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In vivo modulation of gene expression by lentiviral transduction in “Human Immune System” Rag2⁻/⁻γc⁻/⁻ mice

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CHAPTER 3

Abstract

Over the last two decades, several humanized mouse models have been used to experimentally analyze the function and development of the human immune system. Recent advances have led to the establishment of new murine-human chimeric models with improved characteristics, both in terms of human engraftment efficiency and in situ multilineage human hematopoietic development. We describe here the use of newborn BALB/c Rag2^{-/-}γc^{-/-} mice as recipients for human hematopoietic progenitor cells to produce “human immune system” (HIS) (BALB-Rag/γ) mice, using human fetal liver progenitors. The two major subsets of the human dendritic cell lineage, namely BDCA2^{+}CD11c^{+} plasmacytoid dendritic cells and BDCA2 CD11c^{+} conventional dendritic cells, can be found in HIS (BALB-Rag/γ) mice. In order to manipulate the expression of genes of interest, the human hematopoietic progenitor cells can be genetically engineered ex vivo by lentiviral transduction before performing xenograft transplantation. Using this mouse model, the human immune system can be assessed for both fundamental and preclinical purposes.
1. Introduction

It is particularly challenging to get experimental access to the human immune system (HIS) in vivo, for practical and ethical reasons. To meet these concerns, “humanized” animals models have been established over the last two decades, to specifically address questions regarding human immunology. In particular, mouse models have been privileged, because of easy manipulation, easy breeding and relatively low cost, as compared for instance to non-human primates. Confronted with xenograft transplantation barriers, several pioneering groups have screened various immunodeficient mouse strains, and multiple human xenograft transplantation models have been developed, depending on the desired outcome and features (1-6). Among these, the SCID-hu (Thy/Liv) system, which combines both human fetal liver and thymus engraftment into severe combined immunodeficiency (SCID) mice, has been shown to be valuable for the study of HIV pathogenesis in vivo (7), but this model is intrinsically limited by poor accumulation of human cells – mainly T cells – in peripheral lymphoid organs (8, 9). As an alternative, immunodeficient mice using the NOD genetic background (e.g. NOD/SCID, NOD/SCID/γ2m−/− and NOD/SCID/γc−/− mice) have been efficiently reconstituted with hematopoietic stem cells (HSC) isolated from human umbilical cord blood (UCB). Despite improvements, human T cell development and accumulation was described as being limited in such humanized animals, especially in the peripheral lymphoid organs of the recipient mice (10-14). More recently, “BLT” (bone marrow, liver, thymus) mice have been produced by co-engrafting bone marrow HSC and Thy/Liv organoid into NOD/SCID mice and were proven efficiently repopulated by human cells, although technically more challenging (15).

By reasoning that the extent of engraftment by human progenitor cells could be limited by the use of adult mice, we and others have developed a new experimental strategy, namely the inoculation of human HSC into newborn immunodeficient mice (16-19). In particular, the use of newborn BALB/c Rag2−/−γc−/− immunodeficient mice for injection of human HSC-enriched (CD34+) cell populations gives rise to robust human reconstitution, with de novo multilineage hematopoietic reconstitution and marked human thymopoiesis (17, 18). The resulting animals are referred to as “HIS (BALB-Rag/γ) mice” (3), or “human adaptative immune system Rag2−/−γc−/− mice” (huAIS-RG) (20). Efficient engraftment of BALB/c Rag2−/−γc−/− mice is age dependent (18) and necessitates sublethal total body irradiation prior to intra-hepatic inoculation of CD34+ HSC-enriched cell suspensions. HSC suspensions can be prepared from various origins such as fetal liver, umbilical cord blood, fetal bone marrow,
adult bone marrow and mobilized peripheral blood, starting from the richest source of HSC. Before xenograft transplantation, HSC can be manipulated ex vivo by lentiviral vector-mediated transduction to enforce increased or reduced expression of genes of interest in the HSC and their progeny in vivo (18, 21-23). Besides classical over-expression and functional knock-down systems (e.g. RNA interference-based), HIS (BALB-Rag/\(\gamma\)) mice can be constructed using HSC transduced with “Tet-on” lentiviral vectors, which render gene expression or down-regulation inducible upon in vivo doxycycline treatment (M. Centlivre et al., manuscript submitted). Once HIS (BALB-Rag/\(\gamma\)) mice are produced, their effective engraftment level is monitored by flow cytometry on blood samples, not before 6 weeks post-inoculation, in order to identify the animals with satisfying reconstitution level for subsequent experimental use. Such animals can be constructed to dissect mechanisms of human hematopoiesis. For instance, HIS (BALB-Rag/\(\gamma\)) mice were used in our laboratory as a model to study human plasmacytoid dendritic cell (pDC) development, by demonstrating that the SpiB transcription factor is required for proper pDC ontogeny (21). We showed that HIS (BALB-Rag/\(\gamma\)) mice contain a population of IgM\(^+\)IgD\(^-\)CD27\(^+\) marginal zone-like B cells, which develop in a NOTCH2-dependent fashion (22). We also observed that human hematopoiesis in HIS (BALB-Rag/\(\gamma\)) mice is sensitive to addition of human cytokines, such as IL-7 (A.U. van Lent et al., manuscript submitted) and IL-15 (N.D. Huntington et al., manuscript submitted). Furthermore, we described that in vivo treatment of HIS (BALB-Rag/\(\gamma\)) mice with a superagonist anti-human CD28 antibody leads to accumulation of human thymocytes and CD4+FoxP3+ regulatory T cells (24). These results indicate that HIS (BALB-Rag/\(\gamma\)) mice are particularly adapted to investigate the mechanisms underlying the development of specific human hematopoiesis-derived cell subsets, as well as a pre-clinical model for therapeutic approaches.

