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Extensive double humanization of both liver and hematopoiesis in FRGN mice☆

Elizabeth M. Wilson[a], J. Bial[a], Branden Tarlow[b], G. Bial[a], B. Jensen[a], D.L. Greiner[c], M.A. Brehm[c], M. Grompe[a,*]

[a] Yecuris Corp., Tigard, OR, USA
[b] Oregon Health & Science University, Portland, OR, USA
[c] Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA

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Abstract Preclinical research in animals often fails to adequately predict the outcomes observed in human patients. Chimeric animals bearing individual human tissues have been developed to provide improved models of human-specific cellular processes. Mice transplanted with human hematopoietic stem cells can be used to study human immune responses, infections of blood cells and processes of hematopoiesis. Animals with humanized livers are useful for modeling hepatotropic infections as well as drug metabolism and hepatotoxicity. However, many pathophysiologic processes involve both the liver and the hematolymphoid system. Examples include hepatitis C/HIV co-infection, immune mediated liver diseases, liver injuries with inflammation such as steatohepatitis and alcoholic liver disease.

We developed a robust protocol enabling the concurrent double-humanization of mice with mature hepatocytes and human blood. Immune-deficient, fumarylacetoacetate hydrolase (*Fah*$^{-/-}$), *Rag2*$^{-/-}$ and *Il2rg*$^{-/-}$ deficient animals on the NOD-strain background (FRGN) were simultaneously co-transplanted with adult human hepatocytes and hematopoietic stem cells after busulfan and Ad:uPA pre-conditioning. Four months after transplantation the average human liver repopulation exceeded 80% and hematopoietic chimerism also was high (40–80% in bone marrow). Importantly, human macrophages (Kupffer cells) were present in the chimeric livers.

Double-chimeric FRGN mice will serve as a new model for disease processes that involve interactions between hepatocytes and hematolymphoid cells.

Introduction

Despite our ability to genetically engineer animals to mimic human physiology under normal conditions and in disease, animal models often fail to fully recapitulate human processes. For this reason chimeric animals engrafted with human cells and/or tissues play a very important role in biomedical research, including stem cell biology. Xenografting of human cells began with tumor transplants and first became possible when immune deficient strains of mice became available (Rygaard and Povlsen, 1969). Currently, many different kinds of primary cells can be permanently engrafted in mice, among them hematopoietic stem cells,

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* Corresponding author.

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thymic epithelium, neurons and hepatocytes. Hematopoietic chimeras generated by human stem cell transplants have been particularly important as they can be used to study human immunological and inflammatory processes in vivo (Greiner et al., 1998; Pearson et al., 2008; Shultz et al., 2007). When blood stem cell transplantation is combined with engraftment of thymic epithelium, significant aspects of the cellular immune response can be recapitulated.

The liver is the primary site of intermediary and xenobiotic metabolism in mammals and has particular relevance for pharmaceutical drug development. However, major differences exist in metabolic pathways between humans and animals, especially the small rodents used extensively for preclinical drug testing. Furthermore, many common human infectious diseases are hepatotropic and cannot be modeled in rodents. These include hepatitis B and C as well as malaria. To overcome these limitations several mouse models with humanized livers have been generated (Grompe and Strom, 2013). All of these models are based on hepatotoxic processes in the mouse liver that generate a selective growth advantage for transplanted human cells. Replacement indices of >90% can be achieved (Azuma et al., 2007; Hasegawa et al., 2011; Hu et al., 2013; Dandri et al., 2001; Tateno et al., 2004). Liver chimeric mice have been used to study infections (Dandri et al., 2001; Bissig et al., 2010; Mercer et al., 2001; Morosan et al., 2006; Vaughan et al., 2012; Kawahara et al., 2013), drug metabolism (Tanoue et al., 2013; Sanoh et al., 2012), gene therapy (Lisowski et al., 2014) and hepatic stem cells (Duncan et al., 2012; Takebe et al., 2013; Zhu et al., 2014). Importantly however, many normal and pathophysiologic processes in the liver involve interactions between blood derived cells and hepatic epithelial cells. All liver infections have a very important inflammatory component. In addition, inflammation plays a substantial role in fatty liver disease induced by alcohol or obesity and also has significant effects on insulin resistance in type 2 diabetes. In addition to the cellular immune system the liver resident macrophages, termed Kupffer cells, are involved in mediating pathophysiologically important processes.

