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Optimizing the in vitro generation of human monocyte-derived dendritic cells: a comparison between monocyte adherence versus magnetic separation.

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Abstract:	Dendritic Cells (DCs) are antigen presenting cells of the immune system that play a crucial role in lymphocyte responses, host defense mechanisms, and pathogenesis of inflammation. Isolation and study of DCs have been important in biological research because of their distinctive features. Although they are essential key mediators of the immune system, DCs are very rare in blood, accounting for approximately 0.1 - 1% of total blood mononuclear cells. Therefore, alternatives for isolation methods rely on the differentiation of DCs from monocytes isolated from peripheral blood mononuclear cells (PBMCs). The utilization of proper isolation techniques that combine simplicity, affordability, high purity, and high yield of cells is imperative to consider. In the current study, two distinct methods for the generation of DCs will be compared. Monocytes were selected by adherence or negatively enriched using magnetic separation procedure followed by differentiation into DCs with IL-4 and GM-CSF. Monocyte and MDDC viability, proliferation, and phenotype were assessed using viability dyes, MTT assay, and CD11c/ CD14 surface marker analysis by imaging flow cytometry. Although the magnetic separation method yielded a significant higher percentage of monocytes with higher proliferative capacity when compared to the adhesion method. Overall, the findings have demonstrated the ability of both techniques to simultaneously generate monocytes that are capable of proliferating and differentiating into viable CD11c+ MDDCs after seven days in culture. Both methods yielded > 70% CD11c+ MDDCs.

	Therefore, our results provide insights that contribute to the development of reliable methods for isolation and characterization of human DCs.
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TITLE:
Optimizing the *in vitro* generation of human monocyte-derived dendritic cells: a comparison between monocyte adherence versus magnetic separation.

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Human, monocyte-derived dendritic cells, monocytes, isolation techniques, immune system, magnetic separation.

SHORT ABSTRACT:
This study compares two different methods of human monocyte isolation for obtaining *in vitro* dendritic cells (DCs). Monocytes will be selected by adherence or negatively enriched by magnetic separation. Monocytes will be differentiated into monocyte-derived dendritic cells (MDDCs) with IL-4 and GM-CSF. Monocyte yield and viability along with MDDC viability, proliferation and CD11c/CD14 surface marker expression will be compared between both methods.

LONG ABSTRACT:
Dendritic cells (DCs) are antigen presenting cells of the immune system that play a crucial role in lymphocyte responses, host defense mechanisms, and pathogenesis of inflammation. Isolation and study of DCs have been important in biological research because of their distinctive features. Although they are essential key mediators of the immune system, DCs are very rare in blood, accounting for approximately 0.1 – 1% of total blood mononuclear cells. Therefore, alternatives for isolation methods rely on the differentiation of DCs from monocytes isolated from peripheral blood mononuclear cells (PBMCs). The utilization of proper isolation techniques that combine simplicity, affordability, high purity, and high yield of cells is imperative to consider. In the current study, two distinct methods for the generation of DCs will be compared. Monocytes were selected by adherence or negatively enriched using magnetic separation procedure followed by differentiation into DCs with IL-4 and GM-CSF. Monocyte and MDDC viability, proliferation, and phenotype were assessed using viability dyes, MTT assay, and CD11c/ CD14 surface marker analysis by imaging flow cytometry. Although the magnetic separation method yielded a significant higher percentage of monocytes with higher proliferative capacity when compared to the adhesion method. Overall, the findings have demonstrated the ability of both techniques to simultaneously generate monocytes that are capable of proliferating and differentiating into viable CD11c+ MDDCs after seven days in culture. Both methods yielded > 70% CD11c+ MDDCs. Therefore, our results provide insights that contribute to the development of reliable methods for isolation and characterization of human DCs.

INTRODUCTION:
Dendritic cells (DCs) are essential mediators of the innate and adaptive immune systems. They function to induce primary immune responses and facilitate the development of immunological memory. These cells are primarily responsible for antigen capture, migration and T cell stimulation and are therefore referred to as professional antigen presenting cells (APCs) [1]. Manipulation of DCs could be utilized across a wide variety of research fields and in the clinical setting to treat different inflammatory diseases such as HIV [6,7] , cancer [8], autoimmune diseases [9], and allergic responses [10]. DCs are also being used for substance abuse research in order to solve

unknown mechanisms and pathways such as those associated with alcohol dependence [11-14], drug dependence [13,15], and the combination of HIV infection and substance abuse [16-19]. These ongoing studies and future research studies in the field of immunology make *in vitro* generation of DCs extremely important for research. However, there are several difficulties associated with isolating DCs from human blood as they only constitute 0.1 – 1% of total blood mononuclear cells [20].

To date, some of the well-established methods for the generation of DCs *in vitro* consists of plastic or glass adherence of monocytes [21,22], density gradient centrifugation [23], specific marker based separation such as magnetic activated cell sorting[22], fluorescent activated cell sorting [24], positive selection of CD14+ monocytes using dextran-coated magnetic nanoparticles[25], and rapid isolation of highly purified monocytes using fully automated negative cell selection[26]. However, the best method of choice remains controversial. Therefore, to improve DC generation techniques, several methods have been developed in which the purity of these cells can be greatly increased by differentiation from purified CD34+ progenitor cells and monocytes isolated from peripheral blood mononuclear cells (PBMCs) [27]. As mentioned prior, a widely used and popular method for generating monocyte derived dendritic cells (MDDCs) is to explore the ability of monocytes to adhere to glass or plastic (adherence method) [21,22,27]. The adherence method is a rapid and straightforward method that does not require the use of complex equipment. However, some disadvantages of this process include lymphocyte contamination, low flexibility, and monocyte transient manipulation [28]. An alternative method to the adherence method is the magnetic isolation of monocytes from total PBMCs , particularly with the use of a human monocyte enrichment kit, which is designed to isolate monocytes from PBMCs by negative selection [26]. During this procedure, unwanted cells are targeted for removal with tetrameric antibody complexes and dextran-coated magnetic particles. The advantage of this isolation method is that the unwanted labeled cells are separated using a magnet while target cells can be freely poured off into a new tube without the need for columns. To date, with the availability of specific monoclonal antibodies that can label unique cell populations, the magnetic separation technique has become not simply an additional method, but a necessity for the isolation of rare cells in the field of immunology. For instance, techniques such as magnetic cell sorting with commercially available paramagnetic MACS-nanoparticles have facilitated the development of new approaches for research and clinical applications [22,29]. Furthermore, recent research studies comparing DC generation from monocyte adherence and MACS technology methods have demonstrated a higher DC purity and viability using MACS separated monocytes [22,30].

