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ANIMAL MODELS

Human Hepatocytes and Hematolymphoid Dual Reconstitution in Treosulfan-Conditioned uPA-NOG Mice

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Human-specific HIV-1 and hepatitis co-infections significantly affect patient management and call for new therapeutic options. Small xenotransplantation models with human hepatocytes and hematolymphoid tissue should facilitate antiviral/antiretroviral drug trials. However, experience with mouse strains tested for dual reconstitution is limited, with technical difficulties such as risky manipulations with newborns and high mortality rates due to metabolic abnormalities. The best animal strains for hepatocyte transplantation are not optimal for human hematopoietic stem cell (HSC) engraftment, and vice versa. We evaluated a new strain of highly immunodeficient nonobese diabetic/Shi-*scid* (severe combined immunodeficiency)/IL-2Rγ_c^{null} (NOG) mice that carry two copies of the mouse albumin promoter-driven urokinase-type plasminogen activator transgene for dual reconstitution with human liver and immune cells. Three approaches for dual reconstitution were evaluated: i) freshly isolated fetal hepatoblasts were injected intrasplenically, followed by transplantation of cryopreserved HSCs obtained from the same tissue samples 1 month later after treosulfan conditioning; ii) treosulfan conditioning is followed by intrasplenic simultaneous transplantation of fetal hepatoblasts and HSCs; and iii) transplantation of mature hepatocytes is followed by mismatched HSCs. The long-term dual reconstitution was achieved on urokinase-type plasminogen activator–NOG mice with mature hepatocytes (not fetal hepatoblasts) and HSCs. Even major histocompatibility complex mismatched transplantation was sustained without any evidence of hepatocyte rejection by the human immune system. *(Am J Pathol 2014, 184: 101–109; http://dx.doi.org/10.1016/j.ajpath.2013.09.008)*

In Europe, Australia, and North America at least 25% of HIV-infected persons have a concomitant hepatitis C virus (HCV) infection, and 5% to 10% are co-infected with chronic hepatitis B virus (HBV).[1] Although the incidence of monoinfections (HIV, HBV, and HCV) is declining because of prophylaxis, vaccination, and newly available treatments, co-infections of HIV with HBV/HCV are still problematic, and the medical care of these co-infected patients remains a difficult task.[1,2] These human-specific co-infections require a small animal model to study double infections, such as HIV/HCV or HIV/HBV, and to test new antiviral/antiretroviral therapeutics.

A search is ongoing for the best strain of mice for dual reconstitution and available tissue sources. Among multiple mouse models for human hepatocyte transplantation, the most robust and effective for mouse liver cell depletion and human hepatocytes engraftment are tyrosine catabolic enzyme fumarylacetoacetate hydrolase (Fah) mutants[3] and transgenic mice with a tandem array of murine urokinase genes under the control of the albumin promoter (Alb-uPA) on a CB-17-*scid*-bg background.[4] The advantages (up to 99% of human hepatocyte reconstitution) and the disadvantages (colony maintenance, limited time window for transplantation, and mouse health problems) of these mouse

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models for liver repopulation with human hepatocytes have been extensively discussed.[5–8] Several models for human hematopoietic stem cell (HSC) transplantation are based on the nonobese diabetic (NOD) and BALB/c background mouse strains, known as NOG (NOD/Shi-*scid*/IL-2Rγ_c^{null}),[9] NSG (NOD/*scid*/$\gamma_C^{-/-}$/SzJ),[10] and double knockout for Rag2+IL-2Rγ_c.[11,12] A successful co-transplantation of fetal human liver cells and HSCs has been reported in double knockout BALB/c mice with the transgenic expression of the FK506 binding protein–caspase 8 fusion gene driven by the albumin enhancer/promoter.[13] Despite partial success, the manipulation of newborn animals and the use of fetal tissues create technical and ethical problems.

An important issue for any model that is based on human tissue is the source and type of hepatocytes that can be used for transplantation, which must be syngeneic (matched) with HSCs for immune system reconstruction in experimental animals. The fetal liver provides both types of cells for transplantation. A possible choice is fetal liver cells with a progenitor phenotype that expresses epithelial cell adhesion molecule (CD326).[14] The adult hepatocytes can be transplanted with high efficiency and can be sustained long enough[6]; however, the matched sources of HSCs are limited. These cells must be isolated from the same donor bone marrow or peripheral blood. Thus, no existing model is ideal for dual reconstitution.

