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Macrophage differentiation and polarization into an M2-like phenotype using a human monocyte-like THP-1 leukemia cell line

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TITLE:

Macrophage Differentiation and Polarization into an M2-Like Phenotype using a Human Monocyte-Like THP-1 Leukemia Cell Line

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SUMMARY:

M2-like tumor-associated macrophages (TAM) are associated with tumor progression and poor prognosis in cancer. This protocol serves as a detailed guide to reproducibly differentiate and polarize THP-1 monocyte-like cells into M2-like macrophages within 14 days. This model is the basis to investigate the anti-inflammatory effects of TAM within the tumor microenvironment.

ABSTRACT:

Tumor-associated macrophages (TAM) can switch their expression and cytokine profile according to external stimuli. This remarkable plasticity enables TAM to adapt to ongoing changes within the tumor microenvironment. Macrophages can have either primarily pro-inflammatory (M1-like) or anti-inflammatory (M2-like) attributes and can continually switch between these two main states. M2-like macrophages within the tumor environment are associated with cancer progression and poor prognosis in several types of cancer. Many different methods for inducing differentiation and polarization of THP-1 cells are used to investigate cellular and intercellular mechanisms and the effects of TAM within the microenvironment of tumors. Currently, there is no established model for M2-like macrophage polarization using the THP-1 cell line, and the results of expression and cytokine profiles of macrophages due to certain in vitro stimuli vary between studies. This protocol serves as detailed guidance to differentiate THP-1 monocyte-like cells into M0 macrophages and to further polarize cells into an M2-like phenotype within 14 days. We demonstrate the morphological changes of THP-1 monocyte-like cells, differentiated macrophages, and polarized M2-like macrophages using light microscopy. This model is the basis for cell line models investigating the anti-inflammatory effects of TAM and their interactions with other cell populations of the tumor microenvironment.

INTRODUCTION:

Tumor-associated macrophages (TAM) and their role in chronic inflammation, the onset of cancer, and tumor development are important targets in recent research[1,2]. Peripheral blood monocytes that are recruited to the tissue microenvironment of the developing tumor differentiate into macrophages and can be polarized into two main subtypes of macrophages[3]. The classically activated macrophage represents the primarily pro-inflammatory M1-like phenotype and the alternatively activated M2-like subtype shows predominantly anti-inflammatory characteristics[4]. Macrophages can switch dynamically between these two main phenotypes depending upon their cellular metabolism, with intermediate subtypes having both inflammatory and anti-inflammatory attributes[5]. TAM represents a heterogeneous population of both phenotypes. A tumor-promoting function and poor prognosis in different types of cancers is, however, particularly associated with M2-like macrophages[6–8].

The functional profiles of macrophages and their interaction with other cells within the tumor microenvironment are complex and challenging to capture in a continuously changing environment during ongoing tumor development. Cell lines can provide a homogenous cell population with stable viability in culture, which can facilitate the process of demonstrating defined cellular and intercellular mechanisms. The monocyte-like THP-1 cell line is a legitimate model system for primary human monocytes[9]. This spontaneously immortalized cell line has been obtained from the peripheral blood of a one-year-old infant with acute monocytic leukemia[9,10]. The differentiation and polarization of THP-1 cells have been reported by several studies and have been performed in multiple different ways[11–14]. Activation and, therefore, the polarization of macrophages into an M1-like phenotype is followed by a compensatory anti-inflammatory rebound mechanism, promoting an M2-like phenotype through cytokines produced by inflammatory macrophages, such as interleukin 6 (IL-6) or itaconate[15,16]. This might serve as a break mechanism to attenuate an overshooting inflammatory response following cell activation[17]. The process of differentiating and polarizing monocytes and THP-1 monocyte-like cells into an anti-inflammatory M2-like phenotype is itself also accompanied by pro-inflammatory stimuli that must be overcome. An inflammatory cytokine response can be caused by mechanical stress[18], such as changing media to refeed the cells, or adding chemical compounds to differentiate THP-1 cells, such as phorbol 12-myristate 13-acetate (PMA), and induce production of tumor necrosis factor α (TNFα), interleukin 1β (IL-1β) or IL-6[19]. This altered cytokine expression profile as a response to PMA can affect and prevent subsequent macrophage polarization[20]. Adequate resting periods, as reported before after PMA treatment, allow these inflammatory responses to decrease and facilitate cell polarization into a distinct M2-like phenotype[21].

This protocol demonstrates a method to differentiate and polarize THP-1 monocyte-like cells into an M2-like phenotype of macrophages within 14 days.

PROTOCOL:

NOTE: An overview of the steps described in this protocol is shown in **Figure 1**. The human monocyte-like leukemia cell line THP-1 was purchased. Short tandem repeat analysis was

performed to authenticate the THP-1 cell line. Perform all steps under sterile conditions. The THP-1 monocytic cell line grows in suspension and does not attach to cell culture surfaces. Adherence can be induced by differentiating monocytes into macrophage-like cells through, e.g., mechanical stress or specific treatment with PMA.

1. Culturing and maintenance of THP-1 monocyte-like cells

1.1 Set a timer for 150 s. Remove the frozen vial containing the THP-1 cell line (**Table of Materials**) from the liquid nitrogen and thaw it immediately in a clean water bath (37 °C). Start the timer as soon as the vial is put into the water bath. Loosen the cap to release the pressure that is building up due to the thawing process but ensure that the tube opening does not contact the water, to avoid contamination. The optimal time period for thawing the cells lies between 120–150 s. Continue thawing the cell suspension until an ice chip of the size of about 4 mm is left within the vial; then, proceed to the next step immediately.

