Published in final edited form as:

J Immunol Methods. 2014 May ; 407: 127–134. doi:10.1016/j.jim.2014.04.008.

Production of bone marrow, liver, thymus (BLT) humanized mice on the C57BL/6 *Rag2*^{-/-}*γc*^{-/-}*CD47*^{-/-} background

Kerry J. Lavender, Ronald J. Messer, Brent Race, and Kim J. Hasenkrug*

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840, USA

Abstract

C57BL/6 *Rag2*^{-/-}*γc*^{-/-}*CD47*^{-/-} triple-knockout mice engrafted with fetal human bone marrow, liver, thymus (TKO-BLT) not only develop high levels of multi-lineage hematopoiesis but also organized lymphoid tissues including mesenteric lymph nodes, splenic follicles and gut-associated lymphoid tissues of human origin. A unique advantage of these mice is that they sustain human cell and tissue engraftment long-term without the development of graft versus host disease. Thus they can be used for long-term studies not previously feasible with other models. The production of TKO-BLT mice to obtain healthy mice with high level reconstitution of human cells and tissues requires specialized methods that are presented in detail.

Introduction

The study of many human diseases, especially infectious diseases, would be greatly facilitated by the development of small animal models that closely recapitulate human immune systems. We recently described the development of a new humanized mouse model in which immunodeficient mice are reconstituted with a functional human immune system. Results showed that C57BL/6 *Rag2*^{-/-}*γc*^{-/-}*CD47*^{-/-} Triple Knock Out (TKO) mice that are bone marrow, liver, thymus (BLT)-humanized become highly reconstituted with human immune cells and tissues (Lavender et al., 2013). Furthermore, these TKO-BLT mice are susceptible to infection with HIV and develop HIV-specific immune responses. Importantly, especially for long-term studies, TKO-BLT mice appear resistant to developing clinical signs of graft versus host disease as far out as 29wpt, a complication that commonly occurs in other humanized mouse models as early as 12wpt (Greenblatt et al., 2012). Whether this is an inherent quality of the TKO strain or is due to particular aspects of our humanization procedure remains to be determined.

In this report, the methodological procedures necessary to produce TKO-BLT mice are presented. These include surgical procedures for tissue transplantation with minimal animal mortality due to infection, anesthesia or tissue trauma, a laboratory protocol to enrich

*Corresponding author: Kim J. Hasenkrug khasenkrug@nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

progenitor cells from the donor liver and determination of the appropriate irradiation dose to produce a sufficient niche in the bone marrow for transplanted human precursors to engraft without causing excessive damage to supporting tissues. Also discussed is the optimal number of hematopoietic progenitor cells to transfuse in order to achieve ample engraftment without sacrificing the size of the cohort established from a single tissue donor. The methods in this manuscript will help prevent duplication of the empirical work done to optimize this humanization protocol and maximize the future success of others endeavoring to produce TKO-BLT mice.

2. Materials and Methods

2.1 Mice

To create the TKO mice, C57BL/6 $Rag2^{-/-} \gamma_c^{-/-}$ mice (Goldman et al., 1998; Garcia et al., 1999; Mazurier et al., 1999) were crossed with CD47 null B6.129-CD47^{tm1Fpl/J} mice (Jackson Laboratory, Bar Harbor, ME) and F1 $Rag2^{+/-} \gamma_c^{-/Y} CD47^{+/-}$ males were backcrossed with C57BL/6 $Rag2^{-/-} \gamma_c^{-/-}$ females to produce F2s. Mating of F2 $Rag2^{-/-} \gamma_c^{-/-} CD47^{+/-}$ females and $Rag2^{-/-} \gamma_c^{-/Y} CD47^{+/-}$ males produced the $Rag2^{-/-} \gamma_c^{-/-} CD47^{-/-}$ (TKO) strain. TKO mice may be obtained through NIAID's exchange program operated by Taconic (Hudson, NY) (stock #8470). TKO mice are highly immunodeficient and were maintained on oral antibiotics (such as a combination of Sporanox & Baytril) and acidified water to prevent infections until they were fully reconstituted with human immune systems. Animals were housed under specific pathogen-free conditions using filter-cover cages. Experiments were performed in accordance with the regulations and guidelines of the Animal Care and Use Committee of Rocky Mountain Laboratories, NIAID, NIH.

