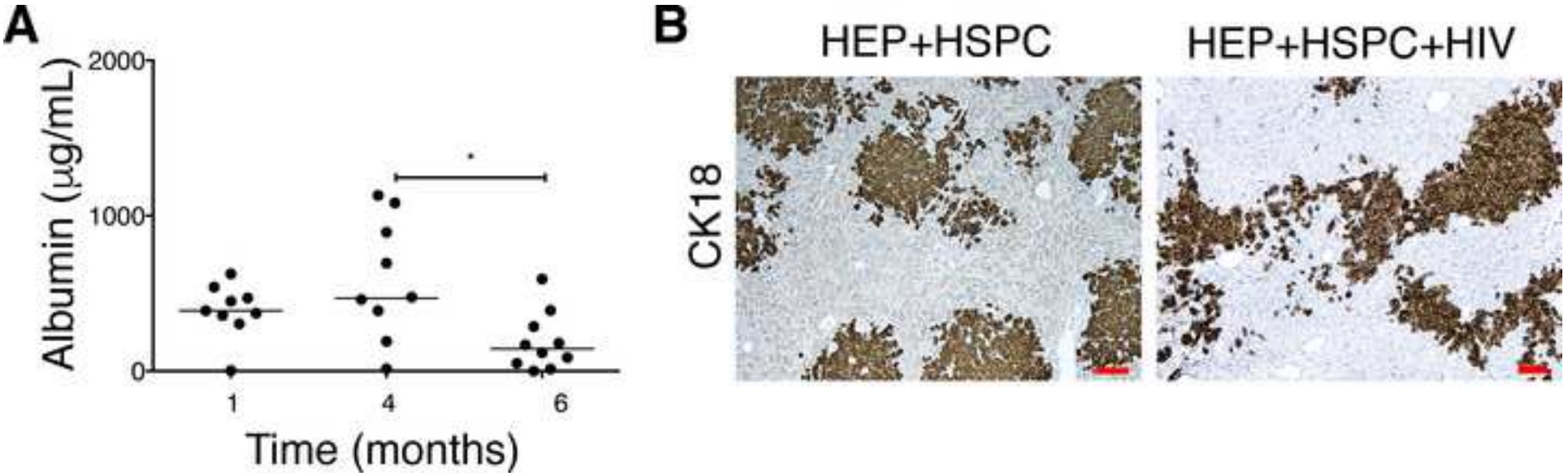
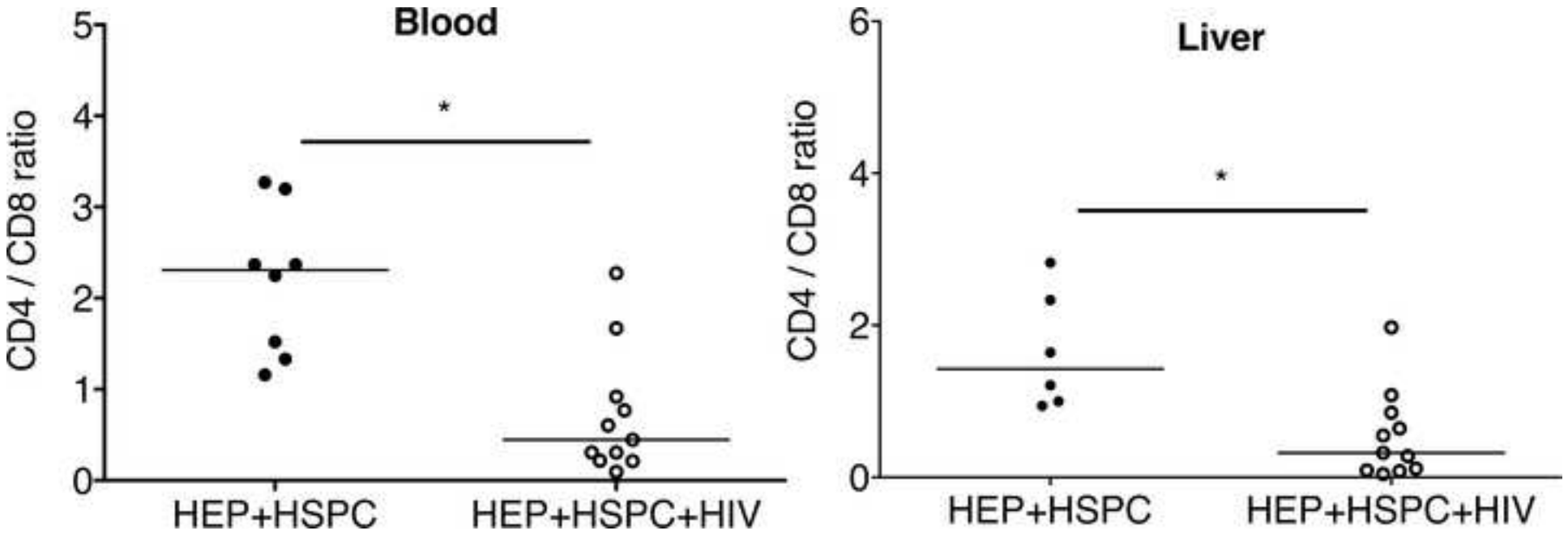


Figure 6



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
27G1/2" needles	BD biosciences	305109	
30G1/2" needles	BD biosciences	305106	
5 mL polystyrene round-bottom tube 12 x 75 mm style	Corning	352054	
BD 1 mL Tuberculin Syringe Without Needle	BD biosciences	309659	
BD FACS array bioanalyzer	BD Biosciences		For purity check of eluted CD34+ cells
BD FACS array software	BD Biosciences		Software to analysis acquired $CD34^+$ cell on FACS array
BD FACS lysing solution	BD Biosciences	349202	To lyse red blood cells
BD LSR II	BD Biosciences		Instrument for acquisiton of flow cytometry samples
BD Vacutainer Plastic Blood Collection Tube	BD biosciences	BD 367874	To collect Cord blood
Bovine Serum Albumin	Sigma-aldrich	A9576	
Buprenorphine			Controlled substance and pain-killer
CD14-PE	BD Biosciences	555398	Specific to human
CD19-BV605	BD Biosciences	562653	Specific to human
CD34 MicroBead Kit, human	Miltenyi Biotec	130-046-702	For isoation of $CD34^+$ HSPC
CD34-PE, human	Miltenyi Biotec	130-081-002	Antibody used for purity check of eluted CD34+ cells
CD3-AF700	BD Biosciences	557943	Specific to human
CD45-PerCPCy5.5	BD Biosciences	564105	Specific to human
CD4-APC	BD Biosciences	555349	Specific to human
CD8-BV421	BD Biosciences	562428	Specific to human

