# Vectofusin-1 based T-cell transduction approach for developing murine CAR-T cells for cancer.

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## Abstract

Gene transfer into human and murine T-cells using viral-based approaches has several promising therapeutic applications including the production of chimeric antigen receptor T-cell (CAR-T) therapy. The generation of murine CAR-T is paramount to test and validate immunocompetent mouse models for CAR-T therapy. Several viral transduction enhancers already exist for gene therapy with few limitations. In this study, we tested vectofusin-1, a short cationic peptide, as a soluble transduction enhancer for gammaretroviral transduction for the generation of anti-CD19 murine CAR-T. We found that in comparison to Retronectin, Vectofusin-1 is an equally optimal transduction enhancer for the generation of murine CAR-T cells.

## Introduction

The chimeric antigen receptor (CARs) are synthetic receptors that comprise a target binding domain, derived mostly from a single-chain variable fragment of an antibody, a hinge/transmembrane region, and a truncated CD3 zeta cytoplasmic domain with or without costimulatory domains[1]. CAR T- cell therapy represents the latest advance in the field of hematological malignancies with unprecedented response rates and survival outcomes seen in patients with relapsed refractory lymphomas[2]. As such, there is a greater need to produce CAR-T cells both for clinical use and for laboratory research purposes for in-vitro and in-vivo validation studies. The generation of CAR-T cells through viral transduction can be optimized through the addition of various cultures additives such as cationic polymers (polybrene)[3], dextran[4], cationic lipids (lipofectamine[5]) etc. Retronectin derived from fibronectin, has recently been used for the generation of both human and murine CAR-T cells for clinical applications[6,7] and research purposes. However, Retronectin based transduction protocols are cumbersome as it needs to be surface coated before use and therefore, a new soluble additive capable of enhancing infection is needed. Vectofusin-1, a new cationic amphipathic peptide, is a soluble additive that has been used for transduction of T-cells successfully; however, that has not been tested for the generation of murine CAR-T cells. The goal of this study is to determine if a novel conjunction protein-peptide, such as Vectofuscin-1, can achieve a similar transduction efficiency for the generation of chimeric antigen receptor (CAR) T-cell in mouse T-cells. Here, we compare the vectofusin-1 based approach to a more traditional method of Retronectin protocols and provides step by step approach to generating murine CAR-T cells through both approaches.

## Reagents

- Retronectin (Takara Bio, Catalog No. T100B)
- Vectofusin -1 (Miltenyi Biotec, Catalog No. 130-111-163)
- EasySep™ Mouse T Cell Isolation Kit – (StemCell, Catalog No: 19851)
- Purified anti-mouse CD3ε Antibody – (Biolegend, Catalog No: 100302)
- Purified anti-mouse CD28 Antibody – (Biolegend, Catalog No: 102102)
- EasySep™ Buffer (Catalog No. 20144)
- Non-tissue culture treated 6-well plates
- C57Bl6 mice
- Complete RPMI (cRPMI): RPMI 1640 medium, 10 % fetal bovine serum (FBS), 100 IU/mL of penicillin, 1 μM sodium pyruvate, 10 mM HEPES, 2.5 μM β-mercaptoethanol (added fresh when changing medium), and 2 mM L -glutamine.

- PBS–BSA: PBS, 0.5 % bovine serum albumin or FACS buffer (PBS + 2% FBS)
- Magnet: we use the EasySep™ Magnet, but others are suitable.
- Recombinant murine IL-2 (Peprotech, Catalog No. 212-12)
- Frozen viral supernatant from transduced Phoenix-E cells that produce m1928z retrovirus (see Explanation1)

**Methods**

Day 1: Mouse T cell isolation and activation
Note: Mouse spleens can be frozen in FBS + 5% DMSO for future use as needed.

