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Generation of T cell receptor retrogenic mice.

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Abstract

The ability to express and study a single T cell receptor *in vivo* has been an important aspect of both basic and translational immunological research. Traditionally, this was achieved by using TCR transgenic mice. In the past decade, a more efficient approach for single TCR expression was developed. This relatively rapid and accessible method utilizes retroviral mediated stem cell-based gene transfer and is commonly referred to as TCR retrogenic approach. In this approach, hematopoietic bone marrow precursors are transduced with retroviral vector carrying both the alpha and the beta chains of a T cell receptor. After successful transduction, bone marrow is injected into recipient mice, in which T cell development is driven by expression of the vector encoded TCR. The current unit details the materials and methods required to generate TCR retrogenic mice. The unit is divided into three sections and provides detailed methods necessary for generation of stable retroviral producer cell lines, isolation and optimal transduction of hematopoietic bone marrow cells, and subsequent analysis of TCR retrogenic T cells. A detailed example of such analysis is provided. The current protocol is a culmination of many years of optimization, and is the most efficient approach to date. Bone marrow transduction and transfer into recipient mice can now be achieved in the shorter period of four days. The protocol can be followed in most laboratories with standard biomedical equipment, and is supported by a troubleshooting guide that covers potential pitfalls and unexpected results.

Keywords

T cell; T cell receptor; TCR; retrogenic; retroviral

Introduction

T cell receptors (TCR) mediate T cell development and function, which underlie proper immune responses to infection and cancer, while sometimes also leading to aberrant T cell activation in autoimmunity (Klein, Kyewski, Allen, & Hogquist, 2014). Since the TCR repertoire is highly diverse and supports the development of heterogeneous T cell fates, an oligoclonal system is a useful tool to understand the molecular mechanisms of TCR

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signaling in T cell development and function. To this end, TCR transgenic mice have been widely used to address key questions in T cell biology. Transgenic mice are generated by pronuclear injection of linearized DNA into fertilized mouse egg, where the DNA fragment is incorporated randomly into the genome. The gene becomes a part of the stable genome and can be transmitted to the offspring. The expertise, high-cost, and time necessary for generating TCR transgenic mice has hampered progress, as this approach has not been able to keep up with the need to expand the repertoire of single TCR models. For those reasons, TCR retrogenic mice can serve as a viable alternative to TCR transgenic mice. Unlike the development of TCR transgenic mice, which requires germline modifications, TCR retrogenic mice are generated by retroviral gene delivery into hematopoietic stem cells. Retroviral gene delivery is a quicker process that can be accomplished using standard tissue culture techniques, followed by intravenous delivery of hematopoietic stem cells. Even though, the gene is not transmitted to offspring, and each TCR retrogenic mouse has to be generated *de novo*, TCR retrogenic approach can be advantageous in studies of multiple TCRs. Importantly, retroviral gene delivery method has made the investigation of single TCRs *in vivo* more accessible to a larger number of laboratories and compatible with unlimited number of genetic backgrounds. Our laboratory and others have successfully applied TCR retrogenic approach to study T cell responses to infectious, autoimmune, and model antigens (Abraham, Flickinger, Waldman, & Snook, 2019; Lee, Sprouse, Banerjee, Bettini, & Bettini, 2017; Sprouse et al., 2018; J. D. Yang et al., 2018). Over the years we have improved the efficiency of generating TCR retrogenic mice. This unit describes an updated and shortened method to produce retrogenic mice (Figure 1). To this end, our updated approach reduces the time of bone marrow culture by one day, results in improved recovery of bone marrow cells and increases efficiency and reproducibility of the approach. An example of expected bone marrow transduction efficiency and *in vivo* T cell reconstitution is provided in Figure 2. Collectively, this protocol provides an efficient and reliable tool to study TCR function *in vivo*. We first describe the procedure for generating stable viral producer cell lines (Basic protocol 1). Then, we describe in detail the steps involved in bone marrow isolation, transduction, and transfer into recipient mice (Basic protocol 2). An example of an analysis of TCR retrogenic mice is described in Basic protocol 3.

Basic protocol 1. Generation of stable viral producer cell lines for bone marrow transduction.

The purpose of this procedure is to generate stable retroviral producer cell lines that can be expanded and aliquoted for future multiple bone marrow transductions. The cell line is generated by transduction and subsequent cell sorting based on expression of a fluorescent reporter (FP).

Materials

Complete DMEM (C-DMEM, DMEM (Corning, cat. no. 10–013-CV), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM MEM nonessential amino acids, 5 mM HEPES, 5.5×10^5 units of 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin)

C-DMEM-5 (C-DMEM supplemented with 5% (v/v) FBS)

C-DMEM-5-CIP (C-DMEM with 10μg/ml ciprofloxacin and 5% (v/v) FBS)

Fetal bovine serum (FBS, Atlanta biological, cat. no. S11550), inactivated by incubating at 56 °C for 30 min.

Ciprofloxacin (CIP) (Alfa Aesar, cat. no. AAJ61970–06)

Trypsin Versene (Lonza, cat. no. BW17–161F)

PBS (Ca^{2+}/Mg^{2+} free, Corning, cat. no. 46–013-CM)

TransIT-LT1 Transfection reagent (Mirus, cat. no. MIR 2300)

Polybrene (6 mg/ml stock in H_2O, store at −20 °C) (Sigma, cat. no. H9268)

Magnetic-activated cell sorting (MACS) buffer (1xPBS, 0.5% (w/v) BSA, 2.5 mM EDTA, 25 mM HEPES, sterilize through a 0.22-μm filter and store at 4°C for up to 6 months)

1.5 ml Eppendorf tubes, sterile

15 ml conical tube, sterile

50 ml conical tube, sterile

150 mm cell culture plate, sterile

100 mm cell culture plate, sterile

10 ml syringe, sterile

0.45 μm syringe filter, sterile

293T (ATCC® CRL-3216™) human embryonic kidney epithelial cell line

GP+E-86 ATCC ® CRL-9642™ mouse fibroblast packaging cell line

pEQ-Pam3(-E) packaging plasmid (Holst et al.; Persons, Mehaffey, Kaleko, Nienhuis, & Vanin) (vector available upon request from M.B.)

pVSVG packaging plasmid (Holst et al.) (vector available upon request form M.B.)

MSCV retroviral vector encoding TCR and fluorescent reporter (FP) (Holst et al.; Persons et al.) (vector available upon request from M.B.).

Note: A number of different fluorescent reporters have been used in MSCV vectors, including GFP, mCherry, Ametrine, BFP. The aforementioned FP vectors are available from our laboratory upon request. Alternatively, fluorescent protein reporter could be substituted for a congenic marker, for example Thy1.

Day 1: Plate out HEK293T and GP+E-86 cells.