The study of the interaction between a human immune system and human cell tropic pathogens is of major interest to better understand the roadblocks during the immune response. Similarly to other humanized mouse models, the HIS (BALB-Rag/\(\gamma\)) mice are able to mount cellular and humoral adaptative immune responses, although in a limited fashion, and they contain functional human antigen-presenting cells (17). As expected from the seminal studies with SCID-hu (Thy/Liv) mice, several reports clearly indicate that HIS (BALB-Rag/\(\gamma\)) mice can support productive HIV infection, mimicking some aspects of HIV pathogenesis (25-29). This includes CD4\(^+\) T cell depletion, blood viremia and spreading of the virus into the lymphoid organs. Still, despite productive and sustained HIV infection, no T cell and only rare B cell anti-HIV immune responses were reported so
Of note, similar results have been described in humanized NOD/SCID/γc−/− mice (30, 31) and BLT mice (32). Interestingly, several studies have focused on mucosal transmission of HIV in humanized mouse models. Using the BLT mice, it was shown that HIV can be efficiently transmitted to humanized mice via the rectal or vaginal routes, resulting in a systemic infection (32, 33). Despite significantly less human cells at the mucosal level, similar results have also been obtained in HIS (BALB-Rag/γ) mice (34). These results are very promising, since they give access to in vivo testing of new prophylactic treatments preventing mucosal transmission of HIV infection (33). In this context, it is of interest to note that the proof-of-concept of a gene therapy against HIV using a short-hairpin RNA-based approach was obtained in several humanized mouse models, including HIS (BALB-Rag/γ) mice (23, 35, 36). In the future, it is likely that several other human-specific pathogens will be assessed in humanized mice, as illustrated by a recent study reporting that HIS (BALB-Rag/γ) mice support Dengue virus infection (37). A special interest should be given to pathogens responsible for devastating infectious diseases in developing countries, and humanized mice are particularly appropriate as a bridging tool between fundamental work and clinical testing of potential therapies.

In this protocol, we give a detailed description of the construction of HIS (BALB-Rag/γ) mice, including the methods used to genetically engineer the human HSC ex vivo by lentiviral transduction, in order to manipulate the in vivo expression of genes of interest. It usually takes 6–7 weeks before significant amounts of human lymphocytes can be detected in the peripheral blood of the HIS (BALB-Rag/γ) mice. Blood samples and lymphoid organ suspensions are analyzed by flow cytometry to determine the level of human reconstitution, by measuring the frequency of hematopoiesis-derived (CD45+) human cells and various immune cell populations. At the end of this protocol, we provide details on the outcome of lentiviral transduction and on the major dendritic cell populations found in HIS (BALB-Rag/γ) mice.

2. Materials

2.1. Production of lentiviral supernatants

1. Dulbecco’s Phosphate-Buffered Saline (PBS) buffer without calcium/magnesium (20x stock solution): dissolve 8 g KCl, 8 g KH2PO4, 57.6 g Na2HPO4·2H2O and 320 g NaCl in 2 l of distilled water and adjust the pH to 7.2 with HCl. Dilute the 20x stock solution with distilled water. The 20x and 1x solutions are kept at room temperature.
2. Antibiotic aliquots: PBS buffer as described in section 2.1. item 1, supplemented with 50x10^3 U/ml penicillin and 50 mg/ml streptomycin (powdered penicillin and streptomycin; Roche). Powdered antibiotics are kept at 4-8°C and dissolved aliquots are stored at -20°C.

3. Complete Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), L-glutamine (Gibco-Invitrogen). Store at 4-8°C.

4. Complete IMDM as described in section 2.1. item 3, supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone). Store at 4-8°C.

5. Complete IMDM as described in section 2.1. item 3, supplemented with 10% heat-inactivated FCS and 0.2% (vol:vol) antibiotics (Roche). Antibiotics are prepared as described in section 2.1. item 2. Store at 4-8°C.

6. Opti-MEM medium (Gibco-Invitrogen). Store at 4-8°C.

7. Opti-MEM medium (Gibco-Invitrogen), supplemented with 0.2% (vol:vol) antibiotics (Roche). Antibiotics are prepared as described in section 2.1. item 2. Store at 4-8°C.

8. Human Embryonic Kidney (HEK) 293T cells, which are available at the American Tissue Type Culture Collection (ATCC, reference CRL-11268), are used for lentiviral production.

9. Polystyrene cell culture flasks of 25 cm² (T25), or 75 cm² (T75) if lentiviral supernatant concentration is required.

10. Lentiviral vector construct, e.g. pCDH1 (System Biosciences) for gene over-expression (See Note 1).

11. Conditional packaging system for third generation lentivirus vector production is provided by plasmids encoding HIV products Gag and Pol (e.g. pMDLg/pRRE), Rev (e.g. pRSV-Rev) and components of the envelope for virions production (e.g. pVSV-g) (38) (See Note 1).

12. Lipofectamin-2000 (Invitrogen) as a transfection reagent. FuGENE-6 (Roche) can also be used in the same conditions.


14. Human T lymphocytic SupT1 cells (ATCC, reference CRL-1942) for titration of the lentiviral supernatant.

2.2. Preparation of recipient mice

1. Newborn BALB/c Rag-2⁻/γc⁻/ mice (See Note 2).

2. Sterile laminar flow cabinet, and autoclaved individual ventilated cages, water bottles
and food pellets.
3. Sterile irradiation container in which newborn mice fit. If the device is hermetically closed, it should contain enough air for the complete duration of the irradiation process.
4. An irradiation source (See Note 3).

**2.3. Isolation of nucleated cells from human HSC source**
1. A source of human HSC. Umbilical cord blood (UCB) is withdrawn from the umbilical cord directly after delivery, and fetal liver is obtained from elective abortions with gestational age ranging from 12 to 20 weeks (See Note 4). The raw material can be maintained at 4-8°C overnight in a cold room or in a fridge.
2. PBS buffer as described in section 2.1. item 1.
3. Roswell Park Memorial Institute-1640 (RPMI) medium supplemented with 25 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), L-glutamine (Gibco-Invitrogen). Store at 4-8°C.
4. RPMI medium as described in section 2.3. item 3, supplemented with 2% heat-inactivated FCS and 0.2% (vol:vol) antibiotics. Antibiotics are prepared as described in section 2.1. item 2. Store at 4-8°C.
5. Ethanol 70% and scissors.
6. Stomacher®-80 Biomaster lab system (Seward), Stomacher® bags, and a bag-sealing device (e.g. Sealboy 236 Audion Elektro) (See Note 5).
7. Plastic disposables: 10 ml and 25 ml pipettes, 100 mm x 20 mm polystyrene Petri dishes, 50 ml polypropylene conical tubes.
8. Lymphoprep (Axis Shield). Store at 4-8°C.

**2.4. Enrichment for CD34+ hematopoietic stem cells**
1. RPMI medium as described in section 2.3. item 4.
2. CD34 progenitor cell isolation kit, human (Miltenyi Biotec) (See Note 6).
3. MACS buffer prepared as follows: PBS buffer as described in section 2.1. item 1, supplemented with 0.5% bovine serum albumin (BSA; Sigma) and 2 mM ethylenediamine-tetra-acetic acid (EDTA; Sigma). Store at 4-8°C.
4. MACS Separator, Large Scale (LS) MACS separation columns, MACS pre-separation filters and recovery tubes (Miltenyi Biotec).
2.5. Cytometry cell sorting for CD34+CD38- hematopoietic stem cells

1. RPMI medium as described in section 2.3. item 4.
2. Any FCS-rich medium can be used to harvest the cells during the cell sorting. For instance, use IMDM medium as described in section 2.1. item 5.
3. Fluorescence-activated cell sorter, for isolation of the HSC-enriched population (See Note 7).
4. Fluorochrome-coupled monoclonal antibodies (See Note 8): anti-huCD38 (clone HB-7, BD Biosciences), anti-huCD34 (clone 581, BD Biosciences). Store the antibodies in the dark at 4-8°C.
5. 5 ml polystyrene round-bottom 12 mm x 75 mm tubes.
6. Sterile 50 μm Filcon filters (Becton Dickinson).
7. Sterile 1 ml Plastipak syringes (Becton Dickinson).

2.6. Lentiviral transduction of hematopoietic stem cells

1. Human cell culture medium: complete IMDM as described in section 2.1. item 3, supplemented with 5% normal human serum (NHS; Invitrogen) and Yssel's supplement (50 ml in a 500 ml bottle of IMDM; Diaclone). Store at 4-8°C.
2. Aliquots (2 μg/ml) of the following recombinant cytokines in complete IMDM as described in section 2.1. item 3: human interleukin-7 (IL-7; Tebu-Peprotech), human stem cell factor (SCF; Tebu-Peprotech) and human thrombopoietin (TPO; Tebu-Peprotech).
3. Transduction medium: human culture medium as described in section 2.6. item 1, supplemented with 20 ng/ml of human IL-7, SCF and TPO (1/100 dilution of cytokine stocks described in section 2.6. item 2).
4. PBS buffer as described in section 2.1. item 1, supplemented with 2% heat-inactivated FCS. Store at 4-8°C.
5. Two series of 24-well plates, both non-tissue culture and tissue culture treated.
6. PBS buffer as described in section 2.1. item 1, supplemented with 30 μg/ml retronectin (Cambrex-TaKaRa). Store at 4-8°C.
7. PBS buffer as described in section 2.1. item 1, supplemented with 2% BSA. Store at 4-8°C.
8. RPMI medium as described in section 2.3. item 4.

2.7. Inoculation of hematopoietic stem cells into recipient mice

1. RPMI medium as described in section 2.3. item 4.
2. BD Micro-Fine+ U-100 Insulin 0.5 ml 0.33(29G) x 12.7 mm syringes (BD Biosciences).

2.8. Monitoring of “Human Immune System” (BALB-Rag/γ) mice
1. Microvette® CB300 lithium heparin coated for capillary blood collection (Sarstedt), supplemented with one drop (5-10 μl) of heparin (5x10^3 IU/ml). Each Microvette® is composed of an outer tube and an inner tube embedded in it. The inner tube has a top cap and can receive a plug at the bottom part of the tube. Blood is collected by capillarity from the bottom and the bottom plug is put in position afterwards.
2. 5 ml polystyrene round-bottom 12 mm x 75 mm tubes.
3. PBS buffer as described in section 2.1. item 1.
4. Lymphoprep (Axis Shield). Store at 4-8°C.
5. RPMI medium as described in section 2.3. item 4.
6. Fluorochrome-coupled monoclonal antibodies (See Note 9): anti-huCD45 (clone 2D1, BD Biosciences) for the detection of human hematopoiesis-derived cells, and other antibodies according to the analyzed cell populations. Store the antibodies in the dark at 4-8°C.
7. FACS buffer: PBS buffer as described in section 2.1. item 1, supplemented with 2% heat-inactivated FCS and 0.02% sodium azide (NaN₃). Store at 4-8°C.
8. FACS buffer as described in section 2.8. item 7, supplemented with 0.2mM 4',6-diamidino-2-phenylindole (DAPI; Sigma) for dead cell exclusion. Store in the dark at 4-8°C. Store aliquots 100x concentrated (20 mM) at -20°C.
9. Round bottom 96-well plates.
10. 1.4 ml U-shaped FACS tubes.
11. Fluorescence-activated cell sorter, for analysis of the cell populations (See Note 7).

3. Methods
The production of HIS (BALB-Rag/γ) mice with engineered gene expression is strongly dependent on efficient planning and logistics. It can be summarized as follows: (a) the lentiviral vector supernatant – for enforced, knock-down or inducible expression of one specific gene – is produced in 293T cells; (b) the human CD34⁺CD38⁻ HSC-enriched cell population is isolated by consecutive magnetic and fluorescence-activated cell sortings (MACS and FACS, respectively); (c) the HSC-enriched cell population is transduced in vitro with the lentiviral supernatant; and (d) newborn BALB/c Rag-2⁻/γc⁻/ mice are eventually inoculated with these genetically modified HSC (Figure 1). The steps (a) and (c) are skipped
when “unmanipulated” HIS (BALB-Rag/γ) mice are produced.

In this protocol, we describe isolation of human HSC from fetal liver, but umbilical cord blood (UCB) can be easily used as well (See also Manz et al. in this issue). Similarly, we focus here on the use of newborn BALB/c Rag-2⁻/γc⁻ mice as recipients for humanized mice production, but alternative protocols can be considered for other mouse strains and
specific applications (39). All isolation steps have to be performed in a sterile manner under laminar flow. Both CD34+ (step 3.4) or CD34+CD38− cells (step 3.5) can be used to engraft recipient mice, depending on the available amount and the desired degree of purity. Still, CD34+CD38− cells are more reliable and more potent, and 10-fold less cells are required to obtain good human engraftment, as compared to non-sorted CD34+ cells. Since CD34+CD38− cells yield from UCB is usually limited, we advise to inject CD34+ cells from UCB, whereas CD34+CD38− cells can be routinely isolated from fetal liver. Concerning the number of cells to be injected into the newborn recipients, we have experienced reliable and high reconstitution level when at least $5 \times 10^5$ CD34+ cells and $5 \times 10^4$ CD34+CD38− cells are inoculated per pup, lower numbers resulting in higher variability in the outcome. In the case of ex vivo HSC manipulation by lentiviral transduction, it is of course wise to produce the lentivirus far before use, since virus aliquots can be kept frozen for years (step 3.1). Although both fetal liver CD34+CD38− and total CD34+ cell populations are susceptible to lentivirus-mediated transduction, the use of CD34+CD38− cells is preferred in order to spare manipulation time, expensive reagents and lentiviral supernatants. Alternative lentiviral supernatant production and HSC transduction protocols can be used to adapt to particular conditions and targeted cell type (40).

3.1. Production of lentiviral supernatants

1. Maintain a stock of HEK 293T cells in an incubator at 37°C with 5% CO2, in IMDM 10% FCS medium with antibiotics. From the stock, prepare one flask of HEK 293T cells per lentiviral supernatant to be produced. Seed 1-2x10⁶ cells and 4-6x10⁶ cells per T25 and T75 flask, respectively, in an appropriate volume of IMDM 10% FCS medium without antibiotics. Within one day, the cells should reach 60-70% confluence in the flask and are then ready to use.

2. For each lentiviral vector, prepare one tube with a mixture of DNA plasmids in Opti-MEM medium without antibiotics, as follows: for a T25 flask, mix 2.4 μg of the lentiviral vector, 1.5 μg of pMDLg/pRRE plasmid, 0.8 μg of pVSV-g plasmid and 0.6 μg of pRSV-Rev plasmid, in 0.6 ml of Opti-MEM medium; for a T75 flask, mix 7.1 μg of the lentiviral vector, 4.6 μg of pMDLg/pRRE plasmid, 2.5 μg of pVSV-g plasmid and 1.8 μg of pRSV-Rev plasmid, in 2 ml of Opti-MEM medium. Incubate 5 min at room temperature.

3. For each lentiviral vector, prepare one tube containing 16 μl of lipofectamine-2000 in 0.6 ml of Opti-MEM medium (T25 flask) or 48 μl of lipofectamin-2000 in 2 ml of Opti-MEM medium (T75 flask). Incubate 5 min at room temperature.
4. Add the lipofectamin-containing medium into the DNA-containing medium and mix gently. The final volume of the lipofectamin-DNA mixture is 1.2 ml (T25) or 4 ml (T75). Incubate for 20 min at room temperature.

5. Replace the medium in the flask, to reach a final volume of 2.2 ml (T25) or 6.6 ml (T75) IMDM 10% FCS culture medium without antibiotics. Perform the transfection by adding the lipofectamin-DNA mixture to the culture medium in the flask. Incubate over-night at 37°C with 5% CO2.

6. At day one after transfection, replace the medium as follows: 3.5 ml (T25) or 10.5 ml (T75) of Opti-MEM medium with antibiotics. Incubate over-night at 37°C with 5% CO2.

7. At day two after transfection, harvest the supernatant from the flask and store it at 4-8°C. Replace the medium in the flask: 3.5 ml (T25) or 10.5 ml (T75) of Opti-MEM medium with antibiotics. Incubate over-night at 37°C with 5% CO2.

8. At day three after transfection, harvest the supernatant from the flask, and pool it with the stored supernatant of the previous day. Centrifuge the supernatant to pellet detached HEK 293T cells (450g, 5min). Filter the centrifugated supernatant with a 0.45 μm filter.

9. (optional) If necessary, concentrate the lentivirus on Amicon column. Apply 15 ml of supernatant on the top of the column and centrifuge (1000g, 10min). Most of the supernatant is going through, and the lentiviral virions are retained on the top of the column. Harvest the remaining supernatant on top of the column and adjust the volume if necessary, by adding the desired volume of Opti-MEM medium.

10. Aliquot the lentiviral supernatant (0.3-0.5 ml per tube) and store at -80°C.

11. Titrate the transduction units per ml in the lentiviral supernatant on SupT1 cells. In brief, seed a fixed amount of SupT1 cells (e.g. 2x10⁵) in 24-well plate, in IMDM 10% FCS medium with antibiotics. In each well, add a decreasing amount of the lentiviral supernatant, for instance 100 μl, 30 μl, 10 μl, 3 μl and 1μl, in a final volume of 100 μl. Wash the SupT1 cells after 6h of transduction. Three days after transduction, harvest the SupT1 cells and measure the frequency of GFP⁺ cells by flow cytometry. The titer of the viral supernatant is calculated for each well as follows: transduction units (TU)/ml = [%GFP⁺] x [number of SupT1 cells] x 10 / [used volume of lentiviral supernatant in μl]. Example: if 1 μl of virus on 2x10⁵ SupT1 cells gives 15% GFP⁺ cells, the titer is 3x10⁷ TU/ml. The most accurate titer evaluations are obtained in wells with a GFP⁺ frequency in a 10-30% range (See Note 10).
3.2. Preparation of recipient mice

1. BALB/c Rag-2\(^{-/-}\)γc\(^{-/-}\) mice exhibit profound immunodeficiency and the colony has to be maintained in strict health conditions, e.g. in isolators or individual ventilated cages. To hinder the risk of infection, always work in sterile conditions, e.g. under laminar flow, wearing lab coat, disinfected gloves and clean material. After irradiation or inoculation of the human cells, the mice are transferred back to cages. Therefore, the necessary cage space, sterile food, water and material have to be prepared accordingly.

2. Production of newborn mice has to be planned depending on the frequency and amount of available human HSC. Since engraftment efficiency is age-dependent (18), we recommend the use of newborn mice as early as possible, and always before a week of age (See Note 11). Once pregnant, female mice are removed from the breeding cage and male mice are kept in the breeding colony. The female and her nest are taken out of the breeding colony and manipulated under laminar flow during all subsequent manipulations.

3. Isolate the newborn mice from the breeding cage and transfer them to the sterile irradiation container. Transport the container to the irradiation apparatus and apply sub-lethal total body irradiation to the newborn mice (~3-4Gy) (See Note 3). Bring the newborns back to the cage containing the mother and let them rest 4-24 h before xenograft transplantation.

3.3. Isolation of nucleated cells from human HSC source (fetal liver)

1. Prepare material under laminar flow as follows (per fetal liver): one Stomacher® bag; one 100 mm x 20 mm dish; six 50 ml tubes; one bottle (500 ml) of RPMI 2% FCS medium. (See Note 12 for alternative procedure using UCB as a source of HSC).

2. Pieces of fetal liver are collected in 15 ml plastic tubes containing 2-4 ml of RPMI 2%FCS medium and have to be processed into cell suspension. Transfer the content of one tube into one Stomacher® bag, add 15-20 ml of RPMI 2%FCS medium and seal the bag twice to avoid any liquid loss. Install the bag inside the Stomacher®-80 Biomaster lab system and let it mechanically process the liver pieces for one minute at high speed. The bag now contains a cell suspension mixed with non-dissociated liver stroma.

3. Wash the bag with ethanol 70% and open it with sterile scissors. Transfer the contents of the bag into a 100 mm x 20 mm Petri dish.

4. Pour RPMI 2% FCS medium directly from the bottle to the dish in order to dilute the cell suspension. Let the big pieces sediment for 1-2 min. Using a 25 ml pipette, recover
the supernatant from the dish and distribute equally among the six 50 ml tubes. Always avoid the pieces of stroma at the bottom of the dish. When it becomes difficult to avoid stroma, pour new RPMI 2% FCS medium into the plate and repeat this procedure until the recovered medium is clear. Usually, pouring fresh medium 3 times is enough.

