

# Journal of Visualized Experiments

## Polarization of M1 and M2 Human Monocyte-Derived Cells and Analysis with Flow Cytometry Upon Mycobacterium tuberculosis Infection --Manuscript Draft--

<b>Article Type:</b>	Invited Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE61807R1
<b>Full Title:</b>	Polarization of M1 and M2 Human Monocyte-Derived Cells and Analysis with Flow Cytometry Upon Mycobacterium tuberculosis Infection
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<b>Additional Information:</b>	
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Karolinska Institutet  
Stockholm, June 30, 2020

To: JoVe

Dear Jaydev Upponi,

As per our correspondence and oral conversation in March 2020, we would like you to consider the following manuscript for submission to the Journal of Visualized Experiments entitled "Polarization of M1 and M2 human monocyte-derived cells and analysis with flow cytometry upon *Mycobacterium tuberculosis* infection" by Akhirunnisa Mily *et al.*, which represents original, unpublished work that has not been submitted or accepted elsewhere. Based on the ICMJE recommendations, all authors have contributed to this work and approved the submitted version of this manuscript. No writing assistance has been provided in the preparation of this manuscript.

Although the experimental protocol has been previously described by us in a scientific report recently published in Frontiers Immunology (PMID: 32038652), the current manuscript contains original data based on this protocol. We have focused the analysis to a 10-color flow cytometry of *M. tuberculosis*-infected M1- and M2-polarized macrophages and use novel applications in FlowJo to analyze our data. The basic experimental set up of this M1/M2 flow cytometry protocol can be used as a backbone that is compatible with most old as well as new flow cytometers and can be built upon and tailored according to individual needs including the challenges posed by work with virulent *M. tuberculosis* in a BSL-3 environment. We anticipate that this protocol can be valuable for studies on immunopathogenesis in human TB and may also be adopted for use in other areas of macrophage research.

Yours sincerely,

Assoc. Prof. Susanna Brighenti  
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Stockholm, Sweden

**TITLE:**

Polarization of M1 and M2 Human Monocyte-Derived Cells and Analysis with Flow Cytometry Upon *Mycobacterium tuberculosis* Infection

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**KEYWORDS:**

macrophage, human, polarization, M1/M2, *Mycobacterium tuberculosis*, infection, immune response, flow cytometry

**SUMMARY:**

This protocol provides a method to study *Mycobacterium tuberculosis* infection in human M1- or M2-polarized macrophages based on differentiation of peripheral-blood-monocytes to macrophage-like cells that are infected with the GFP-labeled virulent strain H37Rv, and analyzed with flow cytometry using a 10-color panel including expression of selected M1/M2 markers.

**ABSTRACT:**

Human macrophages are primary host cells of intracellular *Mycobacterium tuberculosis* (Mtb) infection and thus have a central role in immune control of tuberculosis (TB). We have established an experimental protocol to follow immune polarization of myeloid-derived cells into M1 (classically activated) or M2 (alternatively activated) macrophage-like cells through assessment with a 10-color flow cytometry panel that allows visualization and deep-characterization of green-fluorescent-protein (GFP)-labeled Mtb in diverse macrophages subsets. Monocytes obtained from healthy blood donors were polarized into M1 or M2 cells

using differentiation with granulocyte macrophage-colony-stimulating factor (GM-CSF) or macrophage-colony stimulating factor (M-CSF) followed by polarization with IFN- $\gamma$  and lipopolysaccharide (LPS) or IL-4, respectively. Fully polarized M1 and M2 cells were infected with Mtb-GFP for 4 hours before detached Mtb-infected macrophages were stained with flow cytometry at 4- or 24-hours post-infection. Sample acquisition was performed with flow cytometry and the data was analyzed using a flow cytometry analysis software. Manual gating as well as dimensionality reduction with Uniform Manifold Approximation and Projection (UMAP) and phenograph analysis was performed. This protocol resulted in effective M1/M2 polarization characterized by elevated levels of CD64, CD86, TLR2, HLA-DR and CCR7 on uninfected M1 cells, while uninfected M2 cells exhibited a strong up-regulation of the M2 phenotype markers CD163, CD200R, CD206 and CD80. M1-polarized cells typically contained fewer bacteria compared to M2-polarized cells. Several M1/M2 markers were downregulated after Mtb infection, which suggests that Mtb can modulate macrophage polarization. In addition, 24 different cell clusters of different sizes were found to be uniquely distributed among the M1 and M2 uninfected and Mtb-infected cells at 24-hours post-infection. This M1/M2 flow cytometry protocol could be used as a backbone in Mtb-macrophage research and be adopted for special needs in different areas of research.