1. Plate out previously expanded 293T cells at 2×10^6 cells per plate in 10 ml C-DMEM-5 in a 100 mm tissue culture plate one day before transfection.
2. Break out GP+E-86 cells from liquid nitrogen storage. Thaw cells in a 37°C water bath, and plate out in C-DMEM-5 media for the first 24 h. After 24 hours, switch the media to ciprofloflaxin containing C-DMEM-5, and maintain cells in C-DMEM-5-CIP media during expansion.

Note: Maintain 293T and GP+E-86 cells in C-DMEM-5-CIP to prevent mycoplasma contamination. Perform transfection using C-DMEM-5 (no CIP).

Day 2: Transfect 293T cells.

3. Add 500 μl serum free DMEM to a 1.5 ml eppendorf tube, and bring to room temperature.
4. Add 20 μl TransIt reagent directly to cell culture medium without touching the walls of the tube, vortex gently and briefly, and incubate at room temperature for 15 min.

Note: If you are generating several producer lines, you can make the necessary combined volume of transit/DMEM mixture in one batch in a 15 ml conical tube.

5. For each DNA vector, in a separate eppendorf tube combine 4 μg TCR MSCV plasmid, 4 μg pPam-E (pEQ-Pam3(-E)) plasmid, and 2 μg pVSVG. MSCV (murine stem cell virus based vector) plasmid contains an expanded multi-cloning site and a fluorescent reporter (such as GFP or Ametrine) (Holst et al.; Lee, Shevchenko, Sprouse, Bettini, & Bettini; Persons et al.). pVSVG (*env* gene) and pEQ-Pam3(-E)) (*gag/pol* genes) packaging plasmids provide wide tropism and high infectivity (Persons et al.). These plasmids have been used consistently in our laboratory to generate retroviral producers, and can be obtained upon request (Holst et al.; Lee et al.).

Note: DNA purity and concentration are critical. Typically, expect DNA concentrations to be >1 mg/ml for MSCV and pPam-E plasmids, and > 0.5 mg/ml for pVSVG from a standard Midiprep using PureYield™ Plasmid Midiprep System (Promega). DNA concentration and purity are measured once by NanoDrop after plasmid isolation, and assumed to remain stable if plasmid DNA is stored at −80°C.

6. Dropwise add TransIt/DMEM mixture to DNA, gently tap to mix and incubate at room temperature for 15 min.
7. Dropwise add TransIt/DMEM/DNA mixture (~520 μl) to 293T cells plated out in 100 mm plate. Incubate cells at 37°C and 5% CO_2 overnight.
8. Prepare two plates of GP+E-86 cells at 1×10^5 cells in 10 ml C-DMEM-5-CIP in a 100 mm plate. The extra plate serves as a negative control to use for flow cytometric sorting gate strategy.

Day 3: Transduce GP+E-86.

9. In the morning aspirate the media from 293T cells and add 10 ml fresh C-DMEM-5.

Note: Carefully add media to the side of the plate, as 293T cell monolayer can be easily disturbed and lift off from the bottom of the plate.

10. In the afternoon (8–12 h later) collect the media from 293T cells with a sterile 10 ml syringe, attach a 0.45 μm filter to the syringe. Add 10 ml fresh C-DMEM-5 to 293T cells.

11. Aspirate media from the GP+E-86 cells and add 293T viral supernatant to GP +E-86 cells through the 0.45 μm syringe filter.

12. Add 10 μl polybrene (final concentration 6 μg/ml) to GP+E-86 cells, swirl to mix, and place 293T and GP+E-86 cells into 37°C and 5% CO_2 tissue culture incubator.

Days 4–6: Repeat transduction of GP+E86 cells.

13. Repeat transduction on Days 4 and 5, twice a day, 8–12 h apart.

14. On Day 6, repeat one round of transduction in the morning. Discard 293T cells.

15. At the end of Day 6, change the media on GP+E-86 cells with 10 ml C-DMEM-5-CIP.

Note: Cell confluency should be monitored daily. 293T cells will likely become 100% confluent in culture by end of Day 4. If this is observed, after supernatant has been removed for transduction, 293T cells can be split to reduce confluency and improve viability. To split cells, vigorously pipette cells of the bottom of the plate with 10 ml fresh media. Pipette cells up and down to achieve single cell suspension, and discard half of the cells. Replace the media in the plate to achieve a total volume of 10 ml.

Note: It is advisable to monitor the transfection of 293T cells. If using a fluorescent marker (i.e. Ametrine), transfection efficiency can be easily assessed by flow cytometry. At the end of GP+E-86 transduction protocol, pipette about 200 μl of 293T cells from the bottom of the plate, transfer to a flow tube, break cells up into a single cell suspension by pipetting up and down. At this point 293T cells will be confluent, and a relatively small volume (200 μl) is sufficient for analysis. Analyze FP expression by flow cytometry. Day 6 293T transfection level should be about 50–80% FP+.

Day 8: Sort transduced GP+E-86 cells.

16. Aspirate media from GP+E-86 cells, and replace with 3–5 ml PBS. Rock the plate to gently rinse the cells, and aspirate PBS to remove any residual media. Add just enough trypsin to cover the cells (3–4 ml per 150 mm plate), rock the plate to spread trypsin evenly. Transfer cells into the incubator for 1~2 minutes (or leave at RT). Monitor cell layer detachment using a microscope. After the cells come up from the bottom of plate, pipette trypsinized cells up and down

with 5–10 ml C-DMEM-5 to obtain a single cell suspension. Transfer cells into a 50 ml conical tube.

17. Spin down cells at 1200 rpm 4°C for 10 min. Aspirate supernatant and resuspend cells in 800–1000 μl of MACS buffer. Filter cells into an appropriate tube for sorting through a 40 μm filter (i.e. 15 ml conical tube). Prepare tubes with 3 ml C-DMEM-5 for collection of cells from the cell sorter.

18. For sorting, set FSC, SSC, and FP voltage based on the non-transduced negative control GP+E-86 cells (Maciorowski, Chattopadhyay, & Jain). The negative cells should occupy the first quadrant in the FP marker channel. After gating on live cells and singlets based on FSC and SSC, use the FP negative cells to make a predicted FP+ gate. From the FP+ cells (expect 90–100% for a successful transduction), sort top 40–50% FP+ cells (usually top quadrant). Sort at least 50,000 to 100,000 FP+ cells, spin down at 1200 rpm for 10 min, and plate out in a 10 ml C-DMEM-5-CIP in a 100 mm plate.

19. Expand cells to 75% confluency in one or two 150 mm plates with C-DMEM-5-CIP, freeze aliquots, and store in liquid nitrogen. Freeze about 5 aliquots per one 150 mm plate. Maintain at low passage number (1~5 passages) to ensure consistent and efficient bone marrow transduction.