1.2 Transfer the liquid phase of the cell suspension to a 15 mL tube containing 9 mL of warm (37 °C) growth media (**Table of Materials**). Then, transfer 1 mL of the warm medium-cell suspension into the THP-1 vial and back into the 15 mL tube to melt the remaining ice chip and flush the vial to assure that no cells are left behind.

1.3 Mix the suspension gently by pipetting up and down with a 1000 µL pipette. Remove a small sample (approximately 10 µL) to count the cells for viability (using trypan blue for exclusion) while they are spun. Spin down the warm cell suspension at 200 x *g* for 7 min at 37 °C.

1.4 Remove the supernatant completely and resuspend with a certain volume of warm growth medium to achieve a cell density of 5 x 10^5/mL. Mix the suspension gently and transfer 22 mL of volume into T-75 cell culture flasks (**Table of Materials**). Store flasks upright in an incubator at 37 °C with 5% carbon dioxide (CO_2) concentration. Exchange the growth media every 3–4 days.

2. Seeding of THP-1 cells and differentiation into M0 macrophages

2.1 Prepare the cell containing growth medium with the respective cell density to seed cells at a density of 3 x 10^5/mL/well into 24-well cell culture plates (**Table of Materials**). Mix the medium gently and prepare aliquots of 26 mL, each put into a 50 mL tube. Use each 26 mL aliquot for seeding the cells into a respective plate.

2.2 Transfer 1 mL of the cell-containing medium into each well of a 24-well plate. Mix the media gently by pipetting up and down between transfers.

2.3 Prepare a stock solution of PMA (dissolve 1 mg of PMA in 100 µL of Dimethyl Sulfoxide (DMSO) = ~16 mM solution of PMA in DMSO) and dilute it with cold Phosphate Buffered Saline (PBS) to a final working concentration of 10 ng/µL right before cell treatment (**Table of Materials**). Keep the solution on ice and use it immediately. Do not refreeze. Add 100 ng of PMA per well. Let each cell plate sit in the incubator without any further treatment for 72 h.

2.4 After 72 h, remove the growth medium and replace it with 1 mL of fresh growth medium. Do not touch the bottom of the wells with pipette tips. Let the cells rest for another 96 h in the incubator.

2.5 After 96 h, repeat step 2.4 (media change) and let the cells rest for another 24 h.

NOTE: The M0 macrophages are now ready to be used for experiments (**Figure 2**). Immediately prior to treating the cells as part of further experiments, consider a media change with RPMI only (**Table of Materials**) since growth media supplements can cause interference with reagents that are added for cell treatment. In case M2-like macrophages are needed, proceed with section 3.

3. Polarization of M0 macrophages into M2-like macrophages

3.1 Prepare a stock solution of IL-4 and IL-13 (dissolve 20 µg of IL-4 or IL-13 in 200 µL of nuclease-free water) and dilute it to a final working concentration of 2 ng/µL with PBS immediately prior to cell treatment. Keep the solution on ice and use it immediately. Do not refreeze.

3.2 Remove the growth medium and replace it with 1 mL of fresh growth medium. Add 20 ng of interleukin 4 (IL-4) and 20 ng of interleukin 13 (IL-13) per well. Let the cells rest for 48 h in the incubator.

3.3 After 48 h, repeat step 3.2. Let the cells rest for another 48 h in the incubator.

3.4 Remove the growth medium and replace it with 1 mL of fresh growth medium. Let the cells rest for 48 h in the incubator.

NOTE: M2-like macrophages are now ready to be used for experiments (**Figure 2**). Immediately prior to treating the cells as part of further experiments, consider a media change with RPMI only (**Table of Materials**) since growth media supplements can cause interference.

4. Detaching and harvesting macrophages for flow cytometry

NOTE: Use a mechanical method combining cold shocking and cell scraping to detach and harvest the polarized macrophages from plates for flow cytometry.

4.1 Remove the warm cell medium and replace it with a mixture of ice-cold PBS (without calcium and magnesium) and 5% fetal bovine serum (FBS), 1 mL per well. Immediately after this, place the cell plate on ice for 45 min. Do not place the cell plate on ice before the warm cell medium is removed, since this will decrease cell viability significantly. Keep the cells on ice only after inducing cold shock with ice-cold PBS/5% FBS mixture.

4.2 After 45 min on ice, scrape off the cells using mini cell scrapers (**Table of Materials**). Gently transfer the detached macrophages in cold PBS/5% FBS into a 15 mL tube. Keep the tube on ice

at all times until cells are stained.

NOTE: Pool eight wells of cells to reach adequate cell counts for staining.

REPRESENTATIVE RESULTS:

M2-like macrophages were characterized, and M2-polarization was validated using flow cytometry for Cluster of Differentiation markers (CD) CD14, CD11b, CD80 (M1-like marker), and CD206 (M2-like marker). Flow cytometry staining was performed according to the manufacturer's instructions. Macrophages were washed with PBS/5% FBS and incubated with Fcγ-receptor block to avoid unspecific binding. Cells were then stained with FITC-conjugated mouse anti-human CD14 and CD80 antibodies, with PE-conjugated mouse anti-human CD11b antibodies, and with PE-tandem-conjugated mouse anti-human CD206 antibodies and isotype-matched IgG (**Table of Materials**) for 30 min at 4 °C. Four-color flow cytometric analysis and fluorescence quantitation were performed. Gating of cells was carried out, excluding cell debris according to forward scatter and side scatter.

