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## Transduction and expansion of primary T cells in 9 days with maintenance of central memory phenotype --Manuscript Draft--

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**TITLE:**

**Transduction and Expansion of Primary T Cells in Nine Days with Maintenance of Central Memory Phenotype**

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**KEYWORDS:**

CAR-T cells, transduction, expansion, retrovirus, PBMC, central memory

**SUMMARY:**

We outline a 9-day protocol for the transduction and expansion of rhesus macaque peripheral blood mononuclear cells which yields cells with excellent co-expression of the genes of interest in sufficient number for infusion studies of cell efficacy.

**ABSTRACT:**

Emerging immunotherapies to treat infectious diseases and cancers often involve transduction of cellular populations with genes encoding disease-targeting proteins. For example, chimeric antigen receptor (CAR)-T cells to treat cancers and viral infections involve the transduction of T cells with synthetic genes encoding CAR molecules. The CAR molecules make the T cells specifically recognize and kill cancer or virally infected cells. Cells can also be co-transduced with other genes of interest. For example, cells can be co-transduced with genes encoding proteins that target cells to specific locations. Here, we present a protocol to transduce primary peripheral blood mononuclear cells (PBMCs) with genes encoding a virus-specific CAR and the B cell follicle homing molecule chemokine receptor type 5 (CXCR5). This procedure takes nine days and results in transduced T cell populations that maintain a central memory phenotype. Maintenance of a central memory or less differentiated phenotype has been shown to associate with persistence of cells post-infusion. Furthermore, cells produced with this method show high levels of viability, high levels of co-expression of the two transduced genes, and large enough quantities of cells for immunotherapeutic infusion. This nine-day protocol may be broadly used for CAR-T cell and other T cell immunotherapy approaches. The methods described here are based studies presented in our previous publications.

**INTRODUCTION:**

Cellular immunotherapies are emerging as a new means to treat diseases including cancers and infectious diseases. These immunotherapy methods often involve genetically manipulating

therapeutic cells to express specific molecules. For example, chimeric antigen receptor (CAR) T cells are engineered to express a CAR molecule that has an extracellular domain that specifically binds molecules on diseased cells and a signaling domain that triggers the immune cells to kill the diseased cell. CAR T cells are being used successfully in cancer immunotherapies, and have been particularly effective in treating B cell leukemias<sup>1-3</sup>. CAR-T cells are also being developed to treat viral infections such as HIV. HIV-specific CARs target the envelope proteins on the surface of virally infected cells<sup>4</sup>. Immunotherapeutic cells can also be engineered to express homing molecules to target therapeutic cells to specific tissue locations. We have developed vectors that transduce both a virus-specific CAR as well as the lymphoid follicle homing molecule CXCR5<sup>5</sup>.

Viral replication of some viruses, including human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), is concentrated within B cell follicles in secondary lymphoid tissues<sup>6</sup>. B cell follicles are somewhat immune privileged sites in which low levels of virus-specific CTL permit ongoing viral replication<sup>7,8</sup>. For these reasons, targeting HIV/SIV-specific T cells into the follicle through expression of CXCR5 is a strategy for elimination of the follicular reservoir of virally infected cells<sup>9, 10</sup>. Specifically, we are targeting SIV-specific CAR T cells to B cell follicles via CXCR5 co-expression.

In this protocol we describe a method to transduce CAR/CXCR5 and expand PBMCs to produce CAR/CXCR5 T cells of sufficient quantities for therapeutic infusion. Cells transduced with these methods maintain a central memory phenotype. Studies have shown that cells with a less differentiated phenotype, such as central memory T cells and T memory stem cells better persist than more differentiated cells<sup>11,12</sup>. In addition, many protocols designed to produce adoptively transferred cells have relatively long culture times that result in cells that have a more differentiated phenotype and reduced persistence<sup>13</sup>. Furthermore, adoptively transferred cells with long culture times accumulated within the lungs instead of target lymphoid tissues in rhesus macaque<sup>14,15</sup>. In the methods we describe and demonstrate here, we rapidly transduce and expand rhesus macaque PBMCs to produce transduced T cells that maintain a central memory phenotype.

Both CD4 and CD8 T cells are included in our immunotherapy approach. This mixed cell population was chosen because, in clinical trials, the absence of CD4+ T cells in the CD8+ T cell product was correlated with a failure of infused CD8 T cells to persist<sup>16</sup>. The methods described here begin by activating PBMCs with anti-CD3 and anti-CD28 antibodies which potently stimulate the T cell receptor and provide a co-stimulatory signal to avoid clonal anergy<sup>17,18</sup>.

After activation, cells are transduced with a gammaretroviral vector encoding a virus-specific CAR and the lymphoid homing molecule CXCR5. Transduced cells are then expanded using plates designed for non-adherent cell propagation which have a gas permeable membrane at the base. This membrane permits gas exchange at the bottom of the well leading to enhanced cell survival and nutrient availability<sup>19</sup>. Using these methods, sufficient functional cells can be produced in a 9-day timeframe for in vivo infusion studies. While this protocol is designed for transducing and expanding rhesus macaque T cells, with slight modification of the species-specific activating

antibodies, it can be used with cells from other species. These methods are based on our previously published studies<sup>9,20</sup>.

## **PROTOCOL:**

**NOTE:** This rhesus PBMC transduction protocol requires the use of a gammaretroviral vector. Note that gammaretrovirus can either be produced in the lab or outsourced to a viral production company. In lab production can be carried out with the protocol outlined in step 1 and the virus can be titered as outlined in step 2. Whether the virus is produced on site or by a production company, an MOI (ratio of infectious virions to cells in culture) should be determined, as outlined in step 3, for each newly produced viral preparation and the cells of interest.

**CAUTION:** Work with gammaretroviral vectors or other retroviral vectors requires proper safety precautions such as use of lab coats and double layers of gloves. All handling of the virus must occur in the biosafety hood. All pipets should be decontaminated in a solution of 10% bleach for 30 minutes. All liquid infectious waste should be decontaminated with a final concentration of 10% bleach. Secondary containment, with a seal that prevents release of microbiological aerosols, is required for centrifugation

### **1. Producing the gammaretroviral stock**

1.1. Plate 293T cells for transfection. A full virus preparation requires 12 T75 flasks at  $4.5 \times 10^6$  cells/flask in Dulbecco's modified Eagle medium (DMEM) + 10% fetal bovine serum (FBS) without antibiotics.