Cell counting slides	Bio-rad	1450015	
ChargeSwitch gDNA Mini Tissue Kit	Thermofisher scientific	CS11204	for extraction of genomic DNA from ear piece
Cobas Amplicor system v1.5	Roche Molecular Diagnostics		bioanalyzer to measure viral load
Cotton-tipped applicators	McKesson	24-106-2S	
Cytokeratin-18 (CK18)	DAKO	M7010	Specific to human
DMSO (Dimethyl sulfoxide)	Sigma-aldrich	D2650-5X5ML	
Extension set Microbore Slide Clamp(s) Fixed Male Luer Lock. L: 60 in L: 152 cm PV: 0.55 mL Fluid Path Sterile	BD biosciences	30914	Attached to dispensing pippet and to load with HSPC and HEP suspesion
FACS Diva version 6	BD Biosciences		flow cytometer software required for acqusition of sample
Fetal Bovine Serum (FBS)	Gibco	10438026	
FLOWJO analysis software v10.2	FLOWJO, LLC		flow cytometry analysis software
Ganciclovir	APP Pharmaceuticals, Inc.	315110	Prescripition drug
Greiner MiniCollect EDTA Tubes	Greiner bio-one	450475	
Hepatocytes thawing medium	Triangle Research Labs	MCHT50	
Horizon Open Ligating Clip Appliers	Teleflex	537061	To hold the ligating clips
Hospira Sterile Water for Injection	ACE surgical supply co. Inc.	001-1187	For dilution of Buprenorphine (pain-killer)

Human Albumin ELISA Quantitation Set	Bethyl laboratories	E80-129	For assesing human albumin levels in mouse serum
Human hepatocyte	Triangle Research Labs	HUCP1	Cryopreserved human hepatocytes, induction qualified
Iris Scissors, Straight	Ted Pella, Inc.	13295	
Lancet	MEDIpoint	Goldenrod 5 mm	
LS columns	Miltenyi Biotec	130-042-401	Used to entrap $CD34^{+}$ microbeads (positive selection)
Lymphocyte Separation Medium (LSM)	MP Biomedicals	50494	For isoation of lymphocytes from peripheral blood
MACS MultiStand	Miltenyi Biotec	130-042-303	holds Qudro MACS seperator and LS columns
McPherson-Vannas Micro Dissecting Spring Scissors	Roboz Surgical Instrument Co.	RS-5605	Used to make an incision on skin to expose spleen
Micro Dissecting Forceps	Roboz Surgical Instrument Co.	RS-5157	to hold and pull out spleen from peritoneal cavity
mouse CD45-FITC	BD Biosciences	553080	mouse-specific
PBS (Phosphate Buffered Saline)	Hyclone	SH30256.02	
Qudro MACS separator	Miltenyi Biotec	130-090-976	holds four LS columns
RPMI 1640 medium	Gibco	11875093	
StepOne Plus Real Time PCR	Applied Biosystems		Instrument used to genotype
Stepper Series Repetitive Dispensing Pipette 1ml	DYMAX CORP	T15469	Used to dispense HSPC and HEP supension in controlled manner
Suturevet PGA synthetic absorbale suture	Henry Schein Animal Health	41178	Suturing of skin and peritoneum

TaqMan Gene Expression Master Mix	Thermofisher scientific	4369016	
TC20 automated cell counter	Bio-rad	1450102	
TK-NOG mice			Provided by the Central Institute for Experimental Animals (CIEA, Japan; Drs. Mamoru Ito and Hiroshi Suemizu)
Treosulfan	Medac GmbH		Provided by Dr. Joachim Baumgart (medac GmbH)
Trypan Blue	Bio-rad	1450022	
Vannas-type Micro Scissors, Straight, 80mm L	Ted Pella, Inc.	1346	Used to make an incision on skin to expose spleen
Weck hemoclip traditional titanium ligating clips	Esutures	523700	To ligate the spleen post-injection

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TITLE:

Establishment of Dual Humanized TK-NOG Mouse Model for HIV-Associated Liver Pathogenesis

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KEYWORDS:

Dual humanized mice, albumin, hepatocytes, humanized liver, human immune system, human immunodeficiency virus-1 (HIV-1), hematopoietic stem cells, hematopoietic progenitor cells

SUMMARY:

This protocol provides a reliable method to establish humanized mice with both human immune system and liver. Dual reconstituted immunodeficeint mice achieved *via* intrasplenic injection of human hepatocytes and $CD34^+$ hematopoietic stem cells (HSPC) are susceptible to HIV-1 infection and recapitulate liver damage as observed in HIV-infected patients.

ABSTRACT:

Despite increased life expectancy of patients infected with human immunodeficiency virus (HIV-1), liver disease has emerged as a common cause of their morbidity. The liver immunopathology caused by HIV-1 remains elusive. Small xenograft animal models with human hepatocytes and human immune system can recapitulate the human biology of disease pathogenesis. Herein, a protocol is described to establish a dual humanized mouse model through human hepatocytes and $CD34^+$ hematopoietic stem cells (HSPC) transplantation, to study liver immunopathology as observed in HIV-infected patients. To achieve dual reconstitution, male TK-NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} Tg(Alb-TK)7-2/ShiJic) mice are intraperitoneally injected with ganciclovir (GCV) doses to eliminate mouse transgenic liver cells, and treosulfan for non-myeloablative conditioning, both of which facilitate human hepatocyte (HEP) engraftment and human immune system (HIS) development. Human albumin (ALB) levels are evaluated for liver engraftment, and

the presence of human immune cells in blood detected by flow cytometry confirms the establishment of human immune system. The model developed using the protocol described here resembles multiple components of liver damage from HIV-1 infection. Its establishment could prove to be essential for studies of hepatitis virus co-infection and for evaluation of antiviral and antiretroviral drugs.

