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A Chimeric Mouse Model to Study Immunopathogenesis of HCV Infection

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Abstract

Several human hepatotropic pathogens including chronic hepatitis C virus (HCV) have narrow species restriction, thus hindering research and therapeutics development against these pathogens. Developing a rodent model that accurately recapitulates hepatotropic pathogens infection, human immune response, chronic hepatitis, and associated immunopathogenesis is essential for research and therapeutics development. Here, we describe the recently developed AFC8 humanized liver- and immune system-mouse model for studying chronic hepatitis C virus and associated human immune response, chronic hepatitis, and liver fibrosis.

Keywords

Humanized mice; Chronic hepatitis C virus; Liver immunopathogenesis

1 Introduction

Several human pathogens with significant global health impact, including hepatitis C virus (HCV), hepatitis B virus (HBV), malaria and human immunodeficiency virus (HIV) have narrow host species restriction to humans and other higher primates. Recent advancements in stem cell biology and regenerative medicine have enabled the development of several human–murine chimeric animal models containing lymphoid cells or hepatocytes to overcome host species barriers to studying these pathogens in rodents [1–3]. The Alb-uPA transgenic mouse on an immunodeficient background contains the mouse urokinase-type plasminogen activator (uPA) gene under the control of an Alb enhancer/promoter, which allows for high levels of human adult hepatocyte repopulation. However, these human–murine chimeric liver mouse models lack human immune cells, making it impossible to study chronic hepatitis virus-induced chronic liver inflammation and associated fibrosis [1, 4]. Furthermore, homozygous Alb-uPA transgenic animals are unhealthy and die in the absence of hepatocyte transplantation due to hypofibrinogenemia and associated liver damage [1, 5, 6]. Alb-uPA transgenic immunodeficient mice transplanted with human adult hepatocytes can be infected with HCV HBV, resulting in high levels of virus replication [1]. Additionally, another recently developed human–murine chimeric liver mouse model, the

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Competing Financial Interests

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fumarylacetoacetate hydrolase (Fah)-Balb/c-Rag2^{-/-} gamma c^{-/-} mouse allows human adult hepatocytes engraftment and repopulation following liver damage and HCV replication following infection [4, 7, 8].

The development of small animal models that facilitate the elucidation of the mechanisms, by which chronic hepatitis viruses impair human host immune response and promote immunopathology, is a major barrier in biomedical research. To overcome this barrier, we recently developed a humanized mouse model with both human immune system and liver cells. The humanized liver-and immune system-mouse model was developed by transplanting human progenitor liver cells (HPC) and CD34⁺ hematopoietic stem cells (HSC) into immunodeficient AFC8 mice and inducing Caspase 8-mediated murine-specific hepatocytes apoptosis to promote human hepatocytes regeneration. Caspase 8-mediated murine-specific apoptosis was induced using the AFC8 transgene [9] (Fig. 1). The AFC8 model's construct is a ligand (FKBP) inducible Caspase 8 transgene, under transcriptional regulation by the albumin enhancer/promoter, thus enabling murine liver- specific damage [9] (Fig. 1). The AFC8 construct was used to create the AFC8 mice model in the Balb/c-Rag2^{-/-} gamma c^{-/-} mouse [9]. The Balb/c-Rag2^{-/-} gamma c^{-/-} mouse is a robust immunodeficient mouse model, devoid of functional T, B, and NK cells, and supports development of a functional human immune system following intra-hepatic injection of CD34⁺ human hematopoietic stem cells [2, 3, 10]. To construct the humanized liver- and immune system-mouse model we co-transplanted human liver progenitor cells (HPC) and CD34⁺ human hematopoietic stem cells (HSC) into AFC8 transgenic mice and treated them with the FKBP dimerizer (AP20187) to induce human hepatocytes repopulation (Fig. 2). The AFC8-DKO mouse model allows the development of both human liver and immune cells, thereby generating the HSC/Hep humanized mouse model (Fig. 2) [9]. The HSC/Hep mouse model supported HCV infection and generated a human T cell response to HCV. Additionally, HCV infection induced chronic liver inflammation and fibrosis, which correlated with activation of human hepatic stellate cells and expression of human fibrogenic genes [9] (Fig. 3). Furthermore, unpublished results suggest that the HSC/Hep mouse model supports HBV infection and associated immunopathogenesis.

