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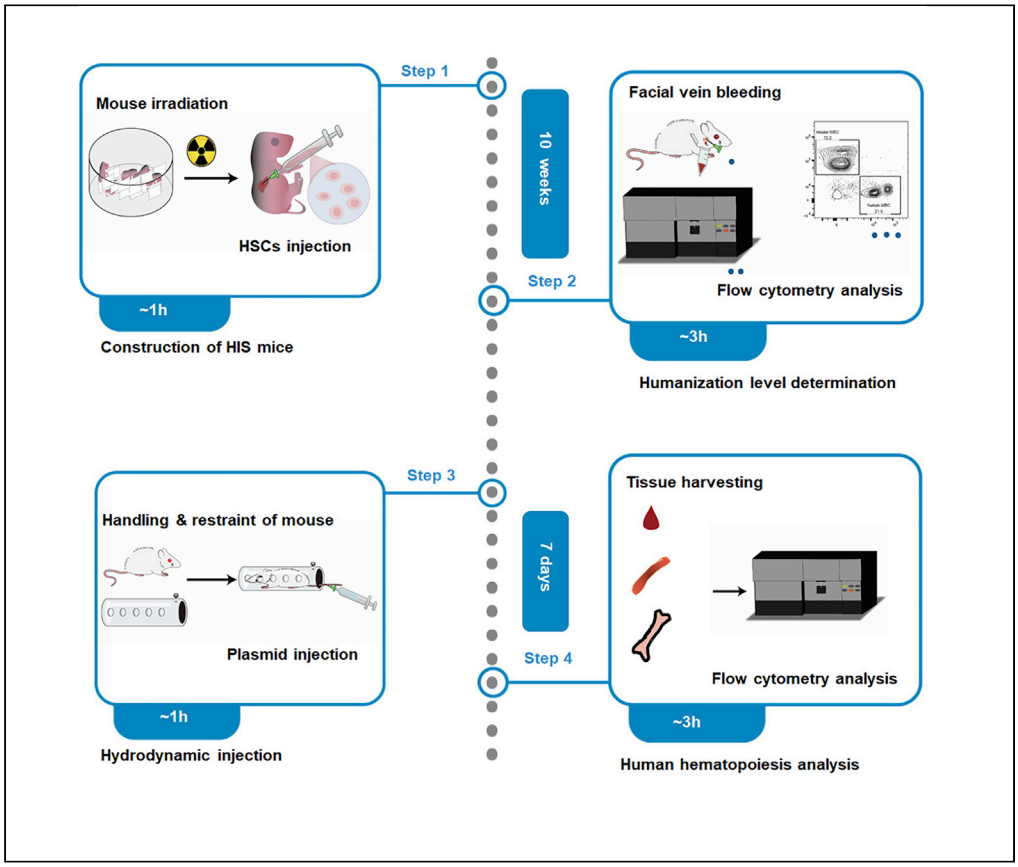
Protocol for generating human immune system mice and hydrodynamic injection to analyze human hematopoiesis *in vivo*



Human immune system (HIS) mice provide a valuable platform to investigate and modulate human hematopoiesis development *in vivo*. Here, we describe detailed protocols for the construction of HIS mice, modulation of human hematopoiesis *in vivo* using hydrodynamic injection of plasmids encoding cytokines of interest, and flow cytometry analysis of humanization levels and human immune subsets. This approach can be easily applied to screen or verify factors that regulate human hematopoiesis and immune system.

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Highlights

The protocol for construction of human immune system mice

Detailed procedure for hydrodynamic injection

Characterization of human immune subpopulations by flow cytometry

In vivo modulation of human hematopoiesis

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Protocol for generating human immune system mice and hydrodynamic injection to analyze human hematopoiesis *in vivo*

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SUMMARY

Human immune system (HIS) mice provide a valuable platform to investigate and modulate human hematopoiesis development *in vivo*. Here, we describe detailed protocols for the construction of HIS mice, modulation of human hematopoiesis *in vivo* using hydrodynamic injection of plasmids encoding cytokines of interest, and flow cytometry analysis of humanization levels and human immune subsets. This approach can be easily applied to screen or verify factors that regulate human hematopoiesis and immune system.

For complete details on the use and execution of this protocol, please refer to Cardoso et al. (2021) and Li et al. (2017).

BEFORE YOU BEGIN

The following steps describe the construction of HIS mice using irradiated immune deficient pups and modulation of human hematopoiesis with hydrodynamic injected plasmids to express cytokines of interest. Over the years, we have successfully deployed this approach in different HIS mouse researches, such as to boost human macrophage cell development (Li et al., 2013), investigate the efficiency and toxicity of high dose IL-2 treatment (Li et al., 2017), or evaluate the influence of IL-10 on emergency hematopoiesis *in vivo* (Cardoso et al., 2021). Nevertheless, the function of genes of interest on murine hematopoiesis or immune cell development can also be investigated by the hydrodynamic injection approach.

Institutional permissions

Investigators should obtain necessary institutional approvals for procedures involving human samples and animals. The cell source of human $CD34^+$ hematopoietic stem cells (HSCs) for creation of human immune system (HIS) mice could either be 14- to 20- week-old fetal liver tissues or cord blood. Adult HSCs could also be used for HIS mice generation, but HSCs from fetal liver or cord blood show better human immune cell reconstruction levels than adult HSCs (Drake et al., 2012; Lepus et al., 2009; Rongvaux et al., 2014). Human $CD34^+$ HSCs described in this protocol were obtained from fetal liver tissues under clinical ethical approval from the ethical committee of Drum Tower Hospital with informed consent (protocol # 2021-488-01). All experiments involving the generation and characterization of HIS mice were approved by an Institutional Animal Care and Use Committee (IACUC) at the Model Animal Research Center in Nanjing University (AP# LY-01).

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CD34$^+$ HSCs preparation

Timing: 3 h

Open the UV light to sterilize the biosafety cabinet and make sure all the materials used are properly sterilized.