Here, we investigated the utility of a new urokinase-type plasminogen activator (uPA)-NOG strain[15] of mice for dual reconstitution and compared different sources of human cells for transplantation. A homozygous line of uPA-NOG mice carries two copies of the transgene array that stably reinforces transgene expression. Perinatal bleeding, embryonic or neonatal lethality, and severe tissue pathology did not occur in homozygous uPA-NOG mice compared with Alb-uPA/*scid* mice. The alanine aminotransferase (ALT) levels were persistently elevated along with evidence of modest hepatic injury by 6 weeks of age in the uPA-NOG homozygotes. Compared with Alb-uPA/scid transgenic mice, which have an age-dependent decrease in uPA expression caused by deletion of the integrated transgene, a relatively low frequency of physical loss of the transgene is observed from uPA-NOG mice. The persistence of the hepatic injury marker should facilitate human hepatocyte engraftment and expansion throughout the life of the mouse.[15] This property of uPA-NOG strain allows for the manipulation of adult animals and expands the window for human cell transplantation. The engraftment of HSCs also requires the creation of a niche in mouse bone marrow for human cells. The widely used total body irradiation increases the risks of severe bacteremia and body weight loss. In this study, we used non-myeloablative conditioning with treosulfan as a safe and well-tolerated alternative to total body irradiation for HSC transplantation.[16–20] Human hepatocyte engraftment at the 3% to 5% level is adequate to perform important studies on intrahepatic pathogens, such as those that cause malaria and hepatitis B. Successful HCV infection was achieved with >10% of hepatocytes being of human origin. Efficient hematolymphoid repopulation in combination with partial liver repopulation is sufficient to study HIV co-infection, and uPA-NOG mice offer this possibility.

Materials and Methods

Animals

The uPA-NOG mice were provided by Central Institute for Experimental Animals (Kanagawa, Japan)[15] and were bred in the animal breeding facility at the University of Nebraska Medical Center. All animal procedures were approved by the University of Nebraska Medical Center Animal Care and Use Committee and were within the guidelines for humane care of laboratory animals. Female hemizygotes ($n = 5$) and male homozygotes ($n = 3$) were obtained, and 158 pups were generated by breeding. The zygosity was determined by the degree of liver damage observed by the serum levels of ALT. Serum ALT levels from 6-week-old males were determined by VetScan VS2 (Abaxis, Union City, CA), and males with elevated ALT were selected for transplantation. Females were used for the next breeding, and we found that homozygous females were able to produce one litter before 4 to 5 months of age when these mice showed phenotypically high ALT levels. NSG mice (The Jackson Laboratory, Bar Harbor, ME; stock no. 005557) were obtained from our breeding colony, which was established in 2005.

Human Cells

HSCs and hepatoblasts were isolated from fetal tissue (Fhbs). Tissues were provided by the University of Washington, Laboratory of Developmental Biology, supported by the National Institutes of Health Award 5R24HD000836 and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (90–117 days of gestation). The tissues arrived 48 hours after collection and were mechanically disrupted, and the resulting fragments were treated with collagenase, hyaluronidase, and DNase at 37°C. The resulting suspensions were washed with medium that contained fetal calf serum, were centrifuged at 50 × *g*, and processed as described.[21] Viability (as evaluated by trypan blue exclusion) always exceeded 80% in the transplanted samples. The fractions contained 1% to 6% asialo glycoprotein receptor-positive hepatocytes as determined by staining with anti-asialo glycoprotein receptor 1–phycoerythrin (PE) antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA).[22] Collected after low-speed centrifugation, the supernatant fluid was used to isolate HSCs. $CD34^+$ HSCs were isolated by using the CD34 MicroBead Kit (Miltenyi Biotec, Auburn, CA), and the purity of the isolated cells evaluated by fluorescence-activated cell sorting (FACS) was >90%. $CD34^+$ cells

were frozen for future transplantation. Adult hepatocytes were obtained from 6-month-old donors. The hepatocytes were cryopreserved and were 80% viable and 40% attachment efficient at the time of transplantation.

Transplantation of Hepatocytes and HSCs

Samples of freshly isolated Fhbs were injected intrasplenically (ispl.) at 2×10^6 cells/mouse.[23] Recipient mice were anesthetized with a xylozine and ketamine combination diluted in distilled water. The left sides of the mice were disinfected with a betadine solution, and a 1.5-cm cut was made 5 mm below the lower edge of the rib cage to enter the peritoneal cavity. The spleen was located and protracted slightly with the blunt-ended forceps, and the lower pole was ligated with a suture. The injection needle of the 1-mL syringe was inserted through the ligation into the spleen, and 100 μL of the cell suspension was injected slowly into the spleen. The needle was retracted, and the ligation was tightened. The spleen was pushed back into the body cavity, and the peritoneum and skin were closed with 3-0 absorbable sutures. The conditioning of the animals for HSC transplantation was done with a non-myeloablative regimen of treosulfan (medac GmbH, Hamburg, Germany), intraperitoneally injected for 3 days at a dose of 1.5 g/kg/day. $CD34^+$ cells were intravenously (i.v.) transplanted into mice at 0.5 to 1×10^6 cells/mouse in 100 μL of PBS via the tail vein with the use of a 28G1/2-gauge needle or with hepatoblasts in 1:1 ratio by intrasplenic injection. Figure 1, A–C, shows the schematic representation of experimental approaches. Five sets of experiments were conducted, and six donor samples and 30 animals were used for approach I. The engraftment was evaluated by the human Alb (hu-Alb) concentration in peripheral blood samples at 4 weeks after surgery. For the second approach of intrasplenic co-transplantation of hepatoblasts and HSCs two samples of donor tissues and 10 animals were used. The third approach included transplantation of cryopreserved hepatocytes after transplantation of cryopreserved mismatched HSCs ($n = 8$).