INTRODUCTION:

Since the advent of antiretroviral therapy, there has been a substantial decrease in deaths related to HIV-1 mono-infection. However, liver disease has emerged as a common cause of morbidity in HIV-infected patients[1,2]. Coinfections of hepatitis viruses with HIV-1 infection are more common, accounting for 10-30% of HIV-infected persons in the United States[3-5].

The host-specificity of HIV-1 and hepatitis viruses limits the utility of small animal models to study human-specific infectious diseases or to investigate multiple aspects of HIV-1-associated liver pathogenesis. Immunodeficient mice that permit the engraftment of human cells and/or tissues (termed humanized mouse models) are acceptable animal models for preclinical studies[6-8]. Since the introduction of humanized mice in the early 2000s, multiple preclinical studies of cholestatic human liver toxicity, human-specific pathogens, including human immunodeficiency virus type one (HIV-1) and HIV-associated neurocognitive disorders, Epstein Barr virus, hepatitis and other infectious diseases, have been investigated in these mice[6,9-11]. Multiple mouse models for $CD34^{+}$ hematopoietic stem/progenitor cells (HSPC) and/or human hepatocyte transplantation have long been developed and have improved over time to study disease pathogenesis of Hepatitis B virus (HBV)-associated liver disease[12-14]. Several models for HSPC and human hepatocyte (HEP) transplantation are based on strains, known as NOG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/JicTac)[8,13], NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/*SzJ*)[15], Balb/C-Rag2$^{-/-}$ γc$^{-/-}$ (Rag2$^{tm1.1Flv}$ Il2rg$^{tm1.1Flv}$/J)[12], and fah$^{-/-}$ NOD rag1$^{-/-}$il2rγnull mouse[16]. However, each model has its own advantages and limitations; for example, AFC8 dual-humanized mice for HEP and HSC on Balb/C-Rag2$^{-/-}$ γc$^{-/-}$ background enables successful engraftment of immune cells and HSC but there is absence of antigen-specific T and B cell response in this model[12]. The major concerns in reconstituting double humanized mice include suboptimal engraftment, lack of suitable models to support different tissues, mismatched conditions, immune rejection or graft-*versus* host disease (GVHD), and technical difficulties, such as risky manipulations with newborns and high mortality rates due to metabolic abnormalities[13].

Although humanized mice have been used for HIV research for many years[17-19], the use of humanized mice to study liver damage caused by HIV-1 has been limited[20]. The authors previously reported the establishment of a dual humanized TK-NOG mouse model and its application in HIV-associated liver disease[8]. This model shows robust engraftment of liver and immune cells and recapitulates HIV infection pathogenesis. This discussion presents a detailed protocol, including the most critical steps in transplantation of human hepatocytes. A description of the HSPC required for successful engraftment of HEP and establishment of functional immune system in TK-NOG mice is also presented. The use of these mice to study HIV-associated liver immunopathogenesis is detailed. TK-NOG male mice carrying a liver-specific herpes simplex virus type 1 thymidine Kinase (HSV-tk) transgene are used. Mouse liver cells expressing this transgene can easily be ablated after brief exposure to a non-toxic dose of ganciclovir (GCV). Transplanted human liver cells are stably maintained within the mouse liver without exogenous drugs[21]. Mice are also pre-conditioned with non-myeloablative doses of treosulfan to create a niche in the

mouse bone marrow for human cells[8]. Immunodeficient TK-NOG mice are intrasplenically injected with HEP and multipotent HSPCs. The mice are then regularly monitored for blood and liver reconstitution by blood immunophenotyping and measurements of serum human-albumin levels, respectively. Mice with successful reconstitution of more than 15% for both human immune cells and HEP are intraperitoneally injected with HIV-1. The effect of HIV on liver can be assessed as early as 4-5 weeks post infection. It is critical to note that, because HIV-1 is used, all necessary precautions must be taken while handling the virus and injecting it into mice.

PROTOCOL:

This protocol has been approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board (IRB) at University of Nebraska Medical Center.

NOTE: Obtain approval from the local IACUC and IRB before performing experiments on animal or human samples.

1. Processing of umbilical cord blood (CB) and isolation of human HSPC

1.1. Perform all steps of the protocols under sterile conditions in laminar flow cabinets.

1.2. Collect human CB in heparinized tubes and store CB samples at room temperature before processing.

1.3. Make CB volume up to 35 mL by adding phosphate buffered saline (PBS) and mix well. Layer the sample on top of the lymphocytes separation medium (LSM) as illustrated in **Figure 1** and centrifuge the LSM with layered CB at 400 x g for 35 min at 4 °C with no brakes.

NOTE: Dilute blood carefully and gently to avoid mixing at the interface.

1.4. Remove the top LSM and plasma layer carefully and transfer the white buffy coat interface into a new tube using a transfer pipette.

1.5. Resuspend the buffy coat in 30-40 mL of ice-cold buffer (Phosphate-buffered Saline (PBS) + 0.5% bovine serum albumin [BSA] + 2 mM ethylenediaminetetraacetic acid [EDTA]). Using a pipette combine 20 µL of the cells suspension with 20 µL of 0.4% trypan blue and pipette 10 µL of the mixture into the outer opening of either of the two chambers of the counting slides and insert the slide in automated cell counter to count the cells.

NOTE: Use ice-cold buffer in all steps, as it helps keep the cells viable.

1.6. Centrifuge the cells at 300 x g for 10 min at 4 °C and aspirate the supernatant carefully. Then add 300 µL of ice-cold buffer.

1.7. Add 100 µL of human FcR blocking reagent and 100 µL monoclonal mouse anti-human CD34 antibody-conjugated microbeads for up to 1 x 10^8 cells (see the **Table of Materials**). Incubate for

Commented [A1]: Some minor edits were made to the structure and language for clarity.

Commented [A2R1]: Thank you for the editing.

Commented [A3]: How?

Commented [A4R3]: The counting was performed on automated cell counter using trypan blue dye exclusion assay. The catalog numbers for counting slides, trypan blue and automated cell counter have been updated in table of materials.

Commented [A5]: Correct?

Commented [A6R5]: Microbeads are supplied as conjugated to monoclonal mouse anti-human CD34 from the supplier and recommended concentration 100 µl for 1x10^8 cells.

Commented [A7]: Concentration?

Commented [A8R7]: Recommended concentration of the microbeads is 100 µl for 1x10^8 cells. Monoclonal mouse anti-human CD34 antibody is not as separate but supplied as conjugated to microbeads.