1. Fetal liver tissue was cut into small pieces (1-2 mm in size) with a scalpel in DMEM.
2. Gently mince the tissues through a 100 μm cell strainer on a 50 mL tube with a 5 mL syringe piston.
3. Spin down the cells at 500 × *g* for 5 min at 4°C.
4. Resuspend the cells with DMEM and the mononuclear cells were further purified using Ficoll by centrifuge at 700 × *g* for 20 min at 20°C, with acceleration/deceleration rates of 5/1 (Eppendorf™ 5810R Centrifuge).
5. Collect the white mononuclear cell layer and centrifuge at 500 × *g* for 5 min at 4°C, then the cells are ready for CD34$^+$ HSCs purification.
6. CD34$^+$ HSCs were enriched using human CD34 MicroBead Kit according to the manuals of the products.
7. Count the live cells using 0.4% trypan blue solution (dilute the sample at 1:1 ration) with a KOVA™ Glasstic™ Slide under microscopy.
8. And the HSCs were cryopreserved in FBS containing 7.5% DMSO at 3 × 10^6 cells/0.5 mL/tube in liquid nitrogen.

Note: It is necessary to obtain approval from the institutional review board for collection of human material before carrying out this protocol.

Endotoxin-free plasmids purification

Timing: 2 days

Plasmids encoding genes of interest should be amplified in advance. Human IL-2 or IL-10 plasmids used for HIS mice hydrodynamic injection were purified from 400 mL E. coli cultured for 12 h using endotoxin-free plasmid isolation kits. The concentrations of plasmids were measured by Nano-300 Micro-Spectrophotometer. And the plasmids were stored at −20°C.

⚠ CRITICAL: Plasmids should be endotoxin-free. Otherwise, it would lead to mouse death after injection.

Breeding immune deficient mice

Timing: Around 4 weeks

The gestation time for mice is 19–21 days. When planning to construct HIS mice, the number of breeding cages needs to be estimated to get enough pups for human HSC injection. Currently, several widely available immunodeficient mouse strains for HIS mice generation have been developed based on BALB/c or NOD strain (Li and Di Santo, 2019; Saito et al., 2020). NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG)(Shultz et al., 2005), NOD.Cg-Prkdc^{scid} Il2rg^{tm1Sug}/JicTac (NOG)(Ito et al., 2002), and NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/NjuCrl (NCG)(Cardoso et al., 2021) mice are similar immunodeficient strains created on NOD mice with different strategies to knockout Prkdc and common cytokine receptor γ chain gene. As such, this protocol is generally applicable for NOD based immunodeficient strains. When applying this protocol to BALB/c.Rag2$^{-/-}$Il2rg$^{-/-}$Sirpa^{NOD} mice (BRGS) (Li et al., 2018; Traggiai et al., 2004), precautions need to be taken for irradiation doses as this strain is more radioresistant.

In our facility, NCG mice obtained from Gempharmatech (T001475) are routinely used. Usually, we put one male and two female NCG mice in one breeding cage, and expect to get 8 pups per litter. Newborn pups (4–6 days old) are used for HIS mouse construction.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD4 (Clone: OKT4) – FITC (1:50 dilution)	BioLegend	Cat#: 317408
Anti-human CD19 (Clone: HIB19) – PE-CF594 (1:50 dilution)	BD	Cat#: 562294
Anti-human CD38 (Clone: HIT2) - PE (1:50 dilution)	BioLegend	Cat#: 303506
Anti-human CD34 (Clone: 581) - BV421 (1:50 dilution)	BD	Cat#: 562577
Anti-mouse CD45 (Clone: REA737) - BV510 (1:50 dilution)	Miltenyi Biotec	Cat#: 130-110-665
Anti-human CD45 (Clone: HI30) - BV605 (1:50 dilution)	BioLegend	Cat#: 304042
Anti-human CD45 (Clone: HI30) - PE (1:50 dilution)	BioLegend	Cat#: 304058
Anti-human CD3 (Clone: UCHT1) - BV650 (1:50 dilution)	BD	Cat#: 563852
Anti-human CD8 (Clone: RPA-T8) - BV785 (1:50 dilution)	BioLegend	Cat#: 301046
Anti-human CD10 (Clone: HI10a) - FITC (1:50 dilution)	BioLegend	Cat#: 312208
Anti-human CD45 (Clone: 2D1) - PE-Cy7 (1:50 dilution)	BioLegend	Cat#: 368532
Anti-human CD123 (Clone: 6H6) - APC (1:50 dilution)	BioLegend	Cat#: 306012
Anti-mouse CD45 (Clone: HI30) - APC-Cy7 (1:50 dilution)	BioLegend	Cat#: 103116
Anti-human CD45RA (Clone: HI100) - BV711 (1:50 dilution)	BioLegend	Cat#: 304138
Chemicals, peptides, and recombinant proteins		
Fixable viability dye eFlour506 (1:400 dilution)	Invitrogen	Cat#: 65-0866-18
RPMI Medium 1640	Biological Industries	Cat#: 01-100-1ACS
DMEM	Biological Industries	Cat#: 06-1055-57-1ACS
Ficoll®-Paque Premium	GE Healthcare	Cat#:17544203
FBS (heat-inactivated)	Gibco	Cat#: 10099141
100× Penicillin/Streptomycin Solution	Gibco	Cat#: 15140122
BD FACS™ Lysing Solution 10× Concentrate	BD	Cat#:349202
Red Blood Cell Lysing Buffer Hybri-Max	Sigma-Aldrich	Cat#: R7757
EDTA	LEAGENE	Cat#: ND0081
Trypan blue	BBI life science	Cat#: A601140-0010
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#: D2650
Human IgG (5 μg/μL, 1:50 dilution)	Sigma-Aldrich	Cat#: I8640
Experimental models: Organisms/strains		
NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/NjuCrl (NCG) 4–6 days old pups (regardless of gender) were used for HIS mice generation; 12 weeks old HIS mice were used for hydrodynamic injection.	Gempharmatech	T001475
Recombinant DNA		
Human IL-10 expression plasmid	Origene	Cat#:SC300099
Human IL-2 expression plasmid	Origene	Cat#:SC125234
Software and algorithms		
NovoExpress Software	Agilent	Version 1.5.6
FlowJo	BD	FlowJo 10.6.2
Other		
Centrifuge	Eppendorf	5810R
Microscopy	Olympus	CX43RF-R
Micro-Spectrophotometer	ALLSHENG	Nano-300
Human CD34 MicroBead Kit	Miltenyi Biotec	Cat#:130-046-702
EndoFree Plasmid Maxi Kit	Vazyme	Cat#: DC202-01
ELISA MAX™ Deluxe Set Human IL-2	BioLegend	Cat#: 431805
ELISA MAX™ Deluxe Set Human IL-10	BioLegend	Cat#: 430604
KOVA™ Glasstic™ Slide 10 with Grids	Kova International, Inc	Cat#: 87144E