## INTRODUCTION:

Macrophages are immune cells that contribute significantly to the regulation of tissue homeostasis, inflammation, and disease pathologies. Being an essential component of innate immunity, the monocyte-macrophage lineage of cells expresses heterogeneous phenotypes in response to altered environmental cues, which reflect their plasticity and adaptation to different anatomical and immunological locations<sup>1</sup>. Depending on the growth factors, cytokines and other mediators present in the microenvironment, macrophages have been categorized into two major reversible populations, each with a different role in bacterial control and clearance<sup>2</sup>: the pro-inflammatory, classically activated M1-polarized macrophages and the anti-inflammatory, alternatively activated M2-polarized macrophages that were originally named to mimic T helper (Th) cell nomenclature<sup>3</sup>. This grouping of immune polarized macrophages is often considered simplistic, as macrophage activation and differentiation is not linear, but more accurately illustrated as a continuum where each population has different characteristics and functional roles in the outcome of disease development and progression<sup>4-7</sup>. Nevertheless, there are numerous experimental advantages with the M1/M2 macrophage model that can be used in several different fields of research.

*Mycobacterium tuberculosis* (Mtb) is the causative agent of tuberculosis (TB) and has been estimated to infect one person every second and is considered the most lethal single infectious agent in the world (Global TB report 2019). Since the respiratory tract is the main route of Mtb infection, alveolar macrophages are the preferred host cells to be infected with Mtb and represent both the primary barriers and the infectious reservoir for Mtb in the lungs. Macrophage polarization in response to different stimuli has been extensively studied over the years<sup>7</sup> and in most of the published work, M1 polarization of monocyte cultures in vitro is induced by Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) together with IFN- $\gamma$  and LPS<sup>8,9</sup>, while M2 polarization is induced with Macrophage Colony Stimulating Factor (M-CSF) and IL-

4<sup>10,11</sup>. The M1 macrophages are potent effector cells that mediate antimicrobial responses against intracellular pathogens and have an essential role in antitumor immunity<sup>12</sup>. M2 macrophages, on the other hand, have an anti-inflammatory function, high phagocytic capacity and are mainly involved in wound healing and tissue repair as well as in parasite infections<sup>12</sup>. Accordingly, M1 macrophages are viewed as more effective in intracellular control of Mtb compared to M2 macrophages<sup>13</sup>. However, Mtb bacteria also have the potential to modulate macrophage polarization to subvert innate immunity<sup>14-17</sup>.

While it is common to generate macrophages from differentiation of monocytes obtained from peripheral blood<sup>18</sup>, macrophages could also be generated from induced pluripotent stem cells (iPSCs)<sup>19</sup> or from bone marrow-derived macrophages from mice<sup>20,21</sup>. These are feasible techniques to study primary macrophage cells obtained from monocyte/macrophage progenitors that will proliferate and differentiate into a homogenous population of mature macrophage-like cells. However, these protocols rarely provide deepened knowledge on the phenotype and function of the cells obtained nor account for the natural heterogeneity observed among macrophages obtained in vivo. As Mtb is a strict human pathogen, there is also an advantage to study Mtb in humanized model systems. Flow cytometry is a powerful technology that offers the possibility to assess multiple phenotypic and functional characteristics of single cells in suspension<sup>22</sup>, something that could be fairly challenging with adherent cells such as macrophages that are also known to be autofluorescent<sup>23,24</sup>. In addition to chemical detachment of firmly adherent macrophages, Mtb infection may pose a significant stress factor to the cells that adds another level of complexity in flow cytometric analyses of Mtb-infected macrophages.