30 min at 4 °C, add 10 mL of ice-cold buffer to wash the cells and centrifuge at 300 x g for 10 min at 4 °C.

NOTE: Scale this up according to cell number if more than 1 x 10^8 cells are present.

1.8. Carefully remove the supernatant, resuspend the pellet in 500 µL of buffer and proceed with the magnetic separation step to enrich HSPC.

1.9. Place positive selection LS column (see the **Table of Materials**) in the magnetic-activated cell sorting field and pass through with 3 mL of buffer.

1.10. Load the sample to the LS column that can entrap microbeads bound to human CD34+ in samples and allow to flow under the influence of gravity into the collection tube.

1.11. Wash the column with buffer 3x and collect the elute in the same collection tube of $CD34^-$ fraction of cells.

1.12. Plunge the column with 5 mL of buffer to elute $CD34^+$ cells into a new collection tube. Repeat the procedure to achieve purity >90%.

1.13. Count eluted $CD34^+$ cells using trypan blue dye in a hemocytometer. After counting, centrifuge the $CD34^+$ cells at 300 x g for 5 min and discard the supernatant.

1.14. Re-suspend cells in a ~~small volume (25 µL) for injection~~ 25 µL of PBS for injection to immediate use in transplantation or cryopreserve at a concentration of 1-2 million / mL in freezing medium (Roswell Park Memorial Institute medium (RPMI 1640 medium) + 50% fetal bovine serum + 10% dimethyl sulfoxide (DMSO)) to further use in transplantation.

NOTE: Always recount viable cells before using in transplantation.

1.15. To check the purity of $CD34^+$ elute, take 50 µL of the suspension and incubate with 10 µL of PE-conjugated anti-human CD34 antibody for 30 min at 4 °C. ~~Acquire on flow cytometer.~~ After the antibody incubation, wash stained cells with PBS and resuspend in 100 µL of PBS before acquiring on flow cytometer. Add an additional tube of cells with no antibody to design the gate in the flow cytometer. Add an additional tube of cells with no antibody to design the gate in the flow cytometer.

1.16. After acquisition, analyze the data by ~~plotting a gate~~ selecting region of interest on forward scatter (FSC) and side scatter (SSC) plot, followed by gating for single cells on FSC-area and FSC-height plots. Gate CD34 positive cells on single cells in PE channel and SSC-area plot. ~~for CD34 positive stained cells.~~

2. ~~Thawing~~ Preparation of human hepatocytes for transplantation

Commented [A9]: Define.

Commented [A10R9]: LS columns have resins that can entrap microbeads bound to human CD34+ cells from the samples.

Commented [A11]: Small volume of what? Buffer?

Commented [A12R11]: PBS (phosphate-buffered saline)

Commented [A13]: How much?

Commented [A14R13]: 1-2 million cells / mL of freezing medium

Commented [A15]: Concentration of antibody?

Commented [A16R15]: We used the PE-conjugated anti-human CD34 (cat 130-081-002) ab at 10 uL in 50 ul of suspension (concentration is not mentioned on the vial). However, the antibody has been replaced with higher concentration by the company and have a cat no. 130-113-179. Concentration of the antibody is not provided by the supplier, but the recommended dilution is 1 ul in 50 ul of cell suspension.

Commented [A17]: Acquire what? Mention gating strategy

Commented [A18R17]: Stained $CD34^+$ cells resuspended in PBS were acquired on flow cytometry. CD34 positive cells are gated on single cells in PE channel and SSC-area plots.

Commented [A19]: Inaccurate phrasing, please revise. What is plotted against what?

Commented [A20R19]: Corrected.

Commented [A21]: I have highlighted this to include it for clarity and continuity

Commented [A22R21]: For the clarity the title and texts have been modified and new subsections are added.

2.1. Remove the cryopreserved hepatocytes from the liquid nitrogen and quickly submerge the vial in the waterbath and thaw for approximately 90-120 sec.

2.2. Remove vial cap and pour thawed hepatocytes into the 50 mL conical tube of wamed thawing medium.

2.3. Suspend the cells by rocking the 50 mL tube by hand for a few seconds.
NOTE: Do not vortex the tube.

2.4. Pellet cells at 100 x g for 8 min at room temperature.

~~Thaw cryopreserved single-donor plateable human hepatocytes in hepatocyte medium at the time of transplantation and pellet at 100 x g for 8 min at 4 °C. thawing~~

2.5. Wash pelleted cells in PBS with 0.1% BSA and pool with either fresh or thawed HSPC (ratio 10:1) in PBS in final volume of 80 µL/mouse.

3. ~~Animal Surgery~~ Animal handling, screening, genotyping and treatment for human HSPC and hepatocytes transplantation

3.1. **Animal handling**

3.1.1. As a result of severe immunodeficiency, breed, house and handle TK-NOG mice under aseptic condition.

3.1.2. Always wear lab coats, gloves, shoe covers, and face mask to prevent infection with potential pathogenic microorganisms.

3.1.3. Use sterile gloves and instruments for surgery and handle animals aseptically throughout surgery.

3.2. **Selecting TK-NOG mice for the experiment**

3.2.1. Maintain the TK-NOG strain colony by breeding female TK-NOG mice with male non-TK-NOG littermates and select transgenic offspring by genotyping.

NOTE: Perform genotyping (see step 3.3) to determine presence or absence of transgene in newborns male and female mice at the time of weaning.

3.2.2. Select males at 6-8 weeks of age for transplantation due to their high sensitivity to GCV-mediated depletion of HSV-TK transgene expressing hepatocytes[21].

Commented [A23]: Please update the section title to represent this section. I made the previous title into substep 3.1 as it pertained only to the first few steps.

Commented [A24R23]: Title of section is updated to "Animal handling, screening, genotyping and treatment for human HSPC and hepatocytes transplantation".

3.2.3. Ear-tag mice at the time of weaning or surgery to ease identification. Note down the weight and health status of animals.

3.3. **Genotyping for the presence of HSV-TK transgene using quantitative real-time PCR**

3.3.1. Perform genotyping at the time of weaning (usually 3-4 weeks of age). For genotyping, cut a piece of the mouse ear in a laminar flow biological safety cabinet to maintain sterility and extract genomic DNA by using a genomic DNA isolation kit.