1. Sacrifice mouse and saturate with 70% ethanol. After left lateral incision below the rib cage, Collect spleen. Store spleens in cRPMI.
2. Place the spleen onto a 40 μM cell strainer placed onto a 50 mL conical tube loaded with 10 mL cRPMI. Using the plunger end of a 1 mL syringe, gently push the spleen through the strainer into the 50 mL tube. Rinse the cell strainer with 10 mL of cRPMI to ensure all spleen cells pass through the strainer into the conical tube.
3. Spin cells at 1500 rpm for 5 min, discard supernatant, and wash the pellet with 5mL PBS one time.
4. Resuspend pellet in 5mL RBC lysis buffer.
5. Incubate at room temperature for 2 mins.
6. Stop RBC lysis with 20 mL of cRPMI. Centrifuge and wash once with PBS.
7. Count cells and prepare cell suspension at a concentration of $1 \times 10^8$ cells/mL in EasySep buffer.
8. Add normal rat serum using the EasySep™ Mouse T-cell Isolation Kit (StemCell Technologies) and Isolation Cocktail, both at 50 μL per mL of cells of splenocytes.
9. Mix well and incubate at room temperature for 10 min.
10. Vortex EasySep™ Streptavidin RapidSpheres™ for 30 s and add to the antibody – splenocyte mixture at 75 μL per mL of splenocytes.
11. Mix well and incubate at room temperature for 2.5 min.
12. Add EasySep buffer to the splenocyte suspension to a total volume of 2.5 mL and mix by gently pipetting up and down.
13. Place the tube into a magnet and set aside at room temperature for 2.5 min.
14. Pick up the magnet, invert, and pour off desired, unbound T cell fraction into a new tube.
15. Spin T cells at 1500 rpm for 5 min, discard supernatant, and resuspend pellet in cRPMI to a final concentration of $1x10^7$ cells/mL.

**Mouse T cell activation**

1. Incubate anti-CD3 antibody at 5 μg/ml and anti-CD28 antibody at 2 μg/ml on a 6-well plate (5mL/well) for 2 hours at 37 °C or overnight at 4 °C (seal the plate).
   # Dynabeads™ Mouse T-Activator CD3/CD28(Catalog No. 11456D) can also be used for mouse T-cell activation and expansion with similar results and expansion.
2. Resuspend T cells in cRPMI to a final concentration of $1x10^7$ cells/mL.
3. Aspirate mAB solution from the plates and add 1ml of T cells per well.
4. Let cells grow overnight at 37°C and 5% $CO_2$.

5. For transduction with RetroNectin, prepare non-tissue culture treated plates by adding RetroNectin ™ (1 µg/µL) (Takara Bio, Otsu, Japan) to enhance transduction efficiency. Add 90 µL of RetroNectin ™ with 6 mL PBS. Dispense 1 mL/well of RetroNectin/PBS into non-tissue culture treated plates. Store the plates overnight at 4 °.
   # The number of wells to be plated with Retronectin depends upon the yield of number of T-cells harvested from the spleen. We prefer to use $1X10^7$ cells per well of 6-well plates.
   # Retronectin coated plates can also be prepared on day of transduction, but require incubation at room temperature for 2 h, followed by blocking with PBS–BSA for 30 min, and washing one time with PBS before same-day use.

**Day 2: First transduction**

<u>RetroNectin based transduction</u>

1. The next day, remove RetroNectin/PBS and block with PBS–BSA for 30 min at room temperature.
2. Remove blocking buffer and wash plates one time with PBS.
3. Thaw the viral supernatant in 37% water bath. Production of gamma-retrovirus production is detailed in the note section. Add 3mL of the viral supernatant to Retronectin-coated wells.
4. Spin at 2000 g for 1h at room temperature.
5. Harvest and centrifuge activated T cells at 1500 rpm for 5 min.
6. Resuspend cells with cRPMI at $1x10^7$ cells/mL and 80 IU/mL of IL-2.
7. Distribute 1 mL of T-cells suspension to each well.
8. Spin plates at 2000 × g for 1 h at room temperature.
9. Incubate plates in a tissue culture incubator at 37 °C (5 % $CO_2$).

<u>Vectofusin-1 based transduction – adapted from manufacturer's instructions</u>

1. Thaw stock aliquot of Vectofusin-1 (1mg/mL stock concentration) at room temperature. Vortex thoroughly before use.
   a. The required final concentration of Vectofusin-1 for transduction is 10 µg/mL in the total culture volume.
2. Add 10ul of vectofuscin-1 to 3 ml of viral supernatant and pipette up and down.
   # we have used lower doses of vectofusin-1 of up to10 ul without compromising on the transduction efficiency.
3. Immediately (not exceeding 10 mins) add the mixture of viral supernatant and Vectofusin-1 to 1mL of cell suspension ($1x10^7$ cells/mL) and pipette up and down.
4. Spin plates at 2000g for 2h at room temperature.
5. Incubate at 37 °C (5 % $CO_2$).
   a. To reach a higher transduction performance, centrifuge cell samples at 400×g for 2 hours at 32 °C followed by static incubation at 37 °C.

**Day 3: Second Transduction 2**

<u>RetroNectin transduction</u>

1. Tilt plates and carefully remove most of media from each wells but be careful to not aspirate cells bound to plates at bottom.
2. Add 3 mL of virus to each well.

3. Spin plates at 2000 × g for 2 h at room temperature.
4. Incubate in a tissue culture incubator at 37 °C (5 % CO 2).

Vectofusin transduction

1. Add 10ul of vectofuscin to 3 ml of viral supernatant and pipette up and down.
2. Tilt plates and carefully remove most of media from each well but be careful to not aspirate cells bound to plates at bottom.
3. Immediately (not exceeding 10 mins) add the mixture of viral supernatant and Vectofusin-1 to each well and pipette up and down.
4. Spin plates at 2000g for 2h at room temperature.
5. Incubate at 37 °C (5 % $CO_2$).

**Day 4 - 7: Expansion and Analysis flow cytometry**

1. Collect cells by thoroughly pipetting up and down and spin at 1500 rpm for 5 minutes and resuspend in 5 mL of cRPMI with 50 IU/mL of IL-2 in a T25 flasks.
2. Incubate in a tissue culture incubator at 37 °C (5% CO2).
3. T cells can be expanded up to Day 7 without further activation. Fresh culture media can be added or exchanged if the media becomes yellow or concentration of cells is more than $2X10^6$ cells /ml.

**Transduction Efficiency Analysis by Flow Cytometry**

1. Harvest and centrifuge transduced T cells at 1500rpm for 5 mins. Warm musRPMI before thawing frozen T cells.
2. Wash once with FACS buffer.
3. Stain with Live/Dead dye and stain with the other appropriate markers: CD4, CD8, CD3, CD45.
4. After washing twice, cells can be immediately analyzed on the flow cytometry.

**Notes:**

1. Activation beads can also be used for activation of T cells; however, they are costly compared to coated antibody methods. We follow the manufacturer protocol with beads to cell ratio at 1:1. Also, we must remove activation beads from T cells before analysis or experimental use by pipetting T cells in a conical tube and thoroughly pipetting up and down to remove the T-cells sticking to beads. Expose the tube to a magnet for 2 min and unbound T cells can be decanted from the tube exposed to the magnet.
2. There are various protocols for transient production of gamma retrovirus from an ecotropic cell lines. However, for large quantities of virus with similar titers, we recommend production of stable virus producers cell lines as shown in the Li et al[8]. For our experiments, we obtained stable virus producing Phoenix-Eco cell lines from Dr. Marco L Davila's lab to generate gamma retrovirus (generous gift).
3. Human IL-2 can be used for both mouse and human T-cells.

**Statistical Analysis:**

Significance of variation between groups was evaluated using a non-parametric two-tailed Student's t test. Test for differences between proportions was performed using two sided Fisher's exact test with $p \leq 0.05$ considered significant.

## Results and Discussion

*Impact of activation beads versus plated antibody on transduction efficiency*

Firstly, we wanted to evaluate the impact of beads versus plated antibodies on the activation of murine T-cells and subsequent transduction efficiency. We took mice spleens (n=3) and murine T-cells were isolated as detailed in the protocol above. $5x10^6$ T-cells were each individually activated with either the mouse T-cell activator beads or plated anti-Cd3/CD28 antibodies. Subsequently, we performed Retronectin based transduction assays with same amount of harvested viral supernatants (3ml) in each well at 24 hours and 48 hours. Viral supernatant was obtained from the stable virus producer cell lines and were pooled before transduction step. This allowed us to make sure that the viral titres were similar between each individual experiments and step.

Our CAR plasmid vector is based on SFG plasmid backbone with anti-mouse CD19 CAR sequence followed by a T2a and GFP sequence as shown in Figure 1A. We evaluated the transduction efficiency in murine T cells by expression of Green fluorescent protein (GFP) by flow cytometry on FITC channel. We found that by Retronectin based transduction assays, similar transduction efficiency was generated between beads (mean=60.40 %, n= 3) and plated antibody anti-CD3/CD28 (mean=55.4%, n=3) T-cell activation methods as shown in the figure 1B and 1C (p=0.14, unpaired t-tests, two-tailed).

*Retronectin is a better transduction enhancer compared to Vectofusin-1 for murine CAR-T*

Next, we compared the Retronectin versus Vectofusin-1 as a transduction enhancer for generation of murine CAR-T cells. For direct comparison, we activated murine T cells isolated from mouse spleens (n=3) with plated anti-CD3/CD28 antibodies. Subsequently, we performed viral transduction on $5X10^6$ T-cells each on day 1 and day 2 with either retronectin or vectofusin-1. We found that vectofusin-1 generated lower transduction efficiency (mean 43.33, n=3) compared to Retronectin (mean=55.57, n=3, p-value 0.001, paired two tailed t-tests) as shown in Figure 2.

*CAR-T cells proliferation and expansion is optimal with Vectofusin-1*

Next, we also evaluated the absolute number of cells at day 5 after transduction with the help of Countess FL automated Cell Counters and computed the median fold expansion from the baseline. We found that by the end of day 5, fold expansion of total T-cells in the media were significantly higher with vectofusin-1 (1.9333 ±0.141 fold) compared to Retronectin (1.4667 ±0.0706) transduction (p=0.008).

DISCUSSION

In our study, we compared vectofusin-1 and Retronectin as transduction enhancers for the generation of murine anti-CD19 CAR-T. We found that the vectofusin-1 generated lesser CAR-T transduction efficiency, as assessed by GFP expression, compared to Retronectin. However, the absolute number of CAR-T cells generated was approximately equal between the two groups due to higher expansion and proliferation of total T-cells under vectofusin-1. The reason for the increased total number of T-cells generated with vectofusin-1 is unclear to us. It is possible that Retronectin labeled plates adhere to T-cells more tightly after spin-down post-transduction and probably limit their mobility and proliferation to a small extent. In conclusion, vectofusin-1 leads to optimal murine CAR-T production, similar to Retronectin for performing in-vitro and in-vivo mouse validation studies.