In this experimental protocol, we have used a previously established human macrophage infection model based on immune polarization of primary peripheral-blood-monocyte-derived cells that are infected with the virulent laboratory Mtb strain H37Rv, and analysed with flow cytometry using a 10-color panel including expression of selected M1 and M2 markers<sup>25</sup>. This protocol provides an efficient and reproducible method to study responses to Mtb infection in M1 or M2 polarized monocyte-derived macrophages. In addition, the use of flow cytometry on adherent Mtb-infected macrophages allows us to study a variety of surface markers associated with conventional M1 and M2 macrophages and their longitudinal response to Mtb infection. Importantly, this protocol can easily be adopted for investigations of infections with other pathogens, in anti-tumor studies or in studies of inflammatory conditions, for drug screening etc. and could also be exploited for assessment of M1/M2 macrophage polarization in human clinical samples.

## **PROTOCOL:**

Human peripheral blood from healthy anonymous blood donors was obtained from the blood bank at Karolinska University Hospital, Huddinge, Sweden (ethical approval Dnr 2010/603-31/4). All experimental steps involving live virulent Mtb were performed at the Biosafety Level-3 (BSL-3) laboratory at the Public Health Agency of Sweden (FOHM), Solna, Sweden.

### **1. Preparation of media, buffers, and bacterial cultures**

NOTE: Details about all the reagents and consumables are provided in the **Table of Materials**.

1.1. RPMI complete medium: Supplement RPMI 1640 with 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES, and 10% heat-inactivated fetal bovine serum (FBS). Avoid antibiotics in the cell culture medium when working with Mtb infection.

1.2. Serum-free RPMI medium: Supplement RPMI 1640 with 1 mM sodium pyruvate, 2 mM L-glutamine and 10 mM HEPES.

1.3. Wash buffer: Make this with PBS containing 0.05% (v/v) Tween-80.

1.4. FACS buffer: Prepare phosphate buffer saline (PBS) containing 2.5% (v/v) FBS and 0.5 mM EDTA.

1.5. Fixation buffer: Add 4% formaldehyde to PBS. Ensure it is freshly prepared before use, e.g., mixed from a stock solution of 37% formaldehyde.

1.6. Permeabilization buffer: Add 0.1% sodium citrate and 0.1% Triton X-100 to deionized water.

1.7. Wash buffer (for immunofluorescence): Make this with PBS containing 0.1% BSA and 0.1% Tween-20.

1.8. Blocking buffer: Add 0.1% BSA and 10% normal goat serum (NGS) to PBS.

1.9. Staining buffer (for immunofluorescence): Prepare by adding 0.1% BSA to PBS.

1.10. TB complete medium: Supplement Middle Brook 7H9 broth with 0.05% (v/v) Tween-80, 0.5% (v/v) glycerol, kanamycin (20 µg/mL), 10% (v/v) Middlebrook oleic acid, albumin, dextrose and catalase enrichment (Middlebrook OADC Enrichment).

1.11. Bacterial cultures: Use the standard virulent Mtb laboratory strain, H37Rv, constitutively expressing green fluorescent protein (GFP), for infection of monocyte-derived cells. This Mtb strain carries a pFPV2 plasmid that contains a gene encoding GFP, as well as a gene for kanamycin resistance. The antibiotic resistance enables continuous selection of plasmid-expressing bacteria in cultures containing kanamycin. Store bacteria in TB complete medium and 70% glycerol (1:1 dilution) at -80 °C.

## **2. Peripheral blood mononuclear cell isolation from buffy coats**

NOTE: Perform all work with human blood (potentially contagious) inside a class II biosafety hood. Inactivate residual blood products with disinfectants for 15 min before discarding. Blood was obtained from healthy volunteers in this case. This in vitro macrophage differentiation protocol was set up to include  $10 \times 10^6$  isolated PBMCs/donor/well. From each donor, one buffy coat contains about 50 mL of a concentrated leukocyte suspension originating from whole blood,

which normally provides  $500\text{--}800 \times 10^6$  PBMCs from which approximately 10% or  $50\text{--}80 \times 10^6$  monocytes can be retrieved.