The current study presents a comparison between two methods for the generation of human DCs from monocytes isolated from PBMCs: 1) monocyte isolation by adherence and 2) monocyte isolation by negative selection using a commercial human monocyte enrichment kit. This study provides evidence to show 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 referenced 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.

In summary, since DCs play a focal role regarding research in the field of immunology, different parameters must be taken into consideration when considering how they are derived and what methods are used to isolate and culture them *in vitro*. Therefore, this study aims to provide insights on two different methods of monocyte isolation and how these methods differentially affect monocyte viability and yield eventually affecting dendritic cell viability, proliferation, and phenotype. These findings will contribute greatly to the field of immunology and will provide a detailed protocol of DC isolation, purification, and characterization.

PROTOCOL:

Overall human blood studies have been reviewed and approved by the Institutional Review Board (IRB) of FIU, IRB protocol approval # IRB-13-0440. Human leukopaks were purchased from the community blood bank in Miami, FL.

1. Isolation of PBMCs by standard density gradient technique

1.1. Perform a 1:1 dilution of blood with 1X-phosphate-buffered saline in a T75 flask.

1.2. Pipette 15 mL of density gradient solution into 50 mL centrifuge tubes and carefully layer (25 – 30 mL/tube) of the diluted blood over this gradient.

1.3. Centrifuge for 20 minutes at 1,200 x g with acceleration of 1 and deceleration of 0.

1.4. After centrifugation, collect the interface layer (white blood cells) into a new 50 mL centrifuge tube and wash cells twice in PBS (3 min at 1,080 x g). Discard supernatant each time.

1.5. Treat cells with ammonium-chloride-potassium lysing (ACK) buffer (to lyse red blood cells). Add 10 mL of buffer and incubate for 15 min at 4 °C.

1.6. Wash cells twice with PBS, centrifuge for 3 min at 1,080 x g and discard supernatant each time.

1.7. Save the pellet, which will contain the PBMCs.

1.8. Proceed with monocyte purification method of choice.

2. **Monocyte purification by adherence method**

2.1. Prepare complete cell culture medium containing L-glutamine (300 mg/mL) and supplemented with penicillin (50 U/mL)-streptomycin (50 µg/mL) and 10% fetal bovine serum.

2.2. Allow PBMCs to adhere for approximately 2 hours by culturing them in a T75 flask at a concentration of 5 x 10^7 cells per 10 mL of complete medium at 37 °C and 5% CO_2 in a humidified incubator.

2.3. After incubation, remove non-adherent floating cells from culture flask and gently wash adherent cells twice with PBS.

2.4. Incubate adherent cells in complete cell culture medium supplemented with 2 µL/mL of human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) stored at a stock concentration of 10 µg/mL.

2.5. Change half of the medium and replenish cytokines every 48 hours.

2.6. Allow 5 – 7 days for the differentiation of monocytes into MDDCs**.**

3. Monocyte purification by magnetic separation method

3.1. Collect PBMCs from standard density gradient technique, pour into a 14 mL polystyrene tube and re-suspend in PBS buffer at a concentration of 5 x 10^7 cells/mL.

3.2. Add human monocyte enrichment cocktail using 50 µL/mL cells, mix well and incubate at 4 °C for 10 min.

3.3. After incubation, add magnetic particles using 50 µL/mL cells, mix well and incubate at 4 °C for 5 min.

3.4. Bring cell suspension up to a total volume of 10 mL by adding PBS buffer, mix well by pipetting up and down 2 – 3 times.

3.5. Place the polystyrene tube without a cap into the magnetic device and set aside at room temperature for 2.5 min.

3.6. After incubation and in one continuous motion, pick up the magnet and pour the desired purified monocyte fraction into a clean 50 mL centrifugation tube.

3.7. Incubate adherent cells in complete cell culture medium supplemented with 2 µL/mL of human granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) stored at a stock concentration of 10 µg/mL.

3.8. Every 48 hours, change half of the medium, spin down at 1,080 x g for 5 minutes and re-suspend pellet with new media (CRPMI) and cytokines.

3.9. Allow 5 – 7 days for the differentiation of monocytes into MDDCs.

4. Trypan Blue exclusion viability assay

Note: Use this technique to obtain cell yield and viability of PBMCs, monocytes, and MDDCs.

4.1. Harvest cells using standard trypsin-EDTA and perform washes of the flasks and the cell pellet using PBS. Depending on the size of the pellet, resuspend in 5 to 10 mL of PBS to dissolve the pellet.

4.2. In a micro-centrifuge tube, perform a 1:1 dilution of trypan blue reagent with diluted cell pellet.

4.3. From this mix, aliquot 10 µL into a cell counting slide and read results using an automated cell counter according to manufacturer's protocol. If an automated cell counter is not available, a similar cell count is possible using a hemocytometer or a manual counter.

5. MTT assay

5.1. Plate cells at a concentration of 4 x 10^4/100 µL of complete media into each well in a 96-well plate. Allow 24 hours for cells to adjust to culture.

5.2. Incubate and perform readings at 0 (day 0), 36 (day 3) and 84 (day 7) hours respectively.

5.3. At completion of desired incubation, remove media and replace with 100 µL of PBS alone.

5.4. Prepare 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT) (5 mg/mL) by adding 10 mL of dimethyl sulfoxide (DMSO) to 1 g of sodium dodecyl sulfate (SDS).

5.5. Add 10 µL (5 mg/mL) of MTT solution to each well and incubate at 37 °C for 2 additional hours.

5.6. After incubation, remove supernatants which contain PBS and unconverted MTT mixture (yellow-color solution).

5.7. Add 100 µL of SDS-DMSO solution to each well and incubate for one additional hour.

5.8. Read absorbance at 540 nm using a microplate reader.

6. Cell surface staining by imaging flow cytometry

6.1. After 7 days of differentiation from purified monocytes, collect MDDCs at a concentration of 1 x 10^6/mL.

6.2. Start cell surface staining by blocking cells using 50 µL of heat inactivated (HI) human serum, incubate at 4 °C for 10 minutes.

