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Higher Serum Alanine Transaminase Levels in Male Urokinase-Type Plasminogen Activator-Transgenic Mice Are Associated With Improved Engraftment of Hepatocytes but not Liver Sinusoidal Endothelial Cells

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The effects of sex on the degree of liver damage and human cell engraftment were investigated in immunodeficient urokinase-type plasminogen activator-transgenic (uPA-NOG) mice. Liver damage, measured by serum alanine transaminase (ALT) levels, was compared in male and female uPA-NOG mice of different ages. Male mice had significantly higher ALT levels than females with a median of 334 versus 158 U/L in transgenic homozygous mice, respectively. Mice were transplanted with human adult hepatocytes or fetal liver cells and analyzed for any correlation of engraftment of hepatocytes, liver sinusoidal endothelial cells (LSECs), and hematopoietic cells with the degree of liver damage. Hepatocyte engraftment was measured by human albumin levels in the mouse serum. Higher ALT levels correlated with higher hepatocyte engraftment, resulting in albumin levels in male mice that were 9.6 times higher than in females. LSEC and hematopoietic cell engraftment were measured by flow cytometric analysis of the mouse liver and bone marrow. LSEC and hematopoietic engraftment did not differ between male and female transplant recipients. Thus, the sex of uPA-NOG mice affects the degree of liver damage, which is reflected in the levels of human hepatocyte engraftment. However, the high levels of LSEC engraftment observed in uPA-NOG mice are not further improved among male mice, suggesting that a lower threshold of liver damage is sufficient to enhance endothelial cell engraftment. Previously described sex differences in human hematopoietic stem cell engraftment in immunodeficient mice were not observed in this model.

Key words: Alanine transaminase (ALT); Endothelial cells; Hepatocytes; Liver; Mice; Transgenic; Urokinase-type plasminogen activator

INTRODUCTION

Transplanting liver cells into immunodeficient mice to "humanize" the liver provides a valuable tool to study human liver biology and pathology[1,2]. Humanized mouse models are continually being improved to allow better replacement of host cells with human cells. Humanization of the liver depends on both the level of immunodeficiency of the mice and the degree to which murine hepatocytes can be depleted to allow engraftment of human hepatocytes. Severe immunodeficiencies are achieved in mice with deleterious mutations of the protein kinase, DNA-activated, catalytic polypeptide (*Prkdc*), and interleukin 2 receptor subunit γ (*Il2rg*) genes that have been bred onto the nonobese diabetic (NOD) background, providing an absence of hemolytic complement (Hc^0) as well as defects in macrophages, dendritic cells, and a permissive signal regulatory protein α (*Sirpa*) allele for human hematopoietic engraftment[3]. Combined, these mutations, characteristic of the NOD-SCID γ (NOD.Cg-$Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/SzJ; NSG) and NOD-Shi-SCID γ (NOD/Shi-*scid*/IL-2Rγ^{null}; NOG) mouse strains, prevent formation of the adaptive immune system and severely compromise innate immune responses. Some other mouse lines employ knockout mutations in the recombination-activating gene 1 (*Rag1*) or *Rag2* genes to produce mice without B or T cells that are phenotypically similar to mice with the $Prkdc^{scid}$ mutation but are less sensitive to DNA damage[4].

Different mouse strains and approaches to enhancing human hepatocyte engraftment in mice have been devised. Mouse strains developed over the last decade for liver research include fumarylacetoacetate hydrolase-deficient ($Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$) mice[5], TK-NOG mice that

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have a herpes simplex virus type 1 thymidine kinase gene expressed under an albumin promotor/enhancer[6], albumin-promoter toxin receptor-mediated conditional cell knockout (Alb-TRECK)/SCID mice[7,8], and AFC8 mice that are transgenic for an FK506-binding protein/caspase 8 fusion protein under an albumin promoter in *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice[9]. These mouse strains all share the characteristic of an inducible form of hepatocyte ablation, which in the absence of drug treatment allows for maintenance of the mouse colony in the absence of severe liver disease. In addition to achieving high levels of human hepatocyte engraftment, cotransplantation of hematopoietic cells has also yielded immune reconstitution in various mouse lines[9,10,11], thus offering the opportunity to study infectious diseases that target hepatocytes in the context of a humanized immune system.

