Video Article

Retroviral Transduction of T-cell Receptors in Mouse T-cells

Shi Zhong[1], Karolina Malecek[1,2], Arianne Perez-Garcia[1], Michelle Krogsgaard[1]

[1]NYU Cancer institute, New York University School of Medicine

[2]Program in Structural Biology, New York University School of Medicine

Correspondence to: Michelle Krogsgaard at krogsm01@nyumc.org

URL: https://www.jove.com/video/2307
DOI: doi:10.3791/2307

Keywords: Immunology, Issue 44, T-cell, T-cell receptor, Retrovirus, Mouse, Transduction, Spleen

Date Published: 10/22/2010

Citation: Zhong, S., Malecek, K., Perez-Garcia, A., Krogsgaard, M. Retroviral Transduction of T-cell Receptors in Mouse T-cells. *J. Vis. Exp.* (44), e2307, doi:10.3791/2307 (2010).

Abstract

T-cell receptors (TCRs) play a central role in the immune system. TCRs on T-cell surfaces can specifically recognize peptide antigens presented by antigen presenting cells (APCs)[1]. This recognition leads to the activation of T-cells and a series of functional outcomes (e.g. cytokine production, killing of the target cells). Understanding the functional role of TCRs is critical to harness the power of the immune system to treat a variety of immunology related diseases (e.g. cancer or autoimmunity).

It is convenient to study TCRs in mouse models, which can be accomplished in several ways. Making TCR transgenic mouse models is costly and time-consuming and currently there are only a limited number of them available[2-4]. Alternatively, mice with antigen-specific T-cells can be generated by bone marrow chimera. This method also takes several weeks and requires expertise[5]. Retroviral transduction of TCRs into *in vitro* activated mouse T-cells is a quick and relatively easy method to obtain T-cells of desired peptide-MHC specificity. Antigen-specific T-cells can be generated in one week and used in any downstream applications. Studying transduced T-cells also has direct application to human immunotherapy, as adoptive transfer of human T-cells transduced with antigen-specific TCRs is an emerging strategy for cancer treatment[6].

Here we present a protocol to retrovirally transduce TCRs into *in vitro* activated mouse T-cells. Both human and mouse TCR genes can be used. Retroviruses carrying specific TCR genes are generated and used to infect mouse T-cells activated with anti-CD3 and anti-CD28 antibodies. After *in vitro* expansion, transduced T-cells are analyzed by flow cytometry.

Video Link

The video component of this article can be found at https://www.jove.com/video/2307/

Protocol

1. Prepare Retroviral Construct

1. Sub-clone the T-cell receptor (TCR) gene of interest into a retroviral vector (Figure 1, example vectors pMSG[7], pMIGII[5,8], pMXs from Cellbiolabs). The TCR α and β chain gene should be on the same vector under the control of the same promoter to ensure equal expression. If the TCR of interest is human, the human constant domains need to be replaced by mouse constant domains[9]. Our observation is that full length human TCRs do not stably express on mouse T-cells.
2. Prepare high quality plasmid DNA using Qiagen Maxi Prep Kit or similar product. The concentration of the plasmid should be at around 1 µg/µL.

2. Transfection and T-cell Isolation

Day -1 (Plate packaging cells)

1. Dislodge Platinum-E (Plat-E, Cellbiolabs) retroviral packaging cells with trypsin-EDTA and neutralize with DMEM media.
2. Centrifuge the cells at 1000 x g for 5 min. Aspirate the supernatant and resuspend the cell pellet in DMEM media.
3. Determine the cell number and dilute the cells at $0.6x10^6$/mL. Plate 10 mL of cell suspension in a 10mm poly-lysine coated tissue culture plate and grow overnight in a cell incubator at 37 °C, 5% CO_2.

Day 0 (Transfection)

4. The next morning, examine the cells under a light microscope. The cells should be approximately 80% confluent.
5. Gently remove the media from the plate. Wash the cells once with 1x PBS and add 10 mL pre-warmed, fresh DMEM media without Penicillin-Streptomycin (Pen/Strep).
(Note: Pen/Strep can interfere with the transfection)

Copyright © 2010 Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License

6. Prepare transfection complex.
   Prepare mix A and B as following:

Mix A: Plasmid DNA	9 µg
pCL-Eco helper plasmid	6.3 µg
OptiMEM	1.5 mL
Mix B: Lipopfectime 2000	60 µL
OptiMEM	1.5 mL

   Incubate A and B separately for 5 minutes at room temperature
   Mix A and B together gently and incubate for 20 minutes at room temperature to form transfection complex.
7. Slowly drip the mixture (total 3 mL) into the Plat-E cells. Gently rock the plate back and forth to distribute the transfection mixture evenly.
8. Incubate cells at 37 °C/5% CO_2 in a cell incubator.
9. After 6-8 hours, replace medium with 10 mL of fresh DMEM medium (with Pen/Strep) for viral production.