6.3. After incubation, centrifuge cells at 720 x g for 5 min, discard supernatant.

6.4. To cell pellet, add respective fluorochrome-conjugated antibodies (*e.g.*, anti-CD11c or anti-CD14) and incubate at 4 °C for 20 minutes protected from light. In separate tubes, prepare single fluorochrome-stained samples ($1 \, x \, 10^6$/ml) since they are needed for compensation.

6.5. After incubation, wash cells twice in 1 mL of PBS buffer and centrifuge each time at 720 x g for 5 min.

6.6. Keeping cells protected from light, re-suspend in PBS buffer at a concentration of $1 \, x \, 10^6$cells/100 µL for flow cytometry analysis.

6.7. In order to gate on viable cells, add 1 µL of DAPI to each tube prior to acquiring the cells on the single cell imaging flow cytometry instrument.

7. Statistical analysis

7.1. Input all data in a spreadsheet.

7.2. Compare results using the student's t-test and/or one-way ANOVA as appropriate.

7.3. Consider differences to be statistically significant if $p < 0.05$.

REPRESENTATIVE RESULTS:

Monocyte yield by magnetic separation is higher compared to monocyte yield by adherence method.

Data presented in Figure 1 display PBMC and monocyte cell counts by the trypan blue exclusion method at the day of isolation of PBMCs and separation of monocytes. On average, monocytes isolated by the adherence method accounted for approximately 6.2 percent of total PBMCs while monocytes isolated by magnetic separation accounted for up to 25 percent of PBMCs. Statistical analysis revealed that the percentage of monocytes yielded by magnetic separation were significantly higher (≥ 4 fold). Data are expressed as mean ± SD of at least three independent experiments. Statistical analysis using student's *t*-test showed a significant difference of monocyte yield when comparing both methods ($p \leq 0.0005$).

Monocyte isolation by adherence and by magnetic separation do not affect monocyte viability.

After monocyte isolation by the two different techniques described above, viability assays were performed to ensure that the methods used were not cytotoxic and affecting the viability of the cells. The data presented in Figure 2 display the average of the percent of viable monocytes isolated by either adherence method or by magnetic separation. Viable cells were counted using the trypan blue exclusion method at the day of isolation. Comparing percent viability of monocytes between the two isolation methods revealed that the difference between both groups was not statistically significant. Additionally, the average of the percent of viable monocytes isolated by adherence was 91.75% ± 1.66 and the average of the percent of viable monocytes isolated by magnetic separation was 87.4% ± 2.75**.** These viability data were assessed from at

least three independent experiments. Statistical significance was determined using student's t-test.

Cells derived from monocytes isolated by magnetic separation show a significant increase in cell proliferation compared to cells derived from monocytes acquired by adherence method.

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. The data presented in Figure 3 demonstrate that both processes of monocyte isolation lead to higher cell proliferation over a period of 7 days as measured by an increased in absorbance at day 0 (adherence method: 0.22 ± 0.02 vs. magnetic method: 0.38 ± 0.02), day 3 (adherence method: 0.28 ± 0.02, magnetic method: 0.55 ± 0.05) and day 7 (adherence method: 0.36 ± 0.01, magnetic = 0.66 ± 0.006). However, unlike the XTT assay (data not shown), the MTT assay revealed a significant increase in cell proliferation of MDDCs from monocytes isolated by magnetic separation compared to MDDCs from monocytes acquired by adherence method at day 0 ($p=0.003$), day 3 ($p=0.006$), and day 7 ($p=0.002$). Cell proliferation was also found to be significantly different between days 0 and 3 ($p= 0.003$), and between days 0 and 7 ($p<0.001$) within the group of MDDCs from monocytes purified through magnetic separation. Furthermore, a significant difference in cell proliferation between day 0 and day 7 in MDDCs from monocytes purified by adhesion ($p=0.008$) was observed. MTT assay was conducted using MDDCs acquired from three different experiments. Statistical significance was determined using ANOVA and student's t-test, $p \leq 0.05$ was considered significant, and p values are noted on the graph unless no statistical significance was found.

Characterization of MDDCs by CD11c and CD14 cell surface staining.

After culturing monocytes for seven days with IL-4 and GM-CSF, MDDCs obtained from monocytes isolated by adherence and magnetic isolation methods were characterized in Figure 4. The characterization revealed low single CD14+/CD11c- phenotype or purely monocytic population in both methods, adherence = 1% ± 1.0 versus magnetic = 1% ± 0.0 and high total CD11c+ phenotype , adherence = 72% ± 11 versus magnetic = 79% ± 19. However, when the total CD14+ population was analyzed further, there was a high yield of cells double positive for CD11c and CD14 in both methods with magnetic separation giving a higher number of CD11c and CD14 double positive population when compared to the adherence method, adherence = 49% ± 7 versus magnetic = 73% ± 15. Although, there was a higher yield of MDDCs with double positive phenotype via magnetic separation, the CD11c positive and CD14 negative population of MDDCs was higher in the adherence method; adherence = 23% ± 7 versus magnetic = 6% ± 7. In Figure 4B, the mean fluorescence intensity (MFI) of each individual marker were looked at in the gated population and showed high intensity of CD14 in CD14+ population, equivalent intensity of both CD11c and CD14 in double positive population and high CD11c intensity in CD11c + and CD14 – population. In summary, even though magnetic isolation gives a higher percentage of CD11c+/CD14+ cells, which may be early stage differentiated MDDCs that have not yet dropped the monocytic marker (CD14), the adherence method gives a higher percentage of CD11c+/CD14-, which may be a late stage differentiated MDDC population. In addition, DAPI staining was performed to confirm viability results and to ensure characterization was performed on live MDDCs. The percent of DAPI negative/live cells (adherence = 76± 7 versus magnetic = 67± 1) are not significantly different between the two

methods confirming both methods do not affect the viability of the MDDCs after seven days in culture.

Figure 1. Monocyte yield by magnetic separation is higher compared to monocyte yield by adherence method. About ~6.2% of monocytes were isolated from PBMCs using the adherence method while ~25% of monocytes were attained using the magnetic separation method. Data are expressed as mean ± SD of at least three independent experiments. Statistical analysis using student's t-test showed a significant difference of monocyte yield when comparing both methods ($p = 0.00005$).

Figure 2. Monocyte isolation by adherence and by magnetic separation do not affect monocyte viability. The average of the percent of viable monocytes isolated by adherence was 91.75% ± 1.66 and the average of the percent of viable monocytes isolated by magnetic separation was 87.4% ± 2.75**.** Data are expressed as mean ± SD percentage of viable cells of at least three independent experiments. No significant difference was observed in cell viability when comparing both purification methods.