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FIGURES

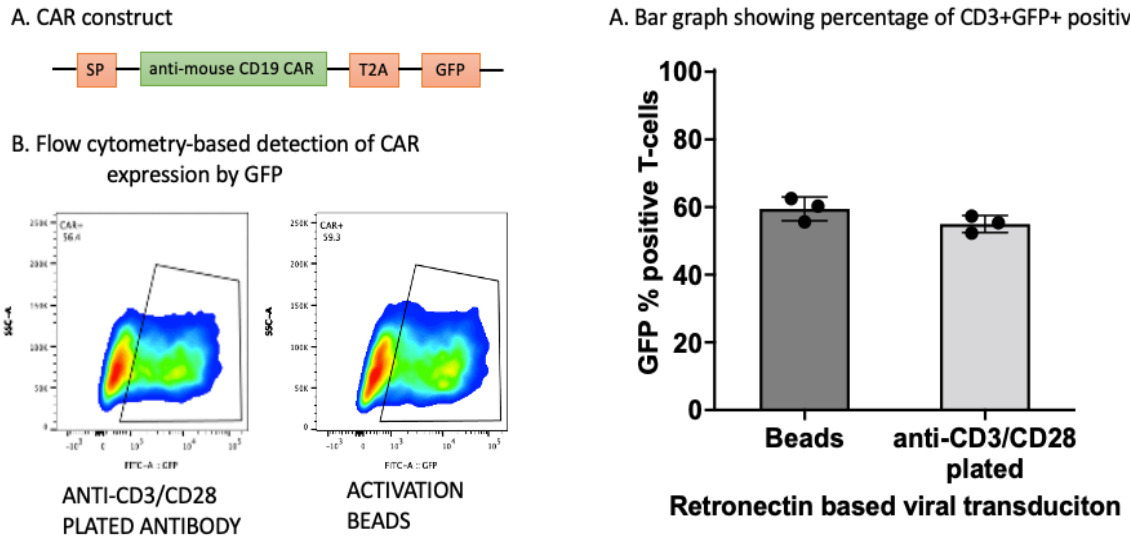


**Figure 1. Comparison of anti-CD3/CD28 plated antibodies versus beads-based activation method for generation of murine anti-CD19 CAR-T.** A) Pictorial representation of CAR vector with GFP segment inserted after a flanking T2A sequence. B) Flow cytometry detection of CAR transduction efficiency in different modalities of activation. C) Bar graph showing percentages of CD3+GFp+ positive cells in Retronectin based viral transduction assays using beads versus plated antibodies activation.

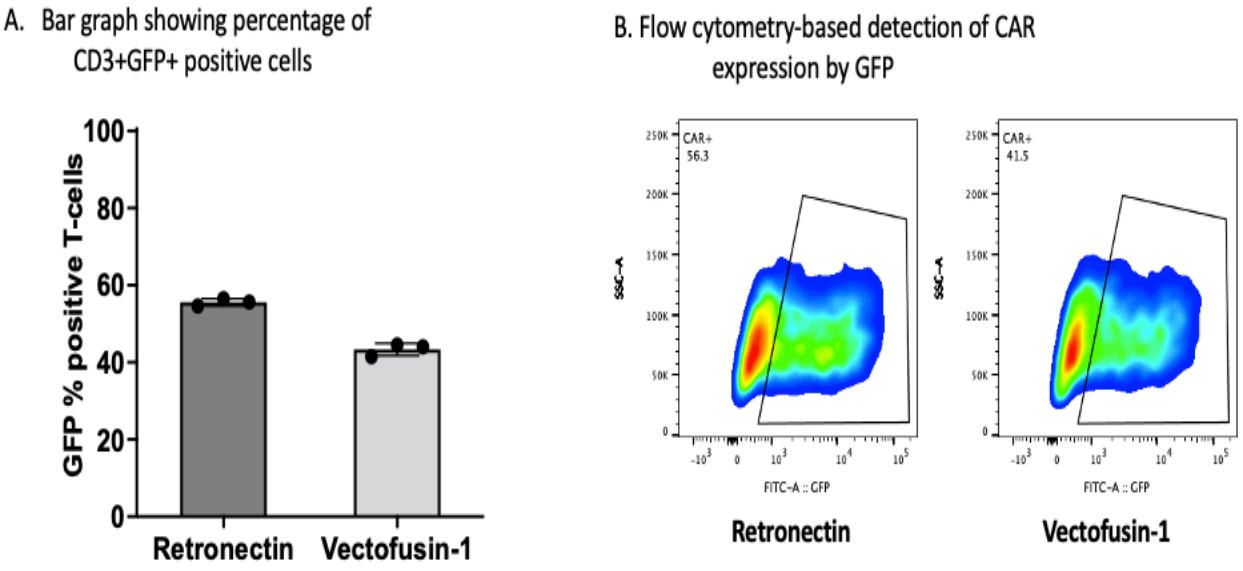


**Figure 2. Comparison of Vectofusin-1 versus Retronectin as transduction enhancers for generation of murine CAR-T cells.** A) bar graph showing percentages of GFP

301 expressing T cells. B) Flow cytometry representation of CAR transduction efficiency with
302 Retronectin versus Vectofusin-1.

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