The earliest efforts at humanization of the mouse liver were made using transgenic mouse lines with hepatocyte-directed expression of urokinase-type plasminogen activator (uPA) that allow for engraftment of human hepatocytes[12]. The constitutive expression of uPA means that early strains could be efficiently engrafted with human hepatocytes, but the mice often died from internal bleeding due to integration of multiple copies of the transgene[13–15]. In 2008, an immunodeficient mouse line was created by Suemizu et al.[16], NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} *Tg(Alb-Plau)11-4*/ShiJic (uPA-NOG), which was designed to overcome the difficulties associated with previous uPA transgenic strains. Mice homozygous for the uPA transgene are viable and can be engrafted with human hepatocytes as well as hematopoietic cells[17,18]. We have also demonstrated that these mice are permissive hosts for the engraftment of liver sinusoidal endothelial cells (LSECs) expressing human factor VIII and so can be used to study these cells as well as model cellular therapy for hemophilia A[19].

Routine breeding of uPA-NOG is performed by crossing uPA-hemizygous females with uPA-homozygous males. Serum alanine transaminase (ALT) levels are measured to deduce the genotype of the animals: hemizygous mice have normal ALT levels, and homozygous mice have notably elevated levels of ALT indicative of liver disease[16]. In this study we performed a retrospective analysis of our ALT phenotype data, which revealed a pattern of sexual differences in uPA-NOG mice that we used to evaluate the effects of variable levels of liver damage on the engraftment of different human cell types.

MATERIALS AND METHODS

Mice

Research on mice was performed with approval of the Institutional Animal Care and Use Committee at Preclinical Medevice Innovations (PMI) (San Carlos, CA, USA). Founder uPA-NOG mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan)[16] and were maintained as described. These mice were maintained by mating male homozygous and female hemizygous mice.

The levels of serum ALT were measured in young adult mice using an ALT-L3K kit (Sekisui Diagnostics PEI Inc., Charlottetown, PE, Canada) on a Cobas Mira Plus Analyzer (Roche Diagnostics, Indianapolis, IN, USA). Mice with ALT levels of ≥100 U/L were considered homozygous for the uPA transgene[19], based on the observation that hemizygous mice have ALT levels well below 100 U/L and homozygous mice have ALT levels well above 100 U/L[16]. Homozygous and hemizygous mice were then separated into cages containing up to five mice for subsequent transplantation or for use in breeding. Mice were not individually marked, preventing correlation of ALT values to engraftment in this retrospective study.

Transplantation of Human Liver Cells

Fetal livers were obtained from elective abortions with the approval of the Committee for Human Research at the University of California, San Francisco, and written consent of the women undergoing the abortion. The gestational age of the anonymous specimens was estimated based on foot length. Midgestation specimens ≤24 weeks of gestation were obtained for this study. Procurement, transport, and cell isolation from fetal livers were performed as previously described[20]. Briefly, fetal livers were procured shortly after the abortion procedure and stored on ice for transport to the laboratory. Enzymatic digestion was used to isolate fetal liver cells, which were depleted of cluster of differentiation 235 alpha-positive ($CD235a^+$) erythrocytes using immunomagnetic beads. Mice were transplanted with cells from a single fetal liver; the cells were never pooled from multiple livers for transplant. Six different fetal liver specimens were used to transplant the mice in this report.

Cryopreserved adult hepatocytes were obtained from Lonza (Walkersville, MD, USA) and Lifeline Cell Technology (Frederick, MD, USA). Mice (4–17 weeks old) were transplanted by intrasplenic injection under deep inhalation anesthesia as per a previously described method[19].

Analysis of Transplanted Mice

Human albumin was measured from mouse serum using the Human Albumin enzyme-linked immunosorbent assay (ELISA) Quantitation Set from Bethyl (Montgomery, TX, USA). Serum was collected from transplanted mice ranging from 4 to 32 weeks after transplant. Serial measurements were made for some cohorts of mice. The ELISA substrate 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Waltham, MA, USA) was used for signal detection, which is based on the detection of horseradish peroxidase activity using the chromogen

3,3′,5,5′-tetramethylbenzidine (TMB). Absorbance was measured at 450 nm using a CLARIOstar plate reader from BMG LABTECH Inc. (Cary, NC, USA).