1.2. Allow the cells to grow overnight at 37 °C before transfection.

1.3. Dilute 60 µL of transfection reagent in 1.5 mL reduced serum media. Prepare one tube for each flask. Incubate for 5 minutes at RT.

1.4. For each flask, prepare a tube containing 1.5 mL of reduced serum media and the following plasmids: 9.0 µg of a transfer plasmid containing the gene(s) of interest, 3 µg of RD114 and 1.2 µg of VSV-G (envelope plasmids), and 3 µg of Gag/pol.

**NOTE:** The envelope and gag/pol genes are needed for retroviral packaging. For our retroviral production, we add both the RD114 envelope and, for added stability, a small amount of VSV-G envelope.

1.5. Add diluted plasmids (step 1.4) to diluted transfection reagent (step 1.3). Mix gently. Incubate for 20 min at room temperature.

1.6. Feed cells 5 mL of fresh complete media and add 3 mL of transfection reagent/plasmid mixture dropwise per flask and incubate for 5–6 h at 37 °C.

1.7. Add an additional 5 mL of fresh medium to the flasks and incubate for 42–43 h at 37 °C.

1.8. After a total transfection time of 48 h, collect media and centrifuge at 1282 x *g* for 3 min at 4 °C to remove cell debris.

1.9. Aliquot into appropriate volumes for future use, flash freeze the virus in an ethanol/dry ice bath and store at -80 °C.

## **2. Titering the gammaretrovirus**

NOTE: Several dilutions of virus may be needed to obtain a valid titer.

2.1. Plate 293T cells at 600,000 cells/well in a 6-well plate and incubate the plates for 24 h at 37 °C.

2.2. Remove the medium from the cells and add desired amount of DMEM + 10% FBS to the 293T (2 mL total volume of virus).

2.3. Add the virus to the corresponding well and swirl gently to mix. For example, add 200 µL, 100 µL, 50 µL and 25 µL respectively to 4 wells. Include a well without virus for flow cytometry (mock sample).

2.4. Incubate for 48 h at 37 °C.

2.5. Trypsinize the 293T cells by adding 0.5 mL of Trypsin-EDTA and incubating for 4 min at 37 °C. Stop the trypsin by adding 1.5 mL of DMEM + 10% FBS. Count the cells and determine viability with an automated cell counter or a hemocytometer.

2.5.1. To use the cell counter, add 10 µL of cells to 10 µL of trypan blue, mix, load the chamber slide, and insert into the counter. Push the “capture” button to count the cells.

2.6. Collect  $0.5\text{--}1 \times 10^6$  cells and evaluate levels of CAR and CXCR5 by flow cytometry (see step 9.5). Expression of CD4 or MBL along with CXCR5 will identify transduced 293T cells co-expressing the CAR and CXCR5. See reactive antibodies in step 9.5.1.

2.7. Calculate viral titer.

NOTE: Choose the sample with a transduction level of 20% or less for calculation of titer.

2.7.1. Use the following formula to calculate titer:

transduction units/mL = (number of cells in culture)(% of cells transduced)/volume of virus added to the culture

**3. Determining the optimal MOI for a newly produced viral preparation and the cells of interest**

3.1. Follow the transduction protocol (sections 4–9) with primary PBMCs and twofold dilutions of the gammaretroviral preparation.

3.2. Assess the level of expression of the genes of interest by flow cytometry (step 9.5).

3.3. Choose a concentration of virus that allows maximal transduction without loss of cell viability.

NOTE: The following steps utilize a gammaretrovirus preparation to transduce primary rhesus PBMC.

**4. Preparing plates for T cell activation by coating wells with anti-CD3 antibodies**

4.1. Prepare anti-macaque CD3 antibody (FN18) by diluting the stock to 10 µg/mL in phosphate-buffered saline (PBS).

4.2. Dispense 2 mL/well in 6-well plates. Incubate at 37 °C for 2 h. Alternatively, the plates can be incubated overnight at 4 °C.

4.3. Aspirate the PBS and unbound antibodies. Rinse wells twice with 2 mL of PBS and use immediately to plate PBMCs.

**5. Stimulating rhesus PBMCs with plate-bound anti-CD3 and soluble anti-CD28**

5.1. Thaw primary rhesus PBMCs in a 37 °C waterbath, with gentle agitation, until just a small amount of ice remains.

5.2. In the hood, gently pipette cells into a conical tube. Rinse the vial with 1 mL of basic medium and add it slowly to the cells. Next, slowly add an additional 9 mL of warm basic medium to the cells.

NOTE: This step can be scaled up but never thaw more than 4 vials at one time.

5.3. Centrifuge at 600 x g for 5 min at 22 °C to pellet the cells. Aspirate the supernatant and then resuspend the pellet in a small amount of growth medium. Choose a volume such that the concentration of cells will exceed 2 x 10<sup>6</sup> cells/mL at this point since the final concentration should be 2 x 10<sup>6</sup> cells/mL.

5.4. Stain cells with trypan blue and count the cells to determine the number of viable cells.

NOTE: This can be done with a standard hemocytometer or an automated cell counter. We use an automated cell counter which displays the viability and number of live cells.

5.4.1. To use the counter, add 10  $\mu$ L of cells to 10  $\mu$ L trypan blue, mix, load the chamber slide and insert into the counter. Push the “capture” button to count the cells.

5.5. Calculate the total number of cells by multiplying cell concentration by volume and then dilute the cells to  $2 \times 10^6$  cells/mL in growth medium. Prior to plating, add anti-CD28 to a final concentration of 5  $\mu$ g/mL in order to provide a necessary co-stimulatory signal for T cell activation.

5.6. Pipette cells into anti-CD3 coated plates. Add  $3\text{--}6 \times 10^6$  cells per well (a good target is  $4 \times 10^6$  cells in 2 mL media per well) and incubate for 2 days at 37 °C in 5% CO<sub>2</sub>.

## 6. Preparing fibronectin-coated plates

NOTE: PBMC and T cells express integrin receptors VLA-4 or VLA-5 and are good targets for fibronectin-mediated transduction.

6.1. Prior to coating the plates, first prepare fibronectin reagent by diluting it 1:100 in sterile PBS.

6.2. Pipette 2 mL of the sterile fibronectin solution into each well of a 6-well plate. Gently rock plates for 2 h at room temperature.

NOTE: Non-treated, cell culture-grade tissue culture plates or dishes should be used in this step.

