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.

Deshan Ren, Wei Liu, Shuai Ding, Yan Li
yanli@nju.edu.cn

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
Protocol

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

Deshan Ren,1,3 Wei Liu,1,3 Shuai Ding,2 and Yan Li1,4,5,*

1MOE Key Laboratory of Model Animals for Disease Study, MOE Engineering Research Center of Protein and Peptide Medicine, The State Key Laboratory of Pharmaceutical Biotechnology, Chemistry and Biomedicine Innovation Center, Model Animal Research Center, Medical School of Nanjing University, Nanjing 210061, China
2Department of Rheumatology and Immunology, Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School, Nanjing, China
3These authors contributed equally
4Technical contact
5Lead contact
*Correspondence: yanli@nju.edu.cn
https://doi.org/10.1016/j.xpro.2022.101217

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 mouse 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).
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 Glassitic Slide under microscopy.
8. And the HSCs were cryopreserved in FBS containing 7.5% DMSO at 3 × 10⁶ 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<sup>scid</sup> Il2rg<sup>tm1Wjl/Sj</sup> (NSG) (Shultz et al., 2005), NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Sug/JicTac</sup> (NOG) (Ito et al., 2002), and NOD-Prkdc<sup>em26Cd52</sup> Il2rg<sup>em26Cd22/NjuCrl</sup> (NCG) (Cardoso et al., 2021) 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<sup>−/−</sup>Il2rg<sup>−/−</sup>Sirpa<sup>NOD</sup> 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: S81) - 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 CD34 (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: H10a) - FITC (1:50 dilution) | BioLegend | Cat#: 312208 |
| Anti-human CD45 (Clone: 2D1) - PE-Cy7 (1:50 dilution) | BioLegend | Cat#: 368532 |
| Anti-human CD45 (Clone: HI30) - APC-Cy7 (1:50 dilution) | BioLegend | Cat#: 306012 |
| Anti-human CD34RA (Clone: H1100) - 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-PrkdcscidIL2rgtm1Kar/NjuCr (NCG) | Gempharmatech | T001475 |
| 4–6 days old pups (regardless of gender) were used for HIS mice generation; 12 weeks old HIS mice were used for hydrodynamic injection. | | |
| **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 | ALSHENG | 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™ Glastic™ Slide 10 with Grids | Kova International, Inc | Cat#: 87144E |

(Continued on next page)
### MATERIALS AND EQUIPMENT

#### FACS Buffer

| Reagent                                | Final concentration | Amount  |
|----------------------------------------|---------------------|---------|
| PBS                                    | n/a                 | 483 mL  |
| Fetal Bovine Serum                     | 2%                  | 10 mL   |
| 100X 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   |
| 100X 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   |
| 100X 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  |
| 100X 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.
**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.

---

**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.
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™ Glastic™ 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
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
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.
32. Dilute 10× BD FACS™ Lysing Solution to 1× with ddH₂O, 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.
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:

\[
\text{cell number per mL} = \frac{\text{cell count of hCD45 from acquired sample}}{\text{acquired volume per sample}} \times \text{total volume per sample} \times 20.
\]

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:

\[
\% \text{ of humanization level} = \frac{\% \text{ of hCD45}}{\% \text{ of hCD45} + \% \text{ of mCD45}} \times 100\%.
\]

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.
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 | Volume |
|------------|--------|
| 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 |
| 10X PBS | 0.1 * (n+2) mL |
| ddH₂O | (n+2) (0.9-A/B) ml |
| Mix the ingredients evenly, then filter through a 0.22 μm sieve. | | |

Figure 4. Gating strategy for FACS analysis of blood samples from HIS mice
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 HybridMax, incubating the cells at room temperature (20°C–25°C) for 10 min.

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 |

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.
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 x 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 ratio) 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 x 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.

66. Add 200 μL ice cold FACS buffer and spin down the cells at 500 x 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 x 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).

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

EXPECTED OUTCOMES

Humanization level

Normally, 10 weeks after intra-hepatic injection with 10⁵ 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 10⁵/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 increased.

| 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            |

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

Table 6. Example cell subpopulations checked in this protocol

| Cell population                      | Immunophenotype |
|--------------------------------------|-----------------|
| Plasmacytoid dendritic cells         | pDCs            |
| Granulocyte-monocyte progenitor      | GMP             |
| Common myeloid progenitor            | CMP             |
| Megakaryocyte-erythrocyte progenitor | MEP             |

CD45⁺ CD34⁺ CD38⁺ CD10⁻ CD45RA⁺ CD123⁺
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.

ACKNOWLEDGMENTS
This study was supported by grants from the National Key Research and Development Program of China (2019YFA0802900), National Natural Science Foundation of China (32070942, 32122035, 32000669), China Postdoctoral Science Foundation grant (2020T130053ZX), and a Jiangsu Provincial Postdoctoral grant (2020Z079). We thank Dr. Margarida Saraiva for critical reading of the manuscript.

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. (2000). Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection. J. Virol. 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., Rahming, S., Mende, N., Renke, S., Hauber, I., Schäfer, 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.

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., Szczepaniak, M., Hossain, J., Ablamunits, V., Kikoles-Smith, N., Herold, K. C., Doris, 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., Chiong, 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 monocytes by M-CSF in humanized mice. J. Immunol. 191, 3192–3199.
STAR Protocols
Protocol

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., Korb, M., Gilles, 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 hematopoietic stem cells. J. Immunol. 174, 6477–6489.

Traggiai, E., Chicha, L., Mazucchelli, 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.