2.1. Load 15 mL of buffy coat blood on top of 15 mL of density gradient medium prepared in 50 mL tube. Slowly overlay blood on top of the density gradient layer by leaning the pipette tip to the wall of the tube.

2.2. Spin the tubes at  $600 \times g$  for 25 min at room temp (RT) with 0 acceleration and 0 deceleration.

NOTE: Close lids carefully before centrifugation and always check the tube holders for potential spill over after centrifugation.

2.3. Remove the top plasma layer with a sterile Pasteur pipette and thereafter carefully collect the mononuclear cell layer into a new 50 mL tube using a sterile Pasteur pipette. Add serum-free RPMI medium to the PBMC pellet to obtain a final volume of 50 mL. Mix carefully by inverting the tube a few times before centrifugation at  $500 \times g$  for 5 min at RT.

2.4. Discard the supernatant carefully and resuspend the cell pellet by flipping the bottom of the tube within the fingers.

2.5. To remove the density gradient medium contamination from the PBMCs, wash cells 2–3 times with serum-free RPMI to obtain a final volume of 50 mL. Centrifuge at  $500 \times g$  for 5 min at RT. Wash until the cell supernatant becomes transparent.

2.6. Discard the supernatant and resuspend the cells in 20 mL of serum-free RPMI medium.

2.7. Count the cells by trypan blue staining, manually using a hemocytometer or using an automated cell counter. Dilute the cell suspension in trypan blue in 1:2 or 1:10 dilution by mixing the cell-trypan blue sample in a 96 well plate e.g.,  $50 \mu\text{L} + 50 \mu\text{L}$  (for hemocytometer counting) or  $10 \mu\text{L} + 10 \mu\text{L}$  (for automated cell counting) and count the cells to obtain the number of live cells/mL.

CAUTION: Trypan blue is toxic and must be discarded in a separate chemical waste.

### 3. Differentiation and polarization of monocyte-derived cells

NOTE: For differentiation and polarization of monocyte-derived cells, a protocol that we previously established for M0, M1-like and M2-like cells as well as fully M1 and M2 polarized cells was followed<sup>25</sup>. For simplicity, only fully polarized M1 and M2 macrophages are described here.

3.1. Use plastic adherence for isolation of monocytes. Briefly, seed freshly isolated PBMCs in a 6 well culture plate at an appropriate concentration, e.g.,  $10 \times 10^6$  PBMCs/well in 2 mL serum-free RPMI medium and incubate at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

3.2. After 2–3 h, remove the non-adherent cells with a pipette and wash the wells 3 times with 1 mL serum-free medium. The attached cells are monocytes and comprises around 10% of the total PBMCs added to the well, i.e.,  $10^6$  monocytes retrieved from  $10 \times 10^6$  PBMCs added per well.

3.3. For macrophage differentiation, prepare a working solution containing 50 ng/mL GM-CSF or M-CSF for M1 and M2 macrophage polarization respectively, added in 2 mL of RPMI complete medium per well. Culture the cells in a 5% CO<sub>2</sub> incubator in 37 °C for 3 days.

3.4. On the day 3, remove 1 mL of the cell culture medium carefully from the top layer of each well and supplement the cell cultures with 1 mL of fresh RPMI complete medium containing the double concentration of M-CSF or GM-CSF to obtain 50 ng/mL final concentration in the wells. Add the growth factors in a pre-made working solution of 100 ng/mL/well.

3.5. On the day 6, add different stimuli for the last 18–20 h of cell differentiation to obtain fully polarized and mature M1 (interferon- $\gamma$ ; IFN- $\gamma$ , and lipopolysaccharide; LPS (E. coli O55:B5)) or M2 (interleukin 4; IL-4) macrophages. For M1 polarization, prepare IFN- $\gamma$  and LPS in RPMI complete medium and add 50  $\mu$ L per well to obtain a final concentration of 50 ng/mL IFN- $\gamma$  and 10 ng/mL LPS in the cell cultures. For M2 polarization, prepare IL-4 in RPMI complete medium and add 50  $\mu$ L per well to obtain a final concentration of 20 ng/mL in the cell cultures.

3.6. For differentiation of M0 polarized macrophages, stimulate the cells with M-CSF only, without any additional cytokines (providing an M2-like phenotype)<sup>25</sup>.

3.7. Check the morphology of monocyte-derived cell cultures regularly with light microscopy to ensure that smaller monocytes are differentiated into larger macrophage-like cells. Also, monitor potential morphological differences between the M1 and M2 polarization, i.e., elongated and stretched M1 cells compared to M2 cells with a more rounded shape<sup>25</sup>.

3.8. On the day 7, transfer the plates with monocyte-derived cells to a BSL-3 laboratory for infection with virulent Mtb.

#### 4. Preparation of Mtb cultures

NOTE: The following steps must be performed in a BSL-3 facility. For all work with virulent Mtb, use protective clothing, respiratory protection, and ethanol resistant gloves.

4.1. Thaw a vial with 1 mL of bacterial aliquot and mix with 9 mL of TB complete medium (1:10 dilution) in a 50 mL filtered cap tube. Culture the suspension in an incubator at 37 °C and 5% CO<sub>2</sub>.

4.2. After 24 h, spin the bacterial suspension at  $2,300 \times g$  for 10 min and carefully pour off the medium. Resuspend the bacterial pellet with 15–20 mL of fresh TB complete medium in a new 50 mL filtered cap culture tube and incubate at 37 °C and 5% CO<sub>2</sub>. Mix the settled-down bacteria in the tube every 2–3 days to maintain a homogenous nutrient supply for all bacterial cells.



4.3. After 7–10 days, mix the bacterial suspension properly by pipetting up and down before transferring to a 50 mL screw cap tube.

4.4. Add 35–40 mL of sterile wash buffer to the 50 mL tube and spin the bacterial suspension at  $2,300 \times g$  for 10 min. Repeat the washing steps once. Resuspend the bacterial pellet in 1 mL of serum-free RPMI medium by pipetting with a micropipette.

4.5. Add another 9 mL of serum-free RPMI medium and sonicate the bacterial suspension inside a class II biosafety cabinet for 5 min at 37 °C, to disrupt the bacterial clumps. Dip the tube repeatedly (3–4 times) in the water bath sonicator to ensure maximum disruption of bacterial clumps. Measure the optical density (OD) of 1 mL of bacterial suspension at 600 nm wavelength using a spectrophotometer placed inside the biosafety cabinet. Use serum-free RPMI medium to set the reference.

4.6. Calculate the number of colony forming units (CFU) using the formula:  $(OD+0.155)/0.161 = Y$ , and  $Y \times 10^7 = Y \times 10^6$  CFU/mL, e.g., an OD value 0.32 provides a bacterial concentration of  $(0.32 + 0.155)/0.161 = 2.95$ ,  $2.95 \times 10^7 = 29.5 \times 10^6$  CFU/mL.

## 5. Mtb infection of monocyte-derived cells

NOTE: The following steps must be performed in a BSL-3 facility.

5.1. Resuspend the bacterial pellet in serum-free RPMI medium in a new sterile 50 mL tube and adjust the final bacterial concentration to approximately  $5 \times 10^6$  CFU/mL.

5.2. Remove the cell culture medium from the wells in the 6 well plate(s) containing monocyte-derived cells. Add 1 mL of serum-free RPMI medium to each well. Add 1 mL of bacterial suspension per well to obtain a multiplicity of infection (MOI) 5:1, i.e.,  $5 \times 10^6$  CFU per  $10^6$  macrophages in 2 mL/well and incubate the plates for 4 h in 37 °C and 5% CO<sub>2</sub>.

5.3. After infection, wash the cells 3 times with 1 mL of sterile wash buffer to remove extracellular bacteria. Tilt the plate and carefully remove the entire wash buffer from the corners. Resuspended the Mtb-infected monocyte-derived cells in 2 mL of RPMI complete medium without antibiotics and proceed to flow cytometry staining or incubate the cells for another 24 h (or other time-points) before flow cytometry.

## 6. Flow cytometry staining of Mtb-infected monocyte-derived cells

NOTE: The following steps must be performed in a BSL-3 facility. The flow cytometry staining could be performed in a 96 well plate instead of tubes.

6.1. Detach the Mtb-infected cells (and uninfected controls) from the wells in the 6 well plate(s) by incubation with 1 mL of FACS buffer per well for at least 30 min at 37 °C and 5% CO<sub>2</sub>.

6.2. Gently pipette up and down a few times to ensure that the cells are detached. If possible, confirm cell detachment with microscopy. Transfer the cell suspension from each well to a screw capped microcentrifuge tube and spin the tubes at 200 x *g* for 5 min. Discard the supernatant carefully by pipetting.

6.3. Wash the cell pellet in each tube twice with FACS buffer and spin the cells at 200 x *g* for 5 min.

6.4. Stain the cells (about  $0.5 \times 10^6$  to  $1 \times 10^6$  cells/tube) with approximately 50  $\mu$ L cocktail of fluorochrome-conjugated anti-human antibodies including TLR2 (AF647), CD206 (APC-Cy7), CD163 (BV605), CD80 (BV650), CCR7 (BV711), CD86 (BV786), CD200R (PE), CD64 (PE-Dazzle 594), HLA-DR (PE-Cy5) (**Table 1**) in combination with viability dye Zombie-UV for 30 min at 4 °C (refrigerator) in the dark.

6.5. Wash the stained cells twice with 400  $\mu$ L of FACS buffer and spin the cells at 200 x *g* for 5 min.

6.6. Fix the stained cells with 200  $\mu$ L of fixation buffer (freshly prepared) for 30 min at RT in the dark to ensure complete inactivation of mycobacteria.

6.7. Wash the cells twice with 400  $\mu$ L of FACS buffer and spin at 200 x *g* for 5 min to remove excess fix buffer.

6.8. Resuspend the fixed cells in 400  $\mu$ L of FACS buffer and transfer the samples into new 1 mL microcentrifuge tubes before taking them out of the BSL-3 laboratory for flow cytometry in BSL-2. Store the stained cells in +4 °C until sample acquisition.

NOTE: Spray the tubes with 70% ethanol before taking them out of the BSL-3 laboratory. Formaldehyde is toxic (carcinogenic) and must be handled in a class II biosafety cabinet. Discard formaldehyde waste in a separate chemical waste.

## **7. Flow cytometric data acquisition and analysis of Mtb-infected monocyte-derived cells**

NOTE: Steps 7.1–7.2 should be performed in advance of the flow cytometry staining described above. To avoid problems with cell clumping and dissociation of fluorochromes after cell fixation, sample acquisition of both Mtb-infected and uninfected cells is performed within 4-10 h after primary antibody staining.

7.1. Before flow cytometry staining described above, compensate the fluorescent signal for each fluorochrome-conjugated antibody listed in the staining panel (**Table of Materials**) using compensation beads (both positive and negative).

7.2. Titrate the antibody dilution for staining of human macrophages to obtain the optimal signal for each fluorochrome.

7.3. Use unstained cells to determine the level of background fluorescence necessary to set gate for the negative cell population allowing for the stained cells to be visualized (macrophages are highly auto fluorescent).

7.4. Acquire a minimum of 50,000 cells/sample in the flow cytometer using the recommended software for data acquisition.

7.5. Export the acquisition files from the flow cytometer in FCS format 3.1.

7.6. Analyze the FCS files in flow cytometry analysis software.

7.7. Gate macrophages according to their forward- and side scatter (FSC and SSC) characteristics and exclude dead cells by live/dead cell gating using the Zombie-UV viability dye.

7.8. Visualize H37Rv-GFP infected macrophages in the FITC channel.

7.9. Identify the frequency of positively stained cells and geometric mean fluorescence intensity (MFI) for all phenotypes (**Table 1**).

## **8. Immunofluorescence staining of Mtb-infected monocyte-derived cells**

NOTE: Mtb infection must be performed in a BSL-3 facility.