6.3. Remove the fibronectin solution by aspiration and then block with 1 mL of sterile 2% bovine serum albumin (BSA, Fraction V) in PBS. Rock the plates at room temperature for 30 min.

6.4. Aspirate the BSA solution and wash the plate with 2 mL of PBS. After aspiration of the PBS, the fibronectin-coated plates can be sealed with seal wrap and stored at 4 °C for up to a week.

NOTE: We usually prepare the plates one day before transduction.

## 7. Transduction of activated rhesus PBMCs

Caution: Work with viral vectors or with rhesus macaque PBMCs requires utilization of proper safety precautions such as use of lab coats and double layers of gloves. All handling of the virus and the cells must occur in the biosafety hood. All pipets should be decontaminated in a solution of 10% bleach for 30 min. All liquid infectious waste should be decontaminated with a final concentration of 10% bleach. Secondary containment with a seal that prevents release of microbiological aerosols, is required for centrifugation.

7.1. Attach the gammaretroviral transducing vector to the fibronectin-coated well.

7.1.1. Warm a centrifuge to 32 °C by running at 2000 x *g* for about 30 min.

264  
265 7.1.2. Warm both serum free and growth media in a 37 °C waterbath.

266  
267 7.1.3. Thaw the virus on ice or by gently swirling the vial in a 37 °C waterbath until only small  
268 amount of ice remains. To avoid degradation of the viral preparation, do not allow the contents  
269 to warm up.

270  
271 7.1.4. Dilute the retrovirus to a predetermined optimal multiplicity of infection (MOI) in serum-  
272 free medium.

273  
274 NOTE: With our gammaretrovirus and rhesus PBMC, we have found that an MOI of 0.5 is  
275 optimal.

276  
277 7.1.4.1. Example dilution: To coat 4 wells with virus and add  $1.5 \times 10^6$  cells,  $(4)(1.5 \times 10^6)(0.5)=3$   
278  $\times 10^6$  TU are needed. If the viral titer is 1.1 TU/mL, then use 2.7 mL virus in 5.3 mL media for a  
279 total volume of 8 mL.

280  
281 7.1.5. Add 2 mL diluted retrovirus to each well of the fibronectin-coated plate.

282  
283 7.1.6. For negative control mock-transduced cells, add media alone to the fibronectin-coated  
284 wells.

285  
286 7.1.7. Place the plates in the pre-warmed 32 °C centrifuge in microplate carriers with biosafe  
287 covers and centrifuge at  $2000 \times g$  for 2 h.

288  
289 NOTE: Virus coated plates can be used immediately or stored, with virus, at 4 °C overnight.

290  
291 7.1.8. For immediate use, aspirate the virus preparation from the wells and add 2 mL of growth  
292 medium. Keep the virus-coated wells from drying.

293  
294 7.2. Plate the stimulated PBMCs.

295  
296 7.2.1. Check the cells using an inverted microscope. The cells should look healthy, meaning that  
297 they are round, bright and refract light. They should also show a clumped appearance indicating  
298 stimulation.

299  
300 7.2.2. Collect the target cells and transfer them to a 50 mL conical tube. Rinse the well once with  
301 1 mL growth media and add to the conical tube. Count the number of living cells with the  
302 automated cell counter as in step 5.4.

303  
304 NOTE: Stimulated cells will be clumped and this clumping may lead to difficulties in accurately  
305 counting the cells.

306  
307 7.2.3. Pellet the cells in the tubes by centrifuging at  $600 \times g$  for 5 min at 32 °C. Aspirate the media

from the cell pellet and resuspend the cells in growth medium at a concentration of  $1.5 \times 10^6$  cells/mL.

7.2.4. To each virus-coated well (prepared in the step 7.1) add 1 mL of cell suspension for a total of  $1.5 \times 10^6$  cells/3 mL. Note that each well already contains 2 mL growth medium. Perform the same steps for mock-transduced cells but plate them onto fibronectin-coated wells which received no virus.

7.2.5. Centrifuge the plates at  $1000 \times g$  for 10 min at  $32^\circ\text{C}$ .

7.2.6. Incubate the plates for 48 h at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ .

## 8. Expansion of the transduced cells

8.1. Draw the contents of the wells up and down with a 5 mL pipet in order to resuspend the cells and then transfer to a 50 mL conical tube.

8.2. Add 1 mL of growth media to each well and draw up and down with a sterile transfer pipet to remove adherent cells.

8.3. Count cells to determine the total cell number and viability using an automated cell counter as in step 5.4.

8.4. If desired, remove  $1 \times 10^6$  cells for flow cytometry to assess gene expression and phenotype. See step 9.5.1 and the **Table of Materials** for recommended antibodies and gating strategy.

8.5. Centrifuge the cells at  $600 \times g$  for 10 min at  $25^\circ\text{C}$ .

8.6. Aspirate the media and resuspend the cells in expansion media to a concentration of  $1 \times 10^6$  cells/mL. Seed each gas-permeable well of a 6-well plate with 5 mL of cells. Carefully layer an additional 25 mL of expansion media per well.

NOTE: We add the cells in a small volume and then layer remaining media so that the cells remain on the bottom of the well.

8.7. Incubate the cells undisturbed at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  incubator for 4 days.

## 9. Collection of expanded cells and evaluating the phenotype of the transduced cell population

9.1. Collect cells from gas permeable wells by removing and discarding 20 mL media. Caution should be taken to avoid disturbing the cells.

NOTE: We expand the cells for only 4 days and collect the cells at this point. However, if additional days of culture are desired, remove 20 mL of media without disturbing the cells and replace it

with 20 mL of fresh expansion media.

9.2. Pipet the remaining media up and down to dislodge the cells. The media should be very cloudy if cells have grown well.

9.3. Using a sterile transfer pipet, rinse each well with 3 mL media to collect any residual cells.

9.4. Count the cells with the automated counter or a hemocytometer to check for viability and cell number.

9.5. Perform flow cytometry to determine transduced gene expression and cell phenotype.

9.5.1. In this example, determine rhesus macaque PBMC phenotype using antibodies directed against CD4 (M-T477), a clone that is reactive with both endogenous rhCD4 and the rhCD4-MBL CAR; against CD3 (SP34-2) and CD8 (RPA-T8) which stain total T cells and CD8 T cells respectively; against the death receptor CD95 (DX2) and the co-stimulatory molecule CD28 (CD28.2) which are used to determine the memory phenotype of the cells; and against CXCR5 (MU5UBEE) and MBL (3E7) to detect the CXCR5 and CD4-MBL CAR on the transduced cells.