The monocyte and macrophage markers CD14 and CD11b were expressed in 70.9% and 74.7% of cells, respectively (**Figure 3**). Cells showed almost no positivity for the M1-like marker CD80 (0.2%) and high surface levels of the M2-like marker CD206 in 62.6% of cells (**Figure 4**).

The macrophages derived from the polarization method described in this protocol show the expression of CD14 as well as CD11b markers. Both markers can but do not have to be expressed in macrophages. A clear positivity for CD206 as an M2-like macrophage marker is expected, while the level of CD80 expression as an M1-like marker should be low. Raggi et al. demonstrated similar results using peripheral blood mononuclear cells (PBMC), that were polarized into M2-like macrophages[22]. The mean expression of CD206 varied between 50%–60%, while the mean expression of CD80 ranged between 20%–25%[22].

Further characterization of the M2-like macrophages created by this protocol was performed using quantitative real-time PCR (qRT-PCR). M2-like macrophages showed an upregulation of IL-6 and C-X-C Motif Chemokine Ligand 10 (CXCL10) compared to THP-1 monocyte-like cells, as well as an upregulation of the anti-inflammatory markers CD206, Interleukin 10 (IL-10) and C-C Motif Chemokine Ligand 18 (CCL18) (results not shown, to be published).

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of the M2-like macrophage cell line model. On day 0, cells are seeded into plates with a growth medium and incubated with PMA for 72 h. On day 3 and day 7 cell medium is changed, which lets the cells rest without PMA for a total of 120 h. On day 8, the growth medium is changed once more, and cells are incubated with IL-4 and IL-13 to induce M2-like polarization. This step is repeated after 2 days, on day 10. On day 12, the last medium change is performed, and M2-like cells rest in the growth medium for another 48 h before being used for experiments (PMA = phorbol 12-myristate-13-acetate; IL = interleukin).

Figure 2: Cell morphology of THP-1 cells, differentiated (M0) macrophages, and M2-like

macrophages using light microscopy. Cells were seeded at 3 x 10^5 per well in a 24-well plate. (**A**,**B**) THP-1 cells are shown at baseline. (**C**,**D**) The differentiated M0 macrophages received PMA treatment for 72 h, growth medium change and a 96-h-resting period. (**E**,**F**) The M2-like macrophages are shown after completed polarization treatment with IL-4 and Il-13 at day 14 of this cell model (20x and 40x magnification; scale = 100 µm).

Figure 3: Flow cytometry fluorescence analysis for CD14 (FITC, FL1-H) and CD11b (PE, FL2-H). (**A**) Density scatter plot, percentages of cells are shown in each quadrant. (**B,C**) The histograms for CD14 and CD11b in M2-like macrophages compared to a negative staining control.

Figure 4: Flow cytometry fluorescence analysis for CD80 (FITC, FL1-H) and CD206 (PE-tandem conjugate, FL3-H). (**A**) Density scatter plot, percentages of cells are shown in each quadrant. (**B,C**) The histograms for CD80 and CD206 in M2-like macrophages compared to a negative staining control.

DISCUSSION:

This protocol on differentiating and polarizing THP-1 monocyte-like cells within 14 days provides a method to obtain macrophages with a distinct M2-like phenotype due to long treatment incubation of cells with adequate resting periods between steps.

Certain steps are critical to this protocol. The doubling time of THP-1 monocytes is approximately 26 h. Cells can be split at a cell density of 9 x 10^5/mL and should be seeded at a density of 3 x 10^5/mL during every split. The split can be performed without removing all the used (old) cell medium – refeeding the cells with only 50% of the fresh medium can indeed lead to faster cell growth because growth factors in conditioned cell medium can enhance cellular proliferation. Not exchanging all the cell media, however, increases the risk of culture contamination and should therefore only be performed during the first passages of freshly cultured cells. Counting the cells is important to be able to culture cells at the right density and to determine the cellular viability after thawing. The proportion of dead cells after thawing cells properly should not exceed 15%.

The seeding of cells into plates requires gentle but thorough mixing of the cell suspension to obtain a consistent cell density in each well. Aliquots are prepared before seeding the cells into culture plates to assure that the volume of the cell-containing the medium is mixed properly. As THP-1 monocytes in the medium tend to sink to the bottom of a vial, the continuous gentle mixing of transfer volume is crucial to achieve a consistent density of cells.

Cell media should be warmed to 37 °C at all times to avoid cold shocks and a pro-inflammatory cellular stress response[23]. Therefore, also for media changes and cell treatment, plates should not be left out of the incubator for more than 15 min. Furthermore, the respective compounds for cell treatment, such as PMA, interleukins, and the PBS for diluting stock solutions prior to treating the cells, should be always kept on ice to avoid degradation.

Live macrophages that differentiated from monocytes by PMA treatment adhere to the surface

of the wells. During media changes and other further treatment steps, pipette tips should not touch the bottom of a well within the plate to prevent cell damage.