8.1. For immunostaining, seed  $2 \times 10^5$  PBMCs/well in 500  $\mu$ L of serum-free RPMI medium into an 8 well chamber slides to obtain  $2 \times 10^4$  monocytes/well. After differentiation and M1/M2 polarization of monocytes, proceed with Mtb infection as described above. Fix the slides after 24 h of Mtb infection with fixation buffer for 30 min. Fixed slides are stored in the freezer at  $-20^\circ\text{C}$  until further analyses.

8.2. Wash the monocyte-derived cells twice with 200  $\mu$ L of PBS for 10 min each.

8.3. Permeabilize the cells with 200  $\mu$ L of permeabilization buffer for 5 min at RT.

8.4. Wash the cells 3 times with 200  $\mu$ L of PBS for 5 min each.

8.5. Wash the cells twice with 200  $\mu$ L of wash buffer for 5 min each.

8.6. Block non-specific binding with 200  $\mu$ L of blocking buffer for 30 min at RT.

8.7. Dilute the primary antibodies 1:100 in 0.1% BSA solution and incubate the M1 cells with an unconjugated CD64 antibody (Clone: 10.1) and the M2 cells with an unconjugated CD163 antibody (polyclonal) for 2 h at RT.

397 8.8. Next, wash the cells 3 times with 200  $\mu$ L of washing solution for 10 min each.

398  
399 8.9. Dilute the fluorescent labeled secondary antibodies 1:1,000 in 0.1% BSA solution and  
400 incubate the M1 cells with an anti-mouse IgG-Alexa Fluor 594 and the M2 cells with an anti-rabbit  
401 IgG-Alexa Fluor 594 for 1 h at RT.

402  
403 8.10. Wash the cells 3 times with 200  $\mu$ L wash buffer for 10 min each.

404  
405 8.11. Remove the chamber grid and place  $\sim$ 20  $\mu$ L of DAPI mounting medium in each well and put  
406 a 1.5 mm coverslip onto each slide.

407  
408 8.12. Seal the coverslip with a layer of nail polish.

409  
410 8.13. Acquire images using a confocal microscope with lasers emitting at 486 nm for excitation  
411 of GFP (green channel), 402 nm for DAPI (blue) and 560 nm for secondary antibody (red)  
412 respectively.

#### 413 414 **REPRESENTATIVE RESULTS:**

415 A schematic illustration of the cytokine stimulations used for polarization of monocyte-derived  
416 cells to M0 (M2-like cells), M1 (fully polarized M1 cells) and M2 (fully polarized M2 cells) is  
417 presented in **Figure 1A**, while representative images of M0, M1 and M2 cell cultures as well as  
418 M1 cultures at day 0, 3 and 7, are shown in **Figure 1B**. Uninfected M0 cells were used to  
419 demonstrate the basic gating strategy (**Figure 2A**). Initially, the myeloid cells ( $\sim$ 85%) were gated  
420 according to their forward scatter (FSC) and side scatter (SSC) properties including the larger cells  
421 with high granularity and excluding the small-sized debris with a low SSC and FSC that are found  
422 at the bottom left corner of the dot plot. In the second plot, doublets (i.e., cell clumps) were  
423 defined as having an increased area but similar height compared to single cells and were excluded  
424 from further analysis. Therefore, only cells proportionate between FSC-Area and FSC-Height  
425 (single cells) were included inside the slanted shape gate. Next, the Zombie-UV viability dye that  
426 stains the cytoplasmic proteins inside the dead cells, was used to exclude the dead cells from  
427 subsequent analysis. As expected, viable uninfected M0 cells were negative for Mtb-GFP  
428 expression visualized in the FITC-channel.

429  
430 Next, we applied the same gating strategy to uninfected as well as Mtb-infected M1 and M2  
431 macrophages at 4 hours post-infection (**Figure 2B,C**). Two sub-populations were detected in the  
432 FCS/SSC gate of uninfected M1 polarized macrophages; one population with a smaller size (FCS)  
433 and higher granularity (SSC) and the other population with a larger size and lower granularity  
434 (**Figure 2B**), while the main gate of uninfected M2 cells appeared more homogenous (**Figure 2C**).  
435 Both M1 and M2 monocyte-derived cells displayed a vertical shift to higher granularity and  
436 reduced cell size upon Mtb infection, which may reflect an increased complexity inside the cells  
437 caused by uptake of intracellular Mtb bacteria (**Figure 2B,C**). Furthermore, the viability stain  
438 revealed an enhanced cell death (17-22%) among the Mtb-infected M1 and M2 cells at a MOI of  
439 5, compared to uninfected M0 cells (99%) (**Figure 2A-C**) or uninfected M1 and M2 cells (data not  
440 shown). Representative data showed that Mtb-GFP expression (i.e., Mtb infectivity) was

substantially higher in M2 (77% GFP-positive cells) compared to M1 (19% GFP-positive cells) cells after 4 hours of infection (**Figure 2B,C**). After 24 hours of infection, Mtb-GFP expression was 43% and 85% in M1 and M2 cells respectively, suggesting that M1 cells had a relatively higher increase in GFP-expression from 4–24 hour after Mtb infection compared to M2 cells, 126% versus 10.4% increase in GFP-expression in M1 and M2 cells from 4–24 hours, respectively.

To characterize the efficacy of M1/M2 polarization in uninfected monocyte-derived cells, dot plots were used to identify M1 cells that were double-positive for CD64 and CD86 (CD64<sup>+</sup>CD86<sup>+</sup>) and M2 cells that were double-positive for CD163 and CD200R (CD163<sup>+</sup>CD200R<sup>+</sup>; **Figure 3A,B**). The selection of M1/M2 markers was primarily made based on the results from our previous work<sup>25</sup> but also from other studies<sup>26-29</sup>. The quadrants for the stained cells, were set using corresponding gates for unstained M1/M2 cells (**Figure 3A**). None of these markers is exclusively expressed by M1 or M2 cells, but the proportion of positive cells as well as the intensity of the surface expression is different. This was particularly evident from the M1 stain where around 95% of M1 cells and 79% of M2 cells were CD64<sup>+</sup>CD86<sup>+</sup>, but the staining intensity was substantially higher in the M1 subset (**Figure 3A**). Whereas 27% of M1 cells were positive for the M2-marker CD200R, only 1% were positive for CD163, providing 0.5% CD163<sup>+</sup>CD200R<sup>+</sup> M1 cells compared to 63% CD163<sup>+</sup>CD200R<sup>+</sup> M2 cells (**Figure 3A**). After 4 hours of Mtb infection, an increase in the frequency of CD200R<sup>+</sup> cells was observed in Mtb-GFP-positive M1 polarized cells (16%), while CD163-expression was reduced in M2 cells (**Figure 3B**). The heat-map demonstrates a high intensity of GFP-expression in CD163<sup>+</sup>CD200R<sup>+</sup> M2 cells, but also in the CD64<sup>+</sup>CD86<sup>+</sup> M2 subset as compared to the corresponding M1 cells subsets (**Figure 3B**). Overall, the shift in expression of the respective M1 and M2 markers is also visualized in the histograms in **Figure 3C**. Furthermore, Mtb-GFP bacteria were also visualized in CD64<sup>+</sup> M1 cells and in CD163<sup>+</sup> M2 cells by confocal microscopy, which supported an enhanced intracellular uptake and/or growth of Mtb inside M2 compared to M1 cells (**Figure 3D**).

To verify the results of the manual gating, we applied dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP). UMAP analysis showed that Mtb infection for 4 hours was not sufficient to affect the polarization of macrophages, in contrast to 24 hours of infection, which resulted in clearly separated clusters of M1 and M2 uninfected and infected cells (**Figure 4A**). Uninfected M1 macrophages displayed higher expression of CD64, CD86, TLR2, HLA-DR and CCR7 compared to M2 macrophages, while uninfected M2 cells exhibited a strong up-regulation of the M2 phenotype markers CD163, CD200R, CD206 and CD80 (