9.5.2. Assess viability was assessed with a fixable viability dye.

9.5.3. Prepare cells for flow cytometry.

9.5.3.1. Make up an antibody master mix for the total number of tubes. Bring up to total volume using PBS.

9.5.3.2. Add the cells to flow tubes. Place  $0.5-1 \times 10^6$  cells per tube. Include negative controls such as unstained cells as well as stained mock transduced cells.

9.5.3.3. Add 2 mL of PBS to the cells, centrifuge at  $400 \times g$  for 5 min at room temperature (RT) and decant.

9.5.3.4. Add 100  $\mu$ L of antibody mix to the tubes and incubate for 30 min at RT.

9.5.3.5. Add 2 mL of PBS, centrifuge at  $400 \times g$  for 5 min at RT and decant.

9.5.3.6. Add 300  $\mu$ L of 1% paraformaldehyde and incubate for 15 min.

9.5.3.7. Centrifuge at  $400 \times g$  for 5 min at RT and decant. Add 300  $\mu$ L of PBS and mix.

9.5.4. On a flow cytometer, acquire 150,000 events for each sample.

9.5.5. Analyze the data with flow analysis software.

NOTE: The gating strategy was previously published<sup>20</sup>.

9.5.5.1. For identification of transduced cells, gate cells on lymphocytes, singlets, live, CD3+, and MBL+CXCR5+.

9.5.5.2. For identification of the central memory population, gate cells on lymphocytes, singlets, live CD3+, CD8+, CD28+ CD95+.

9.6. Use cells as needed. Cells can be used for *in vitro* assays of function, such as a transwell migration assay<sup>9</sup> or *in vitro* cell killing assays<sup>5,9</sup>. Cells can be used for infusion into animals. Alternatively, freeze any remaining cells in 90% FBS with 10% dimethyl sulfoxide (DMSO) for later use or analysis

NOTE: While a target dose for infusion of adoptively transferred cells into a rhesus macaque is not yet established, published adoptive transfer studies in non-human primates have used 0.6 to  $1.2 \times 10^7$  cells/kg<sup>21</sup>, 1 to  $5 \times 10^8$  cells/kg<sup>22</sup> and  $1.4$  to  $8 \times 10^8$  cells/kg<sup>10</sup>. With this range as a guideline, enough cells for infusion can be produced with this 9-day protocol.

## REPRESENTATIVE RESULTS:

### Cell production

Results presented in this publication are similar to those in our previously published work<sup>9,20</sup>. Utilizing the protocol presented here, we expect at least an 8-fold expansion of cells between days 5 and 9 (median 11.1 fold, range 8.7–13 fold). Gas permeable wells were seeded at a starting density of  $5 \times 10^6$  cells and after 4 days of growth, we achieved a median density of  $55.6 \times 10^6$  cells per well (range  $43.5$ – $64.8 \times 10^6$ ) (**Figure 1A**). Cell counting with Trypan Blue exclusion shows that the cells maintain high viability throughout the protocol (day 9 median 85.8%, range 83–95%) (**Figure 1B**). We find animal to animal variability in the ability of the cells to expand; however, with a starting population of  $50$ – $100 \times 10^6$  cells on day 1, we have used this protocol to produce enough cells for rhesus macaque infusion of  $1$ – $2 \times 10^8$  cells/kg.

Co-expression of the transduced genes was monitored with flow cytometry (**Figure 2A**). Expression of the CAR and CXCR5 on the surface of the transduced cells was relatively stable between days 5 and 9 of this expansion protocol. On day 5, a median of 42.8% (range 36.5–46.9%) of the cells were transduced with both CD4-MBL CAR and CXCR5 while on day 9, median expression was 47.6% (range 44.5–64.5%) with a single round of transduction (**Figure 2B**). Although some protocols call for a second round of transduction, we have not seen a benefit in terms of total transduced cells at the conclusion of the protocol (data not shown).

### Cell phenotype

Transduced cells were analyzed by flow cytometry to monitor their memory phenotype. Prior to transduction and expansion, naive, central memory and effector memory populations are identified (data not shown) but the naïve population is lost with culturing and the cells are primarily central memory cells by day 5 (**Figure 3A**) and day 9 (**Figure 3B**) as identified by CD95+

CD28+ expression. The use of this rapid transduction and expansion protocol has produced cells which are primarily central memory cells and thus will be more likely to proliferate and persist when infused into a test animal.

#### FIGURE LEGENDS:

**Figure 1: The transduction and expansion protocol produces abundant transduced cells which are highly viable.** (A) Expansion of the transduced cells from 6 different animals from days 5 to 9 in the gas permeable vessels and (B) viability of the transduced PBMC from the same 6 animals. Trypan blue exclusion using an automated cell counter was used to monitor cell number and viability.

**Figure 2: The transduction protocol produces cells which maintain co-expression of the two transduced genes.** (A) A representative flow plot of rhesus PBMC on day 9 of the transduction and expansion protocol demonstrating expression of both the CD4-MBL CAR and CXCR5. (B) Expression of the CD4-MBL CAR and CXCR5 on transduced rhesus PBMC from 6 different animals on days 5 and 9 in culture as measured by flow cytometry. Gates were set on live, CD3+ cells. Transduced cells are identified as MBL+ CXCR5+.

**Figure 3: The majority of the cells produced in the 9-day protocol have a central memory phenotype.** Representative flow cytometry plots of (A) day 5 and (B) day 9 CAR/CXCR5-transduced rhesus PBMC. Gates were set on live, CD3+, CD8+ cells. Central memory was defined as CD28+ CD95+. Effector memory was defined as CD28-, CD95+.

#### DISCUSSION:

This protocol outlines a T cell immunotherapy production strategy which utilizes a gammaretroviral vector to transduce rhesus macaque PBMC leading to T cell population that expresses an antiviral CAR as well as the follicular homing receptor, CXCR5. With a total of 8 days of ex vivo culture time, viable, functional CAR T cells are produced in a quantity that is within the published range<sup>10,21,22</sup> used for infusion into non-human primates for preclinical efficacy testing.