2.2 Screening for CD47^{-/-} mice

Screening for homozygous CD47 knockout was done by testing blood by flow cytometry. Briefly, 1–5µl of whole blood was collected in heparin and directly stained with anti-mouse CD47, clone MIAP301 (BioLegend, San Diego, CA). CD47 is expressed on red blood cells so no separation of lymphocytes was necessary. TKO mice are CD47 negative. A PCR protocol for CD47 typing is also available from Jackson Labs.

2.3 Preparation for transplantation

Six to 10-week-old mice were used for humanization. Seven to eight week old mice were generally excellent hosts. No more than 24 hours before surgery the mice were anesthetized, shaved over their left flank overlying the kidney, and gavaged with 100µl of water containing 4mg of ascorbic acid. Treatment with ascorbic acid prevents lethal gastrointestinal syndrome resulting from high dose irradiation (Yamamoto et al., 2010). Shaving the mice the day before surgery allowed them to shed the cut hair and made it easier to keep a sterile surgical area. On the morning before surgery the mice were irradiated with either 5.0Gy or 7.5Gy (as indicated) whole body irradiation in a Cs-137 gamma irradiator using an irradiation attenuator (JL Shepherd and Associates) and ambient air ventilation.

2.4 Surgical preparation and anesthesia

We used a team of at least four people to perform surgery, each member at a dedicated station. One member performed prep and anesthesia, one performed surgery, one closed the incision, and one handled anesthesia reversal and recovery. An additional team member prepared stem cells in the lab while surgery was being performed (see below). For anesthesia, the shaved, gavaged and irradiated mice were weighed and administered a solution of 85mg/kg of ketamine and 12mg/kg Xylazine in saline by intraperitoneal injection using a 25ga needle. The anesthetized mice were then placed flat on their right side on a sterile, foam surgical platform and the shaved surgical site washed with antiseptic surgical scrub (Nolvasan, Pfizer, New York, NY) taking care not to over-wet the animals' fur. Artificial tear ointment (Optixcare, CLC Medica, Waterdown, ON, Canada) was then applied to the eyes to prevent dehydration. Fully anesthetized mice (no toe-pinch reflex) were then sterilely draped and placed before the surgeon. Seventeen to 22 week gestational tissues (Advanced Bioscience Resources) were prepared in a biological safety hood by cutting the tissues into 1–2mm³ pieces and placing them in a covered petri dish on ice in matrigel (BD Biosciences, San Jose, CA). The matrigel not only promoted tissue engraftment but also polymerized rapidly at body temperature, which kept the tissue in place after insertion and also helped seal the kidney capsule. The tissues were divided to give the surgeons approximately the same amount of liver tissue as thymus. The remainder of the liver was used to isolate hematopoietic stem cells for injection following recovery from surgery.

2.5 Tissue transplantation surgery

Surgery was performed on a sterile platform with sterile instruments, and surgeons wore surgical garments, head covers, shoe covers, masks, and sterile gloves. Tissue was transplanted under a single kidney for each animal. The kidney was visualized through the skin just below the rib cage and a 6–8mm longitudinal incision (approximately the same length as the kidney) was made through the skin and body wall directly over the kidney by first tenting the skin with forceps and using a single snip of the scissors before elongating the opening to produce a smooth edged incision through first the skin and then the body wall. A small retractor was inserted into the incision and the kidney capsule was gently nicked with a 25ga needle, taking care to avoid cutting the kidney itself. The nicked edge of the capsule was then very gently lifted using small rat tooth forceps before a sequencing pipette tip, that had the end closed and rounded with a flame, was inserted gently under the capsule to make a pocket for the transplanted tissue. A single piece of tissue approximately 1–2mm³, each from the thymus and liver, was then pressed into the pocket under the capsule using a similarly altered sequencing pipette tip. Care was taken not to tear the capsule and to ensure the tissue was placed securely in the capsule pocket.

The body wall was closed by suture and one or two surgical staples closed the skin. Pain management was provided via skin nerve block with local bupivacaine (Hospira, Lake Forest, IL) infiltration. The mice were then injected intraperitoneally with the anesthesia reversal drug atipamezole (1mg/kg in sterile water) (Pfizer, New York, NY) using a 25ga needle and were placed surgical side up in a clean cage. To prevent anesthesia-induced hypothermia, the cages were placed on a 37°C water table until the mice awoke.

