

Chapter 24

Characterization of HIV-1 Infection in the Humanized Rag2^{-/-}γc^{-/-} Mouse Model

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Abstract

Engraftment of immunodeficient mice with a human immune system (humanized mice) provides a model system to study pathogens that target human immune cells. Humanized Rag2^{-/-}γc^{-/-} mice produce the major target cells of HIV-1 and these cells can be detected in primary and secondary lymphoid tissues, as well as in the vaginal and rectal mucosa and brain tissues. This humanized model has already yielded important findings on HIV-1 transmission, mechanisms of pathogenesis, and testing of novel antiviral strategies in vivo. Here, we describe the methods used to infect humanized mice with HIV-1 and to characterize plasma viral load and blood CD4⁺ T cell depletion.

Key words Humanized mice, RAG-hu mice, Animal disease models, Hematopoietic stem cells, Stem cell transplantation, SCID-hu mice, BLT mice, HIV, HIV-1, AIDS

1 Introduction

HIV-1 is highly specific to infecting cells of human origin. Primary human cells and cell lines can be cultured and infected with HIV-1, and much knowledge has been gained about virus replication cycles, cellular pathogenesis, and antiviral drugs as a result. However, there is still much to be learned about HIV-1 pathogenesis and immune responses that cannot be studied in cultured cells. Specifically, living organisms are required for infection because of the diversity of cell types in the whole animal. Likewise, living organisms are capable of mounting immune responses to the virus in order to study virus–host interactions. Although SIV infection in nonhuman primates has been used as a model to study HIV-1 infection in humans, the differences in genetics between both viruses and hosts are problematic for translating those findings directly to HIV-1 in humans. For example, differences in host HLA genes are an obstacle to vaccine studies, and differences in viral genes and/or gene sequences have an impact on pathogenesis.

Humanized mice are an exciting tool to study HIV-1 *in vivo* because they contain human HIV-1 target cells and can readily be infected with HIV-1. Humanized mice have been used to study HIV-1 for over 20 years [1–3]. The newer types of humanized mice are more profoundly immunodeficient than the original SCID mice and are also engrafted with human hematopoietic stem cells, resulting in enhanced engraftment as measured by total number of human cells, diversity of cell types, and distribution and duration of the graft [4, 5]. Human immune cells can be detected in a variety of lymphoid and non-lymphoid organs, and primary human adaptive immune responses (cellular and humoral) to HIV-1 and other pathogens are detectable [6]. Upon challenge with HIV-1 by either direct injection or mucosal routes, humanized mice become infected and viremia can be readily detected and quantified [7, 8]. Virus replication is detected in lymphoid and non-lymphoid organs, including brain tissue [9, 10]. Further, CD4⁺ T cell depletion (the hallmark of AIDS) takes place in both blood and lymphoid organs [8, 11], and animals are responsive to various types of antiretroviral drugs [12]. HIV-1 produces a chronic infection in humanized mice, as in humans [13]. Diverse areas such as virus evolution, vaccine testing, gene therapy, and exploration of various types of pathogenesis are now being explored using the humanized mouse platform [1].

In this chapter we describe methods used to infect humanized mice with HIV-1. We also detail techniques used to measure plasma viral load via quantitative RT-PCR, and methods to quantify and track CD4⁺ T cell depletion in peripheral blood samples.

2 Materials

2.1 HIV-1 Infection of Humanized Rag2^{-/-}γc^{-/-} Mice

1. Biohazard level 2 safety cabinet.
2. 8–10-week post-transplanted Rag2^{-/-}γc^{-/-} mice.
3. HIV-1 inoculum.
4. Complete DMEM medium with 10 % fetal bovine serum and 1× penicillin/streptomycin.
5. 1-cm³ tuberculin syringes with 25-G×5/8-in. needle.