2 Materials

All experiments using live rodents and human tissues must conform to appropriate National and Institutional Guidelines; appropriate biosafety measure commensurate with the infectious pathogen under investigation should be employed.

2.1 Progenitor Liver Cells and Hematopoietic Stem Cells

1. 15–18 weeks gestation period-human fetal liver obtained from Advanced Bioscience Resources, CA (*see Note 1*).
2. Liver Digestion Medium (Gibco).
3. Ficoll.

¹Human tissues should be handled using BSL2-recommended protocols and should be collected and used in accordance with all institutional and governmental ethics guidelines.

4. Human CD34 Indirect MicroBead Kit.
5. Manual or automated cell separators.
6. Running Buffer for cell separator.
7. Rinsing Solution for cell separator.
8. Cell Wash Buffer: make fresh using complete Iscove's Modified Dulbecco's Medium (IMDM) with 2.5 μ L DNase at 5 mg/mL added per 50 mL.
9. Antibody mix for CD34+ HSC yield and purity: Add 2 μ L anti-human CD34 FITC, 2 μ L anti-human CD45 PE, and 0.5 μ L 7-AAD in 50 μ L 2 % Fetal Bovine Serum (FBS)/PBS per sample prior to staining.
10. Antibody mix for human immune repopulation: Add 2 μ L anti-human CD45 FITC, 2 μ L anti-mouse CD45 PE, and 0.5 μ L 7-AAD in 50 μ L 2 % FBS/PBS per sample prior to staining.
11. Media: Make fresh using IMDM supplemented with 10 % of FBS, Penicillin–Streptomycin (Pen-Strep), l-glutamine.
12. Trypan Blue.
13. Hemocytometer.
14. Multicolor Flow Cytometer.

2.2 Transplantation of Progenitor Liver and Hematopoietic Stem Cells

1. 2–5-day-old newborn AFC8 mice (Balb/c-Rag2^{−/−} gamma c^{−/−} mice, expressing active Caspase 8 fused with FK506 binding domain under control of the albumin promoter).
2. X-ray irradiators.
3. Microscope lamps.
4. 0.5 cc insulin syringes with 27-gauge needles.
5. Sulfamethoxazole–trimethoprim.
6. ACK lysis buffer.
7. AP20187 (Induces FKBP/Caspase 8 dimerization and mouse hepatocyte damage and human liver cells repopulation) (Ariad Pharmaceuticals).
8. Avertin.

3 Methods

3.1 Liver Digestion

1. Warm up wash buffer in 37 °C water bath and add 2.5 μ L DNase for every 50 mL of liver digestion medium to create the liver digestion solution; make 100 mL per liver to be digested. Pour entire fetal liver into a 10 cm dish and mince parenchymal tissue (about 1–2 mm³) with scalpels; discard the connective tissue.

Alternatively, the tissue can be dissociated using automated tissue dissociators and following manufacturer's recommended protocol.

2. Transfer the minced tissue into a 50 mL tube and wash the dish with 10 mL liver digestion solution; add wash to the same tube. Fill the tube with liver digestion solution to 40 mL total volume and wrap the tube cap with Parafilm (*see Note 2*).
3. Shake the tube for 5–10 s and place in a 37 °C water bath for 30 min. Shake the tube every 5 min for the duration of the incubation step. The larger tissue pieces will settle to the bottom; remove the liquid portion and filter through a 70 µm filter into a new 50 mL tube and store on ice. Add 35 mL of liver digestion solution to the tube containing larger tissue pieces and repeat digest; filter into a new 50 mL tube.
4. Spin the two tubes with liver cells at $252 \times g$ for 10 min at 4 °C and decant the supernatant. Resuspend the cells with 40 mL wash buffer and combine to one 50 mL tube.