2.6 Stem Cell Transplantation and Bone Marrow Reconstitution

Within 4 hours of surgery the mice were lightly anesthetized by isoflurane inhalation and injected subcutaneously with 0.1mg/kg buprenorphine for pain management and 25–50mg/kg ceftriaxone antibiotic (MWI, Boise, ID) to control any infection (donor tissue may be contaminated with bacteria...see stem cell isolation section). At this time the anesthetized mice were also injected intravenously via the retro-orbital sinus with a 0.5ml phosphate buffered balanced salt solution (PBBS, such as Dulbecco's) containing 7.5U heparin (Solopak Laboratories, Boca Raton, FL) and $\sim 3 \times 10^5$ to 3×10^6 human hematopoietic stem cells (maximum depending on yields from donors and based on the humanization protocol of Melkus et al.)(Merkus et al., 2006) supplemented with 0.5 to 2×10^6 TKO mouse bone marrow cells. Both injections were done using a 25ga needle. The mouse bone marrow cells were needed to prevent anemia as human red blood cells do not develop in these mice. The transplanted bone marrow cells take some time to re-seed the bone marrow and develop red cells so the transplant recipients received additional intravenous transfusions of red cells and precursors using whole splenocytes from TKO mice at 3 and 6 days post-transplantation. One TKO spleen provided sufficient erythrocytes for 20 mice. Cells were transfused into the retro-orbital sinus of mice under isoflurane anesthesia using a 25ga needle. Two transfusions of total splenocytes was typically sufficient but the mice were monitored daily for signs of anemia, evident by lethargy and loss of coloration in the feet and/or nose and mouth. Additional transfusions were carried out as required. The splenocytes were prepared by grinding the spleens through a 100 μ m cell strainer (Fisher Scientific, Waltham, MA) in PBBS. Heparin (7.5U/mouse) was added prior to injection to prevent cell clumping, which could have caused pulmonary emboli. Mice were monitored daily and any mice that displayed signs of distress such as limited mobility or unwillingness to take food or water were examined and treated. Additional buprenorphine for pain control was administered up to 3x per day as needed. Antibiotics were given by subcutaneous injection daily for at least two days. If 100 μ l of medium from the tissue samples gave from zero to a few bacterial colonies on a blood agar plate the sample was considered minimally contaminated, but the mice were nonetheless monitored closely for signs of infection including failure to thrive, lethargy and/or poor hair coat. Mice that displayed such clinical signs or that had more extensive bacterial contamination on the plates were treated with antibiotics for up to seven days.

2.7 Isolation of hCD34⁺ Hematopoietic Progenitor Cells (HPC) from Fetal Liver

One hundred microliters of fluid was withdrawn from the tube containing each fetal tissue and spread onto a blood agarose plate. Plates were incubated overnight at 37°C to check for bacterial contamination.

A section of liver sufficient for transplantation was set aside and the remaining liver was cut into very small pieces, dissecting out any obvious connective tissue, the gall bladder and bile duct for disposal. The minced tissue was then transferred into a sterile container of medium (RPMI-1640 (Invitrogen Carlsbad, CA) plus 10% fetal bovine serum (JR Scientific, Woodland, CA)) supplemented with 1mg/ml collagenase (Roche, Basel, Switzerland) and 0.5U/ml DNase I (New England Biolabs, Ipswich, MA). The tissue was incubated for 1hr at 37°C with gentle stirring and pipetting every 15 minutes to disrupt the tissue. After one hour

the tissue was poured over a sterile metal screen (#60 from Small Parts Inc.) into a sterile beaker at room temperature. Residual tissue was mashed through the screen and then the screen was thoroughly washed to collect as many cells as possible. The liquid was then transferred to 50ml conical tubes, centrifuged and the pellets were combined and pushed through 100µm cell strainers (Fisher Scientific). After centrifugation, a Percoll solution (see below) was added to the cell pellet and mixed well followed by centrifugation at 900rcf for 10min with the brake turned off. Dead cells that formed a layer on the top of the supernatant were carefully removed prior to decanting the remaining liquid. Cells were then resuspended in 50ml MACs buffer (Miltenyi, Auburn, CA) and counted using a glass hemocytometer. After labeling the cells as described in the CD34 Cell Isolation kit protocol (Miltenyi), a 30µm cup filcon (BD Biosciences) inserted into the top of a LS column (Miltenyi) was prepared for approximately every 7×10^8 cells. Column and cup filcon were washed at the same time. Labeled cells were passed over the columns as indicated in the Miltenyi protocol. Once the columns had undergone their final wash, the CD34⁺ fractions were ejected from each column into a 50ml conical containing PBBS. The cells were counted and combined with sufficient TKO bone marrow for the number of mice being transplanted. Cells were then centrifuged and resuspended in PBBS at a volume of ~500µl/mouse including heparin at 15U/ml (SoloPak).