2.2 Quantitative RT-PCR Analysis for Plasma Viral Load

1. Non-heparinized capillary tubes.
2. RNA isolation kit.
3. Sterile RNase-free pipette tips and EDTA-treated microfuge tubes.
4. DEPC-treated H₂O/nuclease-free H₂O.
5. Absolute ethanol (96–100 %).
6. HIV-1-specific PCR primers and TaqMan probe.
7. cDNA reverse transcription kit.

8. PCR tubes.
9. Microcentrifuge.
10. Vortex.
11. Thermal cycler.

2.3 Flow Cytometry for the Evaluation of Human CD4⁺ T Cell Levels

1. Peripheral blood collected from mouse tail vein.
2. EDTA-treated microcentrifuge tubes.
3. 10× ammonium chloride erythrocyte lysing solution: Dissolve 89.9 g of NH₄Cl, 10.0 g of KHCO₃, and 370.0 mg of tetrasodium EDTA in 1 l of ddH₂O. Adjust pH to 7.3. Store at 4 °C in full, tightly closed 50 ml tubes. Prior to use, dilute to 1× with ddH₂O and use immediately.
4. FACS staining buffer: 1× PBS, 0.1 % BSA, and 0.1 % sodium azide. Store at 4 °C.
5. 1× PBS.
6. Affinity purified human Fc receptor binding solution. 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 ddH₂O. Add 2 ml of Human Gamma Globulin. Add 200 μl of 2.4G2 anti-mouse CD16/CD32. Store at 4 °C.
7. Species-specific antibodies for the pan-leukocyte marker CD45: hCD45-PE and mCD45-PE-Cy7 (eBioscience, San Diego, CA, USA).
8. Anti-human CD3 (BioLegend, clone HIT3a, PECy5, 6.25 μg/ml) and CD4 (eBioscience, clone RPA-T4, PECy7, 0.1 μg/μl) conjugated antibodies.
9. 1 % paraformaldehyde in 1× PBS: Paraformaldehyde does not dissolve effectively in PBS. Prepare a stock of 2 % paraformaldehyde in ddH₂O and a stock of 2× PBS in ddH₂O. Mix these solutions together in equal parts and store at 4 °C.
10. 12×75 mm, 5 ml polystyrene round-bottom tubes for flow cytometric analysis.
11. Microcentrifuge.

3 Methods

3.1 HIV-1 Infection of Humanized Rag2^{-/-}γc^{-/-} Mice

1. Prior to infection, mice are monitored for levels of human cell engraftment [6]. It is recommended to infect animals with at least 50 % of engraftment, and at least 8–10 weeks post reconstitution. Institutional approval to work with animals and HIV-1 must be sought prior to initiation of these studies (*see* **Note 1**).

2. To facilitate inoculation of animals, hold and restrain mice accordingly (*see Note 1*).
3. Mice are infected by either intraperitoneal or intravenous injection, using 1-cm³ tuberculin syringes. HIV-1 inoculum is administered in a volume of 0.2 ml, in a dose that can range between 10² TCID₅₀ and 2 × 10⁶ TCID₅₀. For a review of the different isolates and molecular clones of HIV-1 used for infection of humanized mice, *see ref. 1*. In our laboratory, we infect Rag2^{-/-}γc^{-/-} mice in the peritoneal cavity with 1 × 10⁴ TCID₅₀ of HIV-1_{Ba-L}. This HIV-1 stock was obtained from Suzanne Gartner, Mikulas Popovic, and Robert Gallo through the AIDS Research and Reference Reagent Program Division [14].
4. Control animals are mock infected using 0.2 ml of complete DMEM.