For performing flow cytometry with differentiated or polarized macrophages, the cells that are attached to the plates must be harvested. There are either enzymatical or mechanical techniques to detach cells, including trypsinization, treatment with enzyme mixtures with proteolytic and collagenolytic activity, or ethylenediaminetetraacetic acid (EDTA), cold shock, cell scraping, or even detachment through acoustic pressure[24,25]. Enzymatic detachment of macrophages can lead to altered cell surface marker expression and is therefore not the first choice for phenotypic or functional analyses[25]. In contrast to other reports, the experiments performed here showed that the combination of cold shocking and scraping off macrophages showed good cell viability (>90%) using Trypan Blue dye exclusion. Therefore, it is recommended to use this technique to detach the cells, with the annotation that once a cold shock is induced, the cells have to be kept cold on ice at all times.

A limitation of this protocol is the use of a monocyte-like cell line as a basis to mimic macrophage mechanisms in vivo. Cell culture studies using primary macrophages, however, can show variable cellular responses and mechanisms can be masked due to cell heterogeneity[26]. The THP-1 cell line is an established model system for primary human monocytes[9]. Due to the homogenous THP-1 cell population in a controlled culture setting, cellular responses are potentially more precisely reproducible. Furthermore, certain techniques optimize THP-1 cells as a model to resemble primary monocytes. An important step is the resting period of 5 days after PMA treatment, which increases cytoplasmatic volume and cell surface adherence similar to that of differentiated monocyte-derived cells[21].

Another limitation is the creation of a certain M2-like macrophage phenotype that has other characteristics than M2-like macrophages that were used in previous studies. Many different techniques to differentiate and polarize THP-1 cells have been reported, and a lack of baseline characterization complicates interstudy reproducibility[11–14]. Therefore, it is important to characterize the macrophages that are used in a study at baseline, depending on the mechanisms that are investigated. After that, the cellular responses after a respective treatment should be demonstrated.

The M2-like macrophages produced by following this protocol are a solid basis for the investigation of cellular responses in tumor onset and progression in different types of cancers, wound healing, or fibrosis. With this protocol, either M0-macrophages or M2-macrophages can be used *in vitro*, and the cells are suitable to be used in coculture models. This provides a wide variety of applications of a distinct M2-like macrophage phenotype that is robustly controlled *in vitro* over time.

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interpretation of the data.

DISCLOSURES:

The authors declare no potential conflicts of interest.

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Figure 1

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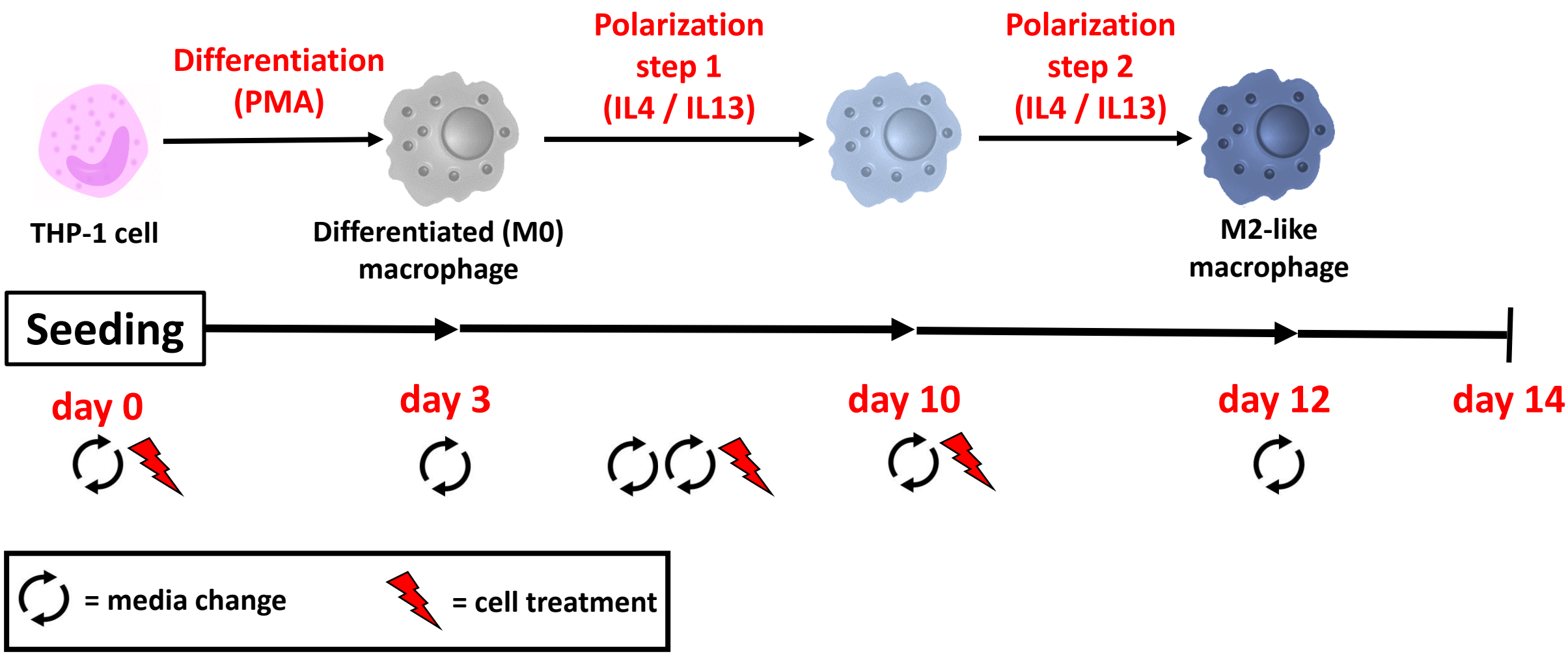