Percoll solution:

- 5.25ml diluted Percoll (Amersham, Pittsburgh, PA) (9 parts Percoll to 1 part 10x PBS)
- 9.75ml PBBS
- 97.5ml 1M Hepes (Life Technologies, Carlsbad, CA)
- 0.01g Heparin (Hospira, Lake Forest, IL)

2.8 Characterization and in vivo tracking of fetal liver HPC

A small aliquot (50–100µl) of the eluted cells was stained with anti-human CD34 APC (clone 581/CD34, Biolegend) and anti-human CD38 PE (clone HB7, eBioscience, San Diego, CA) to assess purity. For tracing experiments a 2.5mM stock solution of CellTrace Violet (Invitrogen) was diluted 1000-fold in serum-free medium containing HPC at 5×10^7 /ml for 10min at 37°C. The reaction was quenched using an equal amount of 10% FBS containing medium before washing and resuspending the cells in PBBS plus heparin (SoloPak) prior to intravenous injection into mice. Tissues from transfused mice were harvested 24 hours later and processed for analysis as previously described (Lavender et al., 2013).

2.9 Isolation of human blood leukocytes and phenotypic analysis

Blood was collected into heparin (15U heparin/ml of blood) by retro-orbital puncture. The initial blood volume that was collected was recorded prior to addition of 1ml of 10x RBC Lysis Buffer (BioLegend) followed by dilution with 9ml of dH₂O. The lysed blood was centrifuged and washed once in PBBS. The supernatant was decanted and the cells were resuspended in the residual diluent. The volume was measured and a 2-fold dilution of the

cells was counted using a standard hemocytometer with Neubauer ruling (0.1mm³ volume) and the count determined using the formula:

$$\frac{\text{Cell count} * 2 \times 10^4 * \text{count volume } (\mu\text{l})}{\text{Initial blood volume } (\mu\text{l})}$$

Purified cells were stained with directly conjugated anti-human CD45-V500, CD3-V450 and CD19-PerCP-Cy5.5 to identify human blood subsets (BD Biosciences). The human blood cell reconstitution/ml of blood was calculated from the total blood count based on the frequency of CD45⁺ cells in the FSC/SSC live gate. Tissues were fixed in 10% formalin then placed in cassettes and processed with a Sakura VIP-5 Tissue Tek, on a 12 hour automated schedule, using a graded series of ethanol, xylene, and ParaPlast Extra. Embedded tissues were sectioned at 5µm and dried overnight at 42°C prior to staining

2.9 Statistical Analysis

Statistical calculations were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).

3. Results

3.1 Purified HPC from fetal liver are primarily CD34⁺CD38⁻ and traffic to multiple tissues after intravenous injection

A critical step in the production of the model was the successful isolation and transfusion of HPC to recipient mice. To calculate the number of fetal liver HPC transplanted to each mouse from the total number of viable cells injected, a small aliquot of the magnetic bead-enriched cells was stained with anti-human CD34 and CD38 for analysis by flow cytometry. Results showed that the technique yielded an enriched population of CD34⁺ progenitor cells, with a majority expressing the CD34⁺CD38^{lo} phenotype of hematopoietic stem cell/multipotent progenitors (HSC/MPP) (Fig. 1A). To determine whether the transfused HPC migrated to important sites of hematopoiesis such as the bone marrow, enriched fetal liver HPC were fluorescently labeled prior to intravenous injection and multiple tissues were harvested 24hr later for analysis by flow cytometry. The labeled HPC were detected in bone marrow, liver and spleen (Fig. 1B).