Coat plate with antibodies

10. Mouse T-cells need to be activated before viral transduction. There are a variety ways to activate T-cells. Here we use plate bound anti-CD3e and anti-CD28 antibodies. Prepare an antibody mixture of 1 µg/mL of anti-CD3e and 2 µg/mL of anti-CD28 in sterile PBS.
11. Dispense 250 µL of the antibody mixture to each well of a 24-well tissue culture plate. The plate can be coated overnight at 4 °C or 2 hours at 37 °C.

Preparation of single-cell suspension from mouse spleen

12. Harvest mouse spleen and transfer to RPMI medium.
13. Place the spleen in a cell strainer. Mash the spleen with a syringe plunger into a 5 cm tissue culture plate. Rinse cells off the cell strainer with sterile PBS.
14. Centrifuge 1000 xg, 5 min.
15. Discard supernatant. Resuspend cell pellet in ACK lysing buffer (2 mL per spleen). Incubate 2 to 3 min at room temperature. Add RPMI medium up to 20 mL and centrifuge 1000 xg for 5 min.
16. Discard supernatant. Resuspend cell pellet in sterile PBS. Determine cell number and centrifuge 1000 xg for 5 min at 4 °C. Proceed to the isolation of T-cell.

Isolation and activation of mouse T-cells

17. Magnetically label the cells according to the product manual (CD8+ T-cell isolation kit from Miltenyi Biotec).
18. Place a LS column (Miltenyi Biotec) in the magnetic field. Apply labeled cell suspension onto the column. Collect flow-through (enriched mouse CD8+ T-cells). Wash the column three times with 3 mL buffer according to the product manual and combine with previous flow-through.
19. Stain the cells with anti-CD3e and anti-CD8a antibodies and analyze by flow cytometry (Figure 2a). For a typical separation, ~8-10% of total cells can be isolated. ~90% of the isolated cells are CD8+ T-cells.
20. Centrifuge the cells at 1000 xg, 5 min. Discard the supernatant. Resuspend the cell pellet in RPMI medium with recombinant human IL-2 (rhIL2, 20 ng/mL) at $1x10^6$ /mL.
21. Just before adding the cells, remove antibody solution from the plate (prepared previously in 2.11). Rinse each well with sterile PBS and remove PBS.
22. Add 1 mL of the cell suspension to each well. Put the plate at 37°C/5% CO_2 in a cell incubator.

3. (Day 2 and Day 3) Infection of Activated T-cells

1. After 2 days of virus production, the medium in the Plat-E cell plate should become yellow. Transfer viral supernatant to a 15 mL conical tube. Add 10 mL of fresh DMEM medium to the plate for further viral production (Optional).
2. Centrifuge virus supernatant at 1000 xg for 5 min to remove cells debris. Carefully transfer virus supernatant to a new tube. Leave some liquid at the bottom of the tube and do not disturb the cells debris.
3. Collect activated T-cells from the plate into a 50 mL conical tube. Determine the cell number. Save some cells into a separate tube to be used as negative (un-transduced) control. Centrifuge at 1000 xg for 5 min and discard supernatant.
4. Resuspend the cell pellet in virus supernatant at 10^6 cells per 1 mL with 20 ng/mL rhIL2 and 10 µg/mL of protamine sulfate. Add 1 mL of cell suspension to each well of a 24 well plate. Resuspend the cells in the control tube in RPMI medium at the same cell density with the same amount of rhIL2 and protamine sulfate. Add the cells to the same plate.
5. Wrap the plate with plastic film. Centrifuge 90 minutes at 2000 xg, at 32 °C with no brake.
6. After the centrifugation, remove the plastic film. Add 1 mL of fresh RPMI medium with 20 ng/mL rhIL2 to each well. Put the plate back to the incubator.
7. (Optional) Higher expression level of TCR can be achieved by collecting the virus supernatant and repeating the infection procedure on day 3.

Copyright © 2010 Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License

4. Expansion of Cells and Evaluation of Transduction Efficiency

1. Examine the transduced T-cells daily. Split the cells at 1:3 ratios when necessary in RPMI medium rhIL2. Do not let the cells overgrow (medium turns yellow).
2. At day 6 or day 7, stain the cells with antibody and MHC tetramer specific for the TCR to evaluate the expression of TCR on T-cell surface. Use the un-transduced T-cells as control (Figure 2c).
3. The transduced T-cells are ready for further downstream applications.