Flow cytometric analysis of human cell engraftment in the livers of mice was performed using reagents and methods previously described[19,21]. Briefly, livers were digested with collagenase IV (Life Technologies, Grand Island, NY, USA), washed, and stained for mouse and human antigens. Cells were stained in blocking buffer consisting of phosphate-buffered saline with 5% mouse serum (Gemini Bio Products Inc., Woodland, CA, USA), 2 μg/ml rat anti-mouse CD16/CD32 (clone 93; BioLegend, San Diego, CA, USA), and 0.01% NaN_3 (Sigma Chemical Co., St. Louis, MO, USA). Liver cells were stained with fluorescent dye-labeled monoclonal antibodies recognizing mouse CD45 (clone 30-F11), mouse TER-119 (clone TER-119), mouse H2-K^d (clone SF1-1.1), human β2-microglobulin (B2M; clone 2M2), human CD14 (clone HCD14), human CD45 (clone H130), and human leukocyte antigen (HLA)-ABC (clone W6/32); all antibodies were purchased from BioLegend. Stained cells were washed twice and suspended in phosphate-buffered saline with 0.3% bovine serum albumin (Roche Diagnostic), 0.01% NaN_3, and 2 μg/ml propidium iodide (Invitrogen, Carlsbad, CA, USA). Samples were analyzed on a BD LSRII flow cytometer (Becton Dickson, San Jose, CA, USA). Data were analyzed using FlowJo software (FlowJo Inc., Ashland, OR, USA). Strong staining with propidium iodide and/or small size based on forward light scatter was used to exclude dead cells and debris from analyses.

Analysis of Gene Transcription in the Liver

Total mRNA was isolated from ~30 mg of mouse liver tissue using RNeasy Mini Kit (Qiagen, Germantown, MD, USA) with the RNAse-free DNase set (Cat. No. 79254; Qiagen). cDNA was synthesized using iScript™ cDNA Synthesis Kit (Cat. No. 1708890; Bio-Rad Laboratories Inc., Hercules, CA, USA).

Gene expression by mouse liver cells of albumin (*Alb*), *B2m*, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was measured by real-time PCR amplification using Applied Biosystems' TaqMan® Gene Expression Assays (Thermo Fisher Scientific). *Alb* was detected using assay identification number Mm00802090_m1 yielding an 89-bp amplicon (NM_009654). *B2m* was detected using assay identification number Mm00437762_m1 yielding a 77-bp amplicon (NM_009735). Gene expression of *Gapdh* was measured by real-time PCR using primers *MusGapdh* F (5′-GCACCACCAACTGCTTAGCCC-3′) and *MusGapdh* R (5′-TCTTCTGGGTGGCAGTGAT G-3′), and 6-carboxyfluorescein (FAM)-labeled probe *MusGapdh* P (5′-TTGTGGAAGGGCTCATGACCAC AGTCC-3′), targeting a 105-bp amplicon. Amplification of *Alb* and *B2m* was performed in a final volume of 15 μl containing PCR buffer, 3.7 mM $MgCl_2$, 1× TaqMan® Gene Expression Assay (Thermo Fisher Scientific), 833 μM each deoxynucleoside triphosphate (Bioline, Tauton, MA, USA), 0.7 U FastStart™ Taq DNA polymerase (Roche Applied Science, Penzberg, Germany), and 5 μl of a 1:10 dilution of the cDNA. Amplification of *Gapdh* was performed the same as for *Alb* and *B2m*, with the exception of the following concentrations: 3.8 mM $MgCl_2$, 667 nM each primer, and 133 nM probe. Each real-time PCR was performed in triplicate amplification wells using the LightCycler 480 instrument (Roche Applied Science) or the 7500 System (Thermo Fisher Scientific). The PCR cycle conditions were as follows: 95°C for 1 min followed by 45 cycles of amplification (95°C for 30 s, 60°C for 1 min). *Alb* expression was quantified using the comparative C_T method relative to the internal controls *B2m* and *Gapdh*.

Statistical Analysis and Data Presentation

Data are presented as notched box and whisker plots in which the notch indicates the median (second quartile) and whiskers extend to the extreme data points. The bottom of the box represents the first quartile and the top the third quartile. Means are shown as diamonds on the plots. The nonparametric Mann–Whitney *U* test was used to determine the significance of differences in the data, with $p \leq 0.05$ considered significant. Statistical analyses and charting were performed using Aabel software (Gigawiz Ltd. Co., Tulsa, OK, USA).