3.3.2. Amplify genomic DNA extracted from tail tissue in 20 µL reaction mixture to screen for HSVtk transgene under control of human albumin promoter by adding 1 µL forward primer 5'-CCATGCACGTCTTTATCCTGG-3', 1 µL reverse primer 5'-TAAGTTGCAGCAGGGCGTC-3', 0.5 µL of FAM probe 5'-FAM-AATCGCCCGCCGGCTGC-MGB-3' and 10 µL of master mix on a real time PCR instrument[22].

3.3.3. Set the real-time PCR settings as follows: 60 °C for 30 s (pre-read stage), 95 °C for 10 min (hold stage), 40 cycles of 95 °C for 15 s and 60 °C for 1 min (PCR stage), and 60 °C for 30 s (post-read stage).

NOTE: Cycle of threshold (Ct) below 22 is considered positive for HSV-TK transgene.

3.4. **Treatment using ganciclovir and treosulfan**

3.4.1. Using 27-gauge needle Inject TK-NOG mice with intraperitoneal GCV injections (6 mg / kg) twice a day at day 7 and at day 5 in 100 µL of saline before surgery to deplete mouse transgenic parenchymal cells (as shown in experimental strategy in **Figure 2**)[23].

3.4.2. On day 3, 2 and 1 before the surgery, precondition mice with non-myeloablative intraperitoneal doses of treosulfan (1.5 g/kg/day) in 100 µL of saline using 27-gauge needle.

3.4.3. One day before the surgery, draw 2-3 drops (~100 µL) of blood from the submandibular vein by pricking with 5 mm lancet and isolate serum by centrifuging (1500 x g for 10 min at 4 °C) for the alanine aminotransferase (ALT) assay to assess the degree of liver damage.

~~3.4.4. Use warm water circulating pads to protect mice from hypothermia after surgery.~~

3.5. **Preparation for the surgery**

3.5.1. Using clippers to shave mouse fur surrounding the incision site at the left of peritoneal wall) before surgery.

3.5.2. Adjust oxygen flow to 1 L / min and isoflurane flow to 3-5% in an induction chamber using a mouse anesthesia machine. Place one mouse at a time in ~~the mice in~~ the induction chamber for anesthesia.

Commented [A25]: How do you ensure sterility? Is the mouse sedated?

Commented [A26R25]: A piece of ear is cut in biological safety cabinet without sedating the mouse.

Commented [A27]: Do you use a PCR kit? Please add it to the table of materials.

Commented [A28R27]: PCR kit is not used. Kit was used to extract genomic DNA only and is mentioned in table of materials.

Commented [A29]: Mention needle gauge and drug volume.

Commented [A30R29]: 27-gauge needle was used to inject 100 ul of GCV dose in saline, intraperitoneally.

Commented [A31]: Mention needle gauge and drug volume.

Commented [A32R31]: 27-gauge needle was used to inject 100 ul of treosulfan dose in saline, intraperitoneally.

Commented [A33]: Mention needle gauge and volume of blood drawn.

Commented [A34R33]: Lancet was used to draw 2-3 drops of blood equivalent to 100 ul of blood by pricking submandibular vein.

Commented [A35]: Add the necessary kit and reagents to the table of materials.

Commented [A36R35]: We obtained ALT results by sending serum samples to Physicians Laboratory Services, Inc. at Omaha.

Commented [A37]: I'm not sure why this is here, should it not be placed after the surgery steps?

Commented [A38R37]: Moved to section 3.6.8

Commented [A39]: How? Using clippers?

Commented [A40]: What is the incision site?

Commented [A41R40]: At the left of peritoneal wall

Commented [A42]: Incorrect units. Is it L/min?

Commented [A43R42]: corrected to L/min

Commented [A44]: How many at one time?

Commented [A45R44]: One mouse at a time.

3.5.3. Attach the one end of sterile extension tube (holding capacity 550 µL of suspension; see the table of materials for specification) to the 30G needle and the other end to a 1 mL syringe.

3.5.4. Fill the syringe with the suspension (80 µL/mouse) of pooled HEP and HSPC (see subsection 2.5) and fit the syringe in the notch of repetitive dispensing pipette and adjust the dispenser to dispense 10 µL in each press.

~~Fill the extension tube with the suspension of pooled HEP and HSPC by using repetitive dispensing pipette 1 mL. Connect one end of the tube to a 30 G needle and another end to the dispensing pipette.~~

3.5.5. Once the mice are anesthetized (usually 3-4 min), switch isoflurane flow to the nose cone and reduce isoflurane flow rate to 1-3%.

3.6. **Intrasplenic transplantation of human HSPC and hepatocytes in mice**

3.6.1. Perform all surgery steps in laminar flow cabinet under sterile conditions.

3.6.2. Place a clean sterile drape over the working surface and scrub the left side of the body of each mouse with 70% ethanol followed by 10% povidone iodine before making an incision.
~~3.6.2.~~3.6.3.

~~Swab the left side of the body of each mouse with povidone iodine before making an incision and swipe with 70% ethanol three times.~~

~~3.6.3.~~3.6.4. Make a small incision (~1-1.5 cm in length and 5 mm deep) on skin, muscle and peritoneum at the left of the peritoneal wall with Vannas-type scissors to enter the peritoneal cavity approximately 5 mm below the lower edge of the rib cage.

~~3.6.4.~~3.6.5. Locate the spleen and pull it slightly with forceps to the operating area for easy access and insert the 30G needle into the lower pole of the spleen.

~~3.6.5.~~3.6.6. Unlock the plunger of dispensing pipette and dispense 10 µL of the volume at a time and limit to 60-80 µL in spleen.

~~3.6.6.~~3.6.7. Retract the needle slowly and clip the spleen with ligating clips using a ligation applier.

~~3.6.7.~~3.6.8. Push the spleen back into the body cavity with cotton-tipped applicators wetted with sterile PBS.

~~3.6.8.~~3.6.9. Close the peritoneum and skin with 6-0 synthetic absorbable sutures.

Commented [A46]: I have highlighted this to include it for clarity and continuity

Commented [A47R46]: New text is added and modified for the clarity and continuity.

Commented [A48]: What are the specifications of the tube? What is the diameter? How long is it?

Commented [A49R48]: Extension set Microbore Slide Clamp(s) Fixed Male Luer Lock. L: 60 in L: 152 cm PV: 0.55 mL Fluid Path Sterile

Commented [A50]: Isoflurane rate?