5. Add RPMI 2% FCS medium to the tubes until you reach approximately 20 ml. Wash the cell suspensions by centrifugation (450g, 5 min). Aspirate the fat-containing supernatant with a pipette or a vacuum system.

6. Resuspend the cell pellet with a 10 ml pipette by adding 13 ml RPMI 2% FCS medium into each tube. Once a homogeneous cell suspension is obtained by repeated pipetting, bring 10 ml Lymphoprep underneath the cell suspension with another 10 ml pipette, carefully avoiding air bubbles that could disturb the formation of a clearly distinguishable interface (See Note 13).

7. Carry the tubes to the centrifuge, carefully avoiding disturbing the interface. Centrifuge the tubes at 1,100g for 15 min with low acceleration and no brake (the centrifugation lasts for ~25 min). After centrifugation, the pellet mostly contains erythrocytes and dead cells, whereas live nucleated cells are concentrated as a cell ring at the interface. Recover the top supernatant and the cell ring from each tubes and pool into new tubes (three tubes into one).

8. Wash the cell suspensions by centrifugation (450g, 5 min) and pool pellets in a total of 10 ml of RPMI 2% FCS medium. Count the amount of nucleated cells in the suspension (See Note 14).

### 3.4. Enrichment for CD34+ hematopoietic stem cells

1. Pellet the nucleated cells by centrifugation (450g, 5 min).

2. Enrichment for the CD34-expressing fraction is done by magnetic sorting, using a two-steps strategy: (a) anti-CD34 hapten-coupled antibody; (b) anti-hapten antibody conjugated with colloidal paramagnetic beads (See Note 6). Perform the first step of indirect MACS labeling: for $10^8$ nucleated cells in the pellet, use 75 μl of RPMI 2% FCS medium to resuspend the pellet and add 25 μl of each “A” reagent (FcR blocking human immunoglobulins; monoclonal hapten-conjugated anti-huCD34 antibody). Keep the tube at 6-12°C for 15 min (in the fridge, as recommended by the MACS kit’s manufacturer).

3. Add 10 ml of RPMI 2% FCS medium in the tube and wash the cell suspensions by centrifugation (450g, 5 min).

4. Perform the second step of indirect MACS labeling: for $10^8$ nucleated cells in the pellet,
use 100 μl of RPMI 2% FCS medium to resuspend the pellet and add 25 μl of the “B” reagent (colloidal super-magnetic MACS MicroBeads conjugated to an anti-hapten antibody). Keep the tube at 6-12°C for 15 min (in the fridge, as recommended by the kit’s manufacturer).

5. During the incubation, install the magnetic separation column and a pre-separation filter on top of it. Prepare the collection tube for the column flow-through. Proceed to filter/column washing with MACS buffer, following the manufacturer’s instructions (See Note 15). For a LS separation column, apply 3 ml of MACS buffer and let it run through.

6. Add 10 ml of RPMI 2% FCS medium in the tube and wash the cell suspensions by centrifugation (450g, 5 min).

7. Resuspend the cell pellet in MACS buffer, using 500 μl of buffer per 10^8 cells.

8. Apply cell suspension through pre-separation column to remove clumps and let the cells pass through the column. Perform column washings with MACS buffer, as indicated in the manufacturer’s hand-guide. For a LS column, apply 3 times 3 ml of MACS buffer on the filter/column assemblage, adding buffer only when the column reservoir is completely empty.

9. Harvest the magnetically labeled cells from the column. Place the column on top of a new 15 ml tube containing 5 ml of RPMI 2% FCS medium. For a LS column, pipette 5 ml of MACS buffer onto the column and apply the plunger on the column. Immediately flush out the CD34-enriched fraction (See Note 16).

10. Wash the cell suspension by centrifugation (450g, 5 min). Resuspend the pellet in a volume of 1 ml of RPMI 2% FCS medium and count the amount of nucleated cells in the suspension (See Note 17).

3.5. Cytometry cell sorting for CD34+CD38- hematopoietic stem cells

1. Pellet the nucleated cells by centrifugation (450g, 5 min).

2. Meanwhile, prepare the monoclonal antibody mixture for cell sorting: mix 1 μl of the anti-CD34 antibody and 1 μl of the anti-CD38 antibody per 10^6 cells (with a minimal final volume of 20 μl).

3. Remove as much supernatant as possible after centrifugation of the cells. Apply the antibody mixture on the dry pellet and resuspend the cells by repeated pipetting. Incubate 10 min on ice (to avoid antibody capping) and in the dark (to avoid fluorochrome bleaching).

4. Add 5 ml of RPMI 2% FCS medium in the tube and wash the cell suspensions by
centrifugation (450g, 5 min).

5. During centrifugation, prepare a set of 5 ml polypropylene tubes: one sorting tube that will contain the sample to be sorted; one recovery tube with 1 ml of FCS-rich medium (e.g. IMDM 10% FCS). For each sample to be sorted, prepare one syringe attached to a 50 μm Filcon filter.

6. After centrifugation, discard the supernatant and resuspend the pellet in RPMI 2% FCS medium to obtain a cell concentration of 5-10x10^6 cells/ml (minimum 0.5 ml) (See Note 18). Transfer the cells to the syringe and flush them through the filter to remove potential sources of clogs for the cell sorter.

7. Sort the human HSC-enriched CD34^+CD38^- population. If possible, maintain the sorted sample and the recovery tube at 4°C, if possible on the used cell sorter.

8. After the cell sorting, centrifuge the recovery tubes (450g, 5 min). Resuspend the cells in an accurate volume of RPMI 2% FCS medium and count the amount of nucleated cells in the suspension (See Note 19).

3.6. Lentiviral transduction of hematopoietic stem cells

1. In a tissue culture treated 24-well plate, seed a maximum of 5x10^5 sorted CD34^+CD38^- HSC per well in 1 ml of the human cytokine-supplemented transduction medium. Incubate the cells overnight at 37°C.

2. Prepare the transduction plate for the next day. For this purpose, coat wells of a non-tissue culture treated 24-well plate with 30 μg/ml retronectin, using 0.5 ml per well. Use one well per lentiviral construct to be used, for a maximum of 5x10^5 cells per well, and determine the number of wells that will be required during lentiviral transduction. Incubate overnight at 4-8°C (or for 1h at 37°C if you prepare this transduction plate the next morning).

3. The next day, recover the retronectin from the transduction plate (it can be re-used at least twice afterwards) and replace it by 0.5 ml of PBS 2% BSA. Incubate for 15-30 min at 37°C.

4. From the culture plate containing the HSC, remove 0.4ml of culture medium from each well and spare in an unused well of the plate. Resuspend the HSC with the remaining 0.6 ml of culture medium and transfer into a 15 ml tube. Wash each well with 0.4 ml of spared culture medium. At this stage, all wells can be pooled into one single tube.