3.2 Liver Progenitor Cells (HPC) Purification

1. Spin the suspension down at $18 \times g$ at 4 °C for 5 min and remove the supernatant. Transfer to a new 50 mL tube and store on ice; this is for CD34+ HSC separation.
2. Resuspend the pellet with 14 mL wash buffer and transfer to a 15 mL conical tube. Spin the suspension down at $18 \times g$ for 5 min at 4 °C and transfer the supernatant to a new tube on ice; this is for CD34+ HSC separation.
3. Resuspend the pellet with 14 mL wash buffer and repeat wash procedure including supernatant collection for CD34+ HSC separation. The pellet should appear white or yellow (not red) in color (*see Note 3*). The light colored pellet contains liver progenitor cells. Resuspend the pellet containing liver progenitor cells with 10 mL wash buffer and transfer into a 15 mL tube. Store tube containing liver progenitor cells on ice until the transplantation step; limit storage to 12 h. Count cells using a hemocytometer after trypan blue staining.

3.3 CD34+ Cell Purification

1. Combine previously collected supernatants “for HSC purification.” Centrifuge the collected supernatants containing CD34+ HSC at $469 \times g$ for 5 min at 4 °C; combine pellets and resuspend in 35 mL of wash buffer.
2. Fill a 15 mL pipette with 14 mL of Ficoll and eject only 11 mL very slowly at constant flow at the bottom of the tube under the cell suspension without disturbing the interface (*see Note 4*). Carefully spin the tube at $1,880 \times g$ for 30 min at room temperature (24 °C). Remove the cells at the interface without disturbing the Ficoll. Transfer the collected cells into a 50 mL tube and add 40 mL of wash buffer.

²Parafilm seal prevents contamination of the sample.

³If the pellet is red, repeat washing step until the pellet appears yellowish or white.

⁴Use extreme caution when ejecting the Ficoll so as not to disturb the interface or create bubbles.

3. Centrifuge the suspension at $469 \times g$ at 4°C for 5 min and discard the supernatant; resuspend the pellet in 10 mL wash buffer. Centrifuge the suspension at $469 \times g$ at 4°C for 5 min and discard the supernatant without disturbing the pellet.
4. Resuspend the cells in 400 μL of wash buffer for every 1×10^8 cells counted using a hemocytometer and trypan blue. Add 100 μL of the FcR Blocking reagent (Human CD34 Indirect MicroBead Kit) for every 1×10^8 cells and mix well. Add 100 μL of the CD34-Hapten-Antibody reagent (Human CD34 Indirect MicroBead Kit) for every 1×10^8 cells and mix well. Incubate cells on ice for 30 min. Add 40 mL of wash buffer to the cells. Centrifuge the cell suspension at $469 \times g$ for 5 min at 4°C ; discard the supernatant. Resuspend the cells in 400 μL of wash buffer for every 1×10^8 cells. Add 100 μL of the Anti- Hapten MicroBeads reagent (Human CD34 Indirect MicroBead Kit) for every 1×10^8 cells; mix well and incubate on ice for 30 min.
5. Resuspend the cells with 40 mL of wash buffer and spin down the cell suspension at $469 \times g$ for 5 min at 4°C and remove the supernatant. Resuspend the pellet in 3 mL of wash buffer and transfer to a 15 mL tube. Repeat wash-resuspension procedures and transfer the residual cells into the same 15 mL tube and put the tube on ice.
6. Sort the cell suspension using an autoMACS[®] cell separator machine and combine positive fractions (CD34+ HSC) (if more than one fraction). Centrifuge the negative and positive fractions separately at $469 \times g$ at 4°C for 5 min and resuspend each fraction in 10 mL wash buffer and store on ice. Determine CD34+ cells purity and yield by staining aliquots of positive and negative fraction cells using CD34+, CD45+ antibodies and 7AAD+ (dead) stain and integrated flow cytometry cell counter system; use human CD34+ HSC or a human CD34+ cell line and isotype antibodies as controls.