Commented [A51R50]: Yes, isoflurane flow rate.

Commented [A52]: Please add a step to mention sterile draping.

Commented [A53R52]: All surgical steps were performed in the laminar flow cabinet under sterile conditions and sterile drapes were used over the working surface (section 3.6.2).

Commented [A54]: %?

Commented [A55R54]: 10% (mentioned in step 3.6.2)

Commented [A56]: How deep? Is this a skin incision or do you incise the musculature as well?

Commented [A57R56]: Skin, muscle and peritoneum incision (5 mm deep) was made with surgical vannas-type scissors.

Commented [A58]: On the left side of the body?

Commented [A59R58]: Yes, the incision has to be on left side.

Commented [A60]: Which needle? The 30 G needle from 3.5.3?

Commented [A61R60]: It is 30G needle.

Commented [A62]: Sterile PBS?

Commented [A63R62]: PBS should be sterile.

~~3.6.9.~~3.6.10. Use warm water circulating pads to protect mice from hypothermia after surgery.

3.7. **Post-operative care**

3.7.1. When the transplanted animal wakens, inject analgesic buprenorphine (0.1 mg/kg), intraperitoneally twice a day for consecutive three days.

3.7.2. Observe animals at least once a day until they return to normal physical condition.

NOTE: Check each animal's body weight, since some mice may lose weight post-surgery. Mice typically regain original weight in one to two weeks.

4. Engraftment validation of human liver by ELISA and human immune system by flow cytometry

4.1. Collect blood samples from the submandibular vein using lancets in EDTA tubes and centrifuge at 1500 x g for 10 min at 4 °C. Isolate serum to check human albumin levels by ELISA to assess engraftment efficiency of mouse liver for transplanted human hepatocytes using human albumin ELISA quantitation set (see table of materials) and follow the manufacturer's instructions.
NOTE: Do not discard the pellet and use the pelleted cells for flow cytometry analysis to evaluate human immune system reconstitution.

NOTE: Evaluate reconstitution of human liver and immune system monthly, starting 1-month post-transplantation by enzyme-linked immunosorbent assay (ELISA) and flow cytometry, respectively.

4.2. Re-suspend the cell pellet without serum in 35 µL of FACS buffer (PBS + 2% FBS) and stained with 5 µL of mouse-specific CD45 (concentration 0.5 mg /mL) and 5 µL each of human-specific antibodies CD45 (0.1 mg / mL), CD3 (0.2 mg/mL), CD8 (0.1 mg / mL), and CD19 (0.5 mg / mL) and 20 µL of CD4 (0.25 mg / mL) and CD14 (0.25 mg / mL) each for 30 min at 4 °C to check the development of functional immune system from $CD34^+$ HSPC.

NOTE: Consider adding one additional tube of unstained cells to determine gating of stained cells.

4.3. After incubation transfer the stained suspension (~100 µL) in polystyrene round-bottom flow cytometry tube and use 2 mL of 1x lysis buffer (see table of materials) by diluting 1 part of 10x lysis buffer to 9 parts of distilled water and incubate 10-15 min to lyse red blood cells.
NOTE: Observe turbidity to evaluate red blood cell lysis. Once the sample becomes clear, lysis is complete.

4.4. After lysis, add 3 mL of FACS buffer in the tube and centrifuge at 300 x g at 4 °C for 5 min to get pellet. Repeat the washing by adding 3 mL of FACS buffer in pellet and centrifuge at 300 x g at 4 °C for 5 min.

Commented [A64]: I'm not sure why this is here, should it not be placed after the surgery steps?

Commented [A65R64]: I have moved that to after the surgery steps (sub section 3.6.10).

Commented [A66]: IP?

Commented [A67R66]: Analgesic was given i.p.

Commented [A68]: Mention antibodies used and their concentrations. Is a blocking agent used? Please add all materials to the table of materials.

Commented [A69R68]: I used the ELISA quantitation set (included in table of materials) according to manufacturer's instructions to measure human albumin levels in serum.

Commented [A70]: Unclear what exactly is done here. Please describe the assays.

Commented [A71R70]: Assays are described continued from section 4.1. I have moved this to the note of section 4.1 for the clarity.

Commented [A72]: What volume?

Commented [A73R72]: 35 µL of FACS buffer was used to make final volume of 100 µL with antibodies cocktail.

Commented [A74]: What is the concentration?

Commented [A75R74]: The antibodies concentrations were as follows:
mouse-specific CD45 (concentration 0.5 mg /mL)
Human CD45 (0.1 mg / mL),
Human CD3 (0.2 mg/mL),
Human CD8 (0.1 mg / mL),
Human CD19 (0.5 mg / mL),
Human CD4 (0.25 mg / mL and,
Human CD14 (0.25 mg / mL)

Commented [A76]: What are their concentrations?

Commented [A77R76]: 10 x lysis buffer from supplier

Commented [A78]: How much buffer? Do you centrifuge? Steps are not described sufficiently.

Commented [A79R78]: FACS buffer (3 mL) was used to wash and followed by one more washing step.

4.5. Fix the cells in freshly made 1% paraformaldehyde (PFA) and acquire stained cells on flow cytometer and analyzed with flow software.

4.6. For the analysis, select lymphocytes gating on a forward scatter (FSC) / side scatter (SSC) plot, followed by single cells gating on FSC-area / FSC-height.

4.7. Further gate for human-specific CD45 (hCD45) on single cell population and include mouse-specific CD45 (mCD45) for exclusion of cells of murine origin. Strategize gating of stained population based on the gating of unstained cells.

4.8. Gate $hCD45^+$ cells to determine $CD3^+$ T cells and $CD19^+$ B cells frequency. Gate T cells to determine CD4 and CD8 subsets. To evaluate monocytes, gate on hCD45 to determine $CD14^+$ monocytes.

5. HIV infection of TK-NOG mice and effect on human liver and immune system

5.1. Handle HIV-1 virus and all infected mice in a designated BSL 3 facility.

CAUTION: Autoclave and discard all HIV-infected wastes in double biohazardous bags.

5.2. Wear personal protection equipment (PPE) including disposable coverall gown, shoe cover, face mask and double gloves at all times while working with the virus.

5.3. Screen mice with reconstitution of more than 15% of human $CD45^+$ cells (tested in subsection 4.7) and presence of human albumin in the serum for HIV-1 infection (tested in subsection 4.1).