5. Centrifuge the cells (450g, 5 min) and harvest the supernatant in a separate tube. This culture medium supernatant is used to resuspend the cells in the desired final volume
(0.3 ml per transduction well). Add cytokines to compensate for the volume of the viral supernatant (0.3 ml), i.e. temporarily spare the cells in culture medium with a 2x cytokine concentration.

6. Prepare a plate for the verification of transduction efficiency. The left-over of culture medium supernatant is distributed in a tissue culture treated 24-well plate (1 well per transduction condition). Add fresh human cell culture medium to reach a final volume of 1 ml per well. It is not necessary to compensate with fresh cytokines, unless their concentration goes below 5 ng/ml. To avoid excessive evaporation in the culture well, distribute 0.5 ml of PBS in the surrounding wells.

7. Remove the PBS 2% BSA from the wells of the transduction plate and wash with 1 ml of PBS. Distribute 0.3 ml of HSC per well in culture medium with 2x cytokines (maximum 5x10^5 cells). Add 0.3 ml of lentiviral supernatant per well (See Note 20). Incubate for 6h at 37°C.

8. After incubation, harvest the cells from the wells into separate 15 ml tubes, and wash the wells with 1 ml RPMI 2% FCS medium. Centrifuge the cells (450g, 5 min), resuspend in an accurate volume of RPMI 2% FCS medium and count the cells.

9. In the plate for the verification of transduction efficiency, seed a small aliquot of each set of transduced HSC in the prepared wells (1-5x10^4 cells per well is sufficient). Incubate the plate for 3 days at 37°C. After 3 days, harvest the cells and measure the transduction efficiency by checking GFP expression by flow cytometry.

### 3.7. Inoculation of hematopoietic stem cells into recipient mice

1. For optimal conditions, plan to inoculate 10^5 CD34^+CD38^- cells per newborn. Pellet the desired amount of cells by centrifugation (450g, 5 min). Resuspend the pellet in the desired volume of RPMI 2% FCS medium (35 µl per newborn mouse to be injected) (See Note 21).

2. Transfer the cells into an 1.5 ml Eppendorf tube, to ensure full access of the needle to the cell suspension.

3. In a laminar air flow cabinet, isolate the newborn mice on a piece of absorbent paper on the bench. For each newborn mouse repeat the following sequence: hold the mouse between thumb and index fingers, with the head down; inject 30-35 µl of cell suspension by intra-hepatic route, i.e. between the thoracic cage and the milk-filled stomach, which appears white through the skin (Figure 2); place the mouse back to its nest in the cage containing the mother. If necessary, toe mark the newborns with sterile scissors.
4. The cage containing the nest and the mother does not require special food diet or water (no antibiotics required). The animals can be weaned normally at 3-4 weeks of age and kept until use.

![Figure 2. Intra-hepatic inoculation of human progenitor cells in newborn mice.](image)

(A) The newborn animals are maintained upside down between two fingers. Using a micro-syringe, the cell mixture is injected in the liver (dark red), between the stomach (white) and the thoracic cage. (B) Schematic positions of the stomach and thoracic cage are indicated. Reproduced from reference (49) with permission from Springer.

3.8. Monitoring of “Human Immune System” (BALB-Rag/\(\gamma\)) mice

1. Peripheral reconstitution of the inoculated mice by human HSC is checked by flow cytometry 7-8 weeks after injection. The fraction of human hematopoiesis-derived is determined by anti-CD45 staining. For each HIS (Rag/\(\gamma\)) mouse, shave one of the hind legs between knee and ankle (lateral side) with a scalpel and make blood arise from the saphenous vein using a needle for limited puncture (Figure 3). Collect the blood drops (~50 \(\mu\)l) by capillarity with the Microvette® inner tubes. Mark the mice for numbering and label the Microvette® accordingly.

2. The blood samples have to be enriched for nucleated cells, using “small scale density gradient” purification. Prepare two series of numbered 5 ml round-bottom tubes. Distribute 1.5 ml of PBS buffer in the group of dilution tubes, and 1.5 ml of Lymphoprep in the group of gradient tubes.

3. Pipette 1 ml of PBS buffer from the first 5 ml round-bottomed tube. Get the inner tube from the Microvette®, open the top cap, horizontally maintain the tube and remove the bottom plug. Put the bottom end of the open Microvette® inner tube against the
wall of the corresponding dilution tube. Pipette out the 1 ml of PBS buffer through the inner Microvette® tube: all the blood that was contained will be washed away. Check inside the plug for remaining blood. Repeat this step for every sample.

4. Transfer the ~1.5 ml of PBS-diluted blood to the gradient tube, on top of the Lymphoprep layer, by careful pipetting. For instance, use a plastic 2 ml pipette and flush drop wise. Repeat this step for every sample.

5. Carry the tubes to the centrifuge, carefully avoiding disturbing the interface. Centrifuge the tubes at 1,100g for 15 min with low acceleration and no brake (the centrifugation lasts for ~25 min). After centrifugation, the pellet mostly contains erythrocytes and dead cells, whereas live nucleated cells are concentrated as a cell ring at the interface. Recover the supernatant and the cell ring into series of new numbered 15 ml tubes.

Figure 3. Puncture of blood at the saphenous vein. (A) The mice are restrained with the head and part of the body inside a cap. The hand should firmly hold the hind leg outside the cap, so that it can be shaved on the external lateral side, between the knee and the ankle. (B) The saphenous vein is exposed and (C) a needle is used to make a small puncture. (D) Blood drops are collected in the heparin-containing tubes by capillarity. Reproduced from reference (49) with permission from Springer.
6. Add 5 ml of RPMI 2% FCS medium in the tube and wash the cell suspensions by centrifugation (450g, 5 min).

7. Meanwhile, prepare the monoclonal antibody mixture for human cell staining: per sample, mix 4 μl of the FITC-coupled and PerCP-Cy5.5 antibodies, and 2 μl of the R-PE-coupled, PE-Cy7, APC and APC-Cy7 antibodies (that is to say 16 μl total per sample in this example).

8. Remove the supernatant after centrifugation of the cells. Resuspend each pellet with 200 μl of FACS buffer and immediately transfer the suspension to individual contiguous wells in a round bottom 96-well plate.

9. Pellet the cell suspensions by centrifugation (450g, 2 min) and remove the supernatant by quick inversion over the sink. Maintain the plate in this position and dry it against a piece of absorbent paper on the bench. Vortex the plate briefly and place it on ice.