3.2 Quantitative RT-PCR Analysis for Plasma Viral Load

1. Inside the hood, place the mouse to be sampled in a rodent restrainer. Pre-warming mice allows for collection of larger blood volumes (*see Note 2*). Carefully clean the tail with an alcohol-soaked cotton pad and allow drying. Hold the tail in a way that the veins can be easily located and cut for tail nick bleeding.
2. Using a clean surgical blade, make a small cut on the tail and immediately collect the blood into non-heparinized capillary tubes. Two capillary tubes (approximately 140 μl of blood) are sufficient to obtain plasma (for viral load) and blood cells (for CD4⁺ T cell counts) from a single blood sample (*see Note 2*).
3. Expel the blood from the capillary tube into an RNase-free, EDTA-treated microfuge tube (*see Note 2*). Store samples on wet ice until RNA extraction. With a clean gauze pad, apply gentle pressure to the wound to stop bleeding and apply styptic powder to promote clotting. Return the animal to its cage.
4. Separate the cellular and plasma fractions by centrifugation at 900 × g for 5 min.
5. Using barrier tips, separate plasma and blood cells into separate RNase-free microfuge tubes. If RNA extraction will be on a different day, store plasma at -80 °C. If RNA is extracted but reverse transcription will be performed at a later time, store extracted RNA at -80 °C.
6. RNA extraction, reverse transcription, and quantitative PCR can be performed with any of the commercially available kits by following the manufacturer's recommendations/protocols. The following list of primer and probe sequences are used in our laboratory for the production of cDNA and quantitative PCR specific for the HIV-1 LTR sequence [15]: *Forward primer*, 5-GCCTCAATAAAGCTTGCCTTGA-3; *Reverse*

primer, 5-GGCGCCACTGCTAGAGATTTT-3; *TaqMan probe*, 5-AAGTAGTGTGTGCCCGTCTGTTRTKTGACT-3 (FAM dye).

The cycling conditions are as follows: 30 min at 48 °C; 10 min at 95 °C; and 50 cycles of 95 °C for 15 s and 60 °C for 1 min.

7. Quantitation of viral load is performed by first calculating the copy number using the results of the quantitative PCR reaction to measure levels of viral cDNA. DNA samples with known copy number are used to calculate copy number of unknown samples, as described. Several additional factors need to be included when calculating the viral load in viral RNA copies/ml plasma (*see Note 3*).

3.3 Flow Cytometry for the Evaluation of Human CD4⁺ T Cell Levels in Peripheral Blood

1. Whole blood is collected as previously described in Subheading 3.2.
2. Remove red blood cells by lysis. Add 1.4 ml of 1× lysis buffer per 0.1 ml of mouse blood.
3. Incubate samples for 3–5 min at room temperature. Then centrifuge at 900×g for 5 min at room temperature.
4. Carefully aspirate and discard the supernatant, and resuspend the pellet in 0.8 ml of cold 1× PBS.
5. Centrifuge at 900×g for 5 min at room temperature. Aspirate and dispose of the supernatant. Resuspend the cell pellet in 0.1 ml of cold FACS staining buffer.
6. Before staining cells with FACS antibodies, pre-incubate the samples for 20 min on ice with 5 μl of human FcγR binding inhibitor. This will block nonspecific interaction of conjugated antibodies with Fc receptors on lymphocytes (*see Note 3*).
7. Stain peripheral blood cells with 3 μl each of hCD45-, hCD3-, and hCD4-conjugated antibodies. Mix gently and incubate samples on ice or at 4 °C in the dark for at least 30 min.
8. At this point, cells must be fixed to neutralize infectious agents (*see Note 1*). Also, fixation will preserve the samples if FACS analysis is planned for a different time. Fix the samples by adding 0.8 ml of 1 % paraformaldehyde in 1× PBS. If FACS analysis is to follow immediately, centrifuge samples at 900×g for 3 min at room temperature.
9. Discard supernatant and resuspend stained cells in 0.15 ml of 1× PBS. Pipet samples up and down until no clumps are present.
10. Transfer stained cells to 12×75 mm, 5 ml polystyrene round-bottom tubes, and proceed to analyze data on a flow cytometer (*see Notes 4–6*). Keep samples dark as much as possible to preserve signal intensity.