Figure 2

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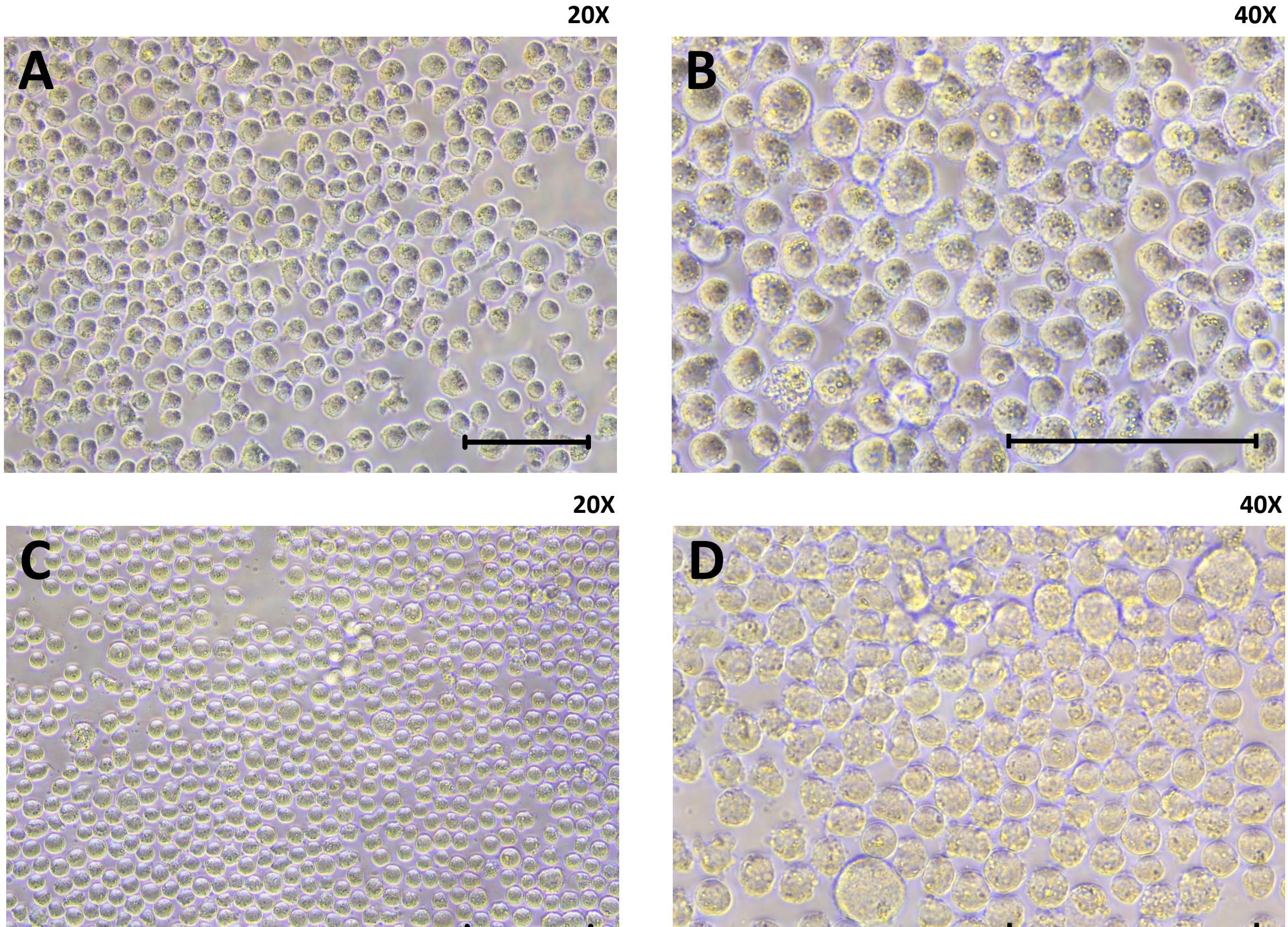