5.4. Inject mice with 1 x 10^3 – 1 x 10^4 tissue culture infectious doses 50 ($TCID_{50}$) HIV-1_{ADA} in a volume of 100-200 µL per mouse, intraperitoneally.

5.5. Euthanize HIV-infected mice 5 weeks HIV post-infection by using isoflurane (isoflurane flow rate > 5%).

NOTE: For safety reasons, wear cut-resistant gloves while handing HIV-infected mice.

5.6. After euthanizing the mice, collect blood by cardiac puncture in mini collect EDTA tubes for isolation of serum to see the effect of HIV-1 on the liver by evaluating human-specific albumin levels by ELISA (see subsection 4.1) and blood cells to check changes in human immune cells using flow cytometry (see subsections 4.2-4.8).

NOTE: Assess peripheral viral load 5 weeks post-infection on a bioanalyzer to confirm if mice are infected.

5.7. After drawing blood, excise liver from the euthanized mice.

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Commented [A81R80]: Human CD45 positive cells was evaluated by flow cytometry as mentioned in revised subsection 4.7.

Commented [A82]: Mention euthanasia method. We don't film anesthesia so I have unhighlighted this.

Commented [A83R82]: Mice were euthanized using isoflurane

Commented [A84]: How is it collected after euthanasia? Heart puncture?

Commented [A85R84]: Blood

Commented [A86]: Mention antibodies (and concentrations) and blocking agents used.

Commented [A87R86]: Please see subsection 4.1.

Commented [A88]: Please describe how the flow cytometry is performed. If you have described this in earlier steps, you can reference them here.

Commented [A89R88]: Referenced the subsections.

Commented [A90]: Exactly what is done? This step is missing several details of what is to be done.

Commented [A91R90]: For viral load we sent 20 µL of the serum in our hospital (University of Nebraska Medical Center) clinic. The step is unhighlighted and included as a note.

5.8. For the liver excision, expose the abdominal cavity by making an incision of 1.5-2 cm and 0.5 cm deep on skin, muscles and peritoneum from the xyphoid. Make a cut perpendicular to the spine between the liver and the diaphragm. Lift the liver and sever any membrane attaching it to stomach and intestine.

5.9. Collect and fix the liver in 4% paraformaldehyde overnight and follow standard immunohistochemical protocol to evaluate effect of HIV on CK18^{+} hepatocytes by using human-specific CK18 antibody[8].

REPRESENTATIVE RESULTS:

The establishment of a dual humanized mouse model with human liver and immune cells can be easily monitored at each step with very simple ELISA and flow cytometry, respectively. Flow cytometry is regularly performed to evaluate the development of functional immune system and to see the effect of HIV infection on immune cells. In dual humanized mice, development of functional immune cells can range from 15% to 90% of the lymphocyte gate. Representative subsets of immune cells are shown in dot plots (**Figure 3**). For evaluation of the engraftment of human hepatocytes, ELISA for human-specific albumin level is performed monthly from mouse serum. Mice engrafted with both HSPC and HEP show human-specific albumin levels ranging from ~7 µg/mL to 377 µg/mL at one month, continuing to grow over the time of observation (6 months) (**Figure 4**). The effect of HIV-infection on human immune cells in blood of dual-humanized mice is monitored by flow cytometry and on HEP in liver by human-specific albumin ELISA. By 5 weeks, HIV-1 causes a decrease in human albumin levels in serum, as assessed by ELISA, and depletion of human CK18^{+} hepatocytes in the liver sections of dual humanized mice, as evaluated by immunohistochemistry (**Figure 5**). A lower ratio of CD4:CD8 by flow cytometry is typically observed in the blood and liver of HIV-infected mice compared to levels before infection noted in the same mouse (**Figure 6**). All reagents and materials important for the protocol are discussed in the **Table of Materials**.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic showing the enrichment of CD34^{+} cells from cord blood. (**A**) Cord blood is layered on Lymphocytes Separation Medium (LSM) and centrifuged to isolate buffy coat. (**B**) LS columns are placed on magnetic stand and rinsed with BSA buffer, followed by adding of buffy coat. Cells positive for CD34 are trapped in columns and CD34^{-} cells are eluted in separate tube. Trapped CD34^{+} cells in column resins are plunged with a plunger and cells are collected in a new tube.

Figure 2. Schematic view of experimental design for dual reconstitution of humanized liver and immune system mice, followed by HIV-1 infection. TK-NOG mice are injected with ganciclovir (GCV) at the dose of 6mg/kg twice a day on day -7 and day -5 followed by treosulfan injection on day -3, -2 and -1. To screen mice for the transplantation (Tx) alanine aminotransferase (ALT) assay is performed one day before the surgery and mice with ALT levels >200 and <600 U/L are selected. After transplantation, mice are checked for reconstitution of human immune system by

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flow cytometry (FACS) and liver reconstitution by assessing albumin level using ELISA. Mice are infected with HIV-1 for 5 weeks before sacrificing.

Figure 3: Flow cytometry analysis gating strategy for human cells distribution of blood. (**A**) First, Lymphocytes are gated on whole blood based on FSC-A and SSC-A. (**B**) Single cells are gated on lymphocytes. (**C**) Human $CD45^+$ leukocytes were gated on single cells using mouse CD45 and human CD45. (**D**) $CD3^+$ T cells and $CD19^+$ B cells are identified on gated $CD45^+$ human leukocytes. (**E**) $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells are identified in gated $CD3^+$ T cells. (**F**) $CD14^+$ monocytes are gated from human $CD45^+$ leukocytes. Results represented here is from one mouse transplanted with dual human hepatocytes and HSPC.

Figure 4: Albumin concentration is measured by ELISA in the serum of dual humanized mice transplanted with both human hepatocytes (HEP) and $CD34^+$ hematopoietic stem/progenitor cells (HSPC) (*n* = 11). Serum is collected at different time at 1, 4 and 6 months post-transplantation and dilutions are made to adjust the unknown samples concentrations in the range of standards. Each symbol represents an individual mouse value. Results represent the median as well as individual values. *P<0.05, by one-way ANOVA. This figure has been modified from Dagur *et al.*[8].