3.2 Levels of peripheral blood reconstitution at 12 weeks post-transplantation relate to the number of transplanted HPC

Each human liver sample that was received yielded a different number of cells for transplantation into a cohort of mice. Similar to the report by Melkus et al. (Merkus et al., 2006) we found we could recover approximately 10⁵ to 3x10⁶ CD34⁺ cells for transplant per mouse and achieved similar levels of engraftment using these amounts (Denton et al., 2012; Lavender et al., 2013). We wished to investigate whether the number of progenitor cells injected into different groups of mice was related to the level of human reconstitution achieved. Typically the mice were stably reconstituted by 12 weeks post transplantation (wpt) so the overall level of human hematopoietic (human CD45⁺) cell reconstitution in the blood at this time point was plotted against the number of purified progenitor cells originally

given (Fig. 2). There was a significant trend toward enhanced reconstitution levels with increasing number of transfused CD34⁺CD38⁻ HSC/MPP (Fig. 2A) or CD34⁺ HPC (Fig. 2B). Cohorts that received greater than 1x10⁶ liver precursor cells per mouse achieved significantly higher levels of peripheral human CD45⁺ cell counts, which averaged around 1x10⁶/ml, compared to cohorts receiving fewer than 1x10⁶ precursor cells per mouse (Fig. 2C). Therefore, to maximize the level of reconstitution without sacrificing the cohort size we aimed to administer at least 1x10⁶ liver precursor cells per mouse and routinely produced cohorts of approximately 40 TKO-BLT mice from a single tissue donor.

3.3 Use of 5.0Gy of irradiation preconditions without compromising peripheral reconstitution levels in C57BL/6 mice

C57BL/6 mice are known to be relatively radiation resistant (Gorantla et al., 2007; Watanabe et al., 2007). Therefore, we compared two preconditioning irradiation doses that were slightly higher than used for the BLT-humanization of radiation-sensitive NOD/SCID strains (Lan et al., 2006; Melkus et al., 2006). Two groups of TKO mice were irradiated with either 7.5Gy or 5.0Gy prior to transplantation with cells and tissue from a single donor, and were then assayed for levels of human reconstitution at 12wpt. Assessment of overall human CD45⁺ cells (Fig. 3A) as well the number and frequency of T cells (Fig. 3B) and B cells (Fig. 3C) in peripheral blood at 12wpt indicated that the 5.0Gy dose of preconditioning irradiation resulted in similar levels of reconstitution as the 7.5Gy dose. Unfortunately, both irradiation doses resulted in many of the mice becoming anemic likely due to damage of the endogenous bone marrow and an inability of the CD47 mutation to protect newly developed human erythrocytes from clearance by TKO phagocytes (Manz and Di Santo, 2009; Hu et al., 2011). Anemia was alleviated by administration of TKO bone marrow along with the human hematopoietic progenitors to serve as a source of murine red blood cells.

3.4 Reducing the preconditioning irradiation and TKO bone marrow dose enhances splenic secondary structure

Analyses were next performed to determine if the amount of preconditioning irradiation and/or TKO mouse bone marrow administered to the mice had any effect on the establishment and organization of the splenic white pulp. Histological examination of spleens from cohorts of mice that received 7.5Gy of preconditioning and 2x10⁶ TKO bone marrow cells showed less than desirable formation and organization of splenic white pulp (Fig. 4A). Reducing the amount of TKO bone marrow cells to 1x10⁶ cells per mouse (Fig. 4B) or reducing the amount of preconditioning irradiation to 5.0Gy (Fig. 4C) resulted in more lymphoid follicles. TKO bone marrow transfusions of less than 1.5x10⁶ cells per mouse were not sufficient to prevent many of the mice from becoming anemic within two weeks of irradiation. Anemic animals were supported by splenocyte transfusions until normal hematopoiesis was established.

Based on these observations a humanization protocol using 5.0Gy of preconditioning irradiation with transplantation of 0.5x10⁶ TKO bone marrow cells per mouse was determined to provide the best reconstitution. The final protocol included two supportive transfusions of TKO splenocytes at 3 and 6 days post transplantation to prevent anemia. This

method produced consistent formation of white pulp and follicular structures in the spleens of TKO-BLT mice (Fig. 4D).