RESULTS

ALT Levels in uPA-NOG Homozygous Mice

ALT levels were measured in 992 mice representing a mixture of uPA-hemizygous and -homozygous mice. Males were found to have higher ALT levels than females (median: 139.5 vs. 53 U/L; $p < 0.001$) (Fig. 1A). Among selected mice with ≥100 U/L ALT, considered homozygous, males also had higher ALT levels than females (median: 334 vs. 158 U/L; $p < 0.001$) (Fig. 1B). These differences were apparent up to 135 days of age.

Engraftment of Adult Human Hepatocytes

Seventy-eight mice were engrafted with cryopreserved human hepatocytes. Human albumin levels in males overall were 9.6-fold higher than in females, indicating higher engraftment (Fig. 2A). Since multiple albumin measurements were made for some mice, the highest human albumin measurement was chosen for the comparison shown in Figure 2A. In Figure 2B, data are shown for the same cohort of mice subdivided based on the time after transplant when the albumin measurements were made. For all analyses ≥8 weeks after transplantation, the levels of albumin were significantly higher in males than in females.

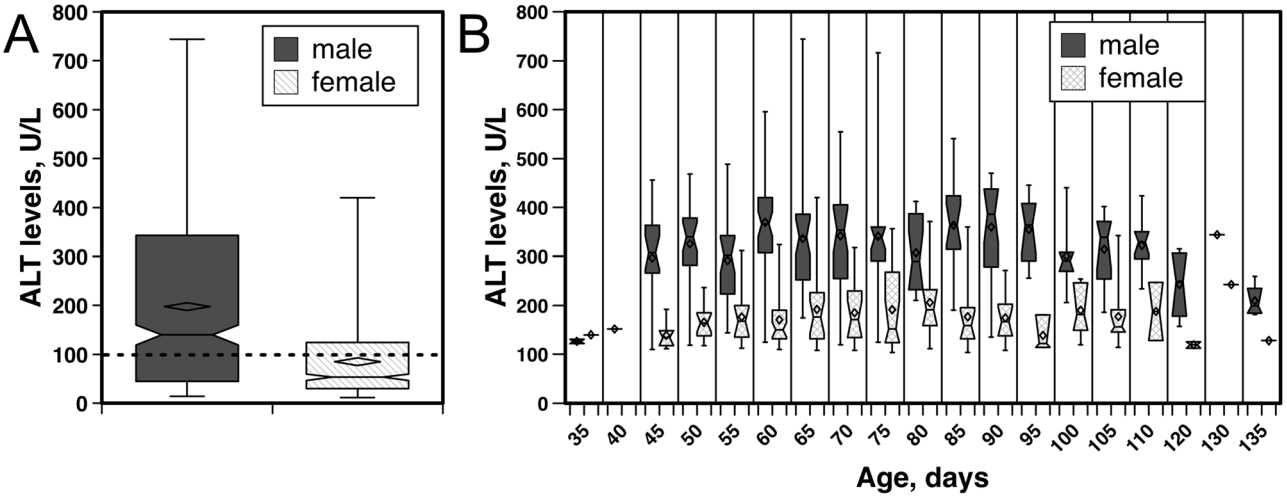


Figure 1. Alanine transaminase (ALT) levels in immunodeficient urokinase-type plasminogen activator-transgenic (uPA-NOG) mice. (A) Total ALT levels in hemizygous and homozygous mice. A total of 516 males and 476 females were evaluated. The dashed line shows a threshold of 100 U/L used for the selection of homozygous mice. (B) ALT levels of homozygous uPA-NOG mice grouped by age. A total of 308 males and 178 females were analyzed.

We also evaluated engraftment of adult hepatocytes based on the source, age, and type of hepatocyte preparation. Hepatocytes from different sources varied in their ability to engraft (Fig. 2C). Normal human primary hepatocytes (hNHEPS) were purchased from Lonza from three different donors (Fig. 2D). These cells are recommended for a number of basic research studies including drug metabolism, pharmacology, and toxicology studies. The hNHEPS preparations are tested to yield viable adherent cells that synthesize albumin. Engraftment was observed with all three donors, and peak human albumin levels are indicated in Figure 2C, as for Figure 2A. Another source of hepatocytes with the capacity for long-term adherent cell growth and suitable for metabolic studies was obtained from Life Line Cell Technology. Mice transplanted with these inducible primary hepatocytes also engrafted. Interestingly, the oldest donors yielded the highest overall albumin levels, in contrast to the often-held belief that younger hepatocytes offer proliferative advantages that make these cells the best choice of donor tissue. However, it is not possible to draw firm conclusions from only four donors. A fifth source of cells suitable only for short-term suspension culture was also transplanted but yielded negligible engraftment. The tendency for higher engraftment in males was observed in the four transplant groups with clear human albumin production and was significant in three of these four cohorts.