Figure 3

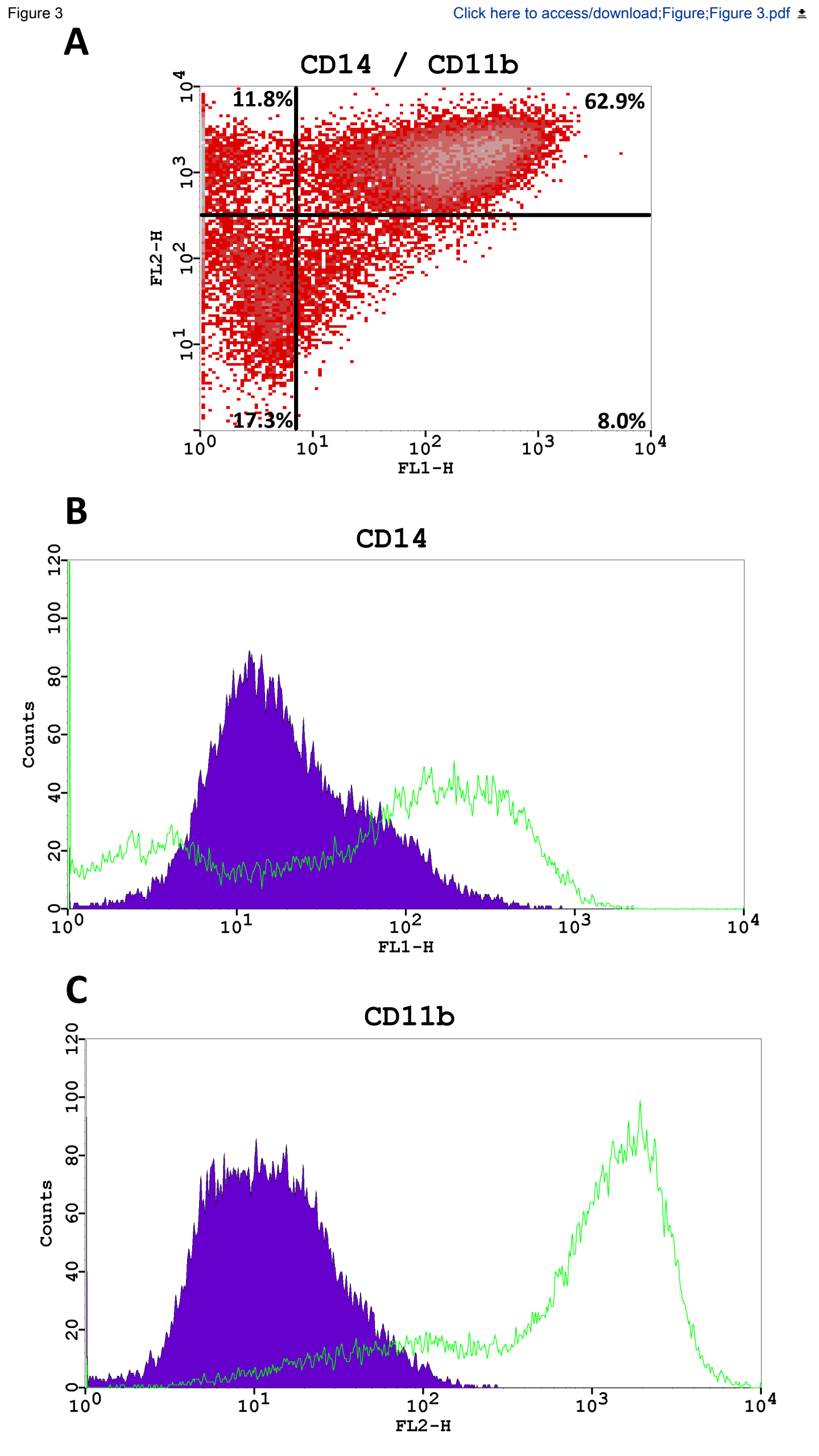

Figure 4

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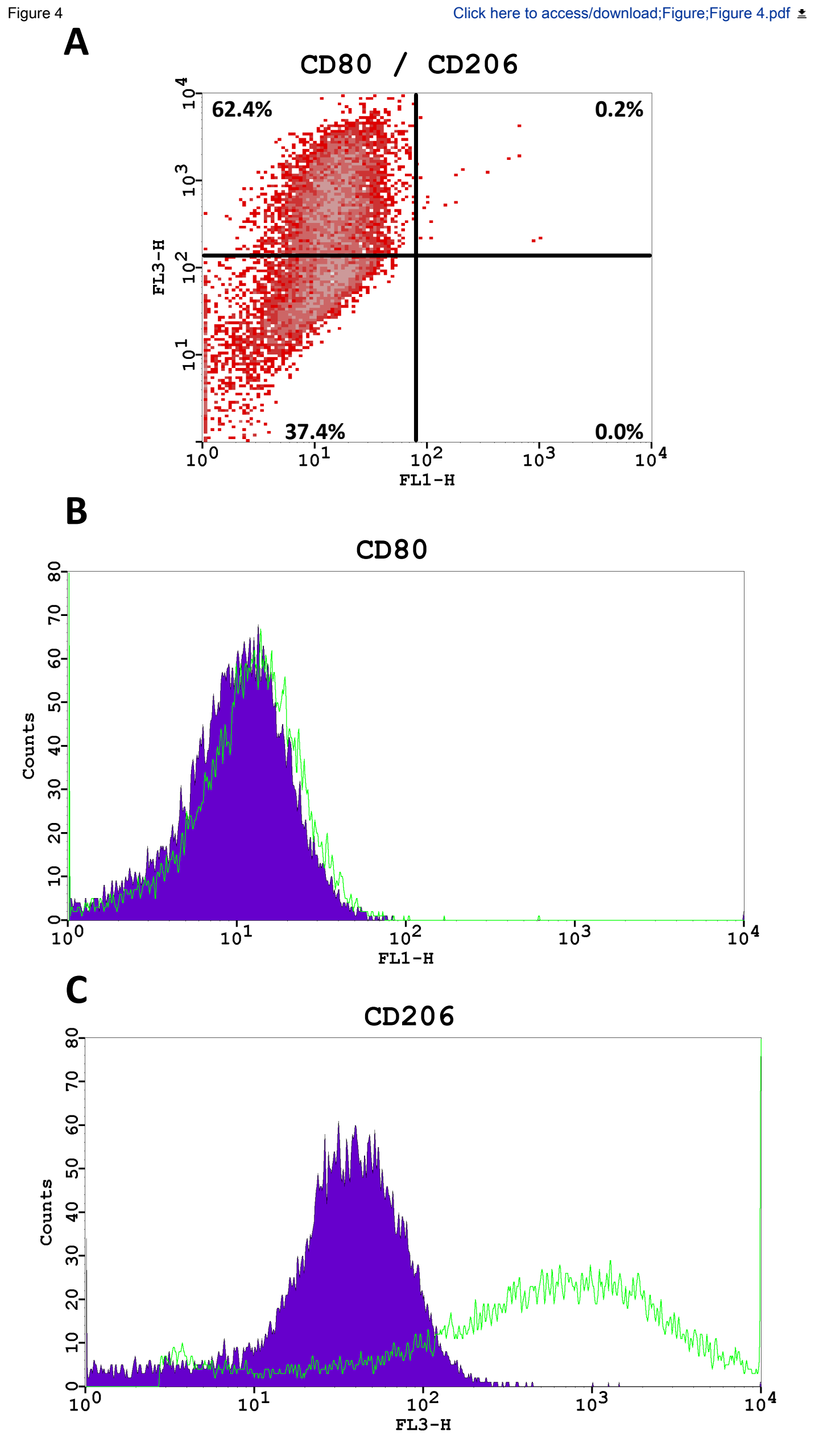

Name of Material/ Equipment	Company	Catalog Number
0.4% trypan blue	VWR, Radnor, USA	152-5061
1.5 mL microcentrifuge tube	USA Scientific, Ocala, USA	1615-5510
10 mL serological pipet	VWR, Radnor, USA	89130-898
1000 µL TipOne pipet tips	USA Scientific, Ocala, USA	1111-2821
15 mL Centrifuge tube	VWR, Radnor, USA	89039-664
20 µL TipOne pipet tips	USA Scientific, Ocala, USA	1120-1810
200 µL TipOne pipet tips	USA Scientific, Ocala, USA	1120-8810
25 mL serological pipet	VWR, Radnor, USA	89130-900
5 mL serological pipet	VWR, Radnor, USA	89130-896
50 mL Centrifuge tube	VWR, Radnor, USA	89039-662
Accutase solution 500 mL	Sigma, St. Louis, USA	A6964
Antibiotic Antimycotic Solution (100x), stabilized	Sigma, St. Louis, USA	A5955-100 mL
Binder CO2 Incubator	VWR, Radnor, USA	C170-ULE3
CytoOne T-75cm flask with filter cap	USA Scientific, Ocala, USA	CC7682-4875
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma, St. Louis, USA	D8537-500 mL
Eppendorf Centrifuge 5804 R (refrigerated)	Eppendorf, Enfield, USA	-
Ethyl alcohol (70%)	-	-
FACSCalibur flow cytometer	BD Biosciences, San Diego, USA	-
Falcon 24-well plate	VWR, Radnor, USA	353504
Fetal Bovine Serum (FBS)	ATCC, Manassas, USA	30-2020
FITC Mouse Anti-Human CD14	BD Biosciences, San Diego, USA	555397
FITC Mouse Anti-Human CD80	BD Pharmingen, San Diego, USA	557226
FITC Mouse IgG1 κ Isotype Control	BD Pharmingen, San Diego, USA	555748
FITC Mouse IgG2a, κ Isotype Control	BD Biosciences, San Diego, USA	553456
Human BD Fc Block	BD Biosciences, San Diego, USA	564220
Human interleukin 13 (IL-13)	R&D, Minneapolis, USA	IL-771-10 µg
Human interleukin 4 (IL-4)	R&D, Minneapolis, USA	SRP3093-20 µg
Labconco Biosafety Cabinet (Delta Series 36212/36213)	Labconco, Kansas City, USA	-
L-Glutamine Solution, 200 mM	ATCC, Manassas, USA	30-2214
Lipopolysaccharide (LPS) from E. coli 0111:B4	Sigma, St. Louis, USA	L2630-100 mg
Mini Cell Scrapers	Biotium, Fremont, USA	22003

Neubauer hemocytometer	Fisher Scientific, Waltham, USA	02-671-5
Nikon Eclipse inverted microscope TS100	Nikon, Melville, USA	-
Nuclease-free water	Invitrogen, Carlsbad, USA	AM9937
Olympus Light Microscope RH-2	Microscope Central, Feasterville, U	40888
P10 variable pipet- Gilson	VWR, Radnor, USA	76180-014
P1000 variable pipet-Gilson	VWR, Radnor, USA	76177-990
P200 variable pipet- Gilson	VWR, Radnor, USA	76177-988
PE Mouse Anti-Human CD11b	BD Biosciences, San Diego, USA	555388
PE Mouse IgG1, κ Isotype Control	BD Biosciences, San Diego, USA	555749
PE-Cy 5 Mouse Anti-Human CD206	BD Pharmingen, San Diego, USA	551136
PE-Cy 5 Mouse IgG1 κ Isotype Control	BD Pharmingen, San Diego, USA	555750
Phorbol 12-myristate 13-acetate (PMA)	Sigma, St. Louis, USA	P8139
Powerpette Plus pipettor	VWR, Radnor, USA	75856-448
Precision Water bath (model 183)	Precision Scientific, Chicago, USA	66551
RPMI-1640 Medium	ATCC, Manassas, USA	30-2001
THP-1 cell line, American Type Culture Collection (ATCC)	ATCC, Manassas, USA	TIB-202