5. Representative Results

It is often advantageous to purify T-cell subsets before transduction although splenocytes can be transduced without purification. High purity T-cell subsets can be obtained using commercial magnetic beads (Figure 2a).

To evaluate the expression level of TCRs on T-cells, antibodies specific for TCR alpha or beta chains can be used. Typically 30% to 80% of the cells can express transduced TCR (Figure 2b) depending on the TCR genes and virus titers. MHC tetramer specific for the TCR can also be used to further verify functional expression of TCRs (Figure 2c).

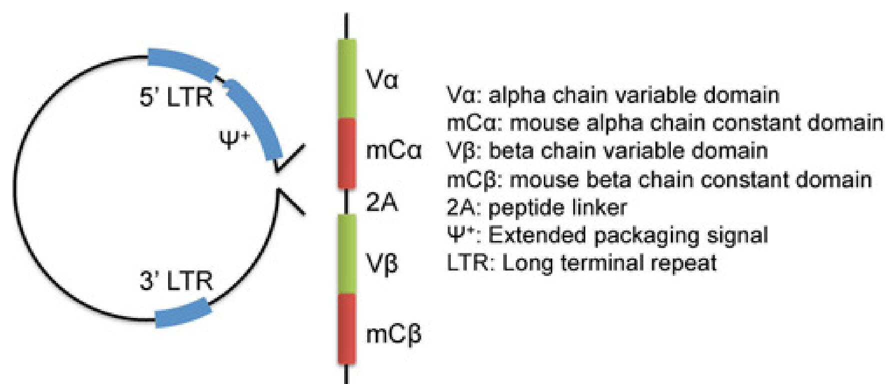
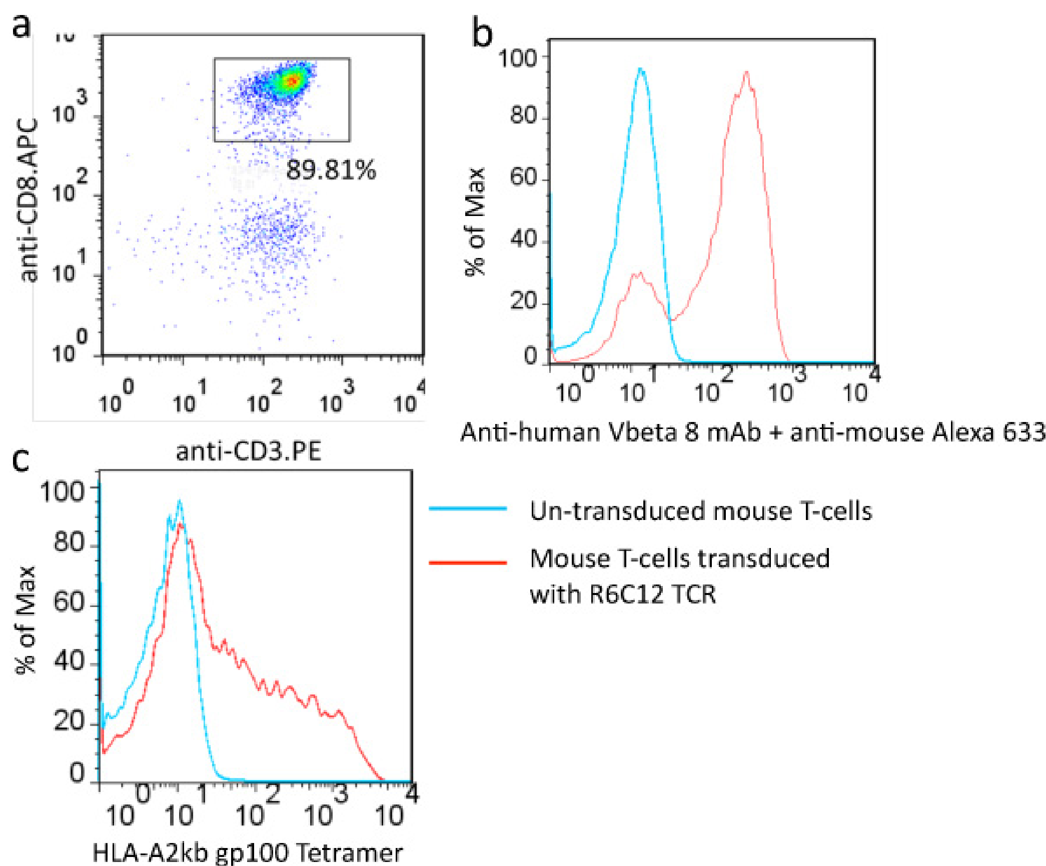


Figure 1. Example of T-cell receptor gene construct sub-cloned into a retroviral vector. Full length alpha and beta chain genes are linked by a self-cleavable 2A peptide linker[8].