In order to better study interactions between human hematolymphoid cells and hepatocytes two groups have previously developed models that permit the simultaneous engraftment of both hepatocytes and hematopoietic stem cells. Washburn et al. used a mouse expressing a drug inducible caspase 8 protein in the liver and were able to engraft fetal hepatoblasts, hematopoietic progenitors and thymus (Washburn et al., 2011). This model was used to study immune responses and fibrosis induced by hepatitis C. However, hepatic repopulation levels were low (~30%) and the human liver epithelial cells were fetal in origin, not displaying fully mature characteristics. More recently, Gutti et al. reported a protocol for the generation of double-chimeric mice using human cells of postnatal origin (Gutti et al., 2014), i.e. cord blood stem cells and mature hepatocytes. The recipient mouse was the uPA-SCID where the selective advantage for human cells is based on transgenic expression of urokinase plasminogen activator (Dandri et al., 2001; Rhim et al., 1994). They compared multiple approaches and generated double chimeras after preconditioning with treosulfan (Gutti et al., 2014). While the degree of hematopoietic chimerism was excellent, the replacement index for hepatocytes was rather low. None of the double-chimeric mice in their report achieved human albumin levels above 1 mg/mL and therefore had repopulation levels of <10%.

Unlike the uPA-SCID model the selection pressure for human hepatocyte expansion can be controlled in immune deficient mice lacking fumarylacetoacetate hydrolase (Fah). Hepatotoxicity is blocked by administration of NTBC in the drinking water or by a low tyrosine diet. Liver repopulation levels of >90% can be routinely achieved in this model. Here we describe a robust protocol in FRGN mice that can produce mice with both >90% liver humanization and extensive lymphohematopoietic chimerism. These dual chimeric mice will serve as a platform for the study of interactions between human hepatocytes and blood cells in vivo.

Results

Generation of the FRGN strain

The non-obese diabetic (NOD) strain of mice has proven ideal for the generation of human hematopoietic chimeras. With an intact immune system these mice serve as a robust model of autoimmunity mediated type1 diabetes (Kolb, 1987). On an immune deficient background, however, they are the gold standard for human hematopoietic chimerism (Shultz et al., 2007). The NOD/SCID/interleukin receptor common γ-chain (Il2rg) knockout (NSG) mouse is used by many laboratories (Ito et al., 2002). Polymorphisms at the Sirp-α locus in NOD mice have been identified as the main reason for the superior hematopoietic engraftment of this strain (Takenaka et al., 2007). In order to create a strain suitable for human hematopoietic engraftment as well as liver humanization, we generated mice triply mutant in the

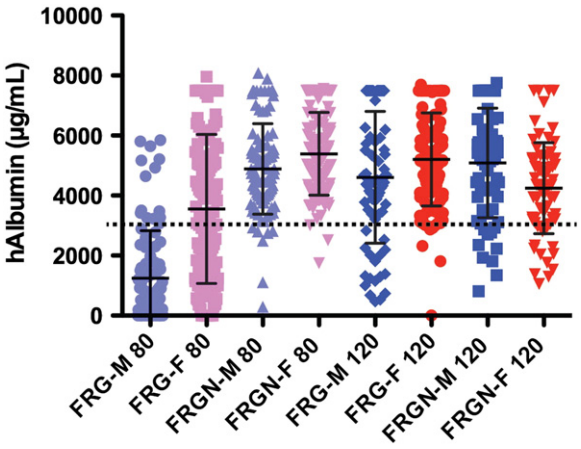

Figure 1 Liver repopulation in FRG vs. FRGN mice. Blood levels of human albumin are shown for male (M) and female (F) FRG and FRGN mice on day 80 or day 120 after hepatocyte transplantation. The dashed line shows the human albumin level (3.5 mg/mL) indicating >80% liver humanization. The mean and standard deviation are given by the black bars. The differences in albumin levels between FRG and FRGN mice are highly significant at day 80 after transplantation ($p = 2.86 \times 10^{-22}$ for males and $p = 6 \times 10^{-5}$ for females).

Fah, *Rag2* and the *Il2rg* genes (FRG mice (Azuma et al., 2007) and genotyped offspring for the NOD Sirp-α allele. After four generations of intercrossing, we obtained mice that were homozygous for all four alleles and expanded this colony of *FRGN* mice. Several large cohorts ($n > 20$ animals for each genotype and gender) of FRG and FRGN mice were transplanted with identical batches of human hepatocytes in parallel ($n = 4$ transplantations) to determine whether the NOD Sirp-α allele conferred an advantage for liver humanization. The overall survival rates during NTBC withdrawal were similar between strains and were ~40% after 5 months (data now shown). However, we observed several significant differences between FRG and *FRGN* mice. First, complete liver repopulation occurred more quickly in the FRGN strain, especially in males. Human albumin levels were clearly higher on day 80 after transplantation in FRGN mice as compared to FRG animals transplanted at the same time with the same batch of cells (Fig. 1). Importantly however, more than 50% of mice of all strains and genders that survived the NTBC withdrawal regimen had human blood albumin levels >3.5 mg/mL four months after transplantation, indicating liver replacement levels of at least 80%. Second, the average litter size was larger with an average of nine per FRGN breeder vs. five pups for FRG breeders (Suppl. Fig. 1). Finally, the average body weights of 8-week-old mice were higher in the FRGN strain for both genders; 22.8 +/− 2.7 g vs. 17 +/− 1.7 g for females ($p = 1.4 \times 10^{-113}$) and 27 +/− 2.6 g vs. 22.2 +/− 2.2 g for males ($p = 6.7 \times 10^{-54}$).