The success of the transduction protocol relies on both healthy, stimulated PBMCs and on quality preparations of gammaretrovirus. In order to achieve successful stimulation and transduction, it is essential that proper care is taken in freezing and transport of the PBMCs after collection. Ideally, if the cells are collected off site, they are shipped in liquid nitrogen and quickly placed in long term liquid nitrogen storage. PBMCs must be mitotically active in order to be successfully transduced by a gammaretrovirus. One should monitor visually for clustering of the cells after stimulation with anti-CD3/anti-CD28. A failure to activate properly will cause low efficiency of transduction. It is also important to monitor the viability in the stimulated cells. Poor viability after the stimulation step usually leads to a less successful transduction. Whether virus preparations are produced in the lab or outsourced, they should be stored at -80 °C in single use aliquots to avoid freeze thaw cycles that can damage the virus and impair transduction efficiency. The virus must be titered so that consistent amounts of virus can be used in each experiment. When using the virus for transduction, thaw the virus quickly and store on ice until needed. The choice of

promoter, enhancer and envelope proteins may all impact transduction efficiency and expression of transgene in target cells. Thus, a MOI must be empirically determined. In addition, the use of a media designed to support T cells and plates with gas permeable wells for expansion have led to excellent expansion of the transduced cells. However, there is animal to animal variability in the cell expansion rate. We recommend a trial study to determine the ability of a particular cell preparation to be transduced and expanded. Expansion levels are consistent in both small-and large-scale transductions.

With the use of this protocol, we have produced up to  $2.5 \times 10^9$  cells for infusion into test animals. Although we have found it unnecessary to scale-up the protocol at this time, the use of 6 well plates is a limitation to large-scale cell preparation and the protocol would require modification to produce greater numbers of cells. Examples of potential modifications include carrying out the transduction in a fibronectin-coated culture bag to increase the number of cells transduced and to utilize a larger gas permeable culture vessel for the expansion step. Although we have not yet made these alterations, they utilize commercially available products and are feasible modifications to the current protocol.

Using this method, we have produced transduced cells from PBMC isolated from uninfected animals, SIV-infected animals and from SIV-infected animals treated with antiretroviral therapy (ART). However, we have noted a reduction in transduction efficiency in the ART treated cells<sup>20</sup>. This reduction is presumably due to the inhibition of reverse transcriptase and/or integrase by the drugs. Transductions of cells from ART-treated animals will require modifications to this protocol such as reducing the intracellular levels of the ART drugs by stopping ART for several days prior to collecting the PBMC or through the use of an alternative vector which is not impacted by the ART drugs commonly in use.

It is important that cells produced for immunotherapy be of a minimally differentiated phenotype so that they will persist post-infusion<sup>12</sup>. Although many protocols for adoptive transfer of cells require long culture times, a reduced ex vivo culture time has been correlated with both a reduction in differentiation and an improvement in CAR T cell function<sup>13</sup>. The relatively rapid timeframe of this transduction and expansion protocol allows maintenance of the desired central memory phenotype while still producing sufficient cells for testing of their immunotherapeutic potential<sup>20</sup>.

The goal of the production strategy for this T cell immunotherapy product is to manufacture T cells that will recognize SIV-infected cells, will traffic to the site of viral replication in the B cell follicle and will persist in the animal resulting in a long-term functional cure without need for anti-retroviral drugs. For translation to a human immunotherapy product, this protocol can be modified to transduce human T cells through the use of human-specific antibodies and cytokines and the implementation of GMP standards with the ultimate goal of a producing a functional cure for HIV.

## **ACKNOWLEDGMENTS:**

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#### **DISCLOSURES:**

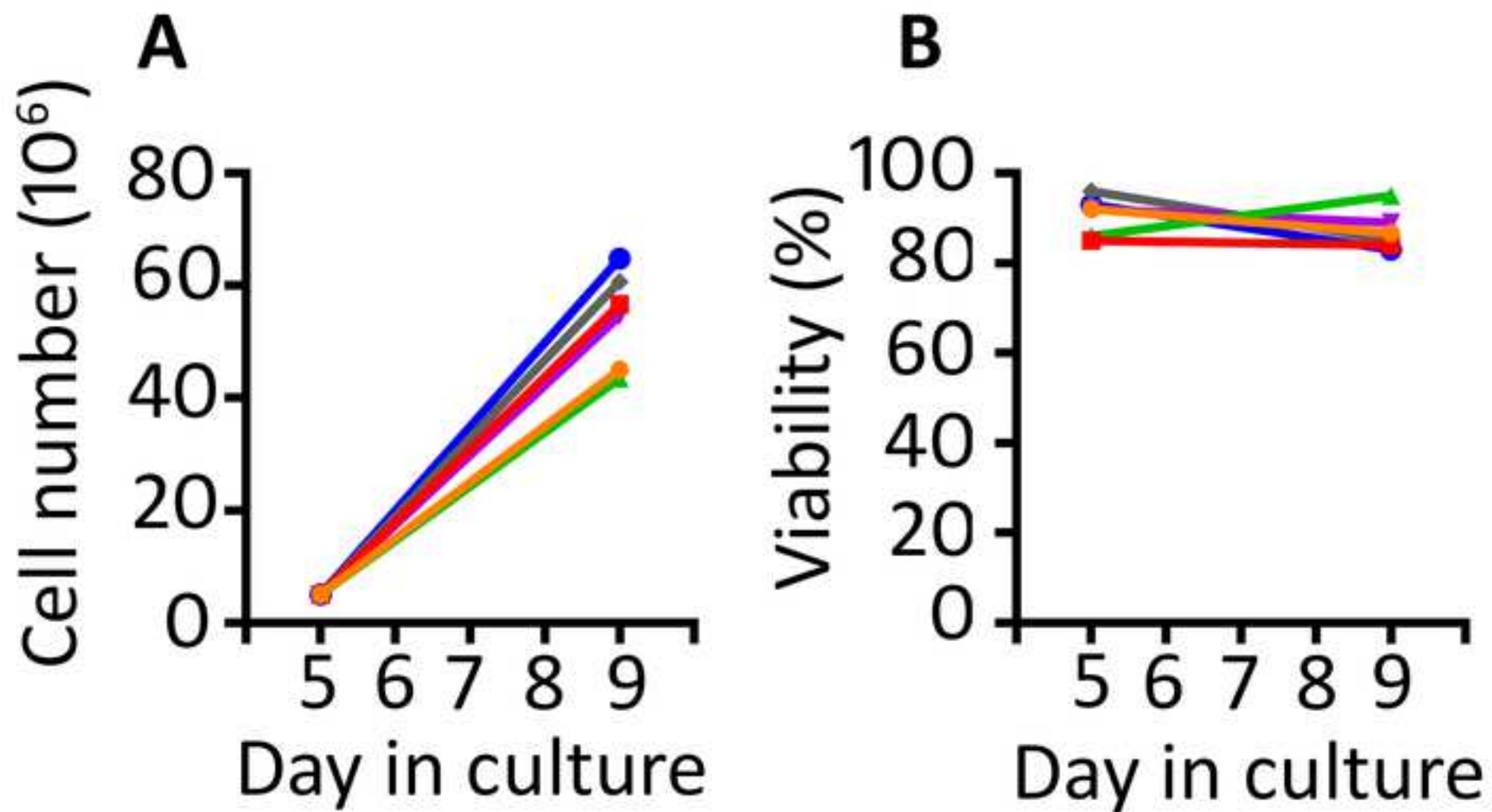
The authors have no conflicts of interest to declare.