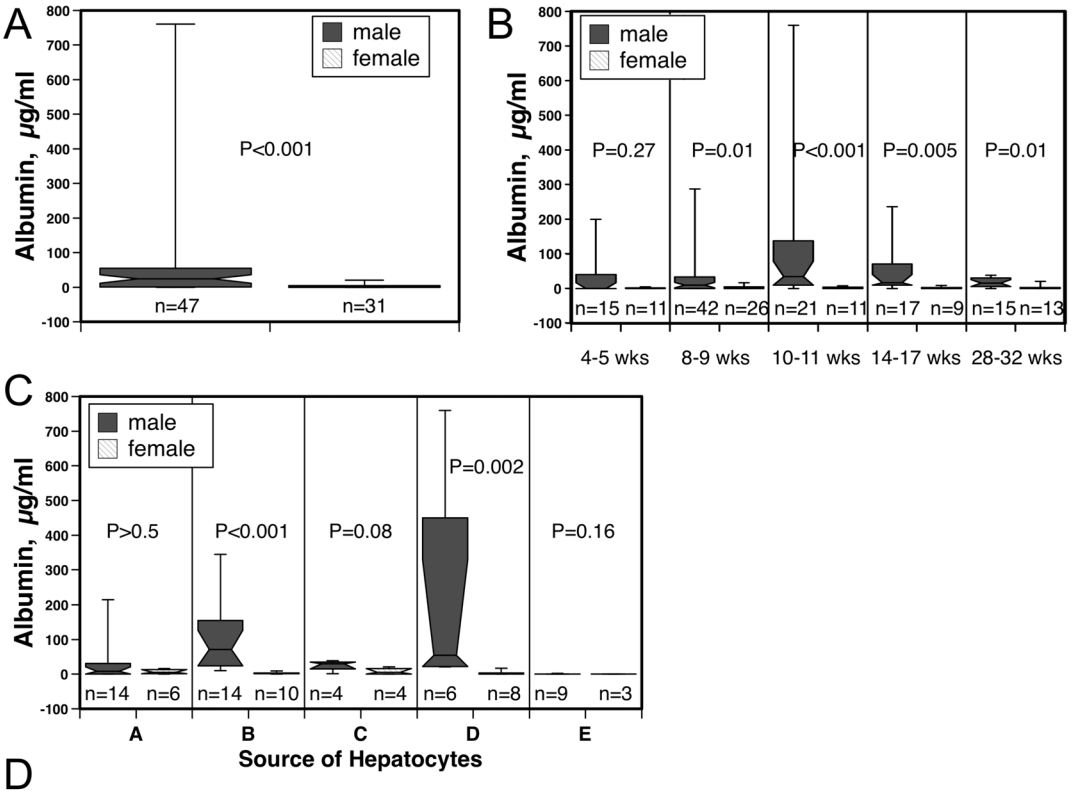
Engraftment of Fetal Liver Cells in uPA-NOG Mice

Noticing that the higher engraftment of adult human hepatocytes in uPA-NOG males was associated with higher ALT levels, we decided to reanalyze previously published results[19] from human fetal liver transplants to investigate any correlation that may exist between recipient sex and engraftment. We have shown that human fetal liver cells transplanted to the uPA-NOG mice resulted into LSEC engraftment in the liver and hematopoietic engraftment in the liver, bone marrow, and spleen[18,19]. Human engraftment in the liver was defined by expression of the pan-human marker B2M and low to no staining of a combination of mouse markers: CD45, TER-119, and H2-K^d (Fig. 3A). Human B2M$^+$ cells were divided into two populations: CD14$^+$ LSECs and CD45$^+$ hematopoietic cells. Percentages of total human cells, LSECs, and hematopoietic cells were compared between male and female recipients. No significant differences were observed in the engraftment of B2M$^+$ cells ($p>0.5$), CD14$^+$ cells ($p=0.49$), and CD45$^+$ cells ($p>0.5$).

Hematopoietic engraftment in the bone marrow defined by expression of human CD45, or pan-human marker HLA-ABC, versus mouse markers CD45, TER-119, and H2-K^d (Fig. 3B) also did not differ between male and female hosts ($p=0.36$ for CD45$^+$ cells and $p>0.5$ for HLA-ABC$^+$ cells).