Development of a protocol for double chimerism

The engraftment of human hepatocytes is enhanced by pre-administration of an adenoviral vector expressing urokinase plasminogen activator (Ad:uPA) (Azuma et al., 2007). Preconditioning of immune deficient mice with human hematopoietic stem cells requires either preparative irradiation or the administration of a DNA-damaging chemical. We sought to develop a protocol that would have wide applicability in many laboratories and not depend on the availability of a radiation source. For this reason we chose to test busulfan, a widely available drug which has previously been used for generating hematopoietic chimeras in both mice (Hayakawa et al., 2009) and humans (Wilkinson et al., 2013; Hassan, 1999). We first performed a dose–response curve to determine the maximum dose that could be used in combination with the standard dose of uPA-adenovirus in FRGN mice. We found that a single dose of 30 mg/kg along with 1.25×10^9 pfu of Ad:uPA per 25 g of body weight was the maximum dose tolerated with 100% survival (data not shown).

We next applied this dosing regimen in the transplant setting (Fig. 2). Two separate cohorts of weanling female FRGN mice ($n = 12$ each, age 3 weeks) were transplanted concurrently with standard doses of adult human hepatocytes (500,000) and CD34+ peripheral blood stem/progenitor cells (200,000). The hepatocytes and blood stem cells were from distinct donors and were not HLA or otherwise matched. The hepatocytes and blood stem cells were mixed and given intrasplenically. After transplantation, NTBC was withdrawn to permit selection of the transplanted hepatocytes. Liver chimerism was monitored by serial measurement of the levels of human albumin in blood and human hematopoietic chimerism was estimated by FACS analysis of peripheral blood. Overall survival of transplanted animals to full liver repopulation was 11/24 = 46%.

Hematopoietic chimerism

Peripheral blood analysis 12–15 weeks after transplantation indicated high levels of liver repopulation along with substantial blood chimerism in all surviving animals (Table 1). We therefore euthanized the animals and performed a detailed analysis of hematopoietic tissues and liver. Blood lineages were analyzed by FACS (see Suppl. Fig. 2, 3) and the liver was subjected to FAH immunohistochemistry. Fig. 3 depicts the overall distribution of human chimerism in spleen, bone marrow and thymus. As shown in Fig. 3, human chimerism in the spleen averaged ~60% with a range from 28 to 85%. Bone marrow chimerism was ~50% and all animals had >90% human cells in the thymus. These numbers are comparable to other studies (Gutti et al., 2014), especially when adult peripheral blood stem cells are used as donors. Human CD20 and CD10 expression was used to ascertain the percentages of mature B- and Pre-B-cells respectively among the human cells. As expected both lineages were found and mature B-cells

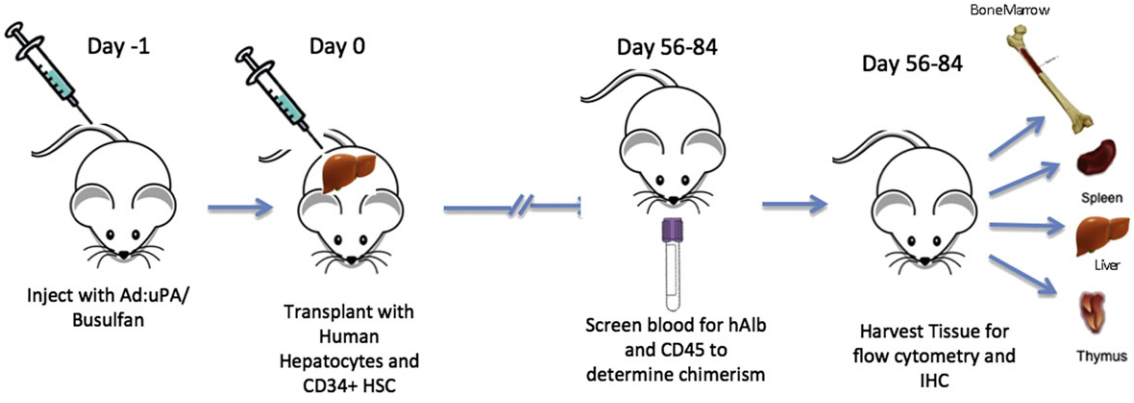

Figure 2 Schematic of dual humanization.

Table 1 Humanization indexes in liver and peripheral blood.

Mouse ID	hAlb (mg/mL)	Estimated human hepatocyte replacement (%)	hCD45 in peripheral blood (%)	Harvest (weeks post transplant)
1	9.2	≥90	22	17
2	6.1	≥90	13	17
3	6.2	≥90	13	17
4	5.0	≥90	13	19
5	5.5	≥90	5	21
6	3.9	80–90	8	21
7	4.2	80–90	7	21
8	6.0	≥90	6	21
9	4.4	80–90	5	21

Table 1: Results from nine FRGN female mice transplanted with human hepatocytes and human CD34+ cord blood stem cells at three weeks of age. Human albumin (hAlb) blood levels of >3.5 mg/mL indicate liver humanization of >80% and >4.5 mg/mL represents >90%. These estimates were validated by FAH immunohistochemistry at harvest (Fig. 4). Chimerism of the peripheral blood mononuclear cells (PBMC) was determined between 17 and 21 weeks post transplant by flow cytometry for the human CD45 surface marker.

dominated in the spleen, whereas the mature/pre-B cell ration (CD20/10) in bone marrow was closer to 1:1 (Fig. 3). The thymus contained mostly T-cells and both CD4 and CD8 human T-cells were present as expected. Myeloid cells were also found. In the bone marrow ~30% of the human cells were positive for the monocyte marker CD64. 8–40% of CD45 positive human bone marrow cells expressed the myeloid markers CD13 or C33. Thus, human hematopoietic cells of both the lymphoid and myeloid lineages were present 4–5 months after transplantation, indicating multilineage engraftment indicative of the presence of human stem/progenitor cells. Overall, the degree of hematopoietic chimerism and the lineage distribution were in concordance with previous publications using postnatal peripheral blood CD34+ cells for transplantation (Brehm et al., 2014).

Liver chimerism

In our model peripheral blood albumin levels of >3.5 mg/mL correlate with overall replacement indices of >80% and here all surviving animals reached at least this level. FAH immunohistochemistry (IHC) was used to measure the degree of liver humanization (Fig. 4). Indeed, the liver replacement index determined by this method exceeded 90% in all mice, confirming the peripheral blood albumin measurements shown in Table 1. We next sought to determine whether human hematopoietic derivatives important for liver biology could be detected. Kupffer cells are essential to many hepatic functions and play a major role in inflammatory responses in this organ (Ajakaiye et al., 2011; Gregory and Wing, 2002; Roberts et al., 2007). CD68 is considered a specific marker for activated Kupffer cells and we therefore used two methods to determine whether human CD68+ cells were present in our chimeras. First, we performed RT-PCR with human-specific primers, not capable of detecting mouse mRNA. As seen in Fig. 5, control RNA from untransplanted FRGN mice had no human CD68 mRNA, whereas double-chimeric mice and samples from human liver explants did. Importantly, FRGN mice that had been given only hepatocytes but not blood did not show any detectable levels of CD68 transcript. We next performed IHC using antibodies with specific reactivity for human proteins. CD45 positive human cells could be seen throughout the

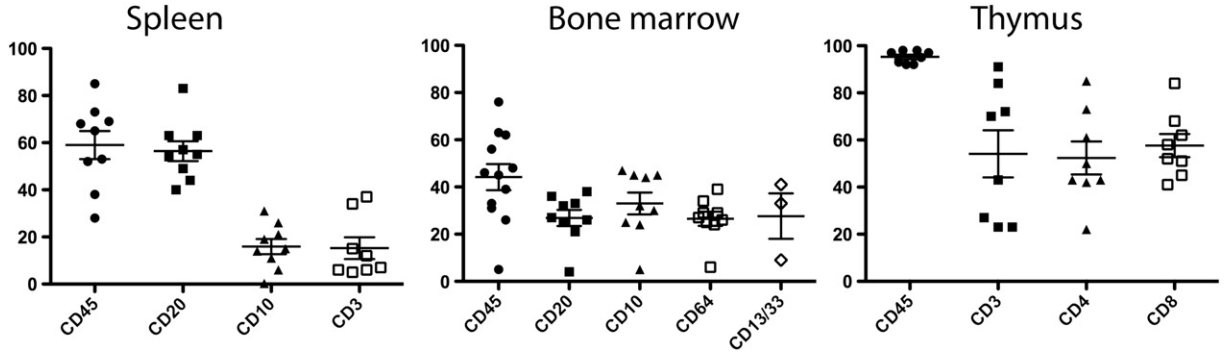

Figure 3 Human hematopoietic chimerism. Scatter plots showing the percentages of human chimerism for CD45, CD20, CD10, CD3, CD4 and CD8 in spleen, bone marrow and thymus of each double humanized mouse. The values were calculated as a percentage of the human CD45 positive cells.

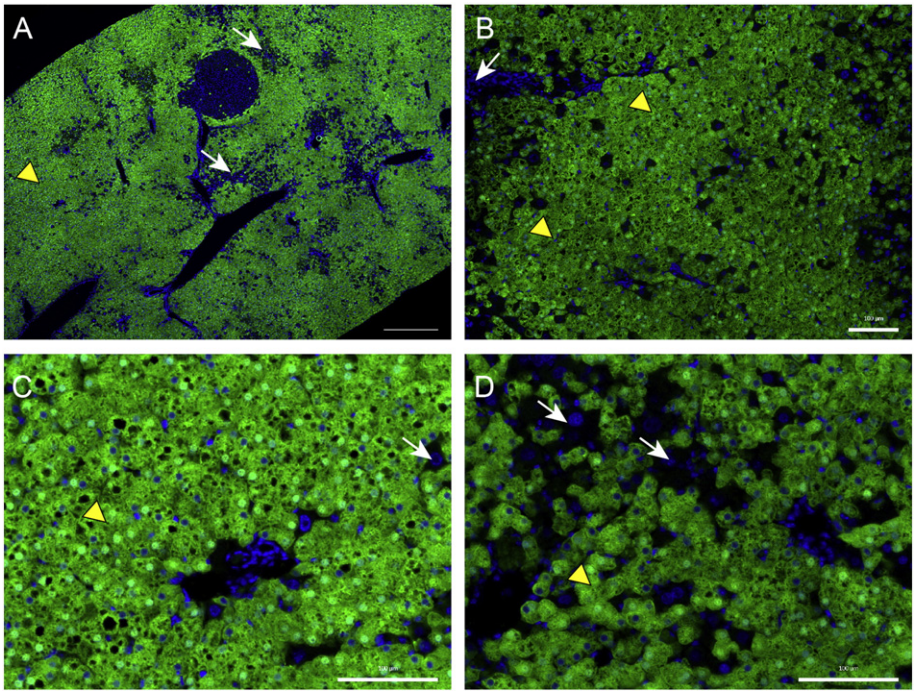

Figure 4 High degree of liver chimerism. Examples of Fah immunohistochemistry (green fluorescence). Nuclei are stained blue with DAPI. A–D: double humanized mice; A = low magnification, stitched from multiple images, scale bar = 500 μm, B,C = 200×; D = 400×, B–D scale bar = 100 μm, Yellow triangles: human hepatocytes; White arrows: remnant mouse cells. The vast majority of hepatocytes are Fah positive and therefore human. A–C display >95% and D ~80–90% humanization.

double-chimeric livers (Fig. 6A). CD68 IHC confirmed the RT-PCR results and activated Kupffer cells were found throughout the parenchyma (Fig.6B). In addition, human T- and B-cells were also found throughout the liver (Fig. 6) as expected from the high degree of hematopoietic chimerism in multiple organs.

Discussion

The FRGN dual chimerism system reported here is the first to achieve both extensive liver humanization with mature hepatocytes and concurrent hematopoietic engraftment in

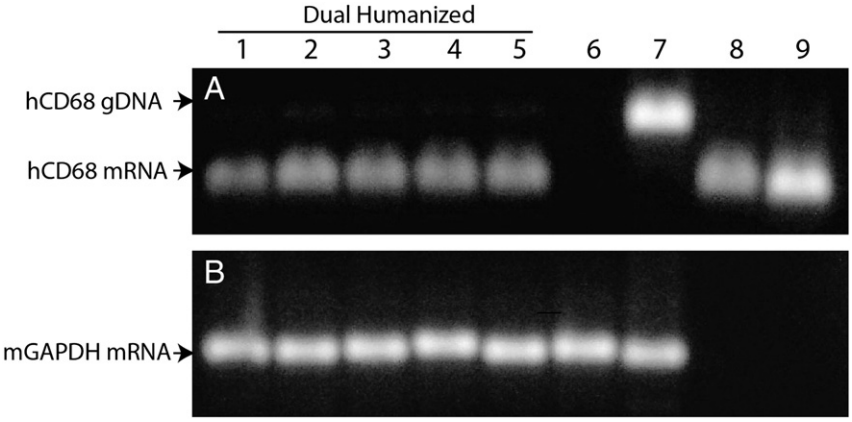

Figure 5 Double humanized liver express human CD68 mRNA. RT-PCR of liver from 5 double humanized mice and controls is shown. Top panel (A): human CD68; bottom panel (B) = mouse GAPDH. Arrows indicate the proper PCR product. Samples 1–5 = double humanized mice; 6 = untransplanted mouse; 7 = liver chimera only (no hematopoietic transplant); 8 non-parenchymal cells from human liver; 9 = primary human hepatocytes. Human CD68 mRNA (376 bp lower band) was expressed in all double-humanized mice and cells isolated from human liver (8,9), but not in untransplanted controls (6) or FRGN mice repopulated with only human hepatocytes (7). A larger band (641 bp) derived from the genomic human CD68 gene was seen in the liver only chimeras (7). All samples harvested from mice (1–7) had murine GAPDH and human liver samples were negative as expected.

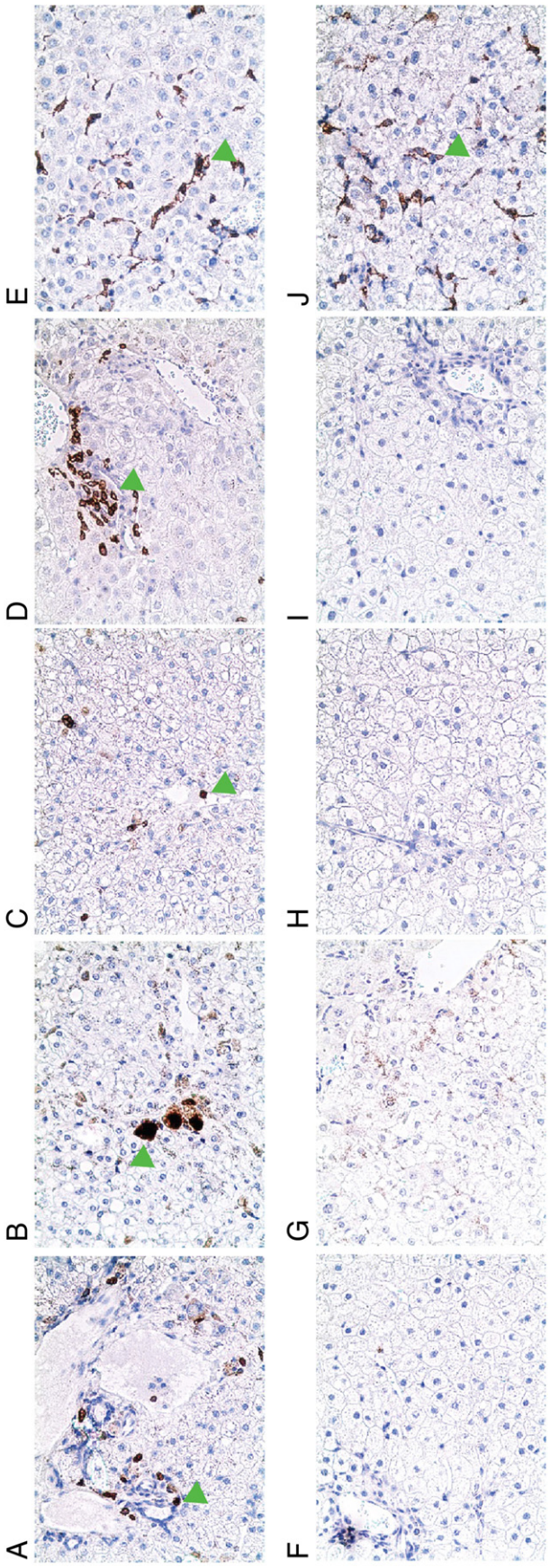

Figure 6 Human hematopoietic cells in the liver of double humanized mice. Immunohistochemistry was done to detect hematopoietic cells in the liver parenchyma (200×). The top row (A–E) depicts liver from a double-chimeric mouse and the bottom row shows liver from a human hepatocyte-only chimera (F–J). Mouse macrophages stained with F4/80 were present in both animals (E,J). However, staining with human-specific antibodies to CD45 (A,F), CD68 (B,G), CD20 (C,H) and CD3 (D,I) showed that only double-humanized livers contained human blood, Kupffer cells, B-cells and T-cells, respectively. Green triangles mark positive staining.

the same animals. Previously reported models of human liver/blood dual chimerism in mice achieved robust hematopoietic engraftment (Washburn et al., 2011; Gutti et al., 2014; Bility et al., 2012). However, the degree of liver repopulation achieved was low. While low hepatocyte replacement indices can support some human liver infections, particularly hepatitis B, a high degree of liver chimerism is essential for any study that involves physiologic or xenobiotic metabolism, pharmacokinetics, drug–drug interactions, transport or toxicology. The precise threshold for each of these applications varies, but generally liver humanization levels of >70% are needed and "more is better" (Grompe and Strom, 2013). In addition, it is important that the human hepatocytes are of postnatal origin and fully mature. Mature human hepatocyte functions in chimeric FRGN mice have been demonstrated by analysis of their gene expression showing absence of fetal markers (Azuma et al., 2007), cytochrome p450 induction (Azuma et al., 2007; Strom et al., 2010), bile acid and lipoprotein metabolism (Ellis et al., 2013). The immature fetal liver cells used by Washburn et al. (2011) lack some critical features of adult drug metabolism. Our overall success rate of double humanization was ~50% of transplanted animals, making the protocol robust enough for the generation of large cohorts of experimental animals.

The FRGN strain developed herein is suitable for hematopoietic reconstitution and in addition to this has some other advantages over our originally reported FRG KO on the C57BL6 strain (Azuma et al., 2007). While the final percentages of highly reconstituted (>80%) mice did not differ significantly between the strains, FRGN mice achieved maximal liver humanization significantly faster. It is interesting that the result parallels those from hematopoietic xenotransplantation and suggests that the interaction between Sirp-α on mouse macrophages and human CD47 on hepatocytes is important for engraftment, as it has been shown for blood transplantation (Takenaka et al., 2007). Not only do FRGN mice repopulate their livers more quickly, but also their breeding efficiency is also higher and their body weight is larger. It is of note that the FRGN strain used herein had not yet been fully backcrossed onto the NOD background and still harbored some C57/BL6 genome. The colony is currently being fully inbred and it is possible that further improvements will result from having completely congenic FRG-NOD strain.

None of the many potential applications of liver/blood double chimeras was validated in this study. Instead, we chose to focus on clearly documenting the degree of chimerism that can be obtained. However, experiments to study interactions between human inflammatory cells and hepatocytes will now be possible in the future. It will be particularly interesting to use our model for Hepatitis C/HIV co-infection (Rockstroh and Spengler, 2004) and metabolically induced steatohepatitis (Kubes and Mehal, 2012), be it by ethanol or a high fat diet.

Thymic epithelium was not humanized in our current generation double-chimeric FRGN mice thus limiting its utility for studying human T-cell responses. For this reason it will be desirable to generate tri-chimeric FRGNs in the future, possibly by transplanting iPSC derived human thymic epithelium (Parent et al., 2013) along with the other tissues.

Materials and methods

Materials

Busulfan (catalog # B2635) and dimethyl sulphoxide (DMSO, catalog #D2650) were purchased from Sigma-Aldrich Chemicals; non-platable human hepatocytes (>70% viable by trypan blue staining) were purchased from BioreclamationIVT (catalog# F00995); human frozen bone marrow CD34+ cells were purchased from AllCells (Catalog# ABM017F); HCM media was purchased from Lonza (Catalog# CC-3198 and CC-3199). The sources of antibodies used for FACS and immunohistochemistry are given in Supplemental Table 1. HBSS, fetal bovine serum and 4% paraformaldehyde were purchased from Fisher Scientific.

Bone marrow transplantation of FRG and FRGN breeders

In order to avoid the complications of immune deficiency during breeding, all breeder animals are transplanted as weanlings (~3 weeks of age) by retro-orbital injection of one million nucleated bone marrow cells from strain matched immune proficient adult donor animals (C57/BL6 or NOD/ShiLtJ, Jackson Laboratories). The transplanted animals are set into breeding at six weeks of age.

Sirp-α genotyping assay

PCR is performed on genomic DNA using a three primer PCR. The C57BL6 SIRP-α PCR product is 350 bp and the NOD SIRP-α product is 180 bp in length.

Primer SIRP-α _ C57BL6 forward: 5′ AGGAGCCACGGGGA AGGAACT 3′; SIRP- α NOD forward: 5′ CAACAGAACACTTTCC TCGAGTTAC 3′; SIRP-α common reverse primer: 5′ AGACC TCTGTTCCCCCTCCAGATTG 3′. The wild type SIRP-α PCR product found in C57BL6 is 350 bp and the product found in the NOD SIRP-α gene is 180 bp.

Dual humanization protocol

Weanling mice were transplanted after preconditioning with both busulfan and a urokinase-expressing adenovirus (Fig. 2).

Busulfan was dissolved in sterile DMSO at 50 mg/mL, diluted to 2.25 mg/mL solution in sterile 0.9% saline and delivered by intraperitoneal injection at 30 μg/g body weight 24 h prior to transplantation. Ad:uPA (Azuma et al., 2007; Vrancken Peeters et al., 1997) was diluted to 1.25×10^9 plaque forming units (pfu)/100 μL in sterile 0.9% saline and filtered using an Acrodisc Syringe filter with 0.45 μ HT Tuffryn membrane. Each mouse was anesthetized using isoflurane and given 5×10^7 pfu per gram of body weight via retro-orbital vein injection 24 h before transplantation.