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
29 gauge 1 mL BD insulin syringe	BD	Cat#: 328421
2.5 mL syringe	Shanghai Kindly Medical Instruments Co., Ltd	NA
23 gauge needle	Shanghai Kindly Medical Instruments Co., Ltd	NA
26 gauge needle	Shanghai Kindly Medical Instruments Co., Ltd	NA
1.5 mL anticoagulant tube	Jiangsu Xinkang Medical Instrument Co., Ltd.	NA
U-bottom 96-well plate	Jet Bio-Fil	Cat#: TCP002096
Scissors and forceps	NA	NA
100 μm cell strainer	Jet Bio-Fil	Cat#: CSS013100
70 μm cell strainer	Jet Bio-Fil	Cat#: CSS01370
0.22 μm sieve	Millipore	Cat#: SLGPR33RB
15 mL centrifuge tube	Jet Bio-Fil	Cat#: CFT011150
50 mL centrifuge tube	Jet Bio-Fil	Cat#: CFT011500
FACS tube	BD	Cat#: 352008

MATERIALS AND EQUIPMENT

FACS Buffer

Reagent	Final concentration	Amount
PBS	n/a	483 mL
Fetal Bovine Serum	2%	10 mL
100× Penicillin/Streptomycin Solution	1%	5 mL
500 mM EDTA	2 mM	2 mL
Total	**n/a**	**500 mL**

Prepare in a biological safety cabinet and store at 4°C up to 6 months.

Cell Buffer

Reagent	Final concentration	Amount
RPMI 1640	n/a	483 mL
Fetal Bovine Serum	2%	10 mL
100× Penicillin/Streptomycin Solution	1%	5 mL
500 mM EDTA	2 mM	2 mL
Total	**n/a**	**500 mL**

Prepare in a biological safety cabinet and store at 4°C up to 6 months.

Cell medium

Reagent	Final concentration	Amount
RPMI 1640	n/a	445 mL
Fetal Bovine Serum	10%	50 mL
100× Penicillin/Streptomycin Solution	1%	5 mL
Total	**n/a**	**500 mL**

Prepare in a biological safety cabinet and store at 4°C up to 6 months.

Injection medium

Reagent	Final concentration	Amount
RPMI 1640	n/a	495 mL
100× Penicillin/Streptomycin Solution	1%	5 mL
Total	**n/a**	**500 mL**

Prepare in a biological safety cabinet and store at 4°C up to 6 months.

Blocking buffer		
Reagent	Dilution	Amount per sample
Fixable viability dye eFlour506	1:400	0.125 μL
IgG (5 μg/μL)	1:50	1 μL
FACS buffer	n/a	48.875 μL
Total	**n/a**	**50 μL**
Prepare prior to use.		

Flow Cytometer

The flow cytometry samples in this protocol were acquired by a 5 laser Agilent NovoCyte Penteon Flow Cytometer (405 nm Violet, 488 nm Blue, 561 nm Yellow, 637 nm Red, and Ultra violet 349 nm).

> *Alternatives:* Flow cytometers equipped with appropriate lasers available from the researcher's facility, such as BD flow cytometers, Attune Flow Cytometer from Thermo fisher are also suitable for the analysis.

Irradiator

In this project, an RS-2000-PRO-225 X-ray irradiator (Rad Source) is shown as an example for mouse irradiation.

> *Alternatives:* Other irradiators, such as gamma-ray irradiator, are also typically employed for mouse irradiation.

STEP-BY-STEP METHOD DETAILS

Mouse irradiation

> Timing: Approximately 20 min

This step describes the key operations for total body irradiation of NCG pups.

1. Turn on and pre-heat the irradiator.
2. 4–6 days old pups (no more than 15 pups) from a ventilated cage along with some bedding were transferred into one autoclaved plastic box, then close the box lid (Figures 1A and 1B).

> ⚠ CRITICAL: The operation should be conducted under a biological safety cabinet. And the following irradiation process should be finished within 30 min. Otherwise, pups would be suffocated.

3. Wrap the individual box with autoclaved paper bags and take it out of the facility.

> *Note:* If the irradiator is inside the animal facility, jump steps 3 and 7, and follow the procedure dictates of the facility.

4. Put the plastic boxes one at a time into the irradiator (Figure 1C).
5. Set the sub-lethal irradiation dose (63 s, 70 cGy X-ray for NCG mice) and start the irradiation process.

Table 1. Reference irradiation doses for different immunodeficient mouse strains

Mice	Strains	Sublethal irradiation doses	References
SCID mice (Prkdc SCID mutation)	NSG	100 cGy	(Li et al., 2013; Rongvaux et al., 2014)
	NOG	75 cGy	(Andersen et al., 2020)
	NCG	70 cGy	(Cardoso et al., 2021)
	NSGW41	No irradiation is required due to genetic mutation in Kit	(Cosgun et al., 2014)
Rag mice (Rag1/Rag2 mutation)	BRG	3.3 Gy	(Huntington et al., 2011)
	BRGS/BRGST	3 Gy	(Li et al., 2017, 2018)

⚠ CRITICAL: The immunodeficient mouse strains for HIS mice generation could be typically classified into SCID mice or Rag mice regarding to their mutation of Prkdc or Rag1/Rag2 respectively. SCID mice are sensitive to irradiation due to their mutation in Prkdc(Biedermann et al., 1991), whereas Rag mice are relatively resistant to irradiation. Thus, precautions need to be taken when choosing a starting irradiation dose for optimization. The reported sublethal doses of mouse pups from different strains are listed (Table 1). As indicated, for SCID mice, the sublethal irradiation dose test should be set between 60 and 110 cGy, e.g., 60, 70, 80, 90, 100, 110 cGy. While for Rag mice the initial dose for sublethal irradiation escalation test should start from 2 Gy, e.g., 2, 2.5, 3, 3.5, 4 Gy.

6. Take out the plastic box when the radiation process finishes and move on to irradiate the next one.
7. Remove paper bags when entering the animal facility.
8. Transfer the plastic boxes carrying irradiated pups into a biological safety cabinet and open the lids.

Note: Avoid introducing foreign scents or contaminations during the process. Otherwise, the parental mice may kill or stop feeding the pups.