Figure 3. Cells derived from monocytes isolated by magnetic separation show a significant increase in cell proliferation compared to cells derived from monocytes acquired by adherence method. A significant increase in absorbance was observed for both methods at both day 3 and day 7 when comparing the values against its respective controls of day 0. In addition, ANOVA and student's t-test also showed a significant difference in absorbance of MTT uptake when comparing both methods ($p \leq 0.05$). Data are expressed as mean ± SD of three independent experiments.

Figure 4. Characterization of MDDCs by CD11c and CD14 cell surface staining. A) Bar graph representing the different populations (% gated out of single cells). First, DAPI-(alive) cells were gated from total single cells. Then, CD14+/CD11c-, CD11c+, CD11c+/CD14+ and CD11c+/CD14- were gated from DAPI- (alive) population. CD11c + population is the summation of two regions CD11c+/CD14+ and CD11c+/CD14-. B) Representative graphs showing the mean fluorescence intensity (MFI) values for both CD11c and CD14 in every subpopulation of cells. C) Representative scatter plots of cells labelled with CD11c-APC and CD14-APCcy7 gated on DAPI negative (alive) cell population for both magnetic and adherence methods. Representative image gallery of single cells (selected in blue in the scatter plots) belonging to each sub-population of cells, CD11c+/CD14- (gated in orange), CD11c+/CD14+ (gated in red), and CD14+ (gated in purple). In the image gallery of single cells, the red color represents CD11c labelled with APC and yellow color represents CD14 labelled with APCcy7. In the scatter plots, the colors chosen to gate the populations are random and do not co-relate to the actual cell images.

DISCUSSION:

Based on the known difficulties of isolating and generating MDDCs from human blood, the present study aimed to provide a comprehensive comparison of two well-established methods for the generation of MDDCs. The first method compared is a well-established traditional method for generating MDDCs by exploiting the ability of monocytes to adhere to glass or plastic (adherence method) [21,22,27]. The adherence method is fast and cost effective, and does not require

the use of complex equipment. However, some disadvantages of this process include lymphocyte contamination, low flexibility, monocyte transient manipulation [22,30,31]. The second alternative method compared is the magnetic isolation of monocytes from total peripheral blood mononuclear cells (PBMCs) using a human monocyte enrichment kit[26], which is designed to isolate monocytes from PBMCs by negative selection. The advantage of this isolation method is that the unwanted cells are labeled and separated using a magnet while the target cells are freely poured off into a new tube without the need of columns and without directly targeting the cells with antibodies. Regardless of monocyte isolation method (adherent vs. magnetic separation), after both procedures, one of the critical steps within the protocol is the *in vitro* stimulation of monocytes with granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), which are required to generate MDDCs. This is a common and well established protocol to generate MDDCs from blood mononuclear cells without compromising antigen capturing and processing capacity characteristic of immature MDDCs [32]. One of the major limitations of the techniques commonly used for the *in vitro* generation of DCs is the low yield of precursor cells such as the monocytes. As reported above, monocytes isolated by the adherence method accounted for approximately 6.2 percent of total PBMCs, while monocytes isolated by magnetic separation accounted for up to 25 percent of PBMCs. According to the literature, ranges for differential white blood cell count in normal adults for monocytes is from 2–10% [31] while DCs only constitute 0.1 – 1% of total blood mononuclear cells [20]. Therefore, the high yield of monocytes (~25%) obtained after magnetic isolation could be due to impurity of cell population and lack of antibody and/or magnetic particle binding to unwanted cells.

To achieve a high yield and purity of the isolated monocytes, all the critical steps in the protocol should be carefully followed. During isolation of PBMCs from blood by standard density gradient centrifugation, there are a few critical steps. First, pipetting and carefully layering of blood over density gradient solution into 50 mL centrifuge tubes is a step that requires practice since pipetting harshly can cause the mixing of this solution and blood, which can lead to a weak PBMC layer that is hard to isolate, or it can result in the complete mixture of red blood cells with PBMCs. In this step, it is important to maintain a constant and relatively slow flow of pipetting to have a well-defined layer of blood and a distinct PBMC layer. Second, centrifugation in the next step with acceleration 1 and deceleration 0 is very critical. If these settings are not used for the first centrifugation step, it will lead to mixing of the blood with the density gradient solution erratically and will not separate the PBMCs from the rest of the blood components. Third, it is important to incubate the PBMCs with Ammonium-Chloride-Potassium (ACK) lysing buffer for no longer than the optimized time (10-15minutes) since longer incubation with ACK can lead to reduction of viable PBMCs along with red blood cells. In addition, it is also important to carry out the PBS washes after ACK incubation. However, if the red blood cells are not lysed with the first ACK step and they still continue to appear after washes, another round of ACK lysing is highly recommended.

After isolation of white blood cells, there are a few critical steps to keep in mind when performing the adherence method to generate monocytes. First, washing off the floating lymphocytes after two hours of PBMCs incubation is very important since the presence of floating lymphocytes can cause less monocytes to adhere and can also result in lower MDDC purity. Although these washing steps are critical, excessive and harsh washing (more than recommended i.e. twice) can cause washing off the adherent monocytes too, resulting in lower

monocyte yield. Second, incubating the cells with complete medium and cytokines is critical since the cytokines are responsible for differentiation of monocytes into MDDCs. In addition, changing media and replenishing the cytokines every 48 hours is important for the differentiation and survival of the cells. While changing media, it is also crucial to save the floating MDDCs and return them back into culture along with fresh media and cytokines since there are some MDDCs that can detach from the surface during the process of differentiation. It also important to make sure that the monocytes are seeded at the recommended concentration, attached and closely spaced because they need cell to cell contact in order to grow. When performing the magnetic separation method to generate monocytes, there are a few critical steps in order to get a good yield and high purity of monocytes. First, it is critical to maintain the cell concentration recommended by the manufacturer. It is also important to not disturb the polystyrene tube when placed in the magnet, as it may lead to lesser yield and lesser purity. As presented above, this technique leads to a significantly higher monocyte yield (Figure 1) when compared to the adherence method, which may be related to the presence of other cells. Furthermore, when the cells were characterized by flow cytometry, the magnetic method shows a higher percentage of double positive cells (CD11C+/CD14+), which may correlate with the presence of other cells or may also be due to the presence of different stages of MDDC differentiation.

Another important aspect of *in vitro* MDDC generation regardless of yield and purity is the ability to generate viable and proliferative MDDCs. The current study provides evidence to demonstrate that both purification methods induce proliferation of MDDCs (Figure 3) as shown by the increase in cell metabolic activity during seven days in culture. In addition, the generation of MDDCs from monocytes purified by magnetic separation showed a significantly higher rate of proliferation compare to MDDCs generated from monocytes purified by adhesion (Figures 3). These findings are in accordance with previous literature demonstrating that generation of DCs from CD34+ cells is a proliferative process due to the presence of proliferative DC progenitors in human blood [27 33]. However, there are controversial findings demonstrating that monocytes can also differentiate into DCs without any evidence of proliferation[34,35]. It is relevant to point out that there is a lot of controversy about the *in vitro* generation of DCs and regarding whether DCs are generated from proliferating precursors or from differentiation of monocytes. For instance, *in vitro* production of DC from adherent peripheral blood cells have been shown to also enrich for progenitor cells that are capable of proliferation after exposure to GM-CSF [27] and there is also evidence demonstrating that the yield of DCs derived in the presence of GM-CSF and IL-4 cannot be expanded beyond the number of starting monocytes [33]. Overall, the current study demonstrates an increased in proliferation of monocytic cells in culture that by seven days result in a total MDDCs population with > 70% CD11c+ phenotype (adherence = 72% ± 11 versus magnetic = 79% ± 19). It is relevant to point out that when further phenotypic analysis was performed, although not significant, the monocytes isolated by the magnetic separation method resulted in MDDCs with a higher double positive phenotype (adherence = 49% ± 7 CD11c+/CD14+ versus magnetic = 73% ± 15 CD11c+/CD14+) while the monocytes isolated by adhesion resulted in MDDCs with a higher single positive phenotype (adherence = 23% ± 7 CD11c+/CD14- versus magnetic = 6% ± 7 CD11c+/CD14-). MDDCs with a higher double positive phenotype (CD11c+/CD14+) may be under early stages of differentiation while MDDCs with a higher single positive phenotype may be under late stages of differentiation.

In summary, 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. When comparing both methods, the findings have demonstrated the ability of both techniques to simultaneously generate monocytes that are capable of proliferating (Figure 3) and differentiating (Figure 4) into CD11c+ MDDCs after seven days in culture since both methods yielded > 70% CD11c+ MDDCs. However, further analysis looking at maturation markers may prove useful to fully characterize the functional MDDC population. In conclusion, both methods are advantageous alternatives for the isolation of CD11c+ MDDCs and provide an adequate yield for research applications and may even be useful for future clinical applications. The lab is currently focusing on the generation of MDDCs to study the effects of alcohol abuse and other substances of abuse on the innate immune system, in particularly the ability of these substances to modify MDDC phenotype and function. Therefore, the current study will provide a baseline of MDDC characterization and enhance the ability to proceed with further experimentation in order to elucidate the molecular mechanisms mediating monocyte-derived dendritic cell function in the context of substance abuse.

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DISCLOSURES:
All authors disclose no competing financial interests.

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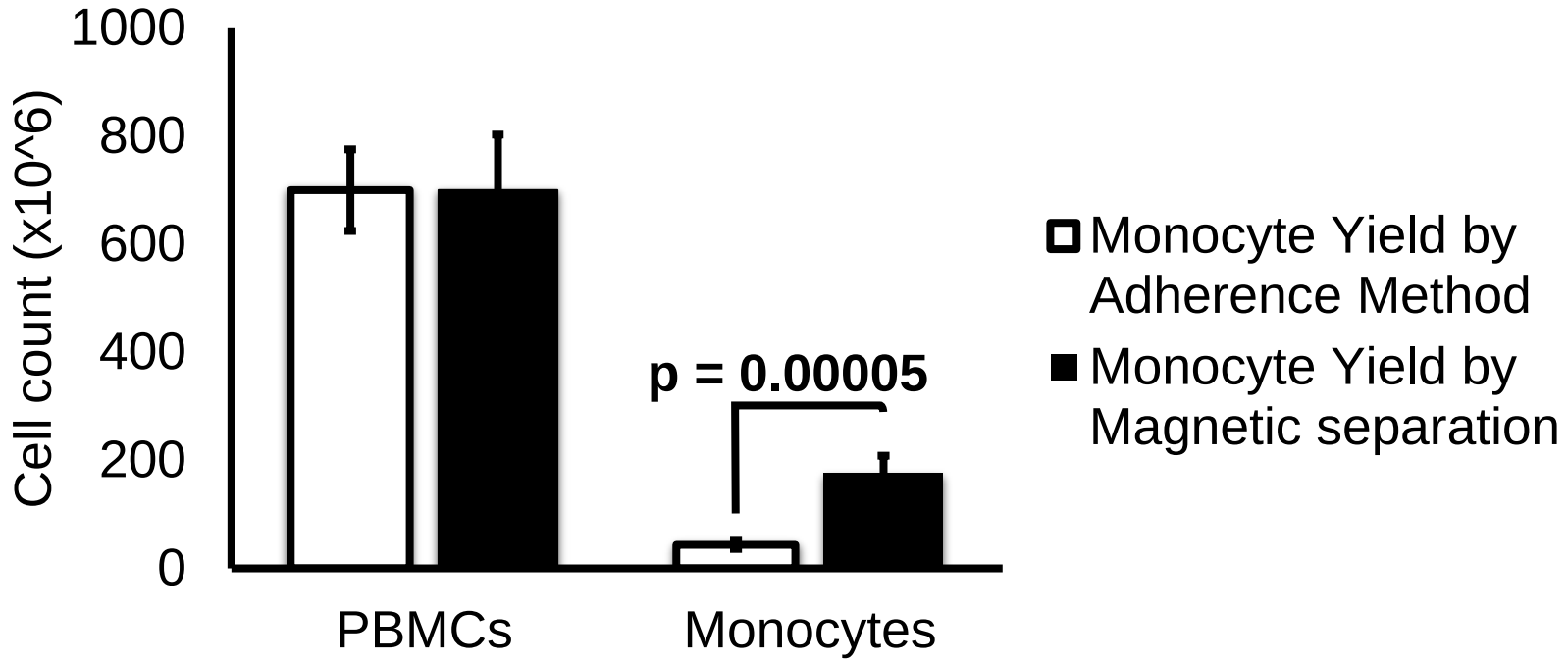