Analysis of the Effects of Sex on Mouse Alb *Gene Expression*

Since the uPA transgene is under the control of the Alb promoter[16], we tested the possibility that differences in *Alb* expression exist between male and female mice that could account for greater expression of uPA and liver damage in male mice. However, when Alb



	Vendor	Cat #; Lot	Cells	Donor
A	Lonza	CC-2591; 8F3379	hNHeps - Human Hepatocytes	Male, 21 months
B	Lonza	CC-2591; 7F3475	hNHeps - Human Hepatocytes	Female, 41 years
C	Lonza	CC-2591; 9F3003	hNHeps - Human Hepatocytes	Male, 2 years
D	Life Line Cell Technology	HC-0012; 2239	Inducible Human Primary Hepatocytes	Male, 55 years
E	Life Line Cell Technology	HC-0010; 2254	Human Primary Hepatocytes for suspension culture	Male,19 years

Figure 2. Engraftment of uPA-NOG mice with human cryopreserved adult hepatocytes. (A) Peak human albumin levels in the serum of male and female mice. (B) Human albumin levels in the serum of male and female mice at different times after transplantation. Peak measurements for individual mice were selected from among these data for comparison in (A) and (C). (C) Peak human albumin levels in male and female mice transplanted with different sources of hepatocytes. (D) Sources of hepatocytes shown in (C).

expression was compared to *B2m* and *Gapdh*, no significant differences were observed between male and female mice (Fig. 4). Liver specimens from untransplanted male and female mice were analyzed. Data are combined from 5 homozygous uPA-NOG, 18 hemizygous uPA-NOG mice, and 5 TK-NOG mice. All mice shared the same NOG genetic background[6,16]. No striking differences in *Alb* expression were observed between the homozygous uPA-NOG mice, with liver damage, and the hemizygous uPA-NOG and TK-NOG mice with no or minimal liver damage (data not shown). Thus, *Alb* expression appears to be similar in male and female mice on the NOG background.

DISCUSSION

Choosing the appropriate animal model for an experiment is an important choice in experimental medicine. Recently, concerns of neglecting sex as an important variable in biomedical research have been raised[22]. Lack of information about the sex of experimental animals can cause problems with reproducibility in scientific studies and dangerous bias in preclinical studies[23]. The US