10. Apply the antibody mixture on the dry pellet by distributing 15.5 μl per well and briefly vortex the plate. Incubate 10 min on ice (to avoid antibody capping) and in the dark (to avoid fluorochrome bleaching).

11. During incubation, prepare one FACS tube per sample on the appropriate rack.

12. At the end of the incubation, distribute 100 μl of FACS buffer per well and wash the plate by centrifugation (450g, 2 min). Remove the supernatant by quick inversion over the sink (see step 3.6.9). Bring the plate back on ice and distribute 50 μl of the DAPI-containing FACS buffer per well. Transfer the content of the wells to their respective FACS tube (e.g. with multi-channel 200 μl pipette) and perform cytometry analysis.

13. We show here an example of transduction efficiency after transduction (Figure 4A) and the recovery of GFP+ human cells in the corresponding HIS (Rag/γ) mice (Figure 4B). Human hematopoiesis is similar the transduced (GFP+) and non-transduced (GFP) cell populations, as shown by frequency of HSC in bone marrow or T cell development in the thymus (Figure 4C/D). The large majority of human cells found in the blood and lymphoid organs of HIS (Rag/γ) mice is composed by B and T lymphocytes, but several populations of dendritic cells are also detected. The two major dendritic cell populations are BDCA2+/CD11c−HLA-DR+ plasmacytoid dendritic cells (pDC) and BDCA2−CD11c+HLA-DR+ conventional dendritic cells (cDC) (Figure 5A). Most of dendritic cells are found in the bone marrow (especially cDC), the spleen and the liver, where pDC can represent up to 30-40% of total human cells. The pDC also express IL-3Rα/CD123 on their surface and cDC express CD40 and B7, as expected from studies in human individuals (Figure 5B and not shown).
4. Notes

1. Gene transfer in immune cell has made extensive use of HIV-derived lentiviral vectors over the last decade, with incremental optimizations and modifications (40, 41). The choice of the vector has to be carefully done, according to specific needs and desired features. For instance, vectors efficiently used for gene over-expression experiments are not necessarily appropriate for RNA interference-mediated knock-down experiments. For bio-safety concerns, the production of the third generation HIV-based virions depends on trans-elements that are provided by several packaging plasmids. The HIV...
products Gag and Pol are required for the formation of the viral capsid, the maturation of the virion, the reverse transcription of the lentiviral vector RNA genome into double-stranded DNA and the subsequent integration of the lentiviral vector DNA into the genome of the transduced cells. Rev binds to the Rev responsive element (RRE) contained in the HIV-derived lentiviral vector and the pMDLg/pRRE packaging plasmid, and drives export of the viral RNA to the cytoplasm before potential splicing events occur in the nucleus. Some plasmids encode these three products, such as

**Figure 5: Dendritic cell development in HIS (BALB-Rag/γ) mice.** (A) The bone marrow of adult HIS (BALB-Rag/γ) mice was analyzed for the presence of plasmacytoid dendritic cells (pDC) and conventional dendritic cells (cDC, also known as myeloid DC), based on the expression of BDCA2 and CD11c, respectively. Of note, BDCA2 and CD11c expression is mutually exclusive, and the CD11c⁺CD14⁺ fraction is described as belonging to the myelo-monocytic (Mo) lineage. (B) The majority of the cDC population found in the bone marrow, spleen and liver of HIS (BALB-Rag/γ) mice expresses the co-stimulatory molecule CD40. All pictures are obtained from 8 to 10-week old HIS (BALB-Rag/γ) mice produced with HSC transduced with a GFP-expressing pCDH1 vector, and no difference was observed between GFP⁺ and GFP⁻ populations.
pCMV.R8.91 (42). The envelope of the produced virions is often based on the vesicular stomatitis virus glycoprotein (VSV-g), but this might be modified, for instance when specific cell types are targeted (40). Simply replace the pVSV-g plasmid by the desired envelope plasmid of your choice when necessary.

2. At least two Rag-deficient (43, 44) and four γc-deficient mouse strains (45-48) have been independently generated so far. Two inbred strains of Rag2−/−γc−/− mice are currently available, respectively in the C57Bl/6 (black) and BALB/c (white) genetic background. Newborn C57Bl/6 Rag2−/−γc−/− mice are not efficiently reconstituted by human HSC. Therefore, the use of BALB/c Rag2−/−γc−/− mice is strongly recommended, although we have been using Rag2−/−γc−/− mice in a mixed BALB/c x 129/OLA background with identical results.

3. We have used both Röntgen X-ray and 137Cs sources, and we have observed similar outcome when applying 3-4Gy total body irradiation on BALB/c Rag2−/−γc−/− mice. The dose has to be adapted to the mouse strain used. For instance, all SCID mouse strains, such as NOD/SCID/γc−/− mice, are known to be extremely irradiation-sensitive and lower dose is required to ensure survival of the animals.

4. Fetal liver is the richest source of human HSC but, depending on local legislation and necessity of informed consent, it may not be easily available for research purposes. Umbilical cord blood is easier to obtain, but the yield of HSC is much lower. Considering the inter-donor variability, decisions have to be to determine how experimental groups of HIS (Rag/γ) mice are produced. There are several alternatives: (a) produce HIS (Rag/γ) mice each time that a source of human HSC is available; (b) freeze each individual source of human HSC and make series of age synchronized HIS (Rag/γ) mice with the individual sources; (c) pool several source donors and make series of age synchronized HIS (Rag/γ) mice with the same “normalized” pool.

The last possibility saves a lot of time and reagents, especially in the situation of recurrent availability of HSC source from numerous (≥ 3) donors. In the method section, we describe the protocol used for processing of one individual fetal liver, and it should be up-scaled accordingly for multiple donors.

5. We list here the material required for automated mechanical preparation of cell suspension. The preparation can also be made manually, simply using a metallic mesh on which the pieces of fetal liver are mechanically processed.

6. Be aware that the “Indirect CD34 MicroBead kit” is used at this step, and not the direct kit (anti-CD34 bead-conjugated antibody). We have observed that the direct kit is not optimally appropriate on fetal liver and UCB cell suspensions, in contrast to post-natal
thymocytes suspensions. Furthermore, manufacturer’s recommendations are to use 1 ml of each reagent per 109 cells in the suspension, but we are routinely using 25% of the recommended volumes without observing yield loss. These adapted volumes are indicated in the method section.

7. We use a FACSAria™ machine (BD Biosciences) for cell populations sorting and a BD LSR II™ machine (BD Biosciences) for cell population analysis, both with 9-parameters (size, scatter and 7 fluorescence colors including DAPI). It has to be noted that any 5-parameters (size, scatter and 3 fluorescence colors) cell sorter is sufficient to isolate HSC enriched populations.