hu-Alb Level Evaluation

The hu-Alb levels in mice that received a transplant were measured every 4 to 5 weeks by using a Human Albumin ELISA (enzyme-linked immunosorbent assay) Quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX). Western blot analysis confirmed the presence of hu-Alb. Animal plasma samples were diluted 1:3 in PBS and were mixed with SDS sample buffer with 5% β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) in 1:1 ratio. The proteins were subjected to SDS-PAGE and were transferred to Hybond-ECL membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The membranes were incubated overnight with mouse monoclonal anti-human serum albumin antibody diluted 1:2000 (Abcam Inc., Cambridge, MA; ab no. 10241) and goat anti-mouse IgG–horseradish peroxidase conjugate (Sc-2005; Santa Cruz Biotechnology Inc.) secondary diluted 1:10,000 for 30 minutes. The immunoblots were developed with the ECL Western Detection System and Hyperfilm ECL (GE Healthcare Bio-Sciences Corp.).

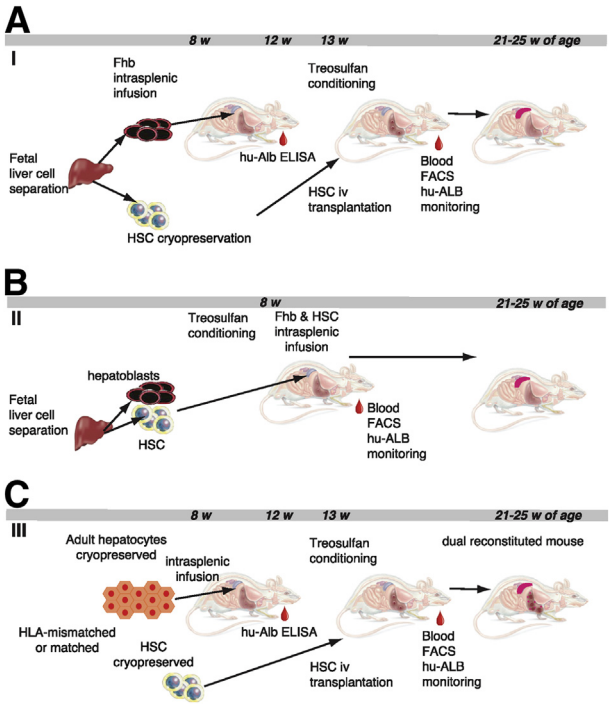

Figure 1 Schematic outlines of the experimental approaches tested in uPA-NOG mice. **A**: Freshly isolated fetal liver cells were infused ispl. The presence of hu-Alb in peripheral blood was confirmed 4 weeks later, and cryopreserved HSCs were administered i.v. after 3 days of chemotherapeutic conditioning with treosulfan. Fetal liver cells did not survive, and the mouse remained mono-reconstituted. The pink color of the spleen represents hematolymphoid reconstitution (**Approach I**). **B**: Isolated liver cells and HSCs were infused in a 1:1 ratio ispl., lower levels of hu-Alb were observed, and the mouse remained mono-repopulated (**Approach II**). **C**: Cryopreserved adult hepatocytes were first transplanted and then mismatched HSCs were derived from the fetal liver. Animals were dual reconstituted with human hematolymphoid cells and hepatocytes (**Approach III**).

Flow Cytometry

Peripheral blood samples were collected from the facial vein in EDTA-coated tubes. Six-color FACS analyses of whole blood samples were performed to monitor changes in the human cell populations. In brief, 100-μL aliquots of whole blood were incubated with respective antibodies for 30 minutes at 4°C. The red blood cells were first lyzed with FACS Lysing Solution (Becton Dickinson, San Jose, CA) and then washed twice with PBS that contained 2% fetal bovine serum. Blood leukocytes were tested for human pan-CD45, CD3, CD4, CD8, CD14, and CD19 markers by multicolor panel. Antibodies and isotype controls were obtained from BD Pharmingen (San Diego, CA), and the staining was analyzed with a FACS DIVA (BD Immunocytometry

Systems, Mountain View, CA). The results were expressed as percentages of the total number of gated lymphocytes. The gating strategy was human CD45⇒CD3⇒CD4/CD8, CD45⇒CD19, and CD45⇒CD14.