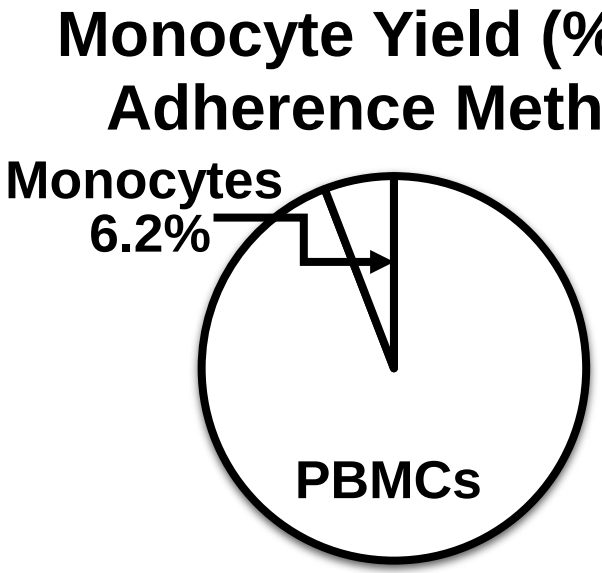

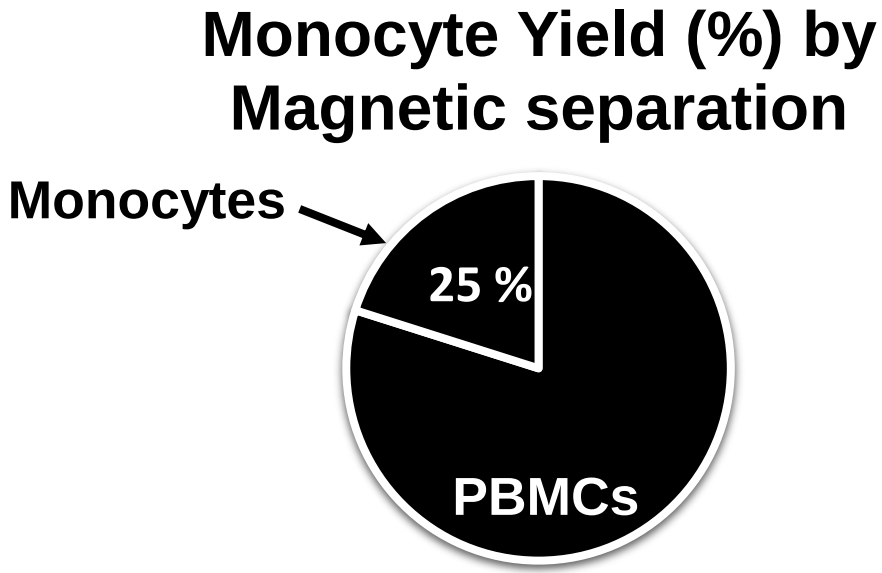

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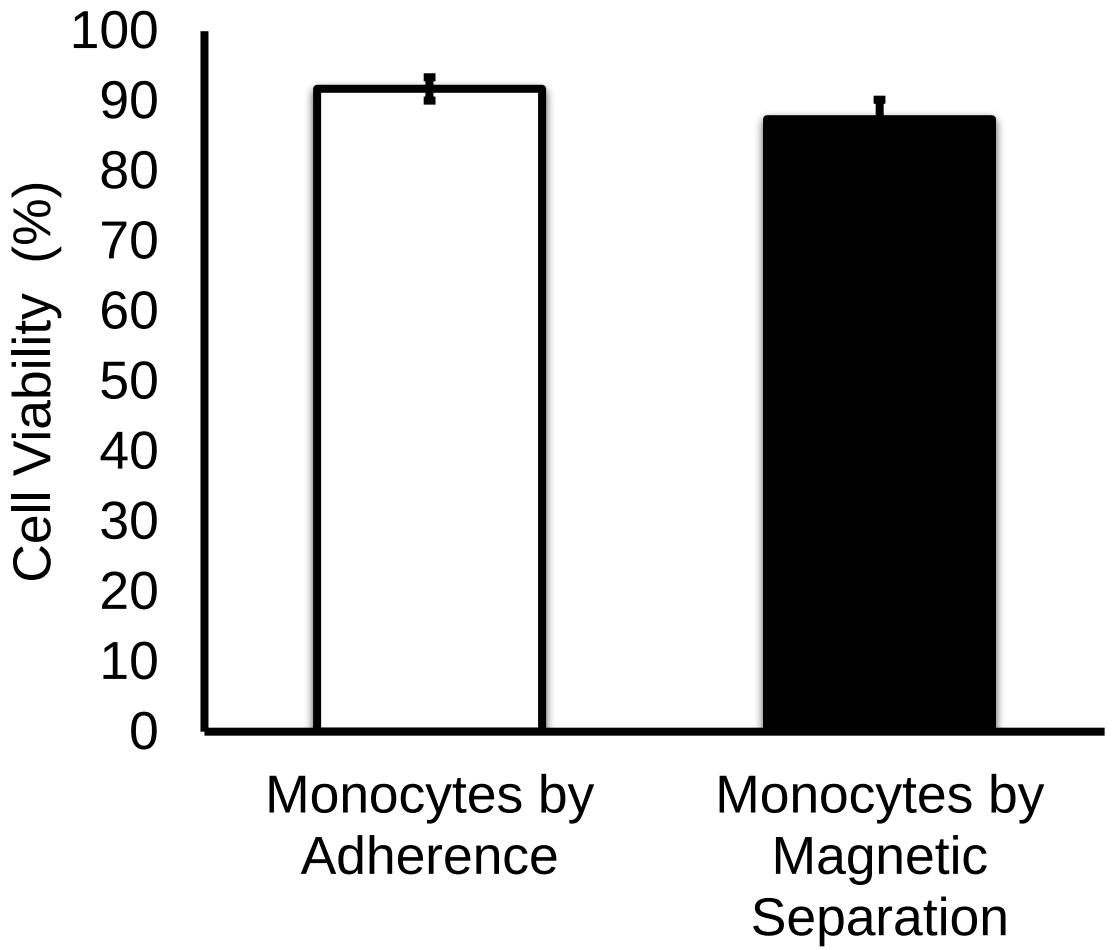

