Chapter 2

Production and Characterization of Humanized Rag2$^{-/-}\gamma c^{-/-}$ Mice

Freddy M. Sanchez, German I. Cuadra, Stanton J. Nielsen, Anne Tanner, and Bradford K. Berges

Abstract

Mice reconstituted with human immune cells represent a model to study the development and functionality of the human immune system. Recent improvements in humanized mice have resulted in multi-lineage hematopoiesis, prolonged human cell engraftment that is detectable in many mouse organs, and the ability to generate de novo human innate and adaptive immune responses. Here, we describe the methods used to produce and characterize humanized Rag2$^{-/-}\gamma c^{-/-}$ mice.

Key words Humanized mice, Animal disease models, Hematopoietic stem cells, Stem cell transplantation, RAG-hu mice, SCID-hu mice, BLT mice

1 Introduction

The preclinical evaluation of therapeutics for a variety of human diseases has relied mainly on the use of small animals and nonhuman primates. Despite the genetic traits conserved between some of these animals and humans, species-specific differences exist. Among these differences are the susceptibility to infection by microbial pathogens, and the host immune response to those infections. The discovery of the severe combined immunodeficiency mutation (*Prkdc*^{scid}) in mice (C.B-17 SCID mice) led to the first attempts to use these animals for the development of effective in vivo models that more accurately resemble the complexity of human biology [1]. One such development has been the "humanization" of mice.

Humanized mice are described as immunocompetent mice capable of transgenically expressing human genes, or immunodeficient mice capable of being engrafted with cells of human origin (typically hematopoietic stem cells, HSCs, or peripheral blood mononuclear cells, PBMCs). These models have provided important findings relevant to various fundamental aspects of human

Irving C. Allen (ed.), *Mouse Models of Innate Immunity: Methods and Protocols*, Methods in Molecular Biology, vol. 1031, DOI 10.1007/978-1-62703-481-4_2,

biology and immunology, including human hemato-lymphopoiesis, innate and adaptive immune responses, autoimmune diseases, infectious diseases, and cancer [2, 3]. The introduction of additional genetic modifications capable of overcoming the limitations (e.g., engraftment barriers) present in the earlier models of humanized mice has permitted a gradual optimization in the generation of such mouse models. Thus, in the past two decades various improved humanized mouse models have been developed [4, 5].

A more recent innovation in the humanization of mice was achieved by crossing mice homozygous for a deletion in the recombination activating gene 2 (Rag2) with mice homozygous for a deletion in the common gamma chain receptor (γc) [6, 7]. $Rag2^{-/-}\gamma c^{-/-}$ mice are incapable of producing mature T, B, and NK cells because Rag2 is required to generate B and T cell receptors and γc is required for cytokine signaling via IL-2 and IL-15 [8, 9]. Since T cells and NK cells play a major role in identification and elimination of foreign cells, this mouse strain is ideal for humanization experiments. Transplantation of human HSCs into $Rag2^{-/-}\gamma c^{-/-}$ mice leads to human multi-lineage hematopoiesis and the development of the major functional components of the human adaptive immune system. Human B and T cells, monocytes/macrophages, and dendritic cells are readily detected in lymphoid organs and in the periphery. Humanized mice have been useful in the study of viral pathogenesis and new treatment strategies for human viruses such as HIV-1, human T-lymphotropic virus, Epstein–Barr virus, human cytomegalovirus, and dengue virus [8, 10–15]. In addition, these mice are capable of producing primary human adaptive immune responses such as human antibody and T cell responses against a variety of viral, bacterial, and other antigenic targets [6, 7, 16].

In this chapter we describe the generation of humanized mice through purification of human HSCs, intrahepatic transplantation into newborn BALB/c $Rag2^{-/-}\gamma c^{-/-}$ mice, and verification of successful engraftment through FACS analysis of peripheral blood samples.

2 Materials

2.1 Purification and Culture of Human Hematopoietic Stem Cells

1. Human $CD34^+$ Selection Kit (Miltenyi Biotec, Auburn, CA, USA, or Stem Cell Technologies, Vancouver, BC, Canada). We have successfully used both kits.
2. Iscove's Modified Dulbecco's Medium supplemented with 10 % fetal calf serum, 2 % penicillin–streptomycin, and 10 ng/ml each of SCF, IL-3, and IL-6. Filter-sterilize the medium and store at 4 °C.

2.2 Transplantation of BALB/c Rag2$^{-/-}$ $\gamma c^{-/-}$ Mice with Human HSCs

1. BALB/c Rag2$^{-/-}$ $\gamma c^{-/-}$ mice (*see* **Note 1**).
2. 28 gauge insulin syringes.
3. Cultured human HSCs.
4. Iscove's Modified Dulbecco's Medium.

2.3 Bleeding Mice to Screen for Human Cell Engraftment

1. Heating pad.
2. Mouse restraint apparatus (Model TV-150; Braintree scientific Inc., Braintree, MA, USA). This device has a groove across the top. A plunger prevents the mouse from escaping.
3. Scalpel blade (surgical blade stainless steel No. 11).
4. Gauze pads.
5. Styptic powder (Kwik-Stop Styptic Powder with Benzocaine; ARC Laboratories).
6. Heparinized microcapillary tubes (Heparinized Micro-hematocrit capillary tubes; Thermo Fisher Scientific Inc., Waltham, MA, USA).
7. Micropipettor with tips.

2.4 FACS Analysis to Detect and Quantify Human Cell Engraftment

1. Antibodies: hCD45-PE-Cy7 and mCD45-PE (eBioscience, San Diego, CA, USA).
2. 10× ammonium chloride erythrocyte lysing solution: Dissolve 89.9 g NH_4Cl, 10.0 g $KHCO_3$, and 370.0 mg tetrasodium EDTA in 1 liter of ddH_2O. Adjust pH to 7.3. Store at 4 °C in full, tightly closed 50 ml tubes. Dilute to 1× with ddH_2O and use immediately.
3. FACS stain buffer: 1× PBS, 0.1 % BSA, and 0.1 % sodium azide. Store at 4 °C.
4. Fc blocking buffer: Human Gamma Globulin (Jackson Immunoresearch Labs, West Grove, PA, USA), Normal Mouse Serum (Jackson Immunoresearch Labs), 2.4G2 monoclonal antibody to murine CD16/CD32 (BD, Franklin Lakes, NJ, USA). Reconstitute Normal Mouse Serum with 5.0 ml of ddH20. Add 2 ml of Human Gamma Globulin. Add 200 μl of 2.4G2 anti-mouse CD16/CD32. Store at 4 °C.
5. 1 % paraformaldehyde in 1× PBS: Paraformaldehyde does not dissolve effectively in PBS. Prepare a stock of 2 % paraformaldehyde in ddH_2O and a stock of 2× PBS in ddH_2O. Mix these solutions together in equal parts and store at 4 °C.
6. Flow cytometer.
7. FACS tubes.

3 Methods

3.1 Preparation of Human HSCs for Transplantation

1. $CD34^+$ human HSCs are purified from human umbilical cord blood or other sources (*see* **Note 2**) using magnetically labeled antibodies according to the manufacturer's protocol. $CD34^+$ cells are cultured for 40–48 h (*see* **Note 3**) post extraction in IMDM supplemented with 10 % FCS, 1× penicillin/streptomycin, and 10 ng/ml each of IL-3, IL-6, and SCF.
2. Resuspend cells by repeated pipetting, since many cells will be semi-adherent. Count cells using a hemocytometer. Samples used for engrafting mice may be divided to engraft multiple mice.
3. Centrifuge samples for 3 min at 900 × *g* and discard the supernatant. Resuspend the cell pellet in serum-free IMDM. Approximately 30–50 μl of re-suspended cells is best for an individual mouse injection. Divide the solution into different samples equal to the number of pups that will be engrafted. We use a minimal dose of 250,000 cells per mouse in order to achieve consistent, high-level engraftment (*see* **Note 4**).

3.2 Conditioning Pups for Transplantation

1. 1- to 5-day-old pups (*see* **Note 5**) are conditioned by gamma irradiation at a dose of 350 rads. Wait at least 1 h between irradiation and cell injection. Care must be taken to prevent animals from being exposed to mouse pathogens during transportation and cell injection (*see* **Note 6**).

3.3 Transplantation of Pups with Human HSCs

1. Add 30–50 μl of $CD34^+$ cells in solution into each syringe. The exact volume depends upon the age and size of the pups (*see* **Note 7**). 30 μl is best for 1-day-old pups. Since some volume is retained in the needle after injection, larger volumes are preferable for older pups in order to prevent loss of cells due to retention of liquid in the syringe.
2. Place pups on their backs and stretch out their bodies to allow visualization of the liver. Since pups are albino, the liver is readily visible. Pups are injected with cells in the liver at a depth of 1–2 mm. Greater depths can result in bleeding from the injection site. Following injection keep the syringe inserted for 20 s to prevent cells from being expelled after needle withdrawal. Upon completion of the injection, place the pups back with their mother.

3.4 Bleeding Mice for FACS Analysis

1. Eight weeks post reconstitution, mice should be screened for human cell engraftment. Warm up the mice by placing them in an empty plastic cage on top of a heating pad. Allow at least 5 min for the mice to sufficiently heat up. The mice are warm enough when their movements are rapid and they are breathing quickly.

2. Remove a mouse from the heating cage and place it in the restraint apparatus. Holding the mouse by the tail, gently pull the mouse (tail first) into the apparatus. Pull the tail along the groove in the top of the apparatus, thus pulling the mouse into the apparatus. Push the plunger into the front of the apparatus so that the mouse is held inside (*see* **Note 8**).
3. Locate the veins on the tail and choose one for tail nick bleeding. Using the scalpel, make a small transverse cut across the selected vein. After the mouse begins to bleed, hold the capillary tube horizontally (to avoid forming air bubbles that can lead to clotting) at the cut site and begin collecting blood. When the capillary is full withdraw it (keeping it horizontal) and place the blood sample into an appropriately labeled microfuge tube.
4. Pinch the tail above the cut site to stop the blood flow and wipe away any excess blood. Scoop out a small amount of styptic powder and apply it to the cut site. Allow enough time for clotting to occur. Place the mouse back into its original cage.
5. Eject the blood from the capillary tube using the micropipettor and draw the capillary tube up and out of the microfuge tube as you eject the blood. This technique will prevent the blood from entering back into the capillary.

3.5 Preparing Blood Samples for FACS Analysis

1. Lyse red blood cells by adding 1.4 ml of erythrocyte lysing solution per 100 μl of blood. Incubate at room temperature for 5–10 min. Centrifuge samples at 900 × *g* for 3 min. Discard the supernatant and resuspend the cell pellet in 100 μl of FACS stain buffer.
2. Add 3 μl of Fc blocking buffer and place samples at 4 °C for 15 min (*see* **Note 9**). Add 3 μl of both mCD45-PE and hCD45-PE-Cy7 to each sample and incubate at 4 °C for 30 min. Keep light exposure to a minimum.
3. Add 900 μl of 1 % paraformaldehyde in 1× PBS to each sample. Spin samples at 900 g for 3 min. Dispose of the supernatant and resuspend the pellet in 150 μl of 1× PBS solution. Transfer samples into FACS tubes and analyze by FACS.

4 Notes

1. There are multiple types of immunodeficient mouse strains that support engraftment of human HSCs and multi-lineage hematopoiesis. The original SCID mouse retains natural killer (NK) cell activity and the SCID mutation can result in leaky production of lymphocytes in older mice; both NK cells and T lymphocytes recognize and reject foreign cells. As a result, strains with greater defects in NK and T cell development are now typically used, including Rag2$^{-/-}\gamma c^{-/-}$ mice, NOD/SCID mice,

NOD/SCID $\gamma c^{-/-}$ mice, and $Rag1^{-/-}$ $\gamma c^{-/-}$ mice. $Rag2^{-/-}$ $\gamma c^{-/-}$ mice are commercially available on a C57BL/6 background, but for unknown reasons these animals cannot be effectively engrafted (BALB/c $Rag2^{-/-}$ $\gamma c^{-/-}$ mice work effectively). Excellent reviews are available that explain the phenotype of each mutation, as well as the history of using these strains to produce humanized mice [3, 17].

2. Three main sources are currently employed to obtain HSCs: umbilical cord blood, fetal liver, and mobilized peripheral blood. Magnetic separation techniques are commonly employed to purify $CD34^+$ cells. Umbilical cord blood is most readily available, but this source yields a low number of cells, at most 1×10^6. Relatively fewer mice can be engrafted per sample due to lower yields. Fetal liver samples have ethical constraints and few suppliers exist, but these samples yield more cells. Fetal liver samples commonly yield greater than 20×10^6 cells. We have no experience using mobilized peripheral blood and this source is rarely used to produce humanized mice [18, 19].
3. $CD34^+$ cells are cultured for 40–48 h in order to obtain maximum expansion of the hematopoietic stem cell population while preventing differentiation of the stem cells. There is no method currently available to culture HSCs without eventual differentiation and loss of potency for engraftment. Density of cells is critical for expansion during culture. Denser cell cultures grow more efficiently than cultures that are less dense. We culture cells in 48-well plates since that provides the appropriate cell density for most umbilical cord blood-derived samples.
4. The number of $CD34^+$ HSCs to inject varies considerably in the literature. In the original paper by Traggiai et al. showing HSC engraftment in $Rag2^{-/-}$ $\gamma c^{-/-}$ mice, they found engraftment with as few as 3.8×10^4 $CD34^+$ HSCs [8]. We typically use at least 2.5×10^5 cells per mouse to achieve consistent, high-level engraftment. Some researchers use up to $1–2 \times 10^6$ cells per mouse [20].
5. Several experiments have shown that age of mice at the time of engraftment has an impact on the level of engraftment achieved. We have found that engraftment levels are superior when $Rag2^{-/-}$ $\gamma c^{-/-}$ pups are less than 5 days of age at the time of irradiation and transplantation. Attempts to engraft older $Rag2^{-/-}$ $\gamma c^{-/-}$ mice result in lower levels of engraftment. Different mouse strains can show effective engraftment with older mice (e.g., NOD/SCID $\gamma c^{-/-}$), but in some cases different conditioning techniques were used [19, 21–23].
6. Immunodeficient mice are housed in specific pathogen-free facilities because they are unable to defend against various types of infections. They are often given antibiotics in their

drinking water in order to prevent bacterial infection. When preparing mice for irradiation, they often have to leave the animal facility; therefore, great care must be taken to keep the animals pathogen-free while in transit so as to avoid contaminating the colony.

7. Intrahepatic injection into newborn mice can be technically challenging. BALB/c mice are albino and hence the liver is readily visible. We typically inject a volume of 30–50 μl of cells per mouse. However, we find that the volume used for cell injection must be smaller for 1-day-old pups; if not the inoculated cells can exit the injection site after withdrawing the needle due to pressure accumulated during injection. For smaller pups, we use an injection volume of 30 μl. Allow the needle to remain in place for 20 s to ensure that the cells will not be expelled from the mouse.
8. Be careful not to catch the mouse's feet between the plunger and the wall of the apparatus. Do not let go of the tail or the mouse may pull the tail inside. Animals can sometimes bury their heads underneath their bodies and suffocate, so make sure that the head stays up for access to fresh air.
9. FACS analysis using cells from chimeric animals is more complicated than using cells from a single organism due to the requirement to block nonspecific antibody binding to both human and mouse cells. We perform initial workup experiments with FACS antibodies on pure mouse blood or pure human blood to verify the accuracy of the staining. We block nonspecific staining by using a combination mouse/human Fc block consisting of anti-mouse CD16/CD32, human gamma globulin, and normal mouse serum (*see* Subheading 2). We typically use mouse monoclonal antibodies for FACS staining and we rarely detect background or cross-species staining.

Acknowledgments

This work was supported by a Mentoring Environment Grant from Brigham Young University.

References

1. Bosma GC, Custer RP, Bosma MJ (1983) A severe combined immunodeficiency mutation in the mouse. Nature 301(5900):527–530
2. Brehm MA, Shultz LD, Greiner DL (2010) Humanized mouse models to study human diseases. Curr Opin Endocrinol Diabetes Obes 17(2):120–125
3. Shultz LD, Ishikawa F, Greiner DL (2007) Humanized mice in translational biomedical research. Nat Rev Immunol 7:118–130
4. Pearson T, Greiner DL, Shultz LD (2008) Humanized SCID mouse models for biomedical research. Curr Top Microbiol Immunol 324:25–51

5. Goldstein H (2008) Summary of presentations at the NIH/NIAID New Humanized Rodent Models 2007 Workshop. AIDS Res Ther 5:3
6. Goldman JP, Blundell MP, Lopes L, Kinnon C, Di Santo JP, Thrasher AJ (1998) Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. Br J Haematol 103(2):335–342
7. Mazurier F, Gan OI, McKenzie JL, Doedens M, Dick JE (1999) A novel immunodeficient mouse model–RAG2 x common cytokine receptor gamma chain double mutants–requiring exogenous cytokine administration for human hematopoietic stem cell engraftment. J Interferon Cytokine Res 19(5):533–541
8. Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti JC, Lanzavecchia A, Manz MG (2004) Development of a human adaptive immune system in cord blood cell-transplanted mice. Science 304:104–107
9. Baenziger S, Tussiwand R, Schlaepfer E, Mazzucchelli L, Heikenwalder M, Kurrer MO, Behnke S, Frey J, Oxenius A, Joller H, Aguzzi A, Manz MG, Speck RF (2006) Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2−/−gc−/− mice. Proc Natl Acad Sci U S A 103:15951–15956
10. Berges BK, Wheat WH, Palmer B, Connick E, Akkina R (2006) HIV-1 infection and CD4 T cell depletion in the humanized Rag2−/−gc−/− (RAG-hu) mouse model. Retrovirology 3:76
11. Berges BK, Rowan MR (2011) The utility of the new generation of humanized mice to study HIV-1 infection: transmission, prevention, pathogenesis, and treatment. Retrovirology 8:65
12. Kuruvilla JG, Troyer RM, Devi S, Akkina R (2007) Dengue virus infection and immune response in humanized $Rag2^{-/-}gc^{-/-}$ (RAG-hu) mice. Virol 369:143–152
13. Smith MS, Goldman DC, Bailey AS, Pfaffle DL, Kreklywich CN, Spencer DB, Othieno FA, Streblow DN, Garcia JV, Fleming WH, Nelson JA, Smith MS (2012) Granulocyte-colony stimulating factor reactivates human cytomegalovirus in a latently infected humanized mouse model. Cell Host Microbe 8:284–291
14. Banerjee P, Tripp A, Lairmore MD, Crawford L, Sieburg M, Ramos J, Harrington W Jr, Beilke MA, Feuer G (2010) Adult T cell leukemia/lymphoma development in HTLV-1-infected humanized SCID mice. Blood 115:2640–2648
15. Berges BK, Akkina SR, Remling L, Akkina R (2010) Humanized Rag2(−/−)gammac(−/−) (RAG-hu) mice can sustain long-term chronic HIV-1 infection lasting more than a year. Virol 397:100–103
16. Chicha L, Tussiwand R, Traggiai E, Mazzucchelli L, Bronz L, Piffaretti JC, Lanzavecchia A, Manz MG (2005) Human adaptive immune system Rag2−/− gamma(c)−/− mice. Ann N Y Acad Sci 1044:236–243
17. Manz MG (2007) Human-hemato-lymphoid-system mice: opportunities and challenges. Immunity 26:537–541
18. Lang J, Weiss N, Freed BM, Torres RM, Pelanda R (2011) Generation of hematopoietic humanized mice in the newborn BALB/c-Rag2(null)Il2rγ(null) mouse model: a multivariable optimization approach. Clin Immunol 140:102–116
19. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, Greiner DL, Handgretinger R (2005) Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immun 174: 6477–6489
20. Kwant-Mitchell A, Ashkar AA, Rosenthal KL (2009) Mucosal innate and adaptive immune responses against HSV-2 in a humanized mouse model. J Virol 83:10664–10676
21. Brehm MA, Cuthbert A, Yang C, Miller DM, DiIorio P, Laning J, Burzenski L, Gott B, Foreman O, Kavirayani A, Herlihy M, Rossini AA, Shultz LD, Greiner DL (2010) Parameters for establishing humanized mouse models to study human immunity: analysis of human hematopoietic stem cell engraftment in three immunodeficient strains of mice bearing the IL2rgamma(null) mutation. Clin Immunol 135:84–98
22. Pearson T, Shultz LD, Miller D, King M, Laning J, Fodor W, Cuthbert A, Burzenski L, Gott B, Lyons B, Foreman O, Rossini AA, Greiner DL (2008) Non-obese diabetic-recombination activating gene-1 (NOD-Rag 1(null)) interleukin (IL)-2 receptor common gamma chain (IL 2 rgamma(null)) null mice: a radioresistant model for human lymphohaematopoietic engraftment. Clin Exp Immunol 154:270–284
23. Rozemuller H, Knaan-Shanzer S, Hagenbeek A, van Bloois L, Storm G, Martens AC (2004) Enhanced engraftment of human cells in RAG2/gammac double-knockout mice after treatment with CL2MDP liposomes. Exp Hematol 32:1118–1125