Immunohistochemistry

Tissues were fixed with 4% paraformaldehyde overnight at 4°C and then embedded in paraffin. Five-micron sections were cut from the paraffin blocks, mounted on glass slides, and subjected to immunohistochemical staining with mouse monoclonal antibodies for HLA (human leukocyte antigen)-DQ/DP/DR (clone CR3/43, 1:100 dilution), CD45 (1:200 dilution), CD68 (1:100 dilution), and cytokeratin 18 (clone DC 10, 1:33 dilution) from Dako (Carpinteria, CA), and α-smooth muscle actin (1:50 dilution) antibodies from Abcam Inc., the M30CytoDEATH (1:10 dilution) antibody was purchased from Roche Applied Science (Indianapolis, IN), the rabbit monoclonal antibody for CD8 (1:100 dilution) antibody was purchased from Abcam Inc,, and the trichrome stain was purchased from ScyTek Laboratories, Inc. (Logan, UT). Polymer-based horseradish peroxidase–conjugated anti-mouse Dako EnVision systems were used as secondary detection reagents and were developed with 3,3′-diaminobenzidine. All paraffin-embedded sections were counterstained with Mayer's hematoxylin. For immunofluorescent staining, secondary anti-mouse and anti-rabbit Alexa Fluor 488 and Alexa Fluor 594 and blue-fluorescent DAPI nucleic acid stain were used (Invitrogen, Eugene, OR). Bright field and immunofluorescent images were obtained with a Nikon Eclipse E800 (Nikon Instruments, Melville, NY) with the use of NIS-Elements F version 3.0 software (Nikon Instruments). Immunofluorescent images were incorporated into a spectral unmixing algorithm (Nuance version 2.10; Advanced Molecular Vision, Lincolnshire, UK) that quantitatively separated the gray-scale images that represent each spectral component.

Results

Outcomes for Liver Cell Transplantation in uPA-NOG Mice Depend on the Experimental Approach

Three experimental approaches for the construction of mice combining human immune and liver cells are presented in Figure 1, A–C. Animals for human liver cell transplantation were selected on the basis of elevated ALT activity in the peripheral blood at 6 to 8 weeks of age (Figure 2A). For the first approach, we transplanted Fhbs via intrasplenic infusion ($2–3 \times 10^6$ cells/mouse). Then, the animals were conditioned with treosulfan, and the cryopreserved HSCs (10^6 cells/mouse isolated from the same donor tissue samples) were injected i.v. The liver repopulation by human Fhbs was monitored by the hu-Alb ELISA up to 30 weeks after surgery. At 4 weeks after Fhb transplantation, hu-Alb levels >1 μg/mL were detected in 14 animals (median,

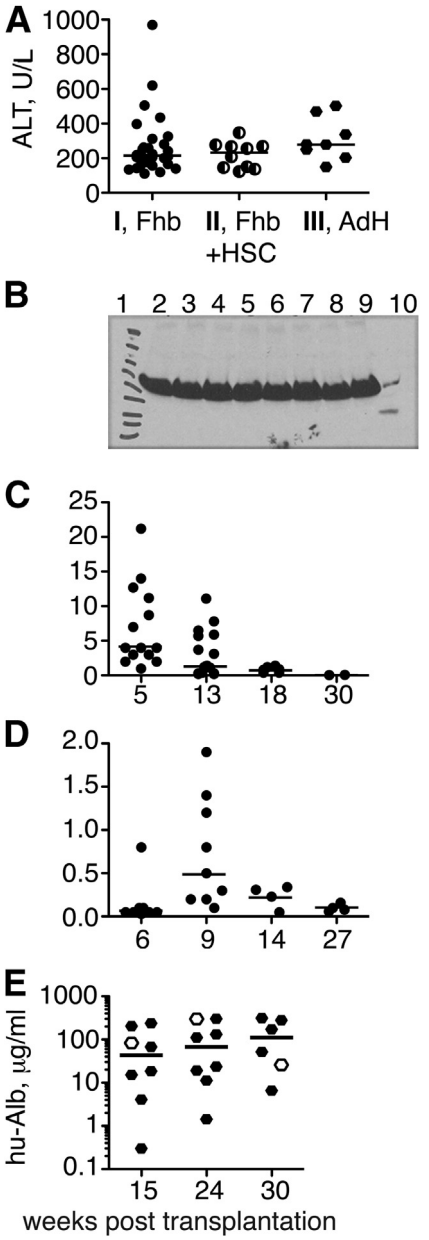

Figure 2 Liver damage levels and the kinetics of human albumin plasma concentration in uPA-NOG mice after fetal or adult hepatocyte transplantation. **A:** The ALT levels in 6- to 8-week-old mice for all three schemes were comparable. **B** and **C:** Scheme I, whereby Fhbs were transplanted first after syngeneic cryopreserved HSC intravenous injection. **B:** Western blot analysis detected human albumin in 5 μL of mouse plasma diluted 1:3. Lanes 2 to 9 represent individual mice at 10 weeks after Fhb transplantation. Lane 10 represents hu-Alb detected in 1 mL of pooled (AB) sera diluted 100 times. **C:** ELISA-based detection of hu-Alb in mouse plasma. **D:** Scheme II, whereby treosulfan-conditioned animals received ispl. a transplant with a combination of two types of cells: Fhbs and HSCs. Fhbs did not sustain in uPA-NOG mouse liver regardless of combination with HSCs or adenovirus expressing human hepatocyte growth factor administration. **E:** Scheme III, whereby adult hepatocytes were transplanted first after treosulfan-conditioning and mismatched HSC intravenous injection. Adult hepatocytes were sustained in the mouse liver in the presence of mismatched hematopoiesis and lymphoid/macrophage repopulation. Open symbols represent mice with reduced hu-Alb concentration at the end of observation. Individual measurements and medians are shown.

4.0 μg/mL; range, from 1.0 to 21.2 μg/mL). Fhb engraftment was also confirmed in eight animals by Western blot analysis at week 10 (Figure 2B). By 18 weeks after surgery, the hu-Alb levels declined, and only six animals had detectable hu-Alb levels (Figure 2C). At the observational end point, the presence of single CK-18$^+$ or human cells positive for apoptotic caspase-cleaved fragment of CK18 (M30) were sporadically found (not shown). The human hematolymphoid tissue development was assessed by blood FACS analysis for the percentage of human CD45$^+$CD3$^+$ (T cells), CD19$^+$ (B cells), and CD14$^+$ (monocytes). All animals successfully established a human immune system.

To improve the engraftment of Fhbs, we used the second approach, in which two types of cells (10^6 cells/mouse freshly isolated Fhbs and HSCs at 1:1 ratio) were co-transplanted by intrasplenic infusion in treosulfan-conditioned mice. We expected that the dynamic interaction between HSCs and Fhbs would support differentiation/maturation into hepatocytes.[24] At week 10 after transplantation, the concentration of hu-Alb in plasma was 10 times lower than with the first approach (median, 0.5 μg/mL; range, 0.1−1.9 μg/mL) (Figure 2D). We assumed that the migration and the engraftment of Fhbs were delayed. Fhbs did not sustain, as in scheme I, and were not detected by immunohistological evaluation of liver tissue samples. However, hematolymphoid reconstitution was successful in all animals.

Finally, as an alternative third approach, the transplantation of adult cryopreserved hepatocytes (2×10^6 cells/mouse) was performed, followed by intravenous injection of major histocompatibility complex mismatched cryopreserved HSCs (0.5×10^6 cells/mouse) in eight animals (Figures 2E and 3). The infusion of mature hepatocytes ensured the stable engraftment and expansion of human cells (Figures 2E and 3, A, B, and I). The median hu-Alb concentration in the peripheral blood 15 weeks after transplantation was 43 μg/mL (range, 0.3−239 μg/mL) and continued to increase up to 30 weeks of observation (median, 111.6 μg/mL; range, 6.6−312 μg/mL). The clusters of CK18$^+$ human hepatocytes were present at the end point of observation, as seen on two representative mice liver tissue slides (Figure 3, A−D). The areas occupied by human CK18$^+$ cells in selected sections were 4.8% to 6.8% of mouse liver tissues. These results showed that adult hepatocytes were able to survive and expand in uPA-NOG mice. Conditioning with treosulfan did not have a negative effect on hepatocyte engraftment. For all three experimental schemes, the increase of the levels of liver damage determined by ALT was similar; however, only the transplantation of mature hepatocytes and HSCs in uPA-NOG mice appeared to be a reliable way to create dual reconstituted mice.

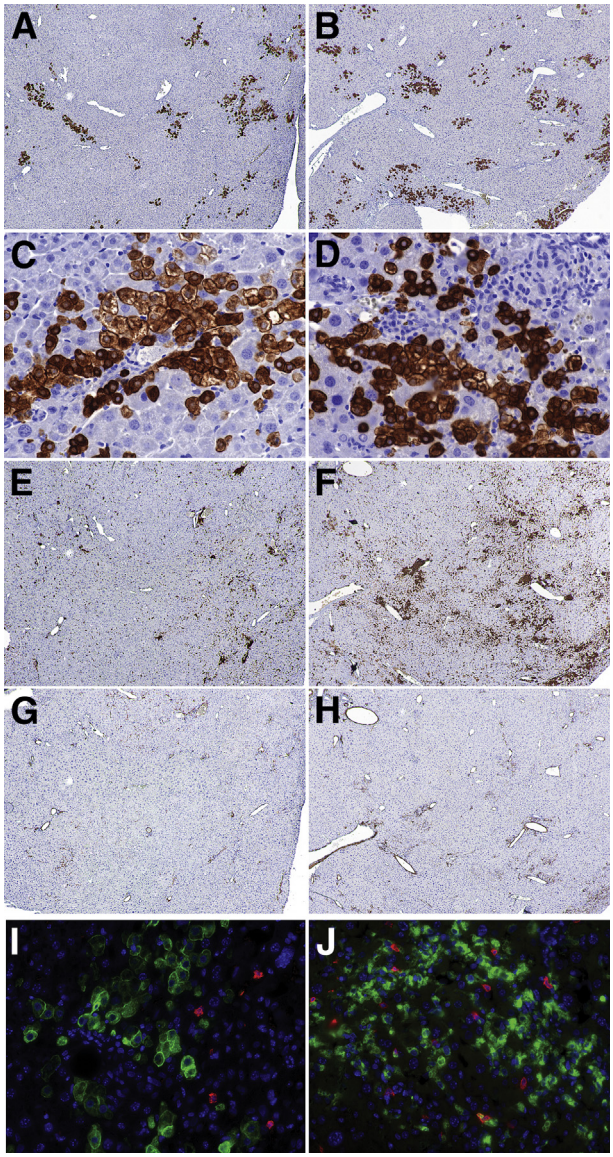

Figure 3 Liver pathomorphology in two representative dual reconstituted uPA-NOG mice. **A** and **B:** Clusters of human hepatocytes were immunostained with human-specific cytokeratine-18. **C** and **D:** Magnified view of selected regions of human hepatocytes is shown. **E** and **F:** Scattering human HLA-DR-positive lymphocytes, macrophages, and Kupffer cells in close proximity in tissue sections of panels **A** and **B**. Significant brown-stained areas are occupied by activated human macrophages/histiocytes (**F**). **G** and **H:** The regions with activated human macrophages were also positively immunostained for α-smooth muscle actin. **I:** Immunofluorescent staining of human CD8$^+$ cells (red) and mismatched hepatocytes by cytokeratine-18 (green). **J:** The accumulation of human CD8$^+$ lymphocytes (red) was not observed around mismatched human hepatocytes and was often found in areas with a significant number of human CD68$^+$ macrophages. Represented are mouse 1561 (**A**, **C**, **E**, and **G**) and mouse 1563 (**B**, **D**, **F**, **H**, **I**, and **J**) with 171.1 μg/mL and 280 μg/mL hu-Alb blood levels at the end point of observation, respectively. Original magnification: ×40 (**A**, **B**, and **E**−**H**); ×400 (**C** and **D**, **I** and **J**).

Hemato-Lymphoid Reconstitution in uPA-NOG Mice

All animals conditioned with treosulfan and which received a transplant with fetal liver-derived CD34$^+$ HSCs successfully developed a human immune system. Human CD45$^+$ cells were detected in the peripheral blood 23 to 30 weeks after HSC transplantation. The proportion of human CD45$^+$ cells for all three schemes was similar (median, approximately 50%−60%) to the lymphocyte gate (Figure 4,