Note: Continuous passaging of producer cell lines can result in outgrowth of cells that produce lower level of viral titer. Maintaining a low passage number (1–5) helps to avoid loss of producer function. At higher passage numbers, retention of vector positive cells can be verified by flow cytometry, and level of FP expression in GP+E-86 producer cell lines can be compared to another viral producer cell line with known high viral titer efficiency. Viral titers can also be compared using easily transfected cell lines, such as mouse fibroblast cell line 3T3 (NIH/3T3 (ATCC® CRL-1658™)) (Holst et al.).

Basic protocol 2. Generation of TCR retrogenic mice.

This protocol describes the procedure to generate TCR retrogenic mice by transferring retroviral transduced bone marrow stem cells into sub-lethally irradiated mice. A single 5-fluorouracil (5-FU) injection is given intraperitoneally to all donor mice 3 days before bone marrow harvest. Bone marrow cells are transduced twice using retrovirus generated by GP+E-86 producer cells (Basic Protocol 1) and transferred into recipients intravenously (Figure 1). The efficiency of transduction is assessed by flow cytometry (Figure 2A).

Materials

Mice NOD.CB17-*Prkdc*^{scid}/J (NOD.*scid*, 5–20 weeks old, used for bone marrow donors), and NOD.129P2(C)-*Tcra*$^{-/-\ tm1Mjo}$/DoiJ (NOD.*TCRα*$^{-/-}$, 6–12 weeks old, used for bone marrow recipients) mice were obtained directly from the Jackson Laboratory. Assume 2:1 donor (NOD.*scid*) to recipient (NOD.TCRα$^{-/-}$) ratio.

5-Fluorouracil (5-FU, NDC 63323-117-51)

PBS (Ca^{2+}/Mg^{2+} free, Corning, cat. no. 46–013-CM)

Hanks' Balanced Salt Solution (HBSS) (Corning/Cellgro, cat. no. 21–020-CV)

HBSS-5 (HBSS supplemented with 5% (v/v) FBS)

C-DMEM-5 (C-DMEM supplemented with 5% (v/v) FBS)

C-DMEM-5-CIP (C-DMEM with 10μg/ml ciprofloxacin and 5% (v/v) FBS)

C-DMEM-20 (C-DMEM supplemented with 20% (v/v) FBS)

Red blood lysis buffer, sterile filtered (8.3 g NH_4Cl, 1g $KHCO_3$, 0.019g EDTA, 1L H_2O)

Mouse IL-3 (Gibco, cat. no. PMC0033L)

Human IL-6 (Gibco, cat. no. PHC0063)

Mouse stem cell factor (mSCF, Gibco, cat. no. PMC2113L)

Amoxicillin trihydrate (Alfa Aesar, cat. no. J61290)

1.5 ml Eppendorf tubes, sterile

15 ml conical tube, sterile

50 ml conical tube, sterile

150 mm cell culture plate, sterile

100 mm cell culture plate, sterile

6-well cell culture plate, sterile

1 ml, 10 ml syringe, sterile

18G, 25G, 27G needle, sterile

0.45 μm syringe filter, sterile

40 μm filter, sterile

All-purpose laboratory plastic wrap (saran wrap)

Surgical instruments (scissors and forceps), sterile

Mouse restrainer

Heat lamp

Irradiator (RS-2000)

Expand viral producer cell line in culture.

1. Thaw out a vial, culture and expand retroviral GP+E-86 producer cell lines (from Basic Protocol 1 step 19) in to obtain sufficient cell number. Typically, we need 3.5×10^6 cells to generate sufficient supernatant for bone marrow transduction and injection of 2 recipient mice. Thaw cells out at least four days prior to bone marrow isolation, and initially plate the cells out in C-DMEM-5. Maintain and expand cells in C-DMEM-5-CIP, and switch back to C-DMEM-5 media during transduction/transfection. Do not let cells overgrow or use cells that have been in culture more than 2 weeks. Maintain cell aliquots at low passage number (1–5).

Note: Immediately after thawing, plate out GP+E-86 cells in C-DMEM-5 media without ciprofloxacin to encourage proliferation. After overnight culture, change media or split cells if necessary, and keep cells in 10 μg/ml ciprofloxacin to avoid mycoplasma contamination during expansion. If you plate out 1×10^6 cells per 150 mm plate on Day 0, you should be able to obtain 70% confluency by Day 3 without the need to passage or split cells. Do not let the viral producer cell lines expand beyond 80% confluency, as this will negatively impact cell viability and viral titer. It is always advisable to monitor cell proliferation and split cells when necessary. The cells usually require splitting every 3–4 days, depending on initial cell density and viability out of liquid nitrogen storage.

Day 0: Inject donor mice with 5-Fluorouracil (5-FU)

2. Weigh the donor mice (5 – 20 weeks old) to determine the amount of 5-FU required. Avoid using donor mice that are too young (< 5 weeks of age) or too old (> 20 weeks of age). To avoid rearrangement of endogenous TCRs and to ensure the expression of a single TCR, it is advisable to use a T cell deficient mouse strain (i.e. *scid*, $Rag^{-/-}$ or $TCR\alpha^{-/-}$ mice) as bone marrow donors. Assume 1 donor to 1 recipient for $Rag^{-/-}$ donors and 2 donors to 1 recipient for *scid* donors.

3. Prepare 10 mg/ml working solution of 5-FU (stock 50 mg/ml) in sterile PBS. Inject 0.15 mg per gram of body weight (i.e. 300 μl per 20 g mouse) of 5-FU intraperitoneally (*i.p.*) using a 27 G needle.

Note: We found that 5-FU administration 72 h prior to bone marrow isolation is the optimal time point. Earlier or later injection will affect bone marrow cell recovery or transduction efficiency. Bone marrow cell yield will fluctuate based on the age, strain, weight, health and genetic background of donor mice. For example, bone marrow cells from scid mice that have a generalized defect in DNA repair tend to yield lower cell number at the end of the transduction protocol. Therefore, the donor to recipient ratio should be adjusted accordingly.

Day 0: Passage producer cells

4. Plate out 1×10^6 GP+E-86 producers in 20 ml C-DMEM-5-CIP in 150 mm tissue culture plate(s). Cell number should reach about 3.5×10^6 cells per plate by Day 3.

Day 3: Extracting and culturing bone marrow cells

5. Sterilize dissection scissors and forceps for bone isolation. Euthanize donor mice according to institutional guidelines. Remove the femur, tibia, humerus and pelvic girdle from the donor mice. Remove any remaining surrounding tissue from the bones. Keep bones hydrated in HBSS-5 on ice.

A detailed protocol with accompanying instructional video for bone marrow isolation and culture has been previously published (Lee et al., 2016).

6. Cut the ends of each bone. Insert a 25 G needle attached to a 10 ml syringe containing HBSS-5 into ends of the bones to flush out the bone marrow. Using the syringe, flush bone marrow through a 40 μm strainer resting in a 50 ml conical tube.

7. Scrape the bone marrow through the 40 μm filter using the plunger from a 1 ml syringe. Rinse the strainer with HBSS-5 to make sure all bone marrow cells are filtered into the 50 ml tube. Passing the bone marrow through the strainer results in single cell suspension and filters out bone fragments and residual tissue.

8. Centrifuge bone marrow cells at 1200 rpm for 10 min at 4°C.

9. Aspirate supernatant and resuspend cell pellet in 1 ml red blood lysis buffer per 50 ml conical tube. After 2 min incubation at room temperature, quench the lysis buffer with 10 ml C-DMEM-20.

10. Combine bone marrow into one 50 ml tube, and centrifuge cells at 1200 rpm for 10 min at 4°C.

11. Aspirate supernatant and resuspend bone marrow in 3–5 ml C-DMEM-20. Count the cells using a Neubauer counting chamber.

The average bone marrow yield should be around $4–10\times10^6$ cells per mouse, but will vary depending on age, sex, body weight, and genetic background.

12. Plate bone marrow cells at a density of 40×10^6 cells per 150 mm plate in 30 ml C-DMEM-20, IL-3 (20 ng/ml), IL-6 (50 ng/ml) and mSCF (50 ng/ml).

13. Incubate bone marrow overnight at 37°C and 5% CO_2.

Note: Keep the bones and bone marrow cell suspension on ice as much as possible during the process. Use 1 ml pipette tips to resuspend cells in red blood cell lysis buffer rather than serological pipettes, as cell pellets can adhere to the inside of serological pipettes.

Day 3: Prepare Retroviral Producer Culture

At this time point, the retroviral producer cells should be at about 70% confluency (3.5×10^6 cells per 150 mm plate). Aspirate media from retroviral producer cells and add 18 ml fresh C-DMEM-5 per plate. If the producer cell line confluency is not optimal by Day 3, retroviral producer cells should be removed from plates using trypsin (as basic protocol step 16) and replated at 3.5×10^6 cells per 150 mm plate. Incubate retroviral producer cells at 37°C and 5% CO_2 overnight.

Note: Do not use ciprofloxacin when generating the viral supernatant for bone marrow transduction. Expect 50–80% bone marrow recovery on day 4. Plan the retroviral producer cell expansion and culture in advance to obtain enough supernatant to plate out recovered bone marrow cells at a density of 1×10^6 cells/ml of supernatant.

Day 4: Collect retroviral supernatant

14. Collect the retroviral supernatant (estimate 18 ml recovery per plate) and spin down at 1200 rpm for 10 min at 4°C, or filter supernatant through a 0.45 μm filter to remove contaminating producer cells. If using the centrifugation approach, remove supernatant from pelleted cells and transfer into a fresh 50 ml conical tube.

15. Add polybrene to the retroviral supernatant at 6 μg/ml and invert several times to mix.

16. Replace media on GP+E-86 cells with 18 ml fresh C-DMEM-5 per 150 mm plate. Continue to culture retroviral producer cells at 37°C and 5% CO_2.

Day 4: Bone marrow transduction day 1

17. Remove non-adherent bone marrow cells from 150 mm plates by pipetting. Use sterile PBS to vigorously wash the plate and collect residual bone marrow cells. Transfer cells into sterile 50 ml tubes.

18. Spin down cells at 1200 rpm for 10 min at 4°C. Aspirate supernatant, resuspend cells in a small volume (1 – 3 ml) of C-DMEM-20 and count. Plate 3×10^6 bone marrow cells in 3 ml of retroviral supernatant (with polybrene) per well in 6-well tissue culture plates (1×10^6 cells/ml). Wrap the plates with plastic wrap to minimize air exchange during the centrifugation. Spin the plates at 2500 rpm for 1 h at 37°C.

19. After spin-transduction, gently aspirate the top 2/3 volume of media from each well and replace it with fresh C-DMEM-20 media containing cytokines (IL-3 (20 ng/ml), IL-6 (50 ng/ml) and mSCF (50 ng/ml)). Place the plates into the incubator overnight.

Note: Gently and carefully remove the supernatant from the side of the wells containing bone marrow (step 38). Most of the bone marrow cells usually remain at the center of the well. Aspirated media can be spun down again to recover residual bone marrow cells. We have observed one-hour spin-transduction is sufficient, and removal of retroviral supernatant immediately after transduction does not significantly impact transduction efficiency. Since virus and polybrene can have negative effects on bone marrow viability, removing viral supernatant immediately after spin improves bone marrow survival and proliferation.

Day 5: Bone marrow transduction day 2

Repeat bone marrow transduction in the same 6-well plate (steps 33–35; 37–38).

20. Spin down the retroviral supernatant at 1200 rpm for 10 min at 4°C to pellet contaminating producer cells, transfer supernatant into a new 50 ml conical tube.

21. Add polybrene (6 μg/ml) to the retroviral supernatant and invert to mix.

22. Carefully aspirate top 2/3 volume of media from each well of the 6-well plates containing bone marrow cells. Add freshly prepared retroviral supernatant at 3 ml per well. Wrap the plates in plastic wrap and spin the plates at 2500 rpm for 1h at 37°C.

23. After centrifugation, gently aspirate the top 2/3 volume of supernatant and replace it with fresh C-DMEM-20 containing cytokines (IL-3 (20 ng/ml), IL-6 (50 ng/ml) and mSCF (50 ng/ml)). Place the plate in the incubator overnight.

Note: Cytokines should be aliquoted and stored at −80°C. The aliquoted cytokines should not be frozen and thawed more than once or kept at 4°C for more than two weeks. Freshly thawed cytokines are necessary to maintain the optimal growth of bone marrow cells.

Day 5: Irradiate recipient mice one day prior to bone marrow injection

In order to ensure single TCR expression, recipient mice with either *scid*, $Rag^{-/-}$ or $TCR\alpha^{-/-}$ genotype can be used. Alternatively, bone marrow can be transferred into lymphoreplete recipients. Lymphoreplete mice should be irradiated with higher doses (900 rads) to ensure elimination of endogenous hematopoietic compartment and efficient donor bone marrow engraftment. Using lymphoreplete mice is challenging because increased doses of radiation can result in poor mouse survival and recovery. Additionally, complete endogenous bone marrow depletion is not always achieved, and could result in a mixed T cell population. If endogenous cells are not completely eliminated, retrogenic T cells can still be tracked in wild type lymphoreplete recipient mice using FP reporter.