Recovery of CD34$^+$ HSCs from cryopreservation

Timing: 30 min

9. Take out one vial of previously cryopreserved CD34$^+$ HSCs from liquid nitrogen (put the tube in liquid nitrogen or dry ice if transfer process is required) and immediately put it into a 37°C water bath.
10. Quickly thaw the cells by gently swirling the vial in the water bath.
11. Take out the vial and spray it with 70% ethanol and then transfer it into a biological safety cabinet.
12. Open the vial and transfer the cells into a centrifuge tube containing 10 mL pre-warmed cell medium.
13. Count the live cells using 0.4% trypan blue solution (dilute the sample at 1:1 ration) with a KOVA™ Glasstic™ Slide under microscopy.
14. Centrifuge the cells at 500 × *g* for 5 min at 4°C.
15. Carefully aspirate the upper supernatant and resuspend cells to the concentration of 10^5 cells/50 μL with RPMI 1640 injection medium.

Intrahepatic HSC injections

Timing: Approximately 1–2 h, about 2 min per mouse

Figure 1. The irradiation process of pups

(A) Transfer pups from a ventilated cage into an autoclaved box.

(B) A plastic box with the lid closed.

(C) Irradiate the pups using a RS-2000 Rad Source Irradiator.

16. Load a 29 gauge 1 mL BD insulin syringe with resuspended HSCs.
17. Take one pup and hold it head down with fingers applying gentle pressure to restrict the movement of pups and expose the abdomen. Then the red-colored liver can be seen just adjacent to the white-colored stomach (Figure 2).
18. Hold the syringe with the other hand and insert the needle at a 45-degree angle into the pup's liver about 3 mm depth.

Note: Always follow the role of handling sharps and needles during the process.

19. Inject 50 μL medium slowly into the liver and hold still for 3 s.
20. Release the pressure of your fingers from the injected pups, then pull out the needle slowly and put the pups back to the plastic box.
21. Repeat the above steps until all the injections are finished.
22. Wean HIS mice at 21 days old and wait for 10 weeks after the HSCs injection before further humanization level analysis.

Note: It takes effort and practice to be proficient at intrahepatic injection, starting from practicing with culture medium/PBS instead of HSCs.

Flow cytometry analysis of humanization level

Flow cytometry analysis of the blood of HIS mice is a convenient and standard approach to obtain the humanization information. This section describes the detailed steps on humanization level determination, including the blood collection and the further immune phenotype analysis using flow cytometry.

Facial vein bleeding of HIS mice

Timing: About 1 min per mouse

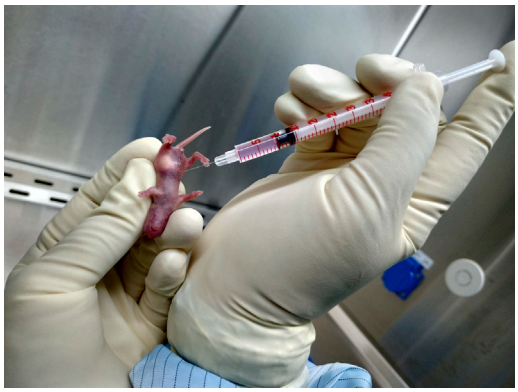

Figure 2. Posture for intrahepatic injection

This step describes the blood draw from HIS mice. Drawing blood from mouse facial veins is routinely employed in our facility. Alternative methods such as retro-orbital blood collection and blood collection from the tail are also applicable. We choose facial vein bleeding method over retro-orbital and tail vein bleeding as this approach causes less stress to the mice and cost less time than other approaches.

23. Take one HIS mouse out of the cage and properly restrain it with one hand.

Note: Properly restraining the mice and applying adequate pressure to the maxillary vein is a prerequisite for a smooth blood draw.

24. Visualize the mark of the maxillary vein: albino NCG mouse has an obvious mark for the maxillary vein, which is viewed as a small bald spot (shown in Figure 3A). The maxillary vein is just under this spot.
25. Puncture it with a 23 gauge needle, withdraw the needle quickly and then collect 50 μL blood using an anticoagulant tube (Figures 3B and 3C).
26. Apply gentle pressure with an alcohol pad on the site of the puncture to stop the bleeding.
27. Dispose the needle into a sharps/biohazard container.
28. Repeat the above steps until the blood draw of all the mice is finished.

Note: Always use a new sterile needle to bleed each HIS mouse. Do not share needles for different mice to avoid cross-contamination.

FACS analysis of the blood samples

Timing: Approximately 1–2 h, it takes about 2 h for 20 mice

29. Prepare fluorescent antibody cocktail containing the antibodies in Table 2.

Note: For a basic detection of human immune cell reconstruction level, antibodies against human CD45, human B cells (CD19/CD20), T cells (CD3) as well as mouse CD45 are necessary. The choice of antibody fluorochrome conjugates in this protocol is optimized for flow cytometer available in our facility. As such, antibody panels should be adjusted according to the parameter of the available flow cytometer.

30. Add 6 μL/well antibody cocktail into a 96 well round bottom plate.
31. Transfer 50 μL of blood from each blood sample to the 96 well round bottom plate and incubate for 30 min at 4°C, protecting from light.

Figure 3. Drawing blood from the submandibular vein

(A) Red arrow indicates the bald area where the submandibular vein locates.

(B) Insert the needle directly into the bald area.

(C) Peripheral blood comes out of the submandibular vein.

32. Dilute 10× BD FACS™ Lysing Solution to 1× with ddH_20, then add 200 μL lysing buffer to each sample after the antibody staining process and incubate for 10 min at room temperature (20°C–25°C).
33. Spin the plate at 500 × *g* for 5 min at 4°C.

Note: Repeat the lysing process if there are red blood cells remaining. Alternatively, the red blood cells can be lysed with ACK (RBC lysis buffer) first and then proceed for antibody staining process.

34. Resuspend the cell pellet in 200 μL of ice cold FACS buffer or cell buffer.
35. Transfer the cell suspension into a flow tube by passing through a 70 μm cell strainer.
36. Store the samples on ice and wait for further flow cytometry acquisition.
37. Acquire the samples to collect the desired volume of sample using available flow cytometer.