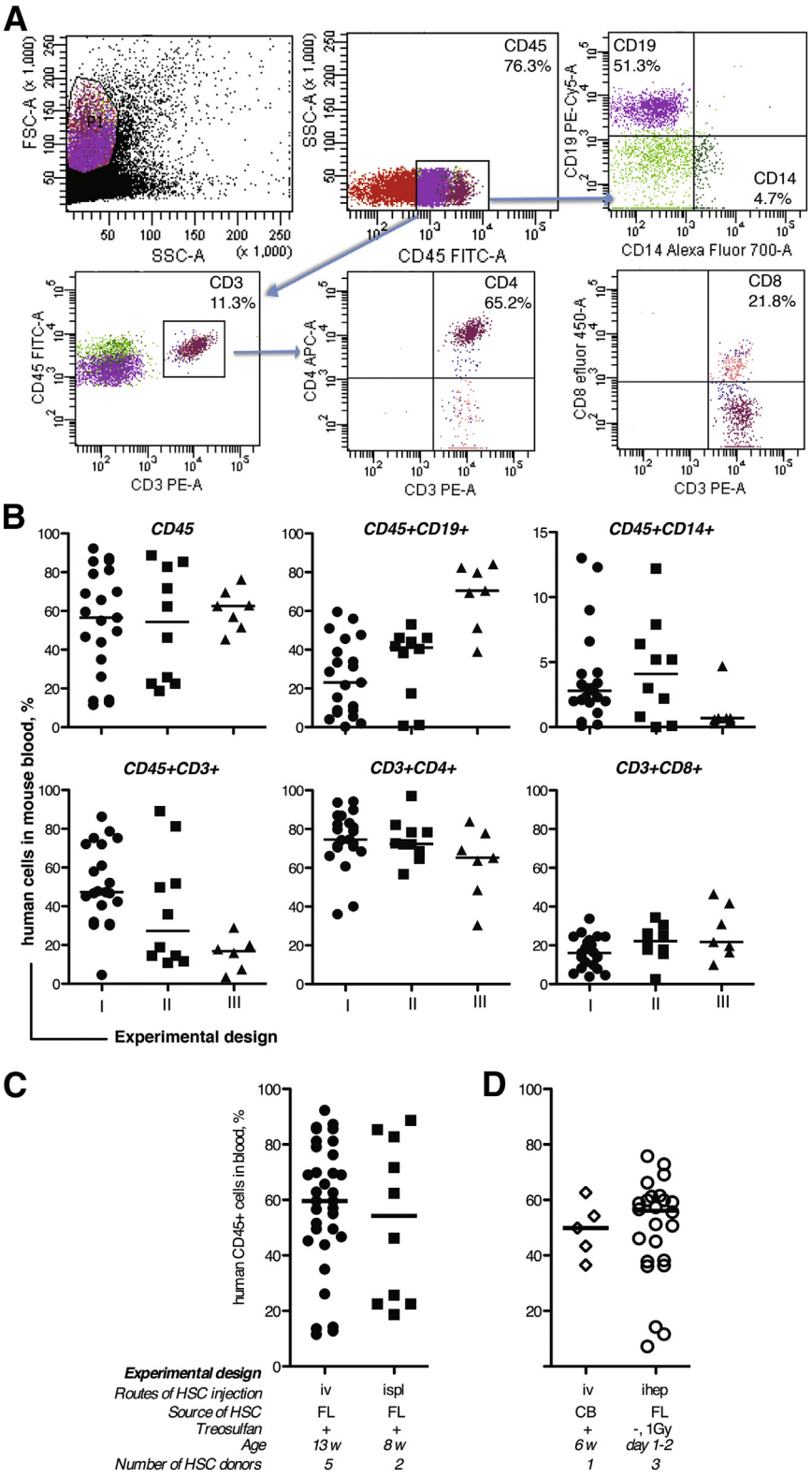

Figure 4 FACS analysis of blood from uPA-NOG mice for human cell population. **A:** Representative plots of six-color panel staining and analysis strategy (50 μL of blood; mouse 1561). **B:** In all experimental schemes of HSC transplantation, the comparable amount of human $CD45^+$ cells and human CD4-to-CD8 cell ratios were found. Scheme I was matched same fetal liver—derived HSCs injected i.v.; Scheme II was same fetal liver—derived cells infused ispl.; and Scheme III was mismatched fetal liver—derived HSCs injected i.v. in mice that received a transplant with adult hepatocytes. The differences in the proportion of B ($CD45^+CD19^+$) and T ($CD45^+CD3^+$) cells between the three approaches was related to the amount of transplanted HSCs and the time of analysis. **C** and **D:** Comparison of peripheral blood lymphoid reconstitution after non-myeloablative conditioning with treosulfan of uPA-NOG and NSG mice. The age and route of HSC administration after treosulfan conditioning in uPA-NOG mice did not affect the levels of reconstitution (**C**). Treosulfan conditioning and HSCs derived from umbilical cord blood intravenous transplantation in NSG mice resulted in comparable reconstitution, as was previously observed with NSG mice transplanted intrahepatically after irradiation at birth[25,26,31–33] (**D**). Individual values and medians are shown. Cy5, cyanine 5; FITC, fluorescein isothiocyanate; FSC, forward scatter; PE, phycoerythrin; SSC, side scatter.

A–D). Furthermore, the proportion of CD4 and CD8 positive T cells within the pool of $CD3^+$ lymphocytes was also similar for animals that received a transplant either i.v. or ispl. with matched or mismatched hepatocytes. For scheme I and II, the animals received a transplant with 10^6 $CD34^+$ HSCs, whereas the animals received a transplant with 0.5 × 10^6 cells in scheme III. The difference in the amount of transplanted HSCs can explain the different human B-cell proportions in the peripheral blood. Such different dynamics could also be donor dependent and is normally observed in NSG/NOG or double knockout strains of mice.[25–30] Treosulfan conditioning was equally efficient for the engraftment

of human CD34$^+$ cells derived from fetal liver and umbilical cord blood (Figure 4D). The level of immune reconstitution was comparable with engraftment achieved after sublethal irradiation of newborn NSG mice.[26,31–34]

Liver Morphology in Dual Reconstituted uPA-NOG Mice

As was shown in the original description of the uPA-NOG mice,[15] the mild liver damage led to fibrotic/cirrhotic changes in these mice by 38 to 40 weeks of age. We analyzed the liver tissue of six dual reconstituted mice among the eight animals that received a transplant. Two animals died between 26 and 30 weeks after hepatocyte transplantation. We found that hemato-lymphoid reconstitution was accompanied by the repopulation of mouse liver with human immune cells. Portal triads were infiltrated with human HLA-DR$^+$ and CD68$^+$ cells. Subsequently, 11 mice had significant parenchymal infiltrates. This liver infiltration by human cells activated macrophages/lymphocytes, which was reminiscent of infection-induced myeloid cell aggregates.[35] In addition to portal stroma, these animals had an increase in the expression of α-smooth muscle actin in the same areas (Figure 3, E–H). Only three of the analyzed mice had a mild collagen deposition (detected by trichrome staining) and human macrophages with digested fragments (not shown). We expected to see cell-mediated rejection in animals reconstituted with adult hepatocytes and mismatched HSCs; however, CD8$^+$ cell expansion or interaction with CK18$^+$ cells was not evident. The livers were populated with human HLA-DR$^+$ macrophages/histiocytes (Figure 3, E and F), and small Kupffer cells were found in the vascular sinuses (Figure 3J). Importantly, in some sham-operated mice and fetal cell-reconstituted mice with higher levels of T-cell repopulation, a significant number of human CD4 and CD8 cells were also present (not shown). In addition, splenic tissues contained well-developed follicular structures composed of human cells. We did not find any CK-18$^+$ cells in spleens. Five animals had foci of human macrophage clusters and multinucleated macrophages, which could be a sign of xenoreaction. None of the animals developed signs of graft-versus-host disease with hair loss, which is often observed in humanized mice that received a co-transplant with fetal thymus/liver[28] and rarely observed on NSG mice that received a transplant with HSCs. Human macrophage infiltrates could be related to the possible increase in the translocation of microbial products in highly immunodeficient animals. Thus, in mismatched co-transplantation of hepatocytes and HSCs, the chances for the cellular immune-mediated rejection of hepatocytes were low. However, human immune cell infiltration associated with xenoreaction or the translocation of bacterial products does exacerbate inherited liver pathology.