24. Warm up the Irradiator machine. Sterilize the irradiation cage. Place recipient mice into irradiation cage and set up the appropriate program to irradiate ($TCR\alpha^{-/-}$ and $Rag^{-/-}$ – 500 rads; scid – 300 rads; wild type, immunocompetent mice – 900 rads). The irradiation process will take about 5 mins per cage.

25. After irradiation, maintain recipient mice on antibiotic water (100 μg/ml amoxicillin) to prevent opportunistic infections.

Note: For optimal protection and survival, it is advisable to initiate antibiotic water treatment one day prior to irradiation.

Day 6: Inject bone marrow

26. Collect bone marrow cells from 6-well plates (from step 42). Wash the plates vigorously with PBS to collect all non-adherent cells. Spin down cells at 1200 rpm for 10 min at 4°C. Resuspend cells in a small volume of PBS (0.2–0.5 ml per 6-well plate) and take a 10 μl aliquote to count cells.

27. Resuspend bone marrow in sufficient volume of PBS to inject 2×10^6 cells in 200–300 μl PBS per mouse.

28. Load bone marrow cells into 1 ml syringes using blunt or large (18G) needles immediately before injection. Inject recipient mice intravenously using 27 G

needles. Warm mice under a heating lamp to stimulate blood vessel dilation and inject 200–300 μl of bone marrow per mouse.

Note: Keep transduced bone marrow in 50 ml conical tubes on ice. Do not preload cells into syringes, and once loaded, inject as quickly as possible, as bone marrow cells settle and clump in the syringe.

29. Monitor mice weekly for symptoms of infection or anemia. Maintain recipient mice on antibiotic water for at least 4 weeks post bone marrow transfer.

Note: Bacterial infections and inflammatory mediators, i.e. interferon gamma, are particularly detrimental to marrow engraftment and proliferation (de Bruin, Demirel, Hooibrink, Brandts, & Nolte, 2013; Matatall et al., 2016). Therefore, it is highly advisable to put recipient mice on antibiotics at least one day prior to bone marrow injection, and to maintain the mice on antibiotic water for at least four weeks post bone marrow transfer.

Basic Protocol 3: Analysis of TCR retrogenic mice.

This protocol describes the procedure used to analyze the bone marrow transduction efficiency and T cell development. Typically, T cell development in TCR retrogenic mice can be observed as early as 5 weeks post bone marrow transfer; although, full reconstitution is not reached until 8–10 weeks post transfer. This protocol utilizes FP expression as an indicator of bone marrow transduction before transfer, and bone marrow reconstitution after transfer. Successful T cell development supported by the retrovirally delivered TCR is evaluated based on TCR expression within FP+ cells in the spleens of recipient mice. Additional T cell markers, including CD8 and CD4, can be used to ensure expected MHCI and MCHII restriction during T cell development.

Materials

PBS (Ca^{2+}/Mg^{2+} free, Corning, cat. no. 46–013-CM)

Hanks' Balanced Salt Solution (HBSS) (Corning/Cellgro, cat. no. 21–020-CV)

HBSS-5 (HBSS supplemented with 5% (v/v) FBS)

Flow cytometry and fluorescence-activated cell sorting (FACS) buffer (1xPBS, 3% FCS, 0.05% NaAz, 3mM EDTA)

Red blood lysis buffer, sterile filtered (8.3 g NH_4Cl, 1g $KHCO_3$, 0.019g EDTA, 1L H_2O)

Anti-Sca1 PerCpCy5.5 (clone D7, eBioscience), anti-cKit APC (clone ACK2, eBioscience), anti-B220 biotin (clone 12A3–6B2, eBioscience), anti-CD11b biotin (clone M1/70, eBioscience), anti-Gr1 biotin (clone RB6–8C5, eBioscience), anti-DX5 biotin (clone DX5, eBioscience), anti-CD4 biotin (clone GK1.5, Biolegend), anti-CD8 biotin (clone 53–6.7, eBioscience), anti-CD4 BV605 (GK1.5, Biolegend), anti-CD8 PECy7 (clone 53–6.7, eBioscience), anti-TCRγδ biotin (UC7-BD5, Biolegend), anti-Ter119 biotin (TER-119, eBioscience), streptavidin-PE (Biolegend)

1.5 ml Eppendorf tubes, sterile

1.2 ml Corning™ Cluster flow tubes

96-well v-bottom plate

frosted microscope slides

Analyze bone marrow transduction efficiency prior to bone marrow transfer

Overall frequency of the FP+ population in the bone marrow culture is usually a sufficient measurement of transduction efficiency, without the need for antibody staining. If desired, additional markers (e.g. Sca-1, cKit and lineage) can be included into the analysis to obtain a more detailed evaluation of HSC transduction (see steps 49–53 and Anticipated Results).

1. Save a small aliquot of bone marrow from basic protocol 2 step 45 (20 μl). At this point, bone marrow can be directly analyzed by flow cytometry for FP expression. For more detailed analysis, stain the cells with anti-Sca1 PerCpCy5.5 (1:200), anti-cKit APC (1:200), anti-CD4 biotin (1:600), anti-CD8 biotin (1:600), anti-B220 biotin (1:600), anti-CD11b biotin (1:600), anti-Gr1 biotin (1:600), anti-DX5 biotin (1:600), anti-TCRγδ biotin (1:600) and anti-Ter119 biotin (1:600) antibodies resuspended in 100 μl FACS buffer for 30 min on ice in v-bottom plate.
2. Spin plate at 1200 rpm for 3 min at 4°C and aspirate buffer.
3. Add streptavidin-PE in 100 μl FACS buffer at 1:500 dilution and incubate for 15 min on ice.
4. After incubation, centrifuge the plate at 1200 rpm for 3 min at 4°C and resuspend the cells in 100 μl FACS buffer to prepare cells for flow analysis.
5. Transfer cells into flow tubes and analyze by flow cytometry (Figure 2A).

Note: Optimal transduction should result in 20–40% FP+ of all live cells (live gate), and could vary depending on the construct and retroviral titer. Avoid using bone marrow with transduction efficiency below 15%, which indicates experimental defects and might lead to low T cell reconstitution.

Analyze bone marrow transduction efficiency after bone marrow transfer

6. Euthanize mice according to institutional guidelines 8–10 weeks post bone marrow transfer. Remove the femur from the mice and flush the bone morrow into 1.5 ml eppendorf tube by using 1 ml syringe filled with HANKS attached to a 25 G needle.
7. Pass bone marrow through the 25G needle several times (by aspirating and expelling back into the Eppendorf tube) to achieve single cell suspension.
8. Centrifuge bone marrow cells at 300g for 5 min in a bench top microcentrifuge. Aspirate supernatant and resuspend cell pellet in 100 μl red blood lysis buffer per 1.5 ml eppendorf tube. After 2 min incubation at room temperature, quench the lysis buffer with 1 ml HBSS-5.