4. Discussion

The production of most humanized mice requires that the host animals be treated with whole body irradiation or another myeloablative regimen to create space in the bone marrow and other hematopoietic niches for transplanted human stem cells to establish residence. The dosage of irradiation must be determined empirically for any given mouse strain with the goal of opening hematopoietic niches without causing excessive damage to radiation sensitive tissues such as the gut (Yamamoto et al., 2010) and supportive stromal networks within lymphoid organs (Tavassoli, 1982). One of the problems associated with reconstituting irradiated mice with human hematopoietic systems is that human red blood cells are highly susceptible to rejection by mouse macrophages through CD47-independent mechanisms (Manz and Di Santo, 2009; Hu et al., 2011). Since erythroid and lymphoid cells both develop from hematopoietic stem cells, the human stem cell transplant needed to be supplemented with mouse stem cells (we use lymphocyte-deficient TKO bone marrow cells) to prevent the lethally irradiated recipients from developing anemia. The human and mouse stem cells also needed to be in the correct quantity and ratio in order to achieve the best colonization of lymphoid niches with human stem cells on one hand, while preventing anemia on the other. Because of the rapid turnover of erythrocytes, anemia developed within days after myeloablative treatment, much quicker than the transfused mouse stem cells could multiply and differentiate to produce sufficient erythroblasts. Thus, in the first several days following irradiation, transplanted hosts were transfused with TKO spleen cells, which contained high concentrations of erythroid progenitors. These transfusions prevented acute anemia until the stem cells had proliferated sufficiently to provide an endogenous source of erythroid progenitors. A major consideration in optimizing the number of mouse stem cells to be transfused was that mouse cells can also migrate to lymphoid tissues and the bone marrow, potentially with better homing efficiency than the co-transfused xenogeneic human cells. This may have been the reason why mice developed spleens engorged with red pulp and contained minor amounts of white pulp with few human lymphoid follicles when mice were administered larger doses of mouse bone marrow. The result was little white pulp in the spleen with few human lymphoid follicles. The strategy detailed here was designed to maximize human lymphoid cell numbers as well as the best possible development of lymphoid tissues containing organized follicles. Although the TKO-BLT mouse is an improved humanized mouse model, especially with regard to the long-term stability of the engrafted human immune system, work still remains to be done to improve the substructure of the lymphoid follicles with regard to B cell and T cell organization (Lavender et al., 2013).

Acknowledgments

This work was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH, USA

References

- Denton PW, Nochi T, Lim A, Krisko JF, Martinez-Torres F, Choudhary SK, Wahl A, Olesen R, Zou W, Di Santo JP, Margolis DM, Garcia JV. IL-2 receptor gamma-chain molecule is critical for intestinal T-cell reconstitution in humanized mice. *Mucosal Immunol.* 2012; 5:555–66. [PubMed: 22569301]
- Garcia S, DiSanto J, Stockinger B. Following the development of a CD4 T cell response in vivo: from activation to memory formation. *Immunity.* 1999; 11:163–71. [PubMed: 10485651]
- Goldman JP, Blundell MP, Lopes L, Kinnon C, Di Santo JP, Thrasher AJ. Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. *Br J Haematol.* 1998; 103:335–42. [PubMed: 9827902]
- Gorantla S, Sneller H, Walters L, Sharp JG, Pirruccello SJ, West JT, Wood C, Dewhurst S, Gendelman HE, Poluektova L. Human immunodeficiency virus type 1 pathobiology studied in humanized BALB/c-Rag2^{-/-}gammac^{-/-} mice. *J Virol.* 2007; 81:2700–12. [PubMed: 17182671]
- Greenblatt MB, Vbranac V, Tivey T, Tsang K, Tager AM, Aliprantis AO. Graft versus host disease in the bone marrow, liver and thymus humanized mouse model. *PLoS ONE.* 2012; 7:e44664. [PubMed: 22957096]
- Hu Z, Van Rooijen N, Yang YG. Macrophages prevent human red blood cell reconstitution in immunodeficient mice. *Blood.* 2011; 118:5938–46. [PubMed: 21926352]
- Lan P, Tonomura N, Shimizu A, Wang S, Yang YG. Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34⁺ cell transplantation. *Blood.* 2006; 108:487–92. [PubMed: 16410443]
- Lavender KJ, Pang WW, Messer RJ, Duley AK, Race B, Phillips K, Scott D, Peterson KE, Chan CK, Dittmer U, Dudek T, Allen TM, Weissman IL, Hasenkrug KJ. BLT-humanized C57BL/6 Rag2^{-/-}gammac^{-/-}CD47^{-/-} mice are resistant to GVHD and develop B and T cell immunity to HIV infection. *Blood.* 2013
- Manz MG, Di Santo JP. Renaissance for mouse models of human hematopoiesis and immunobiology. *Nat Immunol.* 2009; 10:1039–42. [PubMed: 19767720]
- Mazurier F, Fontanellas A, Salesse S, Taine L, Landriau S, Moreau-Gaudry F, Reiffers J, Peault B, Di Santo JP, de Verneuil H. A novel immunodeficient mouse model--RAG2 x common cytokine receptor gamma chain double mutants--requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. *J Interferon Cytokine Res.* 1999; 19:533–41. [PubMed: 10386866]
- Melkus MW, Estes JD, Padgett-Thomas A, Gatlin J, Denton PW, Othieno FA, Wege AK, Haase AT, Garcia JV. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat Med.* 2006; 12:1316–22. [PubMed: 17057712]
- Tavassoli M. Radiosensitivity of stromal cells responsible for in vitro maintenance of hemopoietic stem cells in continuous, long-term marrow culture. *Exp Hematol.* 1982; 10:435–43. [PubMed: 7095018]
- Watanabe S, Ohta S, Yajima M, Terashima K, Ito M, Mugishima H, Fujiwara S, Shimizu K, Honda M, Shimizu N, Yamamoto N. Humanized NOD/SCID/IL2Rgamma(null) mice transplanted with hematopoietic stem cells under nonmyeloablative conditions show prolonged life spans and allow detailed analysis of human immunodeficiency virus type 1 pathogenesis. *J Virol.* 2007; 81:13259–64. [PubMed: 17881441]
- Yamamoto T, Kinoshita M, Shinomiya N, Hiroi S, Sugawara H, Matsushita Y, Majima T, Saitoh D, Seki S. Pretreatment with ascorbic acid prevents lethal gastrointestinal syndrome in mice receiving a massive amount of radiation. *J Radiat Res.* 2010; 51:145–56. [PubMed: 19959877]