3.4 Transplantation

1. Centrifuge CD34+ HSC at $469 \times g$ for 5 min at 4°C and resuspend with wash buffer to 1×10^6 cells per mL. Centrifuge the liver progenitor cells (HPC) at $18 \times g$ for 5 min at 4°C and resuspend with wash buffer to 1×10^6 cells per mL. Store cells overnight at 4°C or proceed to transplantation.
2. Prior to transplantation, centrifuge CD34+ HSC at $469 \times g$ and HPC at $18 \times g$ for 5 min at 4°C ; resuspend each cell type with wash buffer to $0.5\text{--}1 \times 10^6$ cells/17.5 μL . Mix approximately $0.5\text{--}1 \times 10^6$ HSC and $0.5\text{--}1 \times 10^6$ liver progenitor cells (35 μL total volume) for each mouse to be injected; keep cells on ice (*see Note 5*).
3. Irradiate 2–5-day-old newborn AFC8 mice in sterilized paper bags with a single dose of 200 rad using Xrad or other irradiators. Prepare an ice bucket wrapped with autoclaved aluminum foil in the hood. Prepare the warming light for mouse recovery from cold-induced anesthesia. Place a single pup on the foil in the ice

⁵The cell suspension must be prepared at approximately 35 μL volume per mouse, as injecting animals above this volume will result in injury to the animals.

bucket and allow the animal to cool for approximately 1 min to induce a mild anesthesia (*see Note 6*).

4. Resuspend the cells immediately prior to injection and load the syringe (0.5 cc insulin syringe with 27-gauge needle) with the cell suspension at a volume of 30 μ L per mouse. Remove the cooled pup, place on foil, and restrain the pup by placing your index finger between its front legs and your thumb between its hind legs, while applying gentle pressure down and outward, just enough to pull the skin tight without injuring the animal. Vertically position the needle slightly to the left of the right lobe and angle slightly towards the right lobe; push the needle in about halfway then deposit the cells into the right lobe at the rate of 2 s per injection volume. The liver should be visible as a dark region located cranially to the stomach; the stomach is the white color organ in the abdominal region. Release the pressure immediately with your fingers and immediately remove the needle; place the transplanted animal in a cage with the mother. Repeat transplantation steps for the remaining animals.
5. House transplanted animals in a pathogen-free facility and microisolator cages. Feed animals with autoclaved or irradiated food and maintain on acidified autoclaved water with or without sulfamethoxazole–trimethoprim (7.8 mL SMZ per 250 mL of drinking water) on alternate weeks for the duration of the animals' life (*see Note 7*).

3.5 Human Liver and Immune System Reconstitution

1. Wean animals at 3 weeks old and house at 5 humanized animals per cage; maintain autoclaved/irradiated food and acidified auto-claved water with or without sulfamethoxazole–trimethoprim (7.8 mL SMZ per 250 mL of drinking water) on alternate weeks for the duration of the animals' life.
2. Administer AP20187 at a dose of 5 μ g/g body weight to AFC8- humanized mice and appropriate control mice via intraperitoneal injection at 4 weeks post transplantation; repeat injections once a week for 4 weeks.
3. At 12 weeks post transplantation, warm up humanized and control mice with a heat-lamp and bleed by making a single 5 mm cut (nick) on a tail vein with a sterile scalpel. Collect 100 μ L of blood in 1.5 mL sterile Eppendorf tubes containing 100 μ L of 20 mM PBS/EDTA and place on ice; samples can be kept on ice for up to 12 h. Centrifuge the collected blood–PBS/EDTA solution at $469 \times g$ for 5 min at 4 °C. Collect the top portion (plasma) for analysis of human liver reconstitution (human albumin ELISA) and the bottom portion (PBMCs) for analysis of human immune reconstitution (FACs analysis).
4. Resuspend the bottom portion (PBMCs) in $1 \times$ ACK lysis buffer, incubate for 5 min. Centrifuge cells at $469 \times g$ for 5 min at 4 °C and resuspend in 2 % FBS/PBS

⁶Take caution when handling pups in order to prevent contact with unsterilized ice/surfaces.