9. Centrifuge bone marrow cells at 300g for 5 min in a bench top microcentrifuge. Aspirate supernatant, stain and analyze the bone marrow cell as in basic protocol 3 step 49–53 (Figure 2B).

Note: If mice are sublethally irradiated, engrafted donor bone marrow will only account for part of the total bone marrow, and overall bone marrow FP expression will be lower in TCR retrogenic mice compared to pre-injection analysis.

Analyze TCR development in secondary lymphoid tissue after bone marrow transfer

10. Harvest the spleens of mice 8–10 weeks post bone marrow transfer. Keep the spleens hydrated in 10 ml HBSS-5 on ice in 15 ml conical tubes (one spleen per tube). Grind the spleens into single cell suspension between frosted sides of two microscope slides.

11. Centrifuge processed spleens at 1200 rpm for 10 min at 4°C. Aspirate supernatant and add 1 ml red blood cell lysis buffer per 15 ml conical tube. After 2 min incubation at room temperature, quench the lysis buffer with 9 ml HBSS-5.

12. Centrifuge cells at 1200 rpm for 10 min at 4°C. Aspirate supernatant and resuspend in 1 ml FACS buffer. Transfer 100 μl of cell suspension into a single well of a 96 well v-bottom plate.

13. Centrifuge the plate at 1200 rpm for 3 min at 4°C. Aspirate buffer, and resuspend cells in 100 μl FACS buffer with anti-CD4, anti-CD8 and anti-TCR. Stain cells for 30 min on ice.

14. After staining, centrifuge the plate at 1200 rpm for 3 min at 4°C. Aspirate supernatant, resuspend cells in 100 μl FACS buffer, transfer into flow tubes, and analyze by flow cytometry (Figure 2C).

Background

The T cell receptor (TCR) is a critical factor that governs all aspects of T cell development, proliferation, differentiation and peripheral activation (Kalekar et al., 2016; Klein et al., 2014; Snook, Kim, & Williams, 2018). The TCR repertoire is highly diverse, and is estimated to be composed of about 1×10^8 unique TCRs in humans and 2×10^6 in mice (Qi et al., 2014; Zarnitsyna, Evavold, Schoettle, Blattman, & Antia, 2013). The breadth of the overall TCR repertoire gives rise to diverse T cell subpopulations engaged in immune responses against antigenic challenge. TCR transgenic mice have been successfully used to model antigen specific responses. However, the TCR transgenic approach has its limitations. Firstly, generation of TCR transgenic mice is a relatively expensive and time-consuming method (> 6 months, not including the time for vector construction). Secondly, since TCR transgenes are randomly incorporated into the genome, it is possible to generate the so-called “founder effect”, introducing another variable when comparing TCR transgenic strains. Importantly, given the limited number of available TCR transgenic strains and costs involved, it is nearly impossible to use TCR transgenic mice to study multiple TCRs simultaneously. For that reason, TCR retrogenic mice are a more flexible and fast approach

to model the diverse TCR responses that naturally develop from polyclonal T cell populations.

The TCR retroviral expression method originally involved delivery of two vectors expressing TCR alpha and beta chains (L. Yang, Qin, Baltimore, & Van Parijs, 2002). The original two-vector approach was later improved upon by incorporation of 2A “self-cleaving” sequences to allow stoichiometric expression of both alpha and beta chains from the same reading frame in a single vector (Holst et al.). The new method, dubbed “TCR retrogenic” approach, has become a powerful alternative to conventional TCR transgenic mice. The protocol described in this unit has undergone additional improvements to increase its efficiency and speed (Bettini, Bettini, Nakayama, Guy, & Vignali, 2013; Holst et al., 2006; Lee et al., 2016). Once the TCR vector is made, development of a TCR retrogenic mouse typically takes less than 6 weeks. Unlike transgenic TCRs, retrogenic TCR expression cannot be passed on through generations, and new mice must be generated with newly transduced bone marrow. As a result, the potential for the “founder effect” is virtually eliminated. As the retrovirus used in this assay is replication-incompetent, the transduction procedure can be performed in a standard Bsl II tissue culture hood. So far, this method has been successfully applied to express more than 70 TCRs, and was used in over 20 publications in the past three years by multiple laboratories (Abraham et al., 2019; Bettini et al., 2017; Drobek et al., 2018; Giles, Neves, Marshak-Rothstein, & Shlomchik, 2017; Gras et al., 2016; Guan et al., 2017; Guo et al., 2016; Jones, Alli, Li, & Geiger, 2016; Lee et al., 2017; Miao et al., 2017; Milasta et al., 2016; Ng, Leno-Duran, Samanta, Almo, & Strominger, 2019; Packard et al., 2016; Snook et al., 2018; Sprouse et al., 2016; Sprouse et al., 2018; Tan et al., 2017; Van Braeckel-Budimir et al., 2017; Wolf, Emerson, Pingel, Buller, & DiPaolo, 2016; J. D. Yang et al., 2018). More recently, we have adapted the method to express human TCRs in HLA-humanized mice, which has significantly expanded the utility of the method in translational applications (Sprouse et al., 2016).

Despite the advantages listed above, TCR retrogenic mice do have certain limitations. First, the need to make new TCR retrogenic mice for every experiment puts more emphasis on keeping experimental conditions consistent in order to avoid significant batch differences. In our own hands, we have observed changes in viral titer, cytokine quality and animal health conditions to significantly influence the BM reconstitution efficiency, and morbidity and mortality of recipients even when expressing the same TCR. Changes in viral titers could be controlled by using freshly expanded viral producers from early cell passages for each experiment. Consistency can also be achieved by generating viral producers at the same time for multiple TCRs, and using early passages of GP+E-86 viral producer cells lines for each experiment. Consistent FBS lots, cytokine sources, plastics and consumables, mouse age and sex will contribute to improved reproducibility. Additional factors are discussed in in the Critical Parameters and Troubleshooting section. Second, the random insertion of retroviral vectors is potentially genotoxic, like in human retroviral HSC gene therapies (Morgan, Gray, Lomova, & Kohn, 2017), so it is recommended to limit the timeframe of an experiment to no more than 20 weeks after BM injection. Fourth, the irradiation of recipient mice induces inflammation and a mild “cytokine storm”. However, no deleterious consequence of this condition have been observed in our hands (Holst et al., 2006).