Copyright © 2010 Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License

Figure 2. Example of successful isolation and transduction of mouse T-cells. (a) CD8 T-cells were isolated from splenocytes using CD8a + T Cell Isolation Kit (Miltenyi Biotec) and stained with anti-CD3e and anti-CD8a antibodies. Isolated CD8 T-cells were transduced with R6C12 TCR specific for human gp100:209 -217[4] peptide and stained with anti-human Vbeta 8 antibody (b) and HLA-A2kb gp100 tetramer (c).

Discussion

Several steps are critical to achieve optimal results. Plat-E packaging cell line should be kept in healthy condition. If necessary, the cells can be passaged a few times in DMEM medium with 1 µg/mL puromycin, 10 µg/ml blasticidin to prevent loss of retroviral structure protein expression. On the day of transfection, the cells should be ~80% confluent. Too few or too many cells may reduce the virus titer. The virus quality can be checked by testing on cell lines that can be easily transduced. Since TCRs require CD3 complexes to be expressed on the cell surface, we routinely use 58α-/β- hybridoma cells[10](a cell line that does not express endogenous TCR α or β chain, but does express CD3 complex). We obtain nearly 100% transduction efficiency of hybridoma cells when transducing cells with 1 mL of virus supernatant. Finally, the T-cells need to be activated and proliferating because retrovirus can only infect actively dividing cells.

The method presented here for producing antigen-specific T-cells has several limitations. First, it is difficult to reach 100% transduction efficiency for primary mouse T-cells. A portion of the cells do not express the TCR of interest. Second, the transduced TCR α and β chains can mispair with endogenous TCRs[9], which may reduce expression level of the TCR of interest. In addition, the mispaired TCRs may cause autoimmunity *in vivo*[11]. Finally, there is only a limited time frame the transduced T-cells can be used. After around one week, the cells undergo apoptosis unless being re-stimulated. However, cells may become exhausted and lose functions with repetitive re-stimulations.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by grants from NIH (5U01CA137070) and the American Cancer Society (RSG-09-070-01-LIB), a Cancer Research Institute Investigator Award (M.K.), an American Heart Association pre-doctoral fellowship (K.M.) and a Spanish Ministry of Education and Science Fellowship (A.P.G).

We would like the thank Dr. Nicholas Restifo and Dr. Zhiya Yu (NIH/NCI) for helpful suggestions to the protocol.

References

1. Krogsgaard, M. & Davis, M.M. How T cells 'see' antigen. *Nat. Immunol* 6, 239-245 (2005).
2. Berg, L.J. *et al.* Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. *Cell* 58, 1035-1046 (1989).
3. Hogquist, K.A. *et al.* T cell receptor antagonist peptides induce positive selection. *Cell* 76, 17-27 (1994).
4. Yu, Z. *et al.* Poor immunogenicity of a self/tumor antigen derives from peptide-MHC-I instability and is independent of tolerance. *J. Clin. Invest* 114, 551-559 (2004).
5. Holst, J. *et al.* Generation of T-cell receptor retrogenic mice. *Nat Protoc* 1, 406-417 (2006).
6. Morgan, R.A. *et al.* Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 314, 126-129 (2006).
7. Hawley, R.G., Lieu, F.H., Fong, A.Z. & Hawley, T.S. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther* 1, 136-138 (1994).
8. Szymczak, A.L. *et al.* Correction of multi-gene deficiency *in vivo* using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat. Biotechnol* 22, 589-594 (2004).
9. Cohen, C.J., Zhao, Y., Zheng, Z., Rosenberg, S.A. & Morgan, R.A. Enhanced antitumor activity of murine-human hybrid T-cell receptor (TCR) in human lymphocytes is associated with improved pairing and TCR/CD3 stability. *Cancer Res* 66, 8878-8886 (2006).
10. Letourneur, F. & Malissen, B. Derivation of a T cell hybridoma variant deprived of functional T cell receptor alpha and beta chain transcripts reveals a nonfunctional alpha-mRNA of BW5147 origin. *Eur. J. Immunol* 19, 2269-2274 (1989).
11. Bendle, G.M. *et al.* Lethal graft-versus-host disease in mouse models of T cell receptor gene therapy. *Nat. Med* 16, 565-570, 1p following 570 (2010).

Copyright © 2010 Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported License