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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, titering 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,

Pamela J. Skinner, PhD