8. The choice of fluorochromes has to be adapted to the available light filters in the cell sorter. We routinely use anti-huCD38 antibody coupled to R-phycoerythrine (R-PE) and anti-huCD34 antibody coupled to phycoerythrine-cyanine-7 (PE-Cy7). Other markers can be used for exclusion of contaminating lineage-positive cell populations, such as CD3 (T cells), CD19 (B cells), CD56 (NK cells), CD11c (cDC) and BDCA2 (pDC).

9. Similarly to Note 8, the fluorochromes have to be chosen in accordance to the available light filters in the FACS analyzer. As an example, we routinely use fluorescein-isothiocyanate (FITC) anti-huCD19, R-PE anti-huCD8, peridinin chlorophyll protein-cyanin-5.5 (PerCP-Cy5.5) anti-huCD4, PE-Cy7 anti-huCD3, allophycocyanin (APC) anti-BDCA2, allophycocyanin-cyanin-7 (APC-Cy7) anti-huCD45. The proposed staining mixture for reconstitution analysis is adapted to a 9-parameters cytometer, e.g. the BD LSR II™ machine (BD Biosciences) (See Note 7), and is designed for the determination of frequency of B lymphocytes (CD19), pDC (BDCA2) and T lymphocyte subpopulations (CD3, CD4, CD8) within the human hematopoiesis-derived cells (CD45). In the case of a 5-parameters cytometer, we advise to use only anti-CD45, anti-CD3 and anti-CD19 antibodies with the proper fluorochrome combination. The choice of the antibodies has to be adapted to the cell populations of interest, but should always include a CD45-specific antibody to measure to what extent human reconstitution took place and exclude aspecific staining on mouse cells. Cell surface antigens expressed on human cells in HSC-engrafted mice are described in several publications (17, 18, 39). In case of transduction, one channel has to be reserved for analysis of GFP expression (usually the FITC channel), and the antibody mixture has to be redesigned accordingly.

10. SupT1 T cells are used for titration of lentiviral supernatant and exhibit high susceptibility to lentiviral transduction. Be aware that hematopoietic progenitors (or any
other human cell manipulated ex vivo) do not necessarily exhibit the same sensitivity to lentiviral transduction. Furthermore, the required titer for optimal transduction of fetal liver HSC might differ between lentiviral vectors and applications. It is therefore highly advised to always perform an in vitro transduction test on the desired cell population (for instance here, human fetal liver HSC) before starting in vivo mouse experiments with transduced cells. In our experience, we always try to reach a minimum titer around $5 \times 10^6$ TU/mL to ensure good transduction (20-50% GFP+) of fetal liver HSC, but higher titers can be required in some cases, e.g. with vectors expressing short hairpin RNA.

11. There is no strict need for “time-pregnant” female mice, especially in the case of a recurrent human HSC source. Reconstitution is optimal between 1-4 days of age, and efficiency usually drops severely after 5 days of age. We therefore recommend using BALB/c Rag2$^{-/-}$,γc$^{-/-}$ newborn mice that are not older than 5 days of age. Still, we have already observed good reconstitution using 7-day old newborns.

12. Alternatively, UCB can be used instead of fetal liver. In brief, transfer ~60 ml of UCB into a 250 ml flask. Dilute the UCB with ~120 ml of PBS buffer, reaching a total volume of ~180 ml. Prepare twelve 50 ml tubes and distribute ~15 ml of diluted UCB to each tube. Similarly to fetal liver, carefully bring 10 ml Lymphoprep underneath the cell suspension in order to get a clear interface. Centrifuge the tubes at 1,100g for 15 min with low acceleration and no brake. Recover the supernatant and the cell ring from each tube, pool into new tubes. Wash the cell suspensions by centrifugation (450g, 5 min) and pool pellets in a total of 15 ml of RPMI 2% FCS medium. After Lymphoprep gradient, the yield from 60 ml UCB is usually around 50-100x10$^6$ nucleated cells. In contrast to the suspension obtained from fetal liver, contamination by erythrocytes is usually still clearly noticeable by eye (cell suspension is red) but is not a problem for the next steps.

13. In order to avoid waste of plastic disposables, prepare two 50 ml tubes, which contain RPMI 2% FCS medium and Lymphoprep, respectively. Resuspend the cell pellet with the same pipette in the whole 6-tube series, change your 15 ml pipette and next bring Lymphoprep with the same pipette in the whole series of tubes. Simply pour new medium or Lymphoprep directly from the stock bottle into the 50 ml tube when needed.

14. After Lymphoprep gradient, the yield from one medium-sized fetal liver is usually around 100-400x10$^6$ nucleated cells. Important variations are observed, depending on the size of the material, age of the donor and care during tube manipulation.
15. The protocol is described for manual MACS separation. We advise to use LS separation columns, which can hold $10^8$ magnetically labeled cells from up to $2 \times 10^9$ total cells. In theory, smaller columns (e.g. MS separation columns) should fit for UCB samples, but we have frequently observed column clog with such samples. Alternatively, one can consider using an automatic device like AutoMACS™ (Miltenyi Biotec).

16. According to the manufacturer, one can expect a degree of purification of 85-98% CD34$^+$ cells. We routinely reach purification of >90-95% CD34$^+$ cells after the manual MACS separation.

17. After MACS separation, the yield of CD34$^+$ cells is around 0.5-5% of the initial amount of nucleated cells. One can expect 1-10x$10^6$ CD34$^+$ cells per liver and 0.1-1x$10^6$ CD34$^+$ cells per UCB.

18. Volumes and cell concentrations for the cell sorting are convenient for the FACSAria™ sorter (BD Biosciences) and should be adapted to other machines according to manufacturer’s instructions.

19. Be aware that the sorter cell counts are rarely fully accurate. It is therefore reasonable to expect after sorting of CD34$^+$CD38$^-$ cells a yield around 10% of the initial CD34$^+$ cell counts before cell sorting, despite the fact that CD34$^+$CD38$^-$ cells usually represent 20-40% of CD34$^+$ fetal liver cells.

20. We describe here an ideal situation where the lentivirus has been titrated so that 0.3 ml of lentiviral supernatant mixed with 0.3 ml of HSC in culture would result in 50% transduction efficiency. This has to be of course adapted to the lentiviral supernatant titer, and may require dilution of the virus if necessary. The number of cultured HSC in the well has also an influence on the final transduction efficiency, since the multiplicity of infection is dependent on the number of viral particles and the number of target cells.

21. Variability in reconstitution efficiency increases when lower numbers of progenitors are injected. Still, we have routinely obtained similar levels of reconstitution by inoculating $5 \times 10^5$ CD34$^+$ or $5 \times 10^4$ CD34$^+$CD38$^-$ cells per mouse.
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