Table 2. Example staining panel to detect the humanization levels of HIS mice

Antibody	Dilution	Amount per sample
Anti-mouse CD45 BV510	1:50	0.2 μg
Anti-human CD45 BV605	1:50	0.2 μg
Anti-human CD3 BV650	1:50	0.2 μg
Anti-human CD19 PECF594	1:50	0.2 μg
Anti-human CD4 FITC	1:50	0.2 μg
Anti-human CD8 BV785	1:50	0.2 μg

Note: For detailed guidelines and description of flow cytometer set-up when performing flow cytometry experiments please refer to (Cossarizza et al., 2021; Prosser et al., 2021; Rico et al., 2021)

38. Analyze FCS files using flow cytometry analysis software. We routinely use NovoExpress Software or FlowJo for flow cytometer data analysis (a gating strategy is shown in Figure 4).
39. Determine the cell number of human CD45 (hCD45) per mL blood as :

$$cell\ number\ per\ ml = \frac{cell\ count\ of\ hCD45\ from\ acquired\ sample}{acquired\ voulme\ per\ sample} \times total\ volume\ per\ sample \times 20.$$

Note: Here, 20 is the quotient of 1000 μl/50 μl blood.

And calculate the humanization levels of the HIS mice using the following formula:

$$\%\ of\ humanization\ level = \frac{\%\ of\ hCD45}{\%\ of\ hCD45 + \%\ of\ mCD45} \times 100\ \%.$$

The HIS mouse with a hCD45 cell number above 10^5/mL or a humanization level above 10% is used for further experiments. And HIS mice assigned to different groups should have matched gender and similar humanization levels.

Hydrodynamic injection

Timing: Steps 40–41 take about 20 min and steps 42–49 cost about 1 min per mouse

Hydrodynamic injection is a procedure to express proteins of interest in mouse liver by rapid injection of large volume of plasmids DNA solution into the tail vein of mouse. The following steps describe the detailed procedures for hydrodynamic injection, including plasmids solution preparation and tips for the injection.

40. Prepare the plasmid solution using a 50 mL tube as shown in Table 3.

Note: The values for A and B are determined follows: A- The amounts of plasmids injected positively correlate with protein expression levels. But the range of protein expression varies considerably and need to be individually determined. B- Typically, a maxi-prep would yield a stock plasmid concentration of 500 μg/ml to 1500 μg /ml.

⚠ CRITICAL: Plasmids should be endotoxin-free. Otherwise, it would lead to mouse death after injection.

41. Weigh the mice and prepare the injection solution as shown in Table 4.
42. Load the injection solution to a 2.5 mL syringe with a 26 gauge needle.

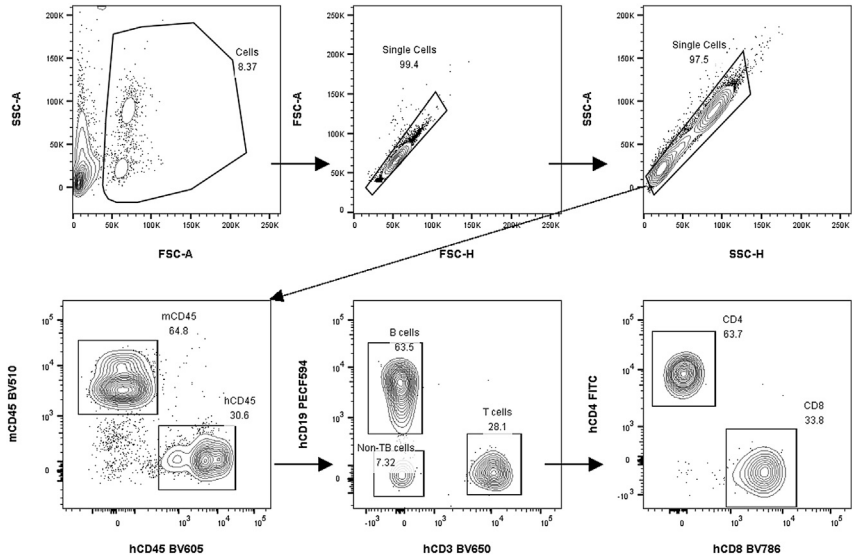

Figure 4. Gating strategy for FACS analysis of blood samples from HIS mice

⚠ CRITICAL: Tap the syringe to remove all the air bubbles in the syringe.

43. Pre-heat the mice with a heat lamp for 15 min to increase the visibility of the lateral caudal veins.
44. Put one HIS mouse in a restraining device and firmly immobilize the mouse (Figure 5A).

Note: Make sure the mouse still breathes freely.

45. Pull and straighten the tail of the mouse with your non-dominant hand.
46. Hold the syringe by the other hand with the bevel of the needle facing upward and nearly parallel to the lateral caudal vein, then insert the needle smoothly into the vein (Figure 5B).
47. Inject the solution quickly within 7 s, otherwise, the transfection efficiency would be extremely low.

Note: Always start the injection from the tip of the tail to ensure that more attempts could be carried out. If the solution cannot be injected completely at a single injection, inject the remaining solution again into the other later tail vein immediately. If two injections cannot be finished within 1 min, the transfection will not be successful. And the mouse should not proceed for further experiments.

48. Immediately release the mouse from the restrainer and put it back into the cage.

Table 3. Guidance for plasmid solution preparation

Parameters	
The number of mice: n The amount of plasmids to be injected per mouse: A μg (usually between 10 and 50 μg) The concentration of plasmid stock: B μg/mL	
The components of the plasmid solution	
Component	Volume
Plasmid stock	(n+2) * A/B ml
10× PBS	0.1* (n+2) mL
ddH_2O	(n+2) (0.9-A/B) ml
Mix the ingredients evenly, then filter through a 0.22 μm sieve.	

Table 4. Guidance for injection solution preparation

Weight of the mouse W	Volume
15 g ≤ W ≤ 25 g	1 mL plasmid solution + (0.1*W-1) mL 1× PBS
W > 25 g	1 mL plasmid solution+ 1.5 mL 1× PBS

⚠ CRITICAL: Remove the mouse from the restrainer immediately after the injection, or it may cause mouse death due to suffocation. The mouse may remain motionless for 10–30 min after hydrodynamic injection due to temporary arrhythmia. Usually, the mouse will recover within 10 min. On the condition that the breathing of the mouse turns out to be really shallow, gently massage the abdomen of the mouse to facilitate breathing.

49. Collect 100 μL of blood from the submandibular vein 24 h after the hydrodynamic injection as described from steps 23–28.
50. Centrifuge the blood at 2,000 × *g* for 10 min at 4°C.
51. Transfer the blood plasma into a new sterilized 0.5 mL tube.
52. Store the blood plasma at −80°C for ELISA analysis.

