First, we would like to thank the editor and reviewers for their comments and suggestions. Below are the responses to the comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

- Manuscript has been proofread by authors.

2. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please **reduce the number of instances of "EasySep**" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

- Lines changed: 419, 211, 419, 498

Similarly, please remove manuscript references to **Ficoll Paque, RPMI 1640 with Glutamax, RoboSep, etc**. Please cite these materials in the Materials Table instead.

- Corrections were made (Lines 178, 157, 189, 200, 208, 217, 266, 286, 438, 439, 442, 445, 453.)

3. Please abbreviate all journal titles.

- Corrections were made

4. Formatting:
-5.4 - phenazine methosulfate is not DMSO
-6.4 – dimethyl sulfoxide is DMSO; step 6.4 should appear before 6.3

- Corrections were made.

5. Grammar:
-Manuscript would benefit from copyediting for typographical and grammatical errors.
-Short & long abstracts – Please use complete sentences and keep the verb tense consistent (i.e. present tense).

- Corrections were made.

-Line 470 – Please rephrase “somehow in accordance.” This sounds as though you expected different results from published literature.

- Corrections were made.

6. Branding should be removed:
-Long abstract, 3.5, Discussion (2X) – EasySep
-2.1 – Glutamax
-3.1 – RoboSep

- Corrections were made .

Reviewers' comments:

Editor's Note: Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Editor's Note: We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

Reviewer #1:
Manuscript Summary:
Two techniques are described in the manuscript to generate in vitro mDCs, plastic adherence and magnetic sorting. The methods are clearly and comprehensively written in superb detail. Based on the data, plastic adherence appears to be an inferior method for producing mDCs for in vitro functional studies.

Major Concerns:
Publications already exist that compare these 2 methods for generating in vitro mDCs. A more interesting study would be to determine if these two techniques lead to different subtypes of mDCs.

- Even though publications exist comparing two methods for generating in vitro mDCs, the magnetic separation used in our study is from a different manufacture and it is a negative selection technique. In addition, for the first time, we analyzed how these two techniques lead to different subtypes of MDDCs as we looked at characterization of CD11c/CD14 MDDC populations by imaging flow cytometry.

Minor Concerns:
Several instances of grammatical errors, and misspellings.

- We went over the manuscript and corrected several grammatical errors and misspellings.

Reviewer #2:
Manuscript Summary:
First studies about that topic were published back in 2001 showing that the viability of DCs produced either by monocyte adherence vs. negatively enriched PBMCs were similar. However, this study shows new techniques for comparison of both production procedures. Additionally it was found that monocyte isolation through magnetic separation yielded in the greatest percentage of highly viable MDDCs with greater functionality. Thus the results of the study may be of scientific interest towards evidence of large scale generation and functionality of DCs intended for DC vaccination in immunotherapy.

Major Concerns:
Major concerns of this study are the lack of exactly described randomization, the production procedures prior to preparation, the statistically exact presentation of results (here: mainly presented in tables), the lack of data on residual cell of both methods and the presentation of cost analysis which may be of interest for the reader in that context.

*Introduction

The Introduction is too long and should be shortened by half. The usage of DCs is well known since many years. The functional aspects and new testing methods of DCs may be of greater interest for the reader (starting from lines 102-129, should be extended) and aspects of costs may also be of interest due to large scale generation of DCs for vaccination for treatment.

- Introduction was shortened.

*Methods

A short description should be given for the presented protocols, which allows the reader to understand the different preparation steps itself prior to monocyte adherence or negative selection magnetic procedure. The protocols itself may be added at the end of the manuscript.

- The preparation prior to monocyte adherence or negative selection magnetic procedure is presented by the "Isolation of PBMCs by standard density gradient technique" (Line 156), the detailed protocol is explained from part 1.1 to part 1.8 (Lines 158-177).

Major concerns:
Does an ethic vote for blood donors exist (ethic vote number)? Or is it the IRB protocol approval number #150 IRB-13-0440?

- Our lab does not have ethic vote for blood donors since leukopaks were commercially obtained from the community blood bank; however, the lab has an existing IRB approved protocol that includes the use of commercially available leukopaks, IRB approval #IRB-13-0440 only. (Lines 152-154).

The description of the production process of monocytes (e.g. leukapheresis, devices, FACS analysis) is missing. As we know there may be differences preparing cells derived from different leukapheresis products depending on residual cells in the product. Validation of methods as it is one of the interesting topics of that manuscript needs a prospectively defined validation procedure; there is no description about it. How many samples were analyzed and compared? Which blood donors donated their blood for the different subsets compared? Were blood samples be randomized for the different preparation procedures? What was the exact procedure in that study to ascertain comparability of results? The methods itself are well established and not new (see line 402).