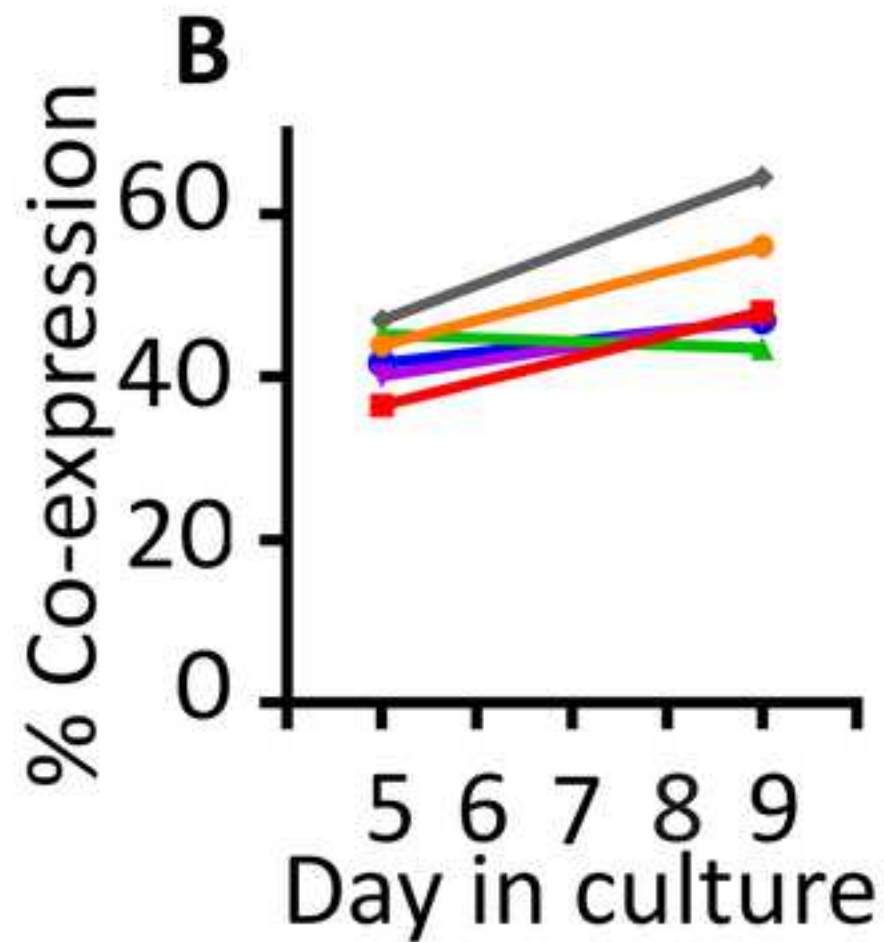
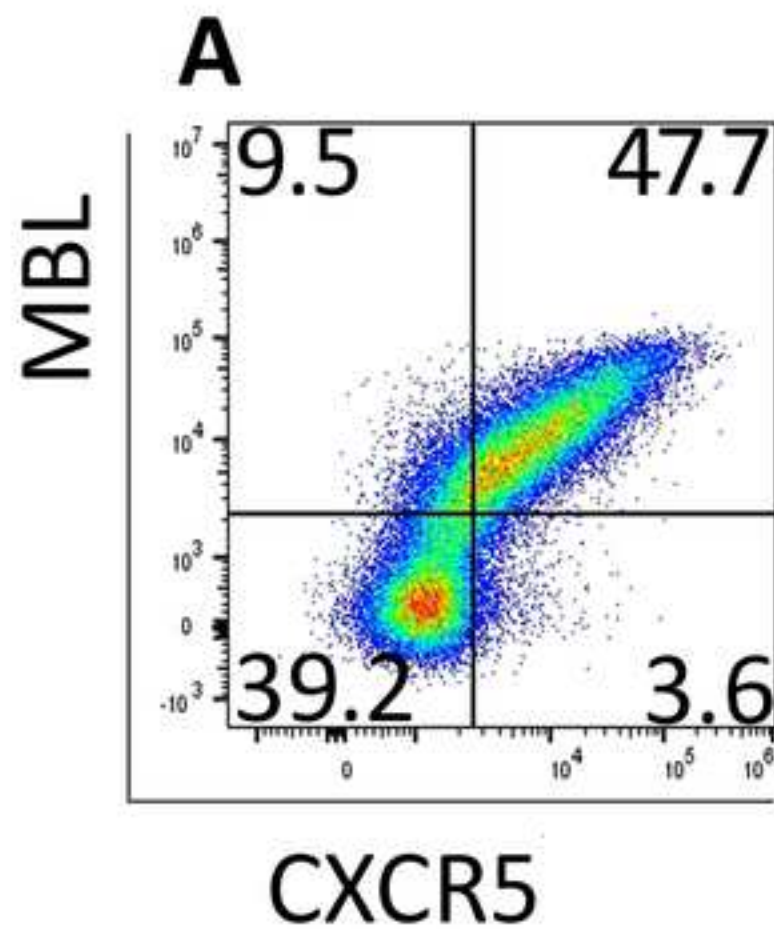
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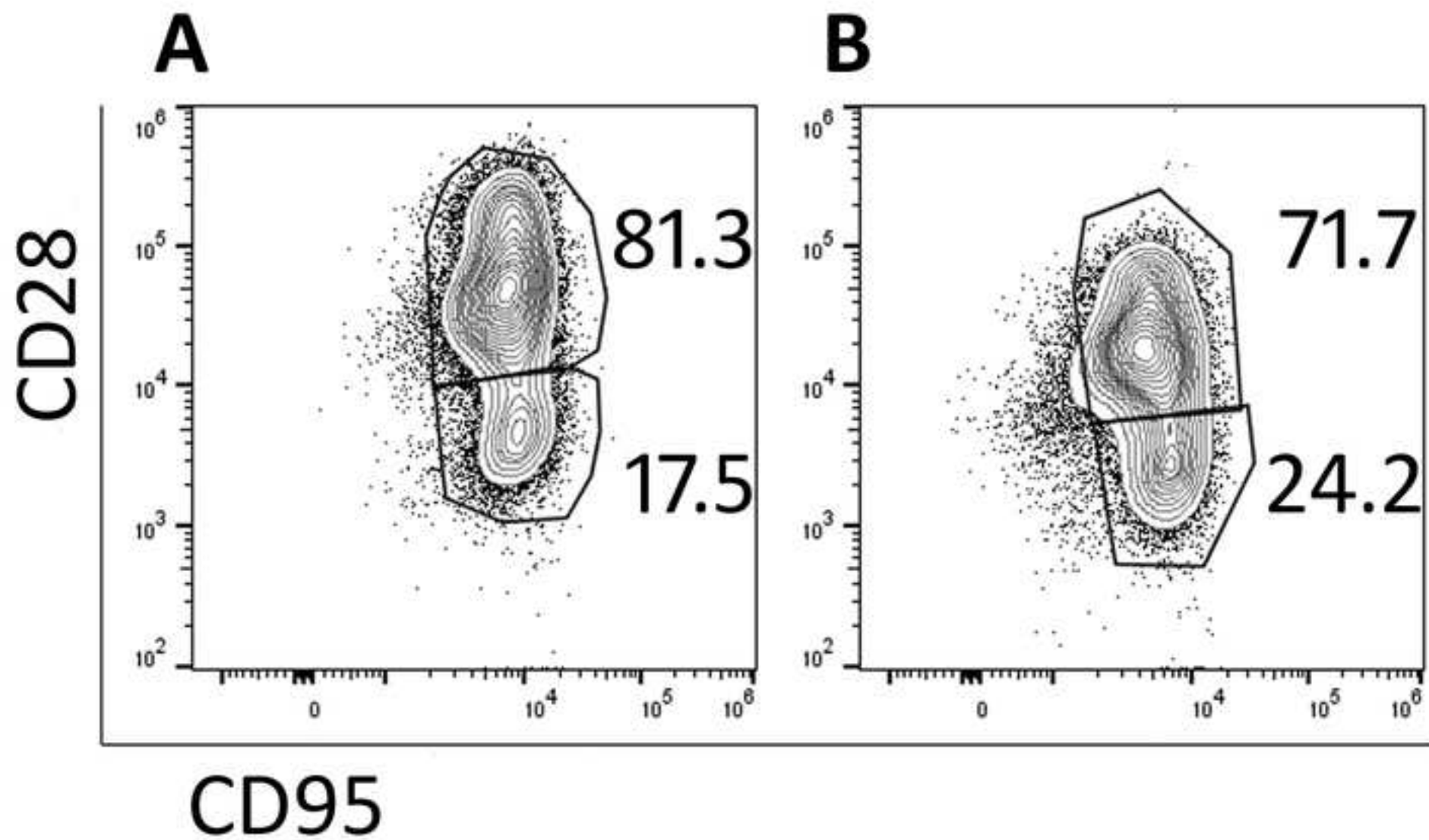
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| Name of Material/ Equipment           | Company                     | Catalog Number |
|---------------------------------------|-----------------------------|----------------|
| <b>Gammaretrovirus preparation</b>    |                             |                |
| 0.025% Trypsin, 0.01% EDTA            | Gibco                       | R-001-100      |
| 293T cells                            | ATCC                        | CRL-3216       |
| 6 well plates, treated                | CytoOne                     | CC7682-7506    |
| DMEM                                  | Gibco                       | 10569-010      |
| Heat-inactivated FBS                  | Hyclone                     | Sh30088.03     |
| Lipofectamine                         | Invitrogen                  | 11668019       |
| Opti-Mem                              | Invitrogen                  | 31985070       |
| pBS-CMV-gagpol                        | Addgene                     | 35614          |
| pMSGV1 containing CAR P2A CXCR5 RD114 | custom order from GenScript |                |
| T75 flasks                            | CytoOne                     | CC7682-4875    |
| VSV-G                                 |                             | pMD.G          |
| <b>T cell stimulation</b>             |                             |                |