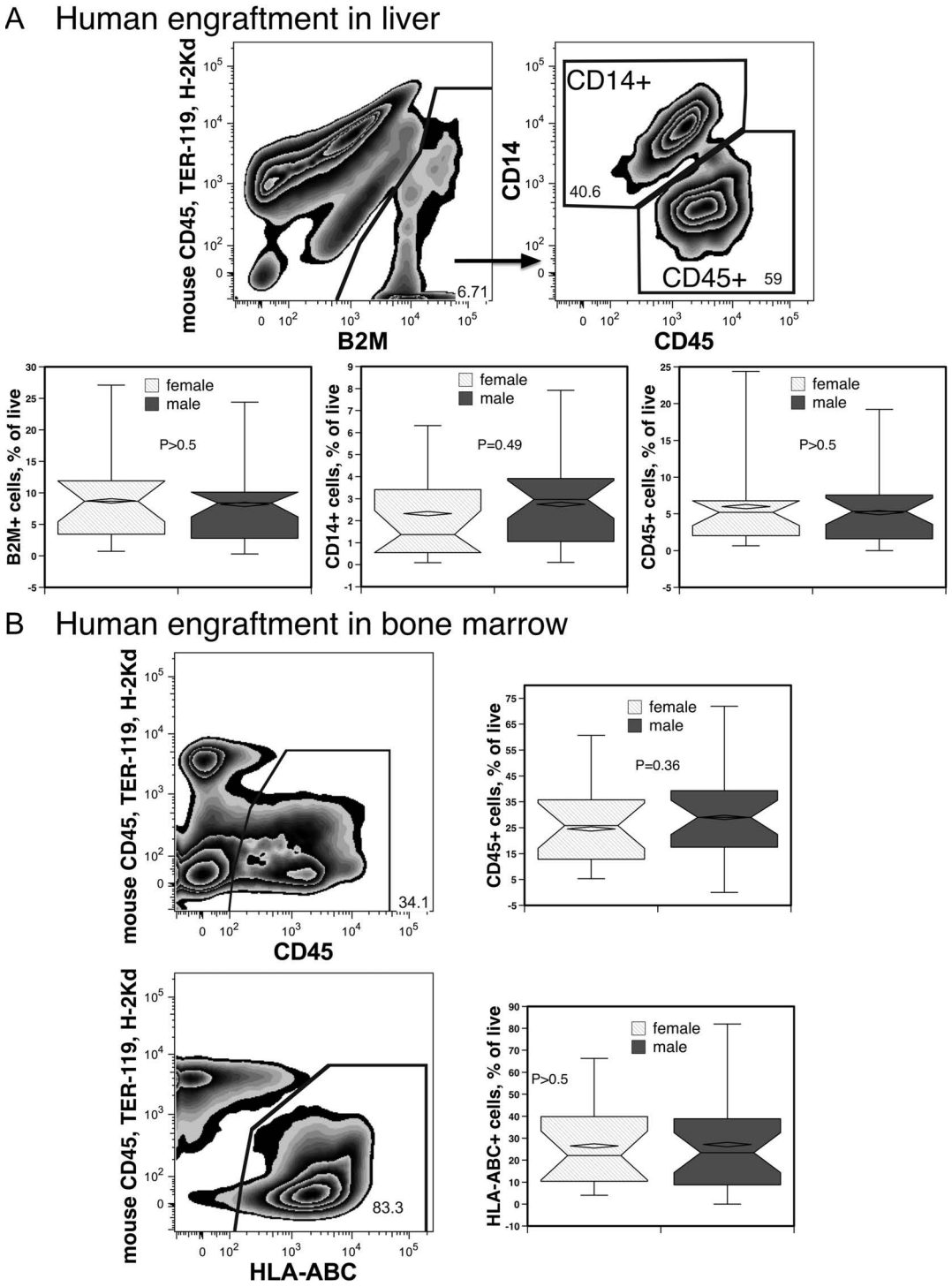


Figure 3. Engraftment of human fetal liver sinusoidal endothelial cells (LSECs) and hematopoietic cells. (A) Engraftment in the liver and (B) bone marrow. A total of 17 females and 27 males were studied. Human markers: B2M, β2-microglobulin; CD14, cluster of differentiation 14 (LSECs); CD45, hematopoietic cells; HLA-ABC, human leukocyte antigen-ABC. Mouse markers: H2-K^d; TER-119, erythroid marker.

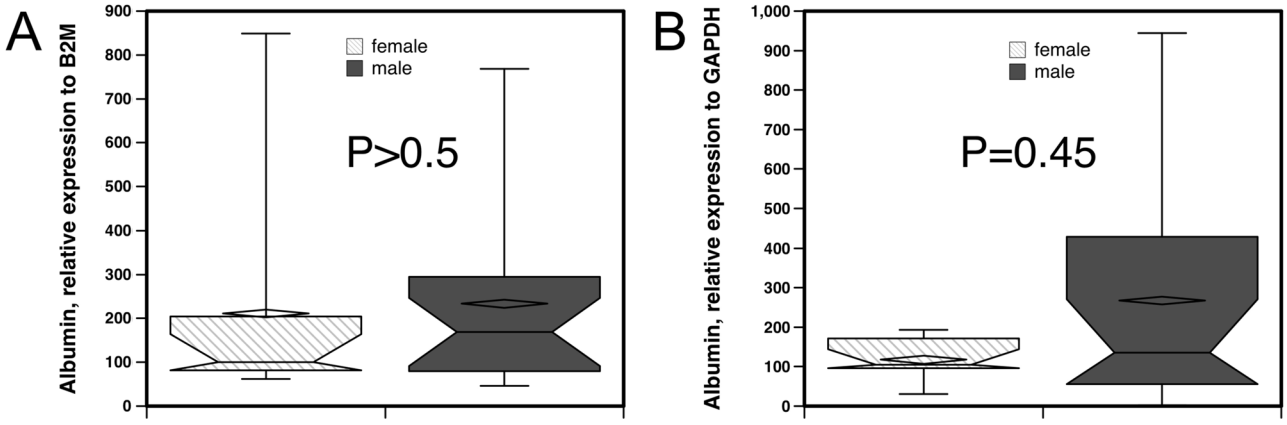


Figure 4. Comparison of albumin (*Alb*) expression in male and female mice. *Alb* expression was compared to (A) *B2m* and (B) glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression. A total of 9 female and 19 male mice were studied ranging in age from 51 to 396 days.