Figure 5: Effect on HIV-1 on albumin levels in serum and depletion of $CK18^+$ human hepatocytes in the liver of dual humanized mice. (**A**) Albumin concentrations is monitored in uninfected mice (*n* = 9) transplanted with both human HEP and HSPC at 1 and 4 months. The mice are infected (*n* = 10) with HIV at 4-5 months post-transplantation and sacrificed 5 weeks post infection. Each symbol represents an individual mouse value. Results represent the median as well as individual values. *P<0.05, by one-way ANOVA. This figure has been modified from Dagur *et al.* [8]. (**B**) Five-micron liver sections from uninfected (HEP+HSPC, left panel) and HIV-infected TK-NOG mice (HEP+HSPC+HIV, right panel) are fixed, paraffin embedded, and stained for anti-human cytokeratin-18 (CK18) antibody. HIV-1 caused depletion of $CK18^+$ hepatocytes are evidenced by less occupied area by the $CK18^+$ human cells. Results represented here is from one uninfected and one HIV-infected mouse transplanted with dual human hepatocytes and HSPC. Scale bars: 100µm.

Figure 6: Ratio of $CD4^+$ cells to $CD8^+$ T cells in peripheral blood, and liver of dual reconstituted uninfected (closed circle, HEP+HSPC, Blood *n* = 7; liver *n* = 6) and HIV-1 infected (open circles, HEP+HSPC+HIV; Blood *n* = 10; Liver n = 11) mice. Results represent the median as well as individual values. *P<0.05, by one-way ANOVA test between HIV-infected and uninfected mice. This figure has been modified from Dagur *et al.* [8].

DISCUSSION:

Liver is compromised and damaged in HIV-infected patients [24]. Experimental small animal models for studying human liver diseases in the presence of HIV-1 is extremely limited, despite the availability of a few co-transplanted animal models with $CD34^+$ HSPCs and hepatocytes [7,12,25]. In *in vitro* experiments, hepatocytes are shown to have low-level of HIV-1 infection [26]. Humanized mice that carry both types of human cells are a desirable model. The liver of mice reconstituted

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with only human immune system has been shown to be affected by HIV-infection under experimental depletion of human regulatory T cells[20,27]. However, the difference in immune and functional properties of mouse and human hepatocytes may underline the differences in their responses to HIV-1 and immune cells. In this review, a protocol is described to reconstitute both human immune system and liver and to address HIV-1-associated liver immunopathology, as observed in human immunodeficiency virus (HIV-1)-infected patients. TK-NOG male mice were selected due to their liver-selective high mRNA expression of HSVtk transgene and susceptibility of GCV toxicity to mouse transgenic liver [21]. Moreover, they can be maintained for long periods after transplantation without the use of exogenous drugs and do not develop spontaneous systemic disease [28]. To establish human immune system and liver reconstitution, ablation of mouse immune system and damage of mouse-specific liver cells are required and achieved using non-myeloablative doses of treosulfan and GCV, as shown previously in TK-NOG male mice [13,23]. Mice are injected with GCV and treosulfan at the age of 6-8 weeks, as the expression of transgene and GCV-induced hepatic injury as assessed by ALT levels are optimal then for providing niche-to-transplanted human cells [21]. Mice showing ALT levels >200 U/L, but less than 600 U/L are usually selected for transplantation. Mice showing ALT levels greater than 600 U/L are at greater risk of death as human hepatocytes not able to rescue damaged mouse liver function.

Currently, dual humanization is shown by transplantation of human $CD34^+$ HSPCs, and fetal liver cells; however, the manipulation of newborn animals creates technical problems [13,14]. HSPC can be derived or isolated from multiple sources such as fetal liver cells (FLC), embryonic stem cells (ESC) and the CB. However, ethical issues constrain the use of ESCs and FLC. The CB has no such restriction and is a most useful alternative to obtain HSPC, as well as being a precious source of primitive hematopoietic stem and progenitor cells that can reconstitute the functional immune system. Cord blood should not be used older than one day to isolate HSPCs, as yield of HSPCs is highly affected. The purity of isolated HSPC needs to be checked before cryopreserving the cells. Cross-contamination of $CD3^+$ T cells is avoided, as it may lead to systemic mouse graft-*versus*-host disease and acute allo-rejection of HEP while transplanting with mismatched cells.

Commercially available hepatocytes were used as a source for liver reconstitution[8,13]. Adult hepatocytes are preferred for establishing liver reconstitution due to their increased efficiency in engraftment and sustainability for a long period of time [29].

The presence of human immune system in mouse model increased albumin levels, as shown previously [30,31]. However, the efficiency of hepatocytes and immune system reconstitution may vary with different source of donor cells and also depend on recipient mouse. So, each mouse needs to be assessed for engraftment and the most critical part is to utilize the antibodies or reagent that are human-specific and do not cross-react with mouse cells. The human- specific reagents and antibodies used in our study are detailed in the table. If antibodies other than provided in the table are used for the study, must be check for the human specificity.

The optimal condition would be the transplantation of syngeneic cells; however, this is technically difficult to achieve. Wherever possible, HSPC and hepatocytes should be pooled from donors with partially matched HLA class 1 antigens (like HLA-A2).

To screen of mice for HIV study, blood is drawn at multiple time points to determine the optimal immune and liver reconstitution, flowcytometry and ELISA are preferred as could be performed with little amount of blood. Blood cells and serum from the same sample could be used for flowcytometry and ELISA, respectively. It is important to make proper dilutions of serum at each time point (1000 - 40,000 range) to evaluate albumin levels, so that the unknown concentrations can be brought to within the range of standard concentrations (kit range 6.25 – 400 ng/mL).

Pro-inflammatory cytokines in response to HIV-1 infection in the presence of human immune system can also be useful in addressing the interaction of hepatocytes and immune cells. The model is useful to show the immunopathogenesis of HIV-1-induced liver disease, given that it recapitulates liver damage in the same manner as in humans, evidenced by a low ratio of CD4:CD8, decrease in albumin levels, human hepatocyte death and liver immune activation. The model also has some limitations such as low level of cytotoxic T cells activity and impaired immunoglobulin class switching. Due to the presence of both human immune system and liver, the model presented here is promising for co-infections studies of HIV-1 and hepatitis viruses, chronic hepatitis infection, to clarify the mechanisms of the anti-hepatitis immune response, and as a cirrhosis model.

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DISCLOSURES:

No competing interests declared.

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