| 6 well plates, untreated              | CytoOne                     | CC7672-7506    |
| Anti-CD28                             | NHP Reagent Resource        | Clone: CD28.2  |
| Anti-macaque CD3                      | NHP Reagent Resource        | Clone: FN18    |
| Phosphate buffered saline             | Gibco                       | 14190-144      |
| Rhesus macaque PBMC or CD8 T cells    | WNPRC                       |                |
| <b>For Fibronectin coating</b>        |                             |                |
| 6 well plates, untreated              | CytoOne                     | CC7672-7506    |
| BSA (Fraction V)                      | HyClone                     | SH 30574.02    |
| RetroNectin (1 µg/µl)                 | TaKaRa                      | T100A          |
| <b>For T cell Expansion</b>           |                             |                |
| G-Rex 6 Well Plate                    | Wilson Wolf                 | P/N 80240M     |
| <b>Media Components</b>               |                             |                |
| β mercaptoethanol                     | Gibco                       | 21985-023      |
| Heat-inactivated FBS                  | Hyclone                     | Sh30088.03     |
| IL-2                                  | NCI Preclinical Repository  |                |
| Penicillin/Streptomycin/Glutamine     | Gibco                       | 10378-016      |
| X-Vivo-15 medium                      | Lonza                       | 04-418Q        |