Fig. 1. Purified fetal liver cells are primarily CD34⁺CD38^{lo} HSC/MPP and traffic to multiple peripheral tissues

A) Example of freshly isolated progenitor cells stained with anti-human CD34 and CD38 antibodies to determine the number of CD34⁺ HPC and CD34⁺CD38^{lo} HSC/MPP administered per mouse. Frequencies are averages from 18 different isolations $\pm$ SD. B)

CellTrace Violet labeled HPC detected by flow cytometry in bone marrow, liver and spleen harvested from humanized mice 24hr after transplantation. Frequencies are averaged from two injected mice.

Fig. 2. The number of transplanted precursors correlates with the level of human reconstitution achieved in peripheral blood of TKO-BLT mice at 12wpt

The number of A) $CD34^+CD38^{lo}$ HSC/MPP and B) total $CD34^+$ HPC transplanted into 12 different groups of mice positively relates with the number of human $CD45^+$ leukocytes per ml of TKO-BLT blood at 12 weeks post transplantation. C) Cohorts of mice that received greater than 1×10^6 $CD34^+$ human progenitors had significantly greater numbers of human $CD45^+$ cells in blood than cohorts receiving fewer progenitors. P values were determined by linear regression analysis (A, B) and unpaired t-test (C).

Fig. 3. TKO-BLT mice demonstrate similar levels of peripheral reconstitution after preconditioning with 5.0Gy or 7.5Gy of whole body irradiation
 Cell frequency and absolute number of A) total human CD45⁺ cells B) CD3⁺ T cells and C) CD19⁺ B cells in the peripheral blood of TKO-BLT mice that were preconditioned with either 5.0Gy or 7.5Gy of whole body irradiation. Data were collected at 12wpt. All mice were created from a single tissue donor. Statistical significance was tested by unpaired t-tests. ns = not significant.

Fig. 4. Lower doses of both TKO bone marrow and preconditioning radiation appear to improve white pulp formation in spleen

Representative 22–25wpt spleen sections from mice that received differing amounts of preconditioning irradiation and TKO bone marrow cells: A) 7.5Gy and 2M cells B) 7.5Gy and 1M cells C) 5.0Gy and 2M cells and D) 5.0Gy and 0.5M cells. Olympus DP72 camera and BX51 microscope. H&E 20x magnification; numerical aperture 0.75. Acquisition software: Olympus cell Sens Dimension 1.4.1.