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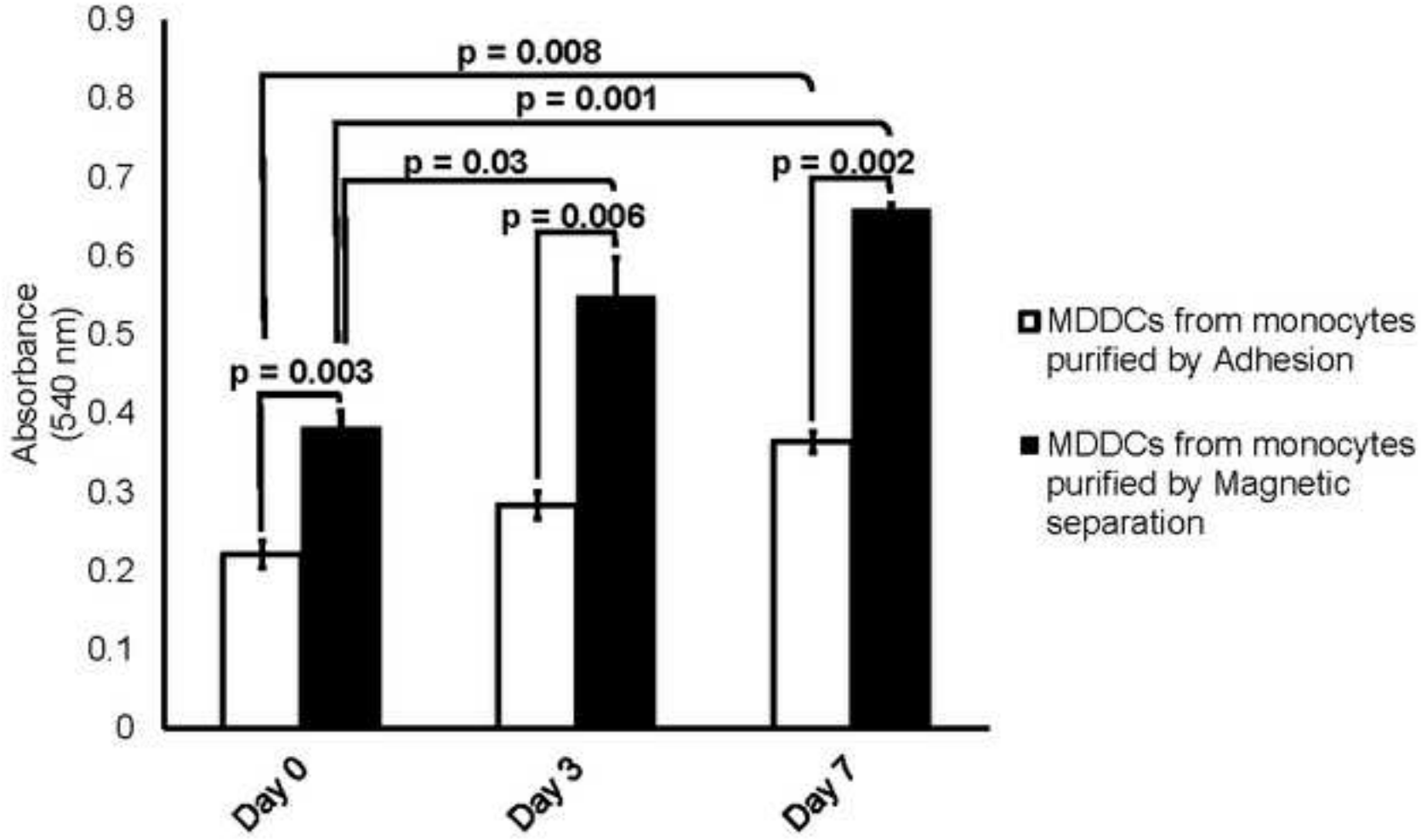

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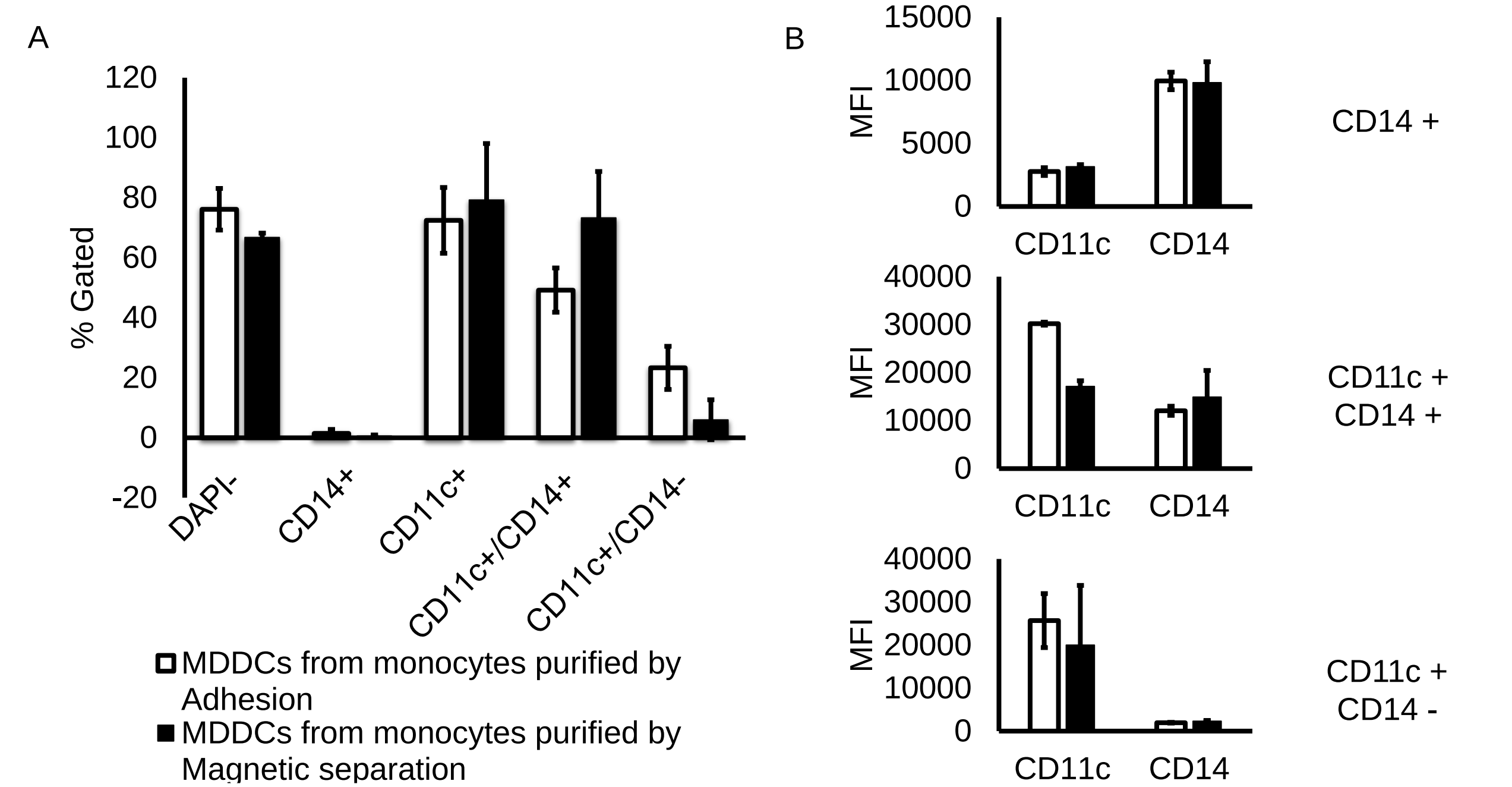