Discussion

The experimental approaches for studies of human-specific co-infections associated with HIV-1 are extremely limited.[1,26] The most desired model is a humanized mouse that carries human hematolymphoid tissue (host for HIV-1, Epstein-Barr virus, human T-lymphotropic virus, cytomegalovirus, etc, viral pathogens, and bacteria) and hepatocytes (as the target cells for hepatitis viruses, malaria parasites).[5,25,36] We introduced uPA-NOG mice, a new mouse strain, for dual reconstitution. uPA-NOG mice are a convenient host and are ready for dual reconstitution at 6 weeks of age. We confirmed the original observation[15] that these animals (males), at 6 to 8 weeks of age, have elevated ALT levels and the ability to engraft permanently adult hepatocytes. The fetal liver is a unique source of two types of cells for dual reconstituted mice. Isolated by standard procedures, fetal HSCs are functional and, with different speed and efficiency, form human hematolymphoid tissue in uPA-NOG mice and in NSG or NOG mice.[37] Unfortunately, the practical application of fetal hepatocytes is highly limited. The reported short-term engraftment success of fetal hepatocytes differentiated *in vitro*[38] or cells differentiated from embryonic stem cells[22] did not warrant the expansion and survival of these cells in the adult mouse liver. As shown in our study, we were successful in the initial engraftment, but the expansion of Fhbs was not sustained despite the ongoing damage of hepatocytes in uPA-NOG mice. Furthermore, co-transplantation with HSCs did not improve engraftment. Several reports showed significant and sustained engraftment of Fhbs by sorting for E-cadherin-positive cells in *Fah*$^{-/-}$Rag2$^{-/-}$ mice.[39] In this strain of mice, liver progenitors, which differentiate into mature human hepatocytes, were successfully infected with HBV. Unfortunately, mice on a *Fah*$^{-/-}$Rag2$^{-/-}$γc$^{-/-}$ background, which potentially could be more appropriate for co-transplantation, were much more fragile, and a significant loss of animals occurred with nonsurgical or surgical manipulations.

We expected that the addition of human vector-delivered hepatocyte growth factor would facilitate the expansion of fetal cells.[40] In an additional experiment, uPA-NOG mice received a transplant with Fhbs along with the intramuscular injection of adenoviral vector-expressing human hepatocyte growth factor (1×10^{11} particles/mouse),[41] and the engraftment of the fetal liver cells was not improved. This observation agrees with previously reported studies on other strains of mice whereby Fhbs can be engrafted but at limited quantities and for a limited time.[14,42,43] The direct comparison of fetal versus adult human hepatocyte transplantation on Alb-uPA$^{+/-}$-Rag2$^{-/-}$γc$^{-/-}$ showed that adult cells repopulate better than fetal cells in BALB/c background mice.[21] Moreover, even though the engraftment reached a relatively significant proportion as shown by Washburn et al,[13] these fetal-derived hepatocytes were not able to efficiently support the replication and secretion of HCV.[25] We cannot exclude that the isolation of mature hepatocytes from fetal livers that express asialo glycoprotein receptor 1 could be a feasible approach to use matched HSCs.[22] However, adult hepatocytes are preferred for establishing a metabolic panel and innate immunity that

allows replication of not only HBV but also HCV. In addition, adult hepatocytes can be transplanted with high efficiency and can be sustained for a long period of time.[6] However, for the last scenario, there is no easily obtainable source of syngeneic HSCs. The ability to obtain two types of cells from the same donor is currently limited to a few centers with human tissue biorepositories. We transplanted mismatched hepatocytes and HSCs, which can be used to evaluate the viral replication of HIV and HBV co-infection in the case of low-hepatic repopulation, or HIV and HCV co-infection with high levels of liver humanization. However, situations with mismatched transplantation of liver in cases of chronic HCV infection are not rare.

The successful engraftment of human HSCs was expected,[9] but we demonstrated that this engraftment could be performed safely at ages >8 to 10 weeks without total body irradiation by using non-myeloablative conditioning with treosulfan.[16–20] In conclusion, uPA-NOG mice serve as the promising tool for dual human liver and hematolymphoid reconstruction, to examine HIV-1–associated co-infections, and to develop new treatment approaches.

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