⁷Failure to keep humanized animals under sterile conditions will result in lower human reconstitution and illness due to opportunistic infections.

containing human CD45, mouse CD45 and 7AAD antibody mixture. Examine human immune reconstitution (% human CD45+ cells/Total CD45+ cells) using flow cytometry analysis. To measure human liver reconstitution, perform human albumin ELISA using plasma and human albumin ELISA kit, following the manufacturer's procedures.

3.6 Virus Inoculation

1. Administer a sub-lethal dose of Avertin (225–240 mg/kg) via intraperitoneal injection to humanized and control mice at 12 weeks post transplantation.
2. Inoculate anesthetized humanized or control animals via retro- orbital injection with 75–100 μ L of human clinical isolates of HCV at $1\text{--}5 \times 10^7$ genome copies/mL or control (PBS, uninfected patients sera, heat inactivated inoculum, etc.) [9] (*see Note 8*).

3.7 Analysis of Infection, Immune Response, and Liver Disease

1. For longitudinal analysis of infection, liver disease, and immune response, bleed animals and analyze viral load, immune response, and liver damage/fibrosis at approximately every 4 weeks following initial bleeding at 2 weeks post inoculation.
2. Monitor animals weekly and sacrifice at approximately 16 weeks post inoculation. Sacrifice animals by administering 800 μ L of Avertin to animals. Collect 500 μ L of blood (serum) and 500 μ L blood plus 500 μ L 20 mM EDTA (plasma and PBMCs) via retro-orbital bleeding.
3. Perform cervical dislocation and collect animal tissues (spleen, lymph nodes, liver, etc.) immediately for DNA, RNA and protein analysis. For cell analysis, place tissues in IMDM medium and keep on ice. For histology and immunohistochemistry fix tissue in formalin and process as paraffin embedded slides. Examine human hepatocyte repopulation in humanized or control tissue at sacrifice point by staining formalin-fixed, paraffin-embedded slides with anti-human albumin antibody or anti-human hepatocyte (HepPar1) antibody. Additionally, examine human hepatocyte differentiation using real-time gene expression analysis of liver RNA. Examine viral load by measuring virus nucleic acids in the serum and liver using established quantitative RT-PCR methods [11].
4. Isolate leukocytes from the spleen, liver, and lymph nodes using standard leukocyte isolation protocols. Determine the frequency, activation state, and number of various human immune cells in the various tissues using standard flow cytometry protocols. Examine anti-HCV T cell immune response by measuring T cell activation and/or expansion following antigen stimulation using standard T cell assays.
5. Examine chronic liver inflammation (hepatitis) by staining paraffin-embedded, formalin-fixed liver slides with antibodies against human immune cells (human

⁸Unpublished results demonstrate that human clinical isolates of HBV at $\sim 1 \times 10^7$ genome copies/mL also infects HSC/Hep humanized mice.

CD45+, human CD3+, human CD68+, etc.) [9]. Examine liver fibrosis by staining the liver with either Sirius red/fast green or Masson's trichrome stains.

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Fig. 1.

Murine specific apoptotic signaling induces temporal liver-specific damage in AFC8 immunodeficient mice. The structure of the AFC8 transgene and the mechanism of Caspase-mediated murine hepatocyte apoptosis following ligand (AP20187) activation

Fig. 2. Liver and immune system humanization in the AFC8 immunodeficient mouse. Overview of hematopoietic stem cell and liver progenitor cell isolation, transplantation, and development of AFC8 humanized HSC/Hep mouse model

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Fig. 3.

Chronic hepatitis virus infection, immune response, and liver immunopathogenesis in the humanized mouse model. Timeline for chronic hepatitis virus infection, human immune response, and liver fibrosis analysis in the humanized mouse model. Blood is collected via tail bleeding for longitudinal analysis of plasma and PBMCs. Blood and tissues (liver, spleen, lymph nodes, bone marrow, and other internal organs) are collected at approximately 16 weeks post inoculation (WPI) for analysis; note extensive liver infiltration of human leukocytes (human CD45+, brown cells—**a**) and associated liver damage fibrosis (blue stain—**b**)