- The validation procedure for MDDC generation from monocytes is demonstrated by the generation of DCs when incubated for 7 days with GM-CSF and IL-4, this in turn is

confirmed with the phenotypic characterization analysis of cell surface markers such as CD14 and CD11c.

- The amount of samples analyzed and compared is well established and specified under their respective experiment.
- Human leukopaks purchased from a community blood bank in Miami, FL were used for all the experiments. Each experiment (n) corresponds to one leukopack and was used to perform both isolation techniques and subsequent analysis. Sample size (n) is specified in the figure legends and at the bottom of each results' section.
- Although the methods for isolation of MDDCs are well established and not new, this study uses additional comparability techniques to statistically show which method of isolation is best suitable for DCs without compromising its viability, cytotoxicity, phenotype, and functionality.

*Results

Major concerns:

Data due to the comparison of two methods are only shown in figures which appears unprecise to the reader. No data is presented about cell yields in different cohorts or as statistical analysis of functional tests (e. g. in tables as median or mean values) which are of high interest as discussed later on. Thus, the exact statistical analysis should be added to the manuscript. Concerning the MTT assay p-values should be added in every data comparison including the test system for analysis (normally or non-normally distributed data).

- Exact statistical data is presented by each experiment under "Representative Results" In this study, we compared which method of isolation is best suitable for MDDCs by the use of other well-established techniques to further compared mDCs' viability, cytotoxicity, phenotype and functionality, representing these results as statistical analysis.
- In this study, MTT assay was used as an independent experiment to test cytotoxicity of MDDCs when comparing both methods of isolation, p-values were addressed under "Representative Results" (Lines 314-332).

Fig. 3: What means absorbance? A short explanation of this method is needed to understand the results. An analysis of residual cells is missing due to the argumentation that the costly negative selection has advantages over the adherence method (line 407).

- In this graph, absorbance represents the light wavelength (590nm) where the reduced purple formazan form of MTT was read by the spectrophotometer. The following statement was added in between lines 316-318: "MTT was used as a colorimetric assay to measure cell proliferation. In living cells, the yellow tetrazole color of MTT is reduced to purple formazan, which is easily measured using a spectrophotometer."

Thus, a cost analysis should be added to compare between both methods due to the discussion (line 405). The magnetic separation is known to be very costly due to large scale generation of DCs for therapeutic usage. Does the advantage of preparation weight out the higher costs of the magnetic isolation procedure? Finally, it might be of interest how to improve especially the low cost adherence method concerning the different preparation steps.

- The cost analysis of both methods is briefly mentioned in the discussion portion of this study since their differences are already well known established. Monocyte magnetic separation technique is more costly than monocyte isolation through adherence, but with many commercially available companies as options for purchasing, it will be difficult to exactly point out an accurate cost analysis comparison for both methods. Our study emphasizes directly on the outcome of MDDCs viability, cytotoxicity, phenotype and functionality by comparing both methods of isolation.

Reviewer #3:

Manuscript Summary:

This manuscript by Figueroa et al., presents two methods for isolating monocytes from human peripheral blood for subsequent differentiation into MDDCs, and compares the efficacy of each of these methods side-by-side. Although I do think the subject of this manuscript could provide a VERY useful JOVE video, to aid in teaching this important technique to other researchers, there are a significant amount of issues regarding explanation of methods, conclusions, and professional writing style. I do think this project could make an excellent resource for those learning how to make MDDCs, but significant improvements must be made to all components of this manuscript to be acceptable for publication.

Major Concerns:

1.) The most important function of DCs, i.e. T-cell priming ability, is not addressed in this paper. This could be easily accomplished with an MLR using CSFE labeled T-cells from a different donor blood sample. It would be a very interesting study and would complete the spectrum of DC biology in comparing the two different protocols for generating MDDCs. This is important as most studies of DCs are focused this specific critical function. This last experiment would provide a full and thorough look at the difference between adherence and negative magnetic sorting monocytes for MDDC generation and result in the manuscript being a much more useful resource for all DC biologists.

- We agree with the reviewer and plan to perform those studies in the future. In the current manuscript instead of testing the antigen presenting functionality of DCs, we opted to test the direct effects of both methods of isolation on surface markers CD11c and CD14.

Another issue with the manuscript is the lack of phenotyping of the MDDCs that arise from adhesion versus magnetic negative selection of PB monocytes. I noticed that the original article DID include this; why was this removed? I think the demonstration that similar populations arise (although the magnetic negative section seemed to result in more mature DCs, higher CD80/86 and CD83), is critical for interpretation of the results. Variation in different DC population could affect, for example, the phagocytic ability of the total MDDC population. Also, what point in the culture were these markers looked at? It would be useful to look at phenotypic similarity of cells isolated by adhesion versus negative sorting right away, and after the GM/IL4 differentiation.

- The markers were looked at after the monocytes differentiate into dendritic cells hence in a period of 5 to 7 days after culture. It is also a great idea to look at the markers for monocytes right after isolation to look at different sub-populations that are isolated with two different methods however our interest lies mainly on the dendritic cells and therefore we waited for them to differentiate to dendritic cells before carrying out

phenotyping with flow cytometry. In addition, after revising the manuscript we focused mainly on two differentiation markers CD14 and CD11c.

3.) The magnetic separation resulted in 23% monocytes from total PBMCs, but in the discussion it is stated that normal whole blood only contains about 2-10% monocytes (line number 423). How do you reconcile this? It would be helpful to have a simple flow cytometry panel for potential contaminating cells, and show how pure your monocyte populations are from the adherence method compared to the magnetic sorting method. It is possible you have some contaminating cells with the negative magnetic sorting, resulting in this high percentage. This is very important to determine and account for, because one of your main conclusions is that you get more monocytes via negative magnetic sorting…but how pure is this population? It would also be useful to provide information about the starting amount of monocytes in a blood sample, and how much of that you can collect with the different methods. This could be an easy flow cytometry right away on the fresh blood, followed by adhesion or negative magnetic selection, and see how much you can collect compared to the original calculated number. It would also be useful to have the information about what percentages of monocytes are in human blood earlier in the manuscript. You mention the rarity of DCs, but if you want to make the point about using monocytes instead, I'd recommend mentioning the information on line 423 earlier in the manuscript.

- These high percentage obtained may be due to impure populations of monocytes from both processes.
- Although monocyte yield and viability by both techniques are already provided and showed in figures 1 and 2; we did not perform flow cytometry panel for the PBMCs or the monocytes since our main focus and cells of interest are the dendritic cells.

4.) In the protocol, it would be helpful to have more information about why certain reagents are needed (i.e. the ACK buffer is to lyse the red blood cells). Also, please explain MTT and XTT (i.e. the information on Line 467 should be earlier - and more information should be provided - see below in minor comments for more information).

- Additional information was added. For simplicity and since both methods measure the same outcome (proliferation/toxicity), we focused the manuscript on MTT assay and only figure 3 was included.

5.) Why is a flow cytometer not used for assessment of FITC-Dextran uptake. An issue with a microplate reader, is you do not know if a limited few cells were very phagocytic, or if the general population of MDDCs were equally phagocytic. Without knowing what populations of MDDCs arise (and if they are phenotypically equivalent between adhesion and magnetic sorting methods), it is difficult to say that the magnetic sorted MDDCs have more phagocytic ability. They may have instead, differentiated a population of DCs with high antigen uptake ability, but not make up the majority of the DCs in the culture…

- We agree with the reviewer; however, when we tried internalization of FITC dextran particles by flow cytometry, we did not observed major differences; therefore, we need to optimize our protocol and we will performed these experiments in future studies. This figure was removed.
- Flow cytometry was performed to analyze surface marker expression (CD11c and CD14) and cell viability (DAPI).

Minor Concerns:
1.) Issues with writing, including grammar, unclear sentences, fragments, and use of 'casual' words. Some examples include:

In Abstract:
-Line 68 - Followed by differentiation into DCs with IL-4 and GM-CSF. (fragment of a sentence - likely needs to be added to the following sentence)

- Corrections were made.

In introduction:
-Line 82: use of the term "genetic" reprograming. DCs, upon maturation, drastically change their transcriptional profile, but are not "genetically" reprogramed - do you mean epigenetic changes perhaps? I believe the use of the phrase "genetic reprogramming" is not scientifically accurate.

- This statement was removed.

-The sentence on lines 92-94 is poorly written/unclear

- Corrections were made.

-Use of "a lot of" in line 137 (and also on line 474)

- Corrections were made.

-Line 142, immunology not needed to be caps.

- Corrections were made

In protocol:
-Part 2.1 (line 176), how much Glutamax? Please provide conc. or percent of total volume.

- Correction was made, line 181.

-For determination of cell viability - please explain harvesting of cells. Do you need to use trypsin so they no longer adhere, or it this not necessary? Section title for cell yield and viability changed, added a sentence about harvesting

-On line 226-7, what exactly do you mean by diluted cell pellet? Please be more specific.

- New line 231-233) Section 4.1

-Part 4.2 - might be useful to provide an option for those without a cell count (i.e. use of hemocytometer and manual counter)

- Added a line about hemocytometer and manual counter, section 4.3.