Importantly, the use of the TCR retrogenic method is not limited to αβ TCR dimers. Retrogenic mice have been used to study γδ TCRs (Guo et al., 2016), invariant NKT cell TCRs (Cruz Tleugabulova et al., 2016) and B cell receptors (Packard et al., 2016). The multicistronic "self-cleaving" 2A peptides can be used to express as many as 4 different proteins in a single reading frame, as was done with the CD3 TCR signaling complex epsilon, gamma, delta and zeta chains (Bettini et al., 2017). Cytokines, cytokine receptors, components of intracellular signaling pathways or their regulators and many other molecules important in biology of hematopoietic lineage could be studied using retroviral gene delivery described in this protocol.

Critical Parameters and Troubleshooting

In order to ensure successful development of TCR retrogenic mice, attention should be paid to several critical steps. Firstly, make sure that retroviral producer cell culture is maintained at below 80% confluency, and is expanded to sufficient cell number prior to bone marrow harvest. High (or low) confluency can result in sub-optimal viral titer and have a negative effect on the efficiency of bone marrow transduction. Secondly, cytokines should be aliquoted and stored at −80°C, and working aliquots should not be kept at 4°C for more than 2 weeks. Remember to warm up the centrifuge before spin-transduction. Wrap the plates containing bone marrow during spin-transduction to avoid excessive air exchange and changes in media pH in response to reduced CO_2 levels. Finally, maintain recipient mice on antibiotic water after irradiation for at least 4 weeks to prevent opportunistic infections. For additional suggestions and solutions to problems refer to the troubleshooting guide (Table 1).

Anticipated Results

Optimal transfection or transduction of 293T, GP+E-86, and bone marrow cells should be monitored by flow cytometry. A representative analysis of bone marrow transduction and reconstitution is depicted in Figure 2. The percent of FP+ cells in this representative experiment within the live/lymphocyte gate is 26.4%. Expect to observe FP expression in 15–30% of bone marrow cells at the end of the transduction protocol. To specifically verify expansion and transduction of progenitor HSCs within the bone marrow culture, additional analysis can be performed using antibodies to lineage and HSC markers. Using this advanced analysis, Lin-Sca1+ HSCs will show higher level of transduction compared to the whole bone marrow culture, as shown in Figure 1A, third panel (49% FP+). If recipient mice are sublethally irradiated, engrafted donor bone marrow will only account for part of the total bone marrow, and overall bone marrow FP expression will be lower in TCR retrogenic mice compared to pre-injection analysis. For example, ten weeks post bone marrow transfer, only 17.9% of stem cells (Lin-Sca1+) were FP+ in NOD.TCRα−/− recipients that received a sublethal dose of 500 rads (Figure 1B). Successful T cell development can be assessed based on accumulation of T cells in peripheral lymphoid organs. FP expression in peripheral organs will depend on the level of bone marrow engraftment, and absence or presence of endogenous B cells, i.e. if the recipient mice are B cell free (*scid* or Rag) or have endogenous BCR rearrangement and FP negative B cell development (i.e. TCRα−/− mice). Example spleen analysis in Figure 2 shows TCR retrogenic spleen containing endogenous B

cells where only a subpopulation of cells are FP+ (Figure 1C). Expect to observe accumulation of T cells in peripheral lymphoid organs starting at 5 weeks post transfer. T cell numbers will plateau at 8–10 weeks post bone marrow transfer.

Time Considerations

Generation of retroviral producer cell lines typically takes 8 days. However, 293T cells need to be expanded and maintained at a healthy confluency before being used for transfection. After transduction and sorting of GP+E-86 cells, additional one to two weeks are necessary to expand the cells. If producer cells are frozen at our recommended density (0.6–1 million per vial), it generally takes four days to reach a cell number of about 3.5 million per vial thawed. Producers should be thawed at least four days prior to bone marrow harvest; however, additional time might be necessary for expansion of cells depending on the target cell number. The time required for bone marrow harvest and processing on Day 3 is dependent on the number of mice used and the skill of the researcher. In general, it takes about 1.5 hours for bone harvest from 20 mice and another 2–3 hours to finish bone marrow processing and culture set up. The transduction procedure on Days 4 and 5 takes about 2 hours from start to finish (centrifugation included). In Basic Protocol 2, recipient mice are irradiated on Day 5 and bone marrow cells are transferred on Day 6. Collecting, transferring bone marrow, and measuring transduction efficiency on Day 6 should take about half a day depending on the scale of the experiment, but should be performed efficiently and with haste. Prolonged bone marrow cell manipulation or storage on ice prior to injection on Day 6 can be detrimental to bone marrow survival.

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Significance Statement

The current protocol details a rapid and efficient way to generate mice expressing a single T cell receptor using retroviral gene delivery into bone marrow progenitor cells. This approach, referred to as “TCR retrogenic mice,” can be applied to the study of antigen specific T cells in a wide range of immunological models and diseases. The flexibility of the method allows generation of mice that express multiple TCRs specific for a wide variety of antigens on a genetic background of choice.

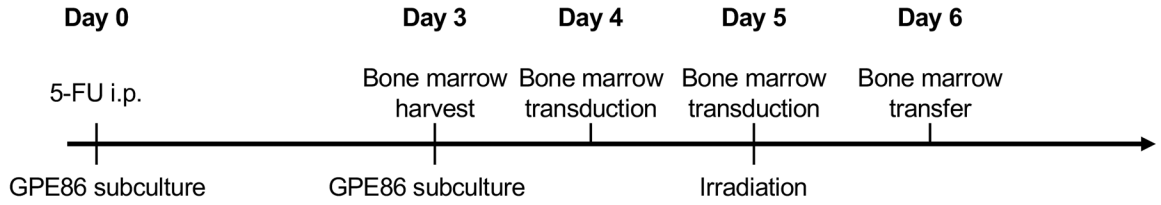

Figure 1.
Time frame for generating TCR retrogenic mice.