Note: ELISA analysis of the plasmid coding protein is done according to the manuals of the corresponding ELISA MAX™ Deluxe Set from Biolegend.

FACS analysis of human hematopoiesis and immune cells

Timing: Approximately 2–3 h, it takes about 3 h for 10 mice

This part demonstrates the basic analysis of human hematopoiesis and immune cells after hydrodynamic injection. Here we take the HIS mice that receive phIL-10 hydrodynamic injection as an example.

53. 7 days post hydrodynamic injection, mice are sacrificed and the spleens are harvested in a 6 well plate containing 5 mL FACS buffer or cell buffer.
54. Gently mince the spleen through a 100 μm cell strainer with a 5 mL syringe piston.
55. Transfer the splenocytes into 15 mL centrifuge tubes and spin at 500 × *g* for 5 min at 4°C.
56. Aspirate the supernatant and resuspend the pellet in 1 mL Red Blood Cell Lysing Buffer Hybri-Max, incubating the cells at room temperature (20°C–25°C) for 10 min.

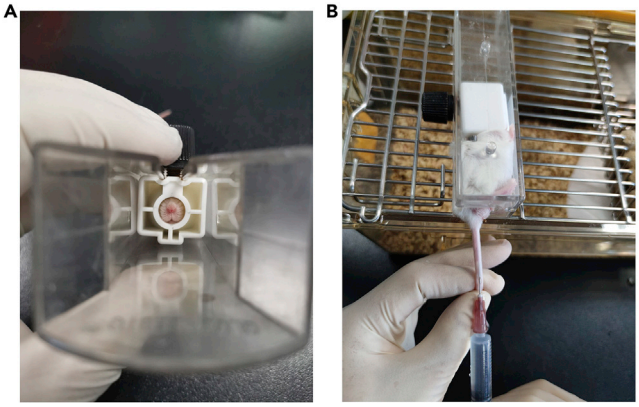

Figure 5. Hydrodynamic injection
(A) Ensure the mouse still can breathe freely after being restrained in the immobilizer.
(B) Insert the needle into the later tail vein and finish the injection within 7 s.

Table 5. Example panel to detect the myeloid progenitors of HIS mice

Antibody	Dilution	Amount per sample
FACS buffer		50 μL
Anti-human CD10 FITC	1:50	0.2 μg
Anti-human CD38 PE	1:50	0.2 μg
Anti-human CD45 PE-Cy7	1:50	0.2 μg
Anti-human CD123 APC	1:50	0.2 μg
Anti-mouse CD45 APC-Cy7	1:50	0.2 μg
Anti-human CD34 BV421	1:50	0.2 μg
Anti-human CD45RA BV711	1:50	0.2 μg

57. Add 9 mL FACS buffer to stop the lysis and filter the cells into a new 15 mL tube by passing through a 70 μm cell strainer.
58. Spin down the cells at 500 × *g* for 5 min at 4°C.
59. Aspirate the supernatant and resuspend the pellet in 5 mL FACS buffer.
60. Count live cells using 0.4% trypan blue solution (dilute the sample at 1:1 ration) with a KOVA™ Glasstic™ Slide under microscopy.
61. Adjust cell concentration and transfer one million live cells per mouse in one well of a 96 well round bottom plate and spin down the cells at 500 × *g* for 5 min at 4°C.
62. Prepare the blocking buffer in 50 μL FACS buffer per sample during centrifugation.
63. Discard the supernatant by flicking the plate over a sink.
64. Resuspend the cells by adding 50 μL blocking buffer and incubate at 4°C for 15 min.
65. Prepare the flow antibody mix as the panel in Table 5.

Note: The cell subpopulations are defined by cell surface markers according to Table 6.

66. Add 200 μL ice cold FACS buffer and spin down the cells at 500 × *g* for 5 min at 4°C.
67. Discard the supernatant, resuspend the cells with previously prepared 50 μL antibody mix and incubate at 4°C for 30 min, protecting from light.
68. Add 200 μL ice cold FACS buffer and spin down the cells at 500 × *g* for 5 min at 4°C.
69. Discard the supernatant, resuspend the cells with 200 μL ice cold FACS buffer.
70. Transfer the cells into a flow tube by passing through a 70 μm cell strainer.
71. Acquire the samples to collect the desired volume of sample with a flow cytometer.
72. Analyze FCS files using NovoExpress Software or FlowJo (representative results are shown in Figure 6).

EXPECTED OUTCOMES

Humanization level

Normally, 10 weeks after intra-hepatic injection with 10^5 $CD34^+$ HSCs, more than 70% of the injected mice are expected with a humanization level over 10% or the CD45 cell number in the blood above 10^5/mL.

Human hematopoiesis and immune system modulation

1 week after plasmid hydrodynamic injection, phIL-10 will lead to emergency myelopoiesis in HIS mice (Cardoso et al., 2021). FACS analysis reveals that both GMP and myeloid cells are significantly

Table 6. Example cell subpopulations checked in this protocol

Cell population	Cell population	Immunophenotype
Plasmacytoid dendritic cells	pDCs	$CD45^+$ $CD34^+$ $CD38^+$ $CD10^-$ $CD45RA^+$ $CD123^+$
Granulocyte-monocyte progenitor	GMP	$CD45^+$ $CD34^+$ $CD38^+$ $CD10^-$ $CD45RA^+$ $CD123^{lo}$
Common myeloid progenitor	CMP	$CD45^+$ $CD34^+$ $CD38^+$ $CD10^-$ $CD45RA^-$ $CD123^{lo}$
Megakaryocyte-erythrocyte progenitor	MEP	$CD45^+$ $CD34^+$ $CD38^+$ $CD10^-$ $CD45RA^-$ $CD123^-$

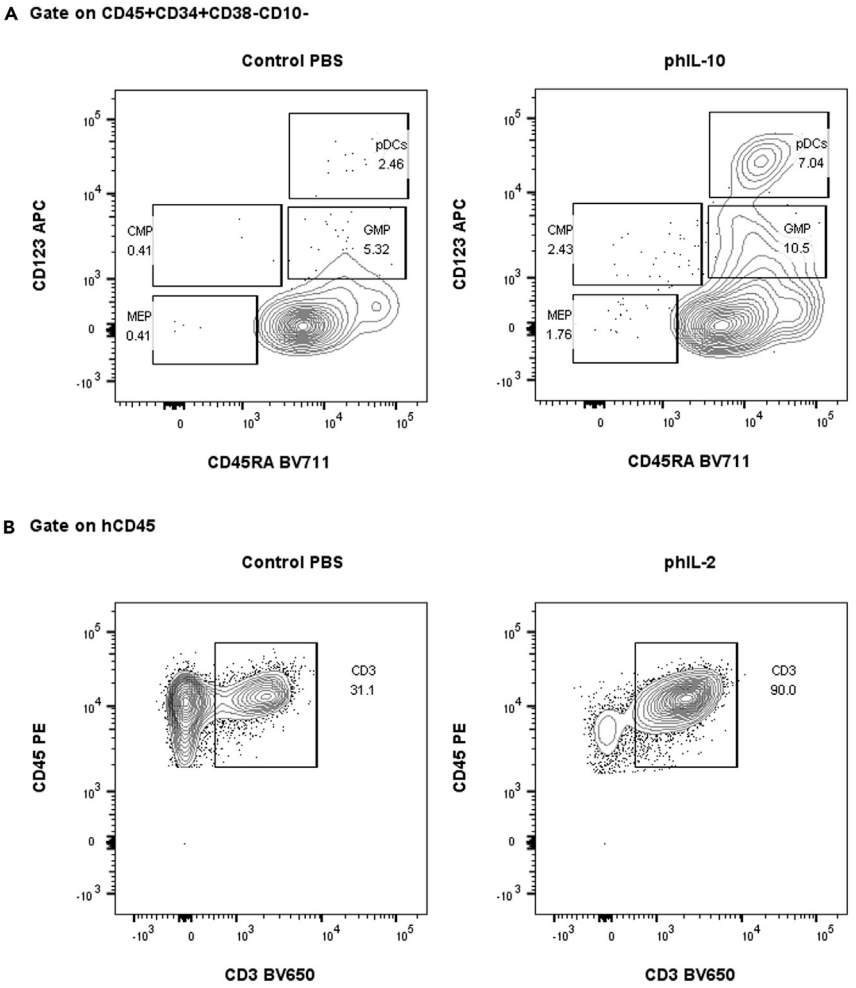

Figure 6. Examples of human hematopoiesis or immune system modulation in HIS mice after hydrodynamic injection

(A) Emergency myelopoiesis in HIS mice after phIL-10 hydrodynamic injection.

(B) Extensive human T cells amplification in phIL-2 hydrodynamic injected HIS mice.

increased as compared to the control group (Figure 6A). And for the HIS mice that receive phIL-2 hydrodynamic injection (Li et al., 2017), the population of spleen T cells will increase dramatically (Figure 6B).

LIMITATIONS

This protocol is convenient for investigating the regulation of human hematopoiesis or immune system by secreted factors such as cytokines and chemokines. It is not suitable to investigate the function of intracellular proteins, e.g., transcriptional factors and enzymes. Hydrodynamic injection is a much simpler and faster approach than transgenic or knock-in mouse models to achieve the expression of the gene of interest *in vivo*. Of note, the expression level is transient. To achieve a longer period of foreign protein expression, pLive plasmids are recommended (Huang et al., 2017). However, both of them result in systemic and supra-physiological expression level of plasmid coding proteins. Even worse, pLive plasmids may lead to human HSC exhaustion in the long-term. Hence, the experiments should be designed carefully to answer particular scientific questions in the right time window.

TROUBLESHOOTING

Problem 1

Pups die before weaning (steps 5 and 8 of step-by-step method details).

Potential solution

If injected pups show growth retardation or die before weaning, it is likely due to the high irradiation dose. Then, the radiation dose escalation experiments should be performed to identify a sub-lethal dose that does not lead to pups' death. In other cases, parental mice will not feed or kill their cubs when they are anxious, so be careful not to disturb the parental mice during the experiment. It's recommended to take some bedding from the cage while transferring the pups and avoid introducing pathogens or foreign scents to the pups.

Problem 2

Variability in HIS reconstitution with different sources of $CD34^+$ HSCs (steps 38 and 39).

Potential solution

HSCs from different donors/sources used contribute most to the data variability. The humanization levels, lineage compositions, and responses to stimuli are relatively stable from HIS mice reconstituted from the same donor. Thus, it is critical to use the same HSC donor/source in the one experiment to minimize the variability, unless the purpose of the experiment is to compare donor to donor, or source to source differences after humanization. According to other researchers and our experience, HSCs from fetal liver show better human immune cell reconstruction level than cord blood and adult HSCs (Drake et al., 2012; Lepus et al., 2009; Rongvaux et al., 2014).

Problem 3

Low level of plasmid encoding gene expression in the serum (step 47).

Potential solution

Make sure the plasmids injection is finished within 7 s, otherwise, the transfection efficiency would be extremely low. Secure the restrainer and straighten the mouse tail when injecting, so that the mouse holds still during the procedure. And a single hydrodynamic injection volume must reach at least 75% of mouse weight to be effective (Liu et al., 1999).

Problem 4

HIS mice die after hydrodynamic injection (steps 40 and 48).

Potential solution

First verify all the plasmids injected are endotoxin-free, make sure endotoxin-free isolation kits are used for plasmids extraction. Ensure the mouse can breathe freely when they were in the mouse restrainer. On the other hand, inflammatory cytokines such as high levels of human IL-2 will lead to physiological toxicity after hydrodynamic injection (Li et al., 2017), chose the right dose of plasmid injection.

Problem 5

Short expression duration of plasmid encoding proteins *in vivo* (step 52).

Potential solution

For a vector with CMV promoter, plasmid expression can be detected within one week after hydrodynamic injection. To achieve a longer expression period, pLIVE® In Vivo Expression Vectors are recommended.

Problem 6

Humanization levels decrease dramatically after hydrodynamic injection (step 53).

Potential solution

If decreased humanization levels are caused by human HSCs exhaustion, the amount of plasmids should be reduced. In addition, for certain cross-species reactive cytokines, such as Flt3L, the injected DNA plasmids may favor the generation of mouse cells than human cells. This will finally lead to extensive abnormal murine hematopoiesis overriding the human hematopoiesis. In this situation, the host mouse genetic background is important. For example, using the Flt3 knockout strain to avoid the competition from mouse cells for supplemented Flt3L (Li et al., 2016).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yan Li (yanli@nju.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No data sets or codes were used in this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.L.; methodology, D.R., W.L., and S.D.; investigation, D.R. and W.L.; writing – original draft, Y.L., D.R., and W.L.; writing – review & editing, Y.L., D.R., W.L., and S.D.; funding acquisition, Y.L. and D.R.; resources, Y.L. and S.D.; supervision, Y.L.