-Title of part 4, 5, and 6. I would recommend staying consistent. In part 4, you title the section from what the assay determines, whereas in parts 5 and 6, you just provide the acronym for the assay. Maybe all sections should be for example: Determination of cell yield and viability (Trypan Blue exclusion)
-Please define MTT assay and XTT assay, and at some point in the protocol (earlier rather than later), explain what the acronym stands for and in simple terms, how does the assay work? What are you directly assessing? This is particularly important as you seem to have two assays to address the same parameter (the MTT assay and the XTT assay) - it would be useful to understand what information each assay is providing and how.

- This was addressed in Part 4 of Major concerns by Reviewer #3 (see above) For simplicity and since both methods measure the same outcome (proliferation/toxicity), we focused the manuscript on MTT assay and only figure 3 was included.

-Additionally, figure 2 and 3 should not be labeled at OD or absorbance @ 540nm. What does that mean? What does the optical density represent? What does the absorbance @540 represent? Can you turn that value into cell number, viability, etc?

- Absorbance represents proliferation.

-Part 5.1, you mention concentration of cells/well, but in what volume? Concentration is usually parts per volume, not parts per aliquot (i.e. the well).

- Changed

-Line 243, adding…DMSO to 1 g of ….SDS

- Changed

-Part 5.5 another 2 additional hours (redundant use of words)

- Changed

-Please explain your calculation more in part 6.6. Explain what each of those values represent and what the final value thus tells you.

- XTT figure has been removed since it measures same parameter as MTT figure 3.

-Part 7.2 - might be useful to specify earlier that you will need two separate 6-well plates, as one will have to be incubating at 37C and the other at 4C (you say in Part 7.1 - incubate the desired cells in a 6-well plate)

- Based on reviewer recommendations to properly perform this measurement using a different method, we decided to remove this figure and focus the manuscript on the characterization rather than the endocytic capacity of the cells.

-Part 7.4 - again, what volume? Concentration of cells/mL

- Changed

-For both the MTT and the XTT assay, the phrase "increase in viability" is used. In truth, I'm not sure you can increase viability, but rather result in cell proliferation, of which the population may contain more viable cells than the seeded population. Do you mean proliferation? This is correctly stated in line 379, as the title for Figure 3 (and subsequently for the Figure 4 title as well) - this should be fixed in the main manuscript text then.

- Corrected to address proliferation

In discussion:
-Line 407, please explain/define monocyte transient manipulation and protein secretion. Protein secretion of what? Cytokines?

- Corrected

-Line 427 - in THE case of isolation…

- Corrected

-Please add commas in sentence from line 428 through 431, or split into two sentences

- Comma added

-Line 438 - unwanted depletion? More like reduction of viable PBMCs due to the lysis buffer I presume?

- Corrected

-Line 439 - On the other side = however?

- Corrected

-Line 452-455: There is also important information mentioned in the discussion, which may be useful to have earlier, in the actual protocol. One such item includes "it is also crucial to save the floating MDDCs and return them back into culture along with fresh media and cytokines…."

- Added to lines 223-224.

-Line 464 - be careful about use of the word "purity", as currently in the manuscript you have not addressed the purity of any of your cell populations.

- Characterization has been added and term purity corrected appropriately throughout.

-Line 470-474 is very long and unclear.

- This was edited

2.) In lines 126-129, you mention that other studies have demonstrated better DC purity and viability using MACS separated monocytes compared to the adherence method. How do your results differ from this study?

- After carefully revising the manuscript, repeating the characterization experiments, and focusing on the surface expression of CD11c and CD14 only. Our results differ from that previous study since both techniques are yielding similar populations of CD14/CD11c MDDCs. There are minor differences, but are not significant. This is explain in great detail in the introduction (lines 128-141). Below is the explanation:

  "The current study presents a comparison between two methods for the generation of human DCs from monocytes isolated from PBMCs including 1) monocyte isolation by adherence and 2) monocyte isolation by negative selection using the EasySep human monocyte enrichment kit. This study provides evidence to support that the negative selection magnetic separation procedure to isolate monocytes generates the highest yield of monocytes with no significant differences in monocyte viability when compared with monocytes isolated by adherence method. In turn, after seven days, the monocytes isolated by magnetic separation differentiated into MDDCs with significantly higher proliferative capacity and higher amount of cells expressing double positive (CD11c+/CD14+) phenotype without affecting MDDC viability. Overall, the current study differs from the previous studies reference above since it demonstrates the ability of both techniques to simultaneously generate monocytes that are capable of proliferating and differentiating into CD11c+ MDDCs (> 70%) after seven days in culture without compromising their viability. In addition, the current approach provides for the first time characterization of different CD11c/CD14 MDDCs populations by imaging flow cytometry."