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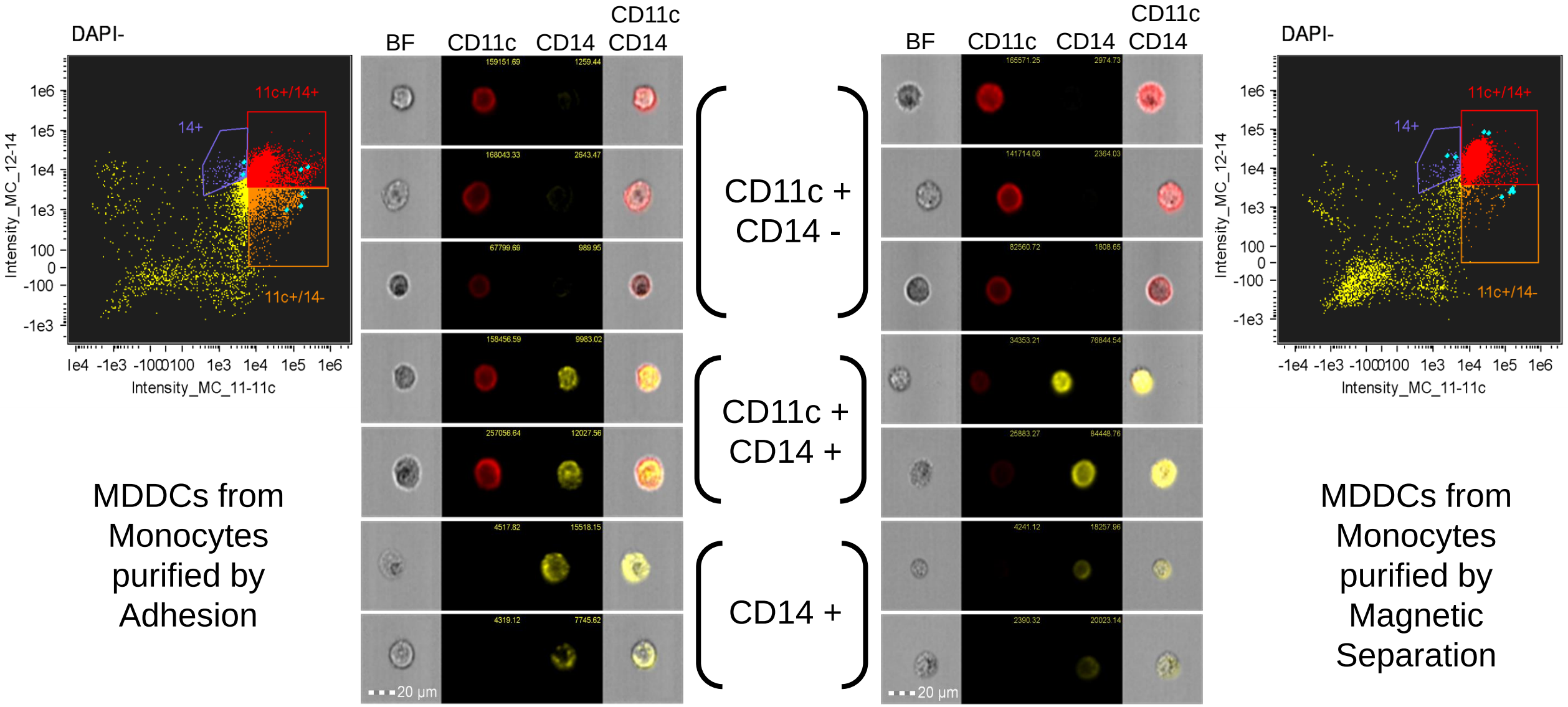

MDDCs from Monocytes purified by Adhesion

MDDCs from Monocytes purified by Magnetic Separation

Name of the Material/Equipment
Ficoll-Paque
Phosphate-buffered saline (PBS)
ACK Lysing buffer
RPMI 1640 medium
Antibiotic-Antimycotic (100X)
Fetal Bovine Serum (FBS)
RoboSep buffer
EasySep Human monocyte enrichment kit
TC20 Automated cell counter
FITC-Dextran
Trypan blue stain (0.4%)
Synergy 2 multi-mode reader
XTT Sodium salt bioreagent (XTT)
Dimethyl sulfoxide bioreagent (DMS)
Thiazolyl blue tetrazolium bromide (MTT)
Sodium Dodecyl Sulfate (SDS)
Phenazine Methosulfate (DMSO)
Inactivated (HI) Human Serum
Accuri C6 Flow Cytometer
FlowSight Amnis Flow Cytometer

Company	Catalog Number
GE Healthcare	17-5442-03
Life Technologies	10010-023
Quality Biological	118-156-101
Life Technologies	22400-089
Life Technologies	15240-062
Life Technologies	16000-044
StemCell	20104
StemCell	19059
Bio-Rad	145-0101
Sigma Aldrich	FD4-100MG
Life Technologies	15250-061
Biotek	7131000
Sigma Aldrich	X4626-100MG
Sigma Aldrich	D8418-500ML
Sigma Aldrich	m5655-500MG
Bio-Rad	161-0302
Sigma Aldrich	P9626-1G
Chemicon	S1-100ML
BD Accuri	653119
EMD Millipore	100300

Comments/Description
Must be used at room temperature

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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."