## Variations of Media used

Basic medium:

X-Vivo 15 medium, 10% heat-inactivated FBS, 1 x  
Penicillin/Streptomycin/L-Glutamine

Expansion medium:

Growth medium + 50  $\mu$ M  $\beta$  mercaptoethanol

Growth medium:

Basic medium + 50 IU/ml IL-2

## Cell counting

Countess cell counting chambers

Invitrogen

AMQAF1000

Countess II FL Automated Cell Counter

Invitrogen

T10282

Trypan blue, 0.4% solution

Invitrogen

T10282

## Flow Cytometry

Alexa Fluor 647 Antibody Labeling Kit

Invitrogen

A20186

anti-CD28-BV605

BD Biosciences

562976

anti-CD3-AF700

BD Biosciences

557917

anti-CD4-FITC

BD Biosciences

556615

anti-CD8-BV788

BD Biosciences

563824

anti-CD95-PerCP Cy5.5

BD Biosciences

561655

anti-CXCR5-PE

eBioscience

12-1985-42

anti-MBL

Invitrogen

MA1-40145-S6

Flow Analysis software

FlowJo, LLC

FlowJo v10

Flow Cytometer

Beckman

CytoFlex

Live/Dead Near IR

Invitrogen

L10119

## Other equipment

Aerosolve canisters to contain aerosol leakage

Beckman

SX4750

Beckman Allegra Centrifuge

Beckman

Sterilgard e3

Cell culture incubator

Thermo Fisher

Everlast 247

Class II Laminar flow hood

Baker

Heracell Vios 160i

Extra-Safe Disposable lab coat

Fisher Scientific

359232

Microplate carriers with biocertified covers

Beckman

SX4750A

Rocking platform

Benchmark

C10228

Swinging bucket rotor

Beckman

X13-R

X-Gen Nitrile gloves

Genesee

## Comments/Description

transfection reagent  
reduced serum media  
A gift from Dr. Patrick Salmon

A gift from Dr. Ed Berger

A gift from Dr. Scott McIvor

Primary cells

Plates with gas permeable wells

**Completion of media by addition of anti-CD28, IL-2 or  $\beta$ -mercaptoethanol should occur on the day of use.**

for conjugation of MBL antibody

**Safety equipment**

**Personal protective equipment**

**Safety equipment**

## Personal protective equipment

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September 18th, 2019

Dear Dr. Dsouza,

Thank you for considering our manuscript “Transduction and expansion of primary T cells in 9 days with maintenance of central memory phenotype” for publication in JoVE (manuscript JoVE60400). We appreciate the comments and suggestions provided by the editor and the reviewers. We are providing a revised manuscript incorporating the suggestions. Below is our point by point responses to the suggestions and concerns raised by the editors and reviewers.

### **Editorial comments:**

**1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.**

We have gone over the manuscript to correct any grammatical or spelling errors

**2. Abstracts: Please remove the citations from the abstract.**

The citations have been removed from the abstract.

**3. Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

We added more detail to the highlighted steps. For example, we originally said “Stain cells with trypan blue and count cells to determine the number of viable cells. This can be done with a standard hemocytometer or an automated cell counter such as the Countess II cell counter.” We now say “Stain cells with trypan blue and count the cells to determine the number of viable cells. This can be done with a standard hemocytometer or an automated cell counter. We use an automated cell counter which displays the viability and number of live cells. To use the counter, add 10 µl of cells to 10 µl trypan blue, mix, load the chamber slide and insert into the counter. Push the “capture” button to count the cells.”

**4. 5.4: Mention gating strategy or cite a reference for flow cytometry. If this is described in a later step, please reference the step number.**

The gating strategy is now described and cited.

**5. 5. 8: please cite a reference for this.**

We have not yet published our studies of cells infused into primates using these methods. So, we have revised the text to cite quantities of cells that others have used in primates and indicate that the described protocol produces cell quantities within this range.

**6. Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.**

- 1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.**
- 2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.**
- 3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.**
- 4) Notes cannot be filmed and should be excluded from highlighting.**

We highlighted the relevant steps and substeps and provide a cohesive narrative from one step to the next.

**7. Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.**

We covered each of the topics indicated in the revised discussion.

**8. References: Please spell out journal names.**

We corrected the reference style and spelled out the journal names.

**9. Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Countess II, RetroNectin, GREX, Live/Dead Fixable Near IR Cell Stain Kit (Invitrogen), FlowJo v10 (FlowJo, LLC), etc**  
**1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table**

**of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.**

We removed the commercial language.

**10. Please define all abbreviations at first use.**

The abbreviations have been defined on first use.

**11. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."**

The figures have not been previously published. However, the data presented in the figures are similar to what we have previously described and we cite these publications at the beginning of the results section.

**Reviewers' comments:**

**Reviewer #1:**

**Major Concerns:**

**1. The preparation of the critical reagent, the retrovirus, was not described or the titration of this reagent. An MOI of 0.5 was advised, but the desired final transduction efficiency depends on that. The procedure for flow cytometry analysis of T cells and the transduced protein is very minimalistic and are crucial to the procedures. Should be added.**

Thank you for pointing out these needed areas of clarification. We have added sections on production of gammaretrovirus vectors, titring of the viral vector preparations and the determination of MOI. The MOI of 0.5 is based on the titer of the viral vector determined by transduction of 293 T cells and empirical findings with transduction of primary PBMCs. We also added a protocol for flow cytometry.

**Minor Concerns:**

**2. In the analysis of T cell subsets, subpopulations are mentioned without defining them by a marker profile. That needs to be added.**

We now describe the T cell subsets and the antibody markers used to define them. In the example we provided in the text, transduced rhesus macaque PBMC phenotypes were determined by using antibodies directed against CD4 (M-T477). This antibody clone is reactive

with both endogenous rhCD4 and the rhCD4-MBL CAR. Antibodies directed against CD3 (SP34-2) and CD8 (RPA-T8) stain total T cells and CD8 T cell subsets, respectively. Antibodies directed against the death receptor CD95 (DX2) and co-stimulatory molecule CD28 (CD28.2) are used to determine the memory phenotype of the cells. Finally, antibodies directed against CXCR5 (MU5UBEE), and MBL (3E7) are used to detect the CXCR5 and the CD4-MBL CAR on transduced cells.

**Reviewer #2:**

**Major Concerns:**

**1. It is unclear why phenotyping reagents CD4, CD8, CD3, and CCR7 are listed in 6.5.1 but are not utilized in the example figures.**

Thank you for pointing this out. We have corrected the list of phenotyping reagents to match what is presented in the figures. We removed CCR7 from the list since it is not used in the data described. We have also, as indicated above, clarified what molecules on T cells each phenotyping antibody binds and why we included them.

Sincerely,

A handwritten signature in black ink, appearing to read 'Pam J. Skinner', with a stylized, cursive script.

Pamela J. Skinner, PhD