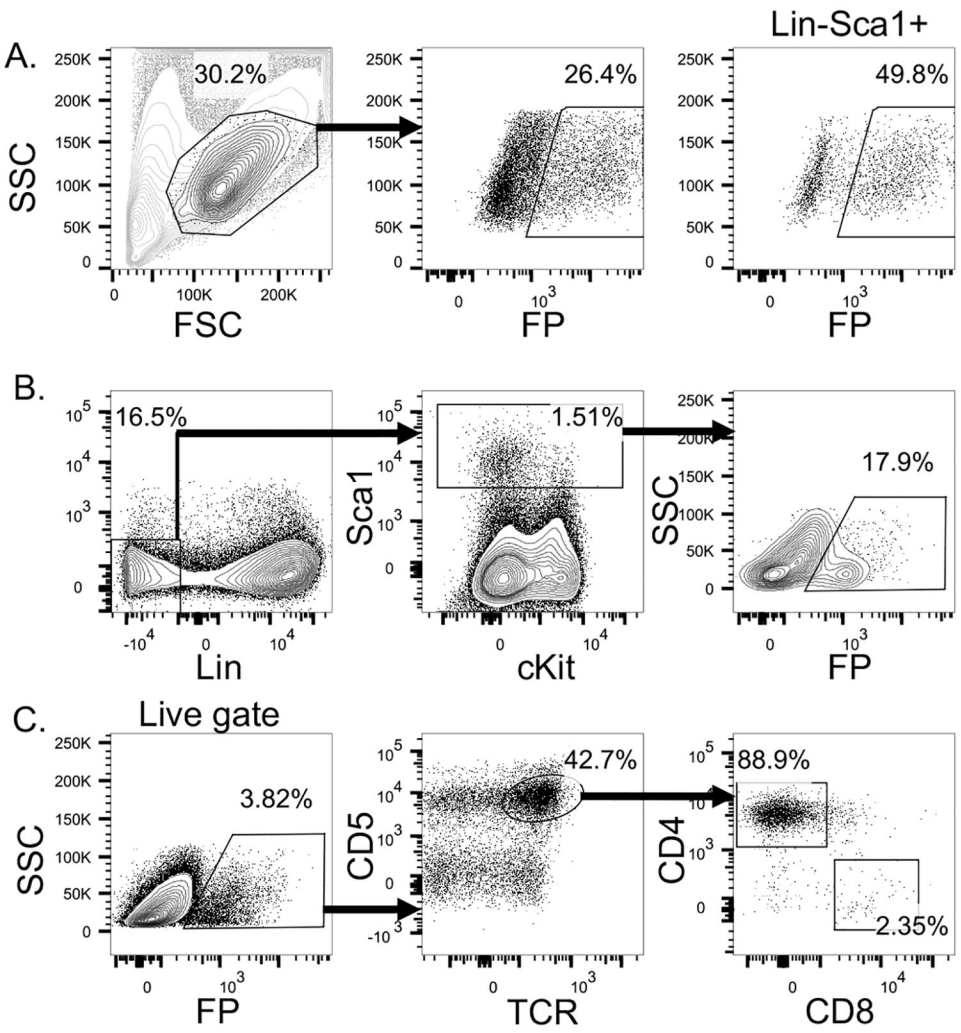

Figure 2.

Representative analysis of bone marrow transduction and reconstitution. NOD.*scid* bone marrow was transduced with MHCII restricted TCR and injected into sublethally irradiated NOD.TCR*a*−/− recipients. **A**. Transduction efficiency of bone marrow cells prior to injection. First panel shows FSC/SSC gating strategy with Lin-Sca1+ HSCs backgated in black. In general, it is sufficient to test overall transduction efficiency as shown in the second panel, without lineage markers staining. However, more precise transduction of HSC precursors can be determined by discriminating Sca1+ HSCs from lineage+ cells, as shown in the third panel. Lin-Sca1+ cells are identified based on CD4-CD8-TCRβ-TCRγδ-B220-CD11b-DX5- and Sca-1+ cells. HSCs usually exhibit higher level of FP expression compared to all cells. **B** and **C**. Analysis of reconstitution efficiency 10 weeks post bone marrow transfer. **B**. Analysis of bone marrow in TCR retrogenic mice. Lin- (CD4-CD8-TCRβ-TCRgd-B220-CD11b-DX5-Ter119-) Sca1+ gate includes both cKit+Sca1+ common lymphoid progenitors with long-term repopulating capacity, and the cKit-Sca1+ HSCs with short-term repopulation capacity. The *in vitro* culture conditions prior to bone marrow injection skew bone marrow progenitors toward cKit-Sca1+ phenotype. **C**. Analysis of the

spleen from a MHCII restricted TCR retrogenic mouse. Second panel shows predominant MHCII restriction in FP+ T cells.

Table 1.

Troubleshooting Guide for Generation of TCR Retrogenic Mice.

Problem	Possible Cause	Solution
Low bone marrow transduction efficiency (less than 15%) *	Low viral titer	Remake GP+E-86 viral producer cells. Sort viral producers based on higher level of reporter expression (at least top 40% FP+). Ensure that plasmid DNA used to generate viral producers is of sufficient concentration (>0.4mg/ml for pVSVg, and >1mg/ml for pMSCV and pPAM-E).
	Bone marrow confluency is too high during transduction.	Ensure that bone marrow is maintained at near confluency throughout expansion and transduction steps.
	Bone marrow cells are not expanding or surviving.	Verify cell viability after first and second transduction. Consider increasing bone marrow confluency, switching to an alternative FBS lot, or using new freshly aliquoted cytokines.
Low bone marrow viability at the end of culture period.	High viral titer (above 35%)	Dilute GP+E-86 supernatant 1:1 with C-DMEM-5.
	Cytokines have lost effectiveness.	Use fresh aliquot, obtain new cytokines.
	FBS lot is not conducive to bone marrow proliferation.	Replace FBS lot.
Mice die within the first week post bone marrow injection.	Opportunistic infections	Use sterile autoclaved cage set up. Switch to a different combination of antibiotics. Provide antibiotics 24 hours prior to irradiation.
	Bone marrow is infected with mycoplasma from contaminated viral producer cells.	Remake retroviral producers from mycoplasma-free cells. Expand producers in CIP containing media.
Mice die within the second week post bone marrow injection.	This is usually associated with low bone marrow viability at the end of culture. Recipient mice might look anemic.	See comments regarding “Low bone marrow viability at the end of culture period”.
	Mice received insufficient numbers of hematopoietic progenitors.	Increase the number of bone marrow cells injected per mouse.
No FP (fluorescent protein) expressing cells in the lymphoid organs after 5 weeks post reconstitution.	Mice received insufficient numbers of hematopoietic progenitors.	Increase the number of bone marrow cells injected per mouse.
	Bone marrow transduction efficiency is low.	See “Low bone marrow transduction efficiency”.
FP expressing cells are present, but none are TCR+.	TCR does not support efficient positive selection of thymocytes. Check for FP expression at the double negative stage of thymic development. Analyze upregulation of CD69 and TCR at the double positive (DP) stage. If FP expression is lower at the DP stage and CD69 and TCR levels are not upregulated, this will suggest a lack of positive selection.	Verify proper TCR complex formation and cell surface expression in a 293T transfection system when co-expressed with CD3εγδζ complex. Low TCR expression levels will lead to reduced TCR expression and lack of T cell development *in vivo*.
	TCR mediates deletion during thymic selection. Analyze upregulation of apoptotic markers, CD69 and TCR expression at the DP stage. Increase in CD69 and TCR during progression through DN/DP/SP developmental stages is driven by TCR signals. Increase in apoptotic markers and loss of FP + TCR+ population at the SP stage suggest negative selection.	

* Percent transduction based on all cells in the bone marrow culture on Day 6 of the protocol.