4 Notes

1. Extreme care must be taken when infecting animals with HIV-1 in order to prevent laboratory personnel exposure. All work with infectious HIV-1 must be performed inside a BSL-2⁺ rated laminar flow hood. Animals must first be administered anesthesia (isoflurane) in order to prevent accidental puncture wounds. In addition, blood draws from HIV-1⁺ animals are another possible source of exposure to lab workers. Infected blood samples intended for FACS analysis must be adequately fixed/inactivated prior to running through the flow cytometer in order to prevent exposure to the cytometer operator. All animal studies involving mice must first receive approval from the Institutional Animal Care and Use Committee prior to initiation. In addition, permission from the Institutional Biosafety Committee must be sought to work with HIV-1.
2. We commonly use a single blood draw to evaluate both viremia (Q-RT-PCR) and CD4⁺ T cell counts (FACS analysis). Placing mouse cages onto a heating pad allows for greater volumes of blood to be collected. In order to prevent coagulation that will interfere with cellular analyses, anticoagulants must be used. We have found that heparin, which is a common anticoagulant, is an inhibitor of some types of PCR. Thus, we use untreated capillary tubes and quickly expel the blood into RNase-free, EDTA-treated microfuge tubes (EDTA is also an anticoagulant).
3. Viral load in humans is typically reported in viral RNA copies per milliliter of plasma, but mouse blood draws are typically restricted to ~150 μ l of whole blood by animal care and use protocols. Thus, the size of plasma samples must be standardized or recorded for each individual sample. 50 μ l of plasma can usually be obtained from each blood draw. In this case, samples must be normalized back to 1 ml by multiplying the final viral load by 20 because 1/20th of 1 ml of blood was used to extract viral RNA. Also, in most cases only a fraction of extracted RNA can be used for cDNA synthesis and quantitative PCR (note that one-step Q-RT-PCR kits are available). To account for only a fraction of RNA used for quantitation, one must also calculate for the fraction not measured by quantitative PCR. Due to these variables, a relatively high limit of detection is achieved despite the fact that quantitative PCR assays can typically detect below five copies of RNA. The following is an example calculation of viral load from raw data: 50 μ l of plasma for RNA extraction = 1/20th of 1 ml of plasma; 10 μ l of RNA (of 50 μ l extracted) used for reverse transcription = 1/5th of RNA used; 5 μ l of cDNA (out of 20 μ l) for quantitative

PCR = 1/4th of cDNA used; thus, $20 \times 5 \times 4 = 400$ normalization factor. The viral load detected by quantitative PCR must be normalized by $20 \times 5 \times 4$ to determine viral load in RNA copies/1 ml of plasma.

4. FACS staining of immune cells adds an additional level of complexity due to cells that express the Fc receptor, which can bind antibodies by the constant, rather than the variable region. Humanized mouse blood samples are even more complicated because Fc receptor-bearing cells are present from two species in the same sample. We perform initial workup experiments with FACS antibodies on pure mouse blood or pure human blood to verify accuracy in 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.
5. Blood samples taken from a mouse are very small, and the number of human leukocytes is also smaller than an equivalent volume of human blood because engraftment does not reach normal human levels. In addition, there is variability in the engraftment rates from mouse to mouse and in the basal CD4⁺-to-CD8⁺ cell ratio in blood prior to infection. Thus, absolute CD4⁺ T cell counts per volume of blood are not used to measure AIDS progression in humanized mice as is common in humans. CD4⁺ T cell depletion in humanized mice is typically measured by calculating the ratio of CD4⁺ T cells out of the total CD3⁺ population, or alternatively by measuring the (CD4⁺ cells)/(CD4⁺ cells added to CD8⁺ cells). In this way, a baseline CD4⁺ ratio can be established for individual mice and then changes in the ratio can be readily calculated after HIV-1 infection.
6. HIV-1 pathogenesis is often more rapid and severe in humanized mice as compared to humans, especially when using CXCR4-tropic HIV-1. The reasons for this finding are not entirely clear, but one possible explanation is that the human antiviral immune response present in humanized mice to combat HIV-1 is thought to be relatively weak.

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