Comments/Description
with 10,000 units penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per mL, sterile-filtered, BioReagent, suitable for cell culture
PBS without calcium chloride and magnesium chloride should be used, since both can alter macrophage polarization
The flow cytometer operates with CellQuest software (BD Biosciences)
Flow cytometry, myeloid cell marker (100 tests)
Flow cytometry, M1 marker (100 tests)
Flow cytometry, isotype control for CD80 (100 tests)
Flow cytometry, isotype control for CD14 (100 tests)
Flow cytometry, Fc block (0.25 mg)

Flow cytometry, myeloid cell marker (100 tests)
Flow cytometry, isotype control for CD11b (100 tests)
Flow cytometry, M2 marker (100 tests)
Flow cytometry, isotype control for CD206 (100 tests)

Dear Dr. Vidhya Iyer,

Hereby we resubmit the revised version of our manuscript “Macrophage differentiation and polarization into an M2-like phenotype using a human monocyte-like THP-1 leukemia cell line”. We considered the comments with care and all editorial and peer review comments have been addressed. Please find the comments and respective answers below.

Thank you for considering our manuscript for publication in JOVE.

Yours sincerely,

Katharina Scheurlen (on behalf of all co-authors)

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

This has been done, treating the abstract and the article separately.

2. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials, e.g., American Type Culture Collection (ATCC TIB202); BD Pharmingen, San Diego, USA; BD FACSCalibur; CellQuest software; accutase;

All commercial language has been removed. Companies and their headquarters are now listed in the Table of Materials.

3. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion (e.g., the note from lines 127-133). Some material in other notes could actually be included in the protocol in the imperative tense (e.g., Prepare a stock solution of PMA …)

The discussion has been moved and notes have been included in the protocol where appropriate.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions

should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The whole protocol is written in the imperative tense now. Notes are used sparingly.

5. 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 ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

More details have been added to the steps and the former “Notes” sections are included in the protocol now, describing steps in the imperative tense.

6. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. This will ensure that filming will be completed in one day. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

The important steps that should be included in the video are now highlighted.

7. Please add some brief description to Figure 2’s legend.

The description has been added.

8. Please remove TM symbols from the text, e.g., Figure 4’s legend.

All symbols have been removed. Product names that include a company branding have been changed.

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names.

The reference format has been adjusted.

Reviewer's comments

Reviewer #1

Major Concerns:
My main concern is that as the author said in the discussion section, the THP1 cells may not fully represent the induction process of mononuclear macrophages derived from the original body.

Thank you for pointing this out - this is a general limitation of the THP-1 cell line model that is often discussed. The discussion section was changed to further explain the THP-1 cell line as a legitimate model for primary monocytes.

The following text has been added on p.7, line 294:
"Cell culture studies using primary macrophages, however, can show variable cellular responses and mechanisms can be masked due to cell heterogeneity [26]. The THP-1 cell line is an established model system for primary human monocytes [9]. Due to the homogenous THP-1 cell population in a controlled culture setting, cellular responses are potentially more precisely reproducible. Furthermore, certain techniques optimize THP-1 cells as a model to resemble primary monocytes."

Minor Concerns:
None mentioned.

-

Reviewer #2:

Major Concerns:
Other M2 markers could have been examined, for example, an IRF4, as well as cell surface markers.

The characterization of macrophages is important to define the cells that are used for experiments at baseline and to create interstudy reproducibility. The representative results that are demonstrated as part of this paper are only part of cell characterization. Further results are described now in the following section (detailed results to be published, manuscript currently under preparation for submission).

The following text has been added on p. 5, Line 215:
"Further characterization of the M2-like macrophages created by this protocol was performed using quantitative real-time PCR (qRT-PCR). M2-like macrophages showed an upregulation of IL-6 and C-X-C Motif Chemokine Ligand 10 (CXCL10) compared to THP-1 monocyte-like cells, as well as an upregulation of the anti-inflammatory markers CD206, Interleukin 10 (IL-10) and C-C Motif Chemokine Ligand 18 (CCL18) (data not shown)."

Minor Concerns:

It might take too long time to rest at M0 stage. The reviewer thought that 48 - 72 hours could be enough for it.

This is an important aspect, since many different time periods of cell rest are reported. The length of cell rest after PMA treatment has a beneficial impact on inflammatory cytokine expression and subsequent macrophage polarization. In addition, a resting period of five days ensures that THP-1 macrophages develop similar characteristics to differentiated monocyte-derived cells concerning cell morphology and surface adherence.

The following text has been inserted on p. 2, Line 77:
"This altered cytokine expression profile as a response to PMA can affect and prevent subsequent macrophage polarization [20]. Adequate resting periods, as reported before after PMA treatment, allow these inflammatory responses to decrease and facilitates cell polarization into a distinct M2-like phenotype [21]."

and on p. 7, Line: 298:
"An important step is the resting period of five days after PMA treatment, which increases cytoplasmatic volume and cell surface adherence similar to that of differentiated monocyte-derived cells [21]."