**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-*Prkdcscid Il2rgtm1Sug* 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 patients1,2. Coinfections of hepatitis viruses with HIV-1 infection are more common, accounting for 10-30% of HIV-infected persons in the United States3-5.

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 studies6-8. 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 mice6,9-11. 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 disease12-14. Several models for HSPC and human hepatocyte (HEP) transplantation are based on strains, known as NOG (NOD.Cg-*Prkdcscid Il2rgtm1Sug*/JicTac)8,13, NSG (NOD.Cg-*Prkdcscid Il2rgtm1Wjl/SzJ*)15, Balb/C-Rag2-/- γc-/- (Rag2tm1.1Flv Il2rgtm1.1Flv/J)12, and fah-/- NOD rag1-/-il2rγnull mouse16. 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 model12. 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 abnormalities13.

Although humanized mice have been used for HIV research for many years17-19, the use of humanized mice to study liver damage caused by HIV-1 has been limited20. The authors previously reported the establishment of a dual humanized TK-NOG mouse model and its application in HIV-associated liver disease8. 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 drugs21. Mice are also pre-conditioned with non-myeloablative doses of treosulfan to create a niche in the mouse bone marrow for human cells8. 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. Perform all steps of the protocols under sterile conditions in laminar flow cabinets.
   2. Collect human CB in heparinized tubes and store CB samples at room temperature before processing.
   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. Remove the top LSM and plasma layer carefully and transfer the white buffy coat interface into a new tube using a transfer pipette.
  2. 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. 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.
  2. Add 100 µL of human FcR blocking reagent and 100 µL monoclonal mouse anti-human CD34 antibody-conjugated microbeads for up to 1 x 108 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 cell number if more than 1 x 108 cells are present.

* 1. Carefully remove the supernatant, resuspend the pellet in 500 µL of buffer and proceed with the magnetic separation step to enrich HSPC.
  2. 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.
  3. 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.
  4. Wash the column with buffer 3x and collect the elute in the same collection tube of CD34- fraction of cells.
  5. Plunge the column with 5 mL of buffer to elute CD34+ cells into a new collection tube. Repeat the procedure to achieve purity >90%.
  6. 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.
  7. 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. 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.~~

1. **~~Thawing~~ Preparation of human hepatocytes for transplantation**
   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. Remove vial cap and pour thawed hepatocytes into the 50 mL conical tube of wamed thawing medium.
   3. Suspend the cells by rocking the 50 mL tube by hand for a few seconds.

NOTE: Do not vortex the tube.

* 1. 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~~

* 1. 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.

1. **~~Animal Surgery~~ Animal handling, screening, genotyping and treatment for human HSPC and hepatocytes transplantation**
   1. **Animal handling** 
      1. As a result of severe immunodeficiency, breed, house and handle TK-NOG mice under aseptic condition.
      2. Always wear lab coats, gloves, shoe covers, and face mask to prevent infection with potential pathogenic microorganisms.
      3. Use sterile gloves and instruments for surgery and handle animals aseptically throughout surgery.
   2. **Selecting TK-NOG mice for the experiment**
      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.

* + 1. Select males at 6-8 weeks of age for transplantation due to their high sensitivity to GCV-mediated depletion of HSV-TK transgene expressing hepatocytes21.
    2. Ear-tag mice at the time of weaning or surgery to ease identification. Note down the weight and health status of animals.
  1. **Genotyping for the presence of HSV-TK transgene using quantitative real-time PCR**
     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.
     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 instrument22.
     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.

* 1. **Treatment using ganciclovir and treosulfan**
     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.
     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. 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.
     4. ~~Use warm water circulating pads to protect mice from hypothermia after surgery.~~
  2. **Preparation for the surgery** 
     1. Using clippers to shave mouse fur surrounding the incision site at the left of peritoneal wall) before surgery.
     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.
     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.
     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.~~

* + 1. Once the mice are anesthetized (usually 3-4 min), switch isoflurane flow to the nose cone and reduce isoflurane flow rate to 1-3%.
  1. **Intrasplenic transplantation of human HSPC and hepatocytes in mice**
     1. Perform all surgery steps in laminar flow cabinet under sterile conditions.
     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.

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

* + 1. 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.
    2. 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. Unlock the plunger of dispensing pipette and dispense 10 µL of the volume at a time and limit to 60-80 µL in spleen.
    4. Retract the needle slowly and clip the spleen with ligating clips using a ligation applier.
    5. Push the spleen back into the body cavity with cotton-tipped applicators wetted with sterile PBS.
    6. Close the peritoneum and skin with 6-0 synthetic absorbable sutures.
    7. Use warm water circulating pads to protect mice from hypothermia after surgery.
  1. **Post-operative care**
     1. When the transplanted animal wakens, inject analgesic buprenorphine (0.1 mg/kg), intraperitoneally twice a day for consecutive three days.
     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.

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

* 1. 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.

* 1. 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.

* 1. After lysis, add 3 mL of FACS buffer in the tube and centrifuge at 300 x g at 4 oC 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 oC for 5 min.
  2. Fix the cells in freshly made 1% paraformaldehyde (PFA) and acquire stained cells on flow cytometer and analyzed with flow software.
  3. 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. 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.
  5. 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.

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

* 1. Wear personal protection equipment (PPE) including disposable coverall gown, shoe cover, face mask and double gloves at all times while working with the virus.
  2. 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).
  3. Inject mice with 1 x 103 – 1 x 104 tissue culture infectious doses 50 (TCID50) HIV-1ADA in a volume of 100-200 µL per mouse, intraperitoneally.
  4. 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.

* 1. 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.

* 1. After drawing blood, excise liver from the euthanized mice.
  2. 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.
  3. 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 antibody8.

**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 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 with only human immune system has been shown to be affected by HIV-infection under experimental depletion of human regulatory T cells20,27. 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 reconstitution8,13. 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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No competing interests declared.

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