Cryopreserved human hepatocytes were thawed and the cryopreservation solution was removed by centrifugation at 100 ×*g* for 5 min at 4 °C followed by resuspension in HCM media. The resuspended hepatocytes were diluted 1:1 in 0.4% trypan blue and the cell number and viability were

determined using a hemocytometer. Hepatocytes were centrifuged again at 100 ×*g* for 5 min at 4 °C and reconstituted in HCM at 10 × 10^6 cells/mL.

Cryopreserved human bone marrow CD34+ cells were thawed and the cryopreservation solution was removed by centrifugation at 500 ×g for 10 min at 4 °C followed by resuspension in HCM. The resuspended CD34+ HSC were diluted 1:1 in 0.4% trypan blue and the cell number and viability were determined using a hemocytometer. The bone marrow CD34+ cells were centrifuged again at 500 ×g for 10 min at 4 °C and reconstituted in HCM at 4 × 10^6 cells/mL.

For cell injection, the mice were anesthetized using isoflurane, the two cell types were mixed 1:1 and each mouse received 500,000 hepatocytes and 200,000 bone marrow CD34+ cells by intrasplenic injection as previously described (Gutti et al., 2014; Rajvanshi et al., 1996; Bumgardner et al., 1998). The mice were then subjected to the standard NTBC withdrawal regimen (Azuma et al., 2007).

Human albumin ELISA

Starting at eight weeks post transplantation human albumin levels are monitored using the Bethyl Laboratory Quantitative Human Albumin ELISA Kit (Catalog #E90-134) according to the manufacturer's protocol. Two microliters of whole blood is collected via the saphenous vein and immediately diluted into ELISA diluent buffer. Whole blood albumin levels of >4 mg/mL correlate with repopulation indices >90% (Azuma et al., 2007).

FACS analysis of tissues

The spleen and thymus were isolated. Each organ was placed inside a 100 μm cell strainer on top a 50 mL conical tube. Using the plunger end of a 5 mL syringe the tissues were pulverized. The cells were collected in the 50 mL tube by rinsing the plunger and strainer with 10 mL of HBSS containing 3% fetal calf serum. The cells were pelleted by centrifugation and any contaminating red blood cells were lysed and removed by additional centrifugation. Between 4 × 10^5 and 1 × 10^6 viable cells were used for staining with each antibody cocktail.

The femora and tibiae from both sides of the mouse were isolated, opened at both ends and flushed with PBS using a 27G needle attached to 1 cc syringe. The eluent was collected in a 50 mL conical tube. The cells were pelleted by centrifugation at 500 ×*g* for 10 min at 4 °C and any contaminating red blood cells were lysed using 1X RBC Lysis (eBioscience, Catalog #00433357) according to the manufacturer's protocol and removed by additional centrifugation. Between 10^5 and 1 × 10^6 viable bone marrow cells were used for staining with each antibody cocktail (Supplementary table 1).

All antibodies used in flow cytometry analysis were purchased from BD Bioscience pre-conjugated with the fluorophore.

Immunohistochemistry

At the time of harvest the liver was fixed in 10% normal buffered formalin for 48 h, rinsed and stored in 70% ethanol until paraffin embedded and sectioned. Human specific antibodies were used to detect the specific immune cells in the dual chimeric mice. Antibodies are listed in supplementary table 1.

RT-PCR

Total RNA was isolated using Trizol™ Reagent (Invitrogen, Life Technologies) from control, liver only and liver/blood chimeric FRGN mice. First strand cDNA synthesis was performed using Life Technology's high capacity RNA to cDNA™ Kit. To confirm the presence of human CD68 (hCD68) transcripts in the dual humanized mice, PCR was performed using AmpliTaq Gold 360 Master mix using the following primers; *hCD68 Forward primer*: 5′ GCCACGGTTCATCC AACAAGCA 3′ and *hCD68 Reverse primer*: 5′ CTGAGCTACA TGGCGGTGGAG 3′. Control PCR reactions using human and mouse specific GAPDH primers were also performed with the same cDNAs using the following primer pairs: *hGAPDH forward*: 5′ GAGAAGGCTGGGGCTCATT TGC 3′; *hGAPDH reverse*: 5′ ACTGTGGCGTGATGGCCGCGG 3′; *mGAPDH forward* 5′ GAGGCCGGTGCTGAGTATGTCG 3′; and *mGAPDH reverse*: 5′ CAAGAAGGTGGTGAAGCAGGCA 3′. The expected PCR products are as follows: human CD68, 376 bp, human GAPDH, 287 bp and mouse GAPDH 531 bp.

Statistics

Statistical analyses were performed using Prism 5.0d software (GraphPad Software Inc.).

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