DECLARATION OF INTERESTS

Y.L. is currently consulting for GemPharmatech Co.

REFERENCES

Andersen, A.H.F., Nielsen, S.S.F., Olesen, R., Mack, K., Dagnæs-Hansen, F., Uldbjerg, N., Østergaard, L., Søgaard, O.S., Denton, P.W., and Tolstrup, M. (2020). Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection. J. Vis. Exp. *155*, e60723..

Biedermann, K.A., Sun, J.R., Giaccia, A.J., Tosto, L.M., and Brown, J.M. (1991). scid mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double-strand break repair. Proc. Natl. Acad. Sci. U S A *88*, 1394–1397.

Cardoso, A., Martins, A.C., Maceiras, A.R., Liu, W., Castro, I., Castro, A.G., Bandeira, A., Di Santo, J.P., Cumano, A., Li, Y., et al. (2021). Interleukin-10 induces interferon-gamma-dependent emergency myelopoiesis. Cell Rep. *37*, 109887.

Cosgun, K.N., Rahmig, S., Mende, N., Reinke, S., Hauber, I., Schafer, C., Petzold, A., Weisbach, H., Heidkamp, G., Purbojo, A., et al. (2014). Kit regulates HSC engraftment across the human-mouse species barrier. Cell Stem Cell *15*, 227–238.

Cossarizza, A., Chang, H.D., Radbruch, A., Abrignani, S., Addo, R., Akdis, M., Andra, I., Andreata, F., Annunziato, F., Arranz, E., et al. (2021). Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). Eur. J. Immunol. *51*, 2708–3145.

Drake, A.C., Chen, Q., and Chen, J. (2012). Engineering humanized mice for improved hematopoietic reconstitution. Cell Mol Immunol. *9*, 215–224.

Huang, M., Sun, R., Huang, Q., and Tian, Z. (2017). Technical improvement and application of hydrodynamic gene delivery in study of liver diseases. Front. Pharmacol. *8*, 591.

Huntington, N.D., Alves, N.L., Legrand, N., Lim, A., Strick-Marchand, H., Mention, J.J., Plet, A., Weijer, K., Jacques, Y., Becker, P.D., et al. (2011). IL-15 transpresentation promotes both human T-cell reconstitution and T-cell-dependent antibody responses in vivo. Proc. Natl. Acad. Sci. U S A *108*, 6217–6222.

Ito, M., Hiramatsu, H., Kobayashi, K., Suzue, K., Kawahata, M., Hioki, K., Ueyama, Y., Koyanagi, Y., Sugamura, K., Tsuji, K., et al. (2002). NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood *100*, 3175–3182.

Lepus, C.M., Gibson, T.F., Gerber, S.A., Kawikova, I., Szczepanik, M., Hossain, J., Ablamunits, V., Kirkiles-Smith, N., Herold, K.C., Donis, R.O., et al. (2009). Comparison of human fetal liver, umbilical cord blood, and adult blood hematopoietic stem cell engraftment in NOD-scid/gammac-/-, Balb/c-Rag1-/-gammac-/-, and C.B-17-scid/bg immunodeficient mice. Hum. Immunol. *70*, 790–802.

Li, Y., Chen, Q., Zheng, D., Yin, L., Chionh, Y.H., Wong, L.H., Tan, S.Q., Tan, T.C., Chan, J.K., Alonso, S., et al. (2013). Induction of functional human macrophages from bone marrow promonocytes by M-CSF in humanized mice. J. Immunol. *191*, 3192–3199.

Li, Y., and Di Santo, J.P. (2019). Modeling infectious diseases in mice with a "humanized" immune system. Microbiol. Spectr. *7*, 1–13.

Li, Y., Masse-Ranson, G., Garcia, Z., Bruel, T., Kok, A., Strick-Marchand, H., Jouvion, G., Serafini, N., Lim, A.I., Dusseaux, M., et al. (2018). A human immune system mouse model with robust lymph node development. Nat. Methods *15*, 623–630.

Li, Y., Mention, J.J., Court, N., Masse-Ranson, G., Toubert, A., Spits, H., Legrand, N., Corcuff, E., Strick-Marchand, H., and Di Santo, J.P. (2016). A novel Flt3-deficient HIS mouse model with selective enhancement of human DC development. Eur. J. Immunol. *46*, 1291–1299.

Li, Y., Strick-Marchand, H., Lim, A.I., Ren, J., Masse-Ranson, G., Dan, L., Jouvion, G., Rogge, L., Lucas, S., Bin, L., and Di Santo, J.P. (2017). Regulatory T cells control toxicity in a humanized model of IL-2 therapy. Nat. Commun. *8*, 1762.

Liu, F., Song, Y., and Liu, D. (1999). Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. *1258*, 1266.

Prosser, A., Dart, S., Larma-Cornwall, I., and Lucas, M. (2021). Flow cytometric characterization of tissue-resident lymphocytes after murine liver and heart transplantation. STAR Protoc. *2*, 100810.

Rico, L.G., Salvia, R., Ward, M.D., Bradford, J.A., and Petriz, J. (2021). Flow-cytometry-based protocols for human blood/marrow immunophenotyping with minimal sample perturbation. STAR Protoc. *2*, 100883.

Rongvaux, A., Willinger, T., Martinek, J., Strowig, T., Gearty, S.V., Teichmann, L.L., Saito, Y., Marches, F., Halene, S., Palucka, A.K., et al. (2014). Development and function of human innate immune cells in a humanized mouse model. Nat. Biotechnol. *32*, 364–372.

Saito, Y., Shultz, L.D., and Ishikawa, F. (2020). Understanding normal and malignant human hematopoiesis using next-generation humanized mice. Trends Immunol. *41*, 706–720.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., et al. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J. Immunol. *174*, 6477–6489.

Traggiai, E., Chicha, L., Mazzucchelli, L., Bronz, L., Piffaretti, J.C., Lanzavecchia, A., and Manz, M.G. (2004). Development of a human adaptive immune system in cord blood cell-transplanted mice. Science *304*, 104–107.