National Institutes of Health has developed new polices requiring applicants to balance males and females in their funded studies and to provide information about the sex of animals in publications[24]. Exceptions can be applied for models that are specific for one sex or when otherwise justified[23].

We performed many transplants of uPA-NOG mice using both male and female recipients unaware that there may be a difference in liver damage between the sexes. Our retrospective analysis clearly shows that male uPA-NOG mice have higher ALT levels correlating with higher engraftment of human hepatocytes than female mice. Serum ALT levels reflect the level of liver damage and, strikingly, predict an engraftment advantage for donor hepatocytes even given the modest differences in ALT levels between male and female homozygous uPA-NOG mice. Expression of the uPA transgene inside hepatocytes causes colocalization of plasminogen and plasminogen activator in the rough endoplasmic reticulum, thought to result in irreparable proteolytic damage of hepatocytes[25]. A number of transgenic mice with liver-directed uPA expression have been created, but, to our knowledge, there are no reports showing higher expression of the uPA transgene in males compared to females. We also found no data reporting elevated ALT levels in male uPA mice. Kato et al. observed that *uPA*/SCID males engrafted better with human hepatocytes, having higher levels of human serum albumin than females, but ALT levels were not reported[26]. Additionally, we observed no differences in *Alb* expression between male and female NOG mice, which could have otherwise accounted for differences in uPA expression under the Alb promoter, thus suggesting that other mechanisms are involved in the greater liver damage observed in males.

Sex differences in liver enzymes in healthy rodents and in rodents exposed to hepatotoxic substances have been observed. In a recent screen of common wild-type mouse strains, Otto et al. observed a number of differences in the levels of plasma proteins between male and female mice, including a significant 15% higher level of ALT in male mice[27]. Additionally, female Fisher 344 rats are more resistant to hepatic toxicity induced by the herbicide diquat than males, according to plasma ALT levels[28]. Male Wistar, Long–Evans, and Sprague–Dawley rats given a methionine choline-deficient diet had higher ALT levels and developed steatohepatitis to a greater degree than females[29]. Additionally, higher ALT levels were also observed in male rats, compared to females, after bile duct ligation[30]. Interestingly, analysis of healthy human adolescents also reveals higher ALT levels in males[31]. The same pattern of differences was also observed in patients with hepatitis C[32], hepatitis B[33], and adolescents with metabolic syndrome[34]. Together, these findings point to a sex-based difference in ALT levels that is shared by rodents and humans, and there is speculation that sex hormones may play a role in these differences[30].

uPA-NOG mice are more easily engrafted by LSECs than immunodeficient mice lacking the uPA transgene[18]. No differences between male and female recipients were observed in regard to LSEC engraftment. The mechanism behind the ease of engrafting LSECs in uPA-NOG mice is not known, but we hypothesize it may be due to the elevated levels of vascular endothelial growth factor measured in the livers of homozygous uPA-NOG mice[19]. This study suggests a threshold effect whereby LSEC engraftment is not further enhanced with greater hepatocyte damage.

Human hematopoietic stem cell engraftment has been reported to be more efficient in female immunodeficient mice than in males[35,36]. Chevaleyre et al. explain this effect, partially, by the fact that females are smaller than males and therefore received a higher cell dose relative

to body weight[36]. In our study, both sexes of uPA-NOG mice engrafted with human hematopoietic cells at similar levels despite uPA-NOG females having lower body weights than males (data not shown). A noteworthy difference between our study and past reports is that our recipient mice did not receive any cytoablative treatment prior to transplant, which reduces the levels of human engraftment that can be achieved. Nonetheless, we have retrospectively compared engraftment in three experiments in which irradiated NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice were transplanted intravenously and found no significant differences between male and female hosts (M.O.M., unpublished data). Both Notta et al.[35] and Chevaleyre et al.[36] used donor cells prepared from umbilical cord blood, whereas our cells were midgestation liver or bone marrow cells. We have observed greater engraftment using fetal cells compared to neonatal cells and speculate that the superior growth of fetal cells may mask any modest effect of the sex of the host mice.

This study is an example of the importance of performing sex-balanced experiments and evaluating outcomes by the sex of the animal. However, future studies employing hepatocyte transplantation in uPA-NOG mice may be justified in using only male mice to favor higher engraftment levels. Understanding the biological mechanisms underlying sex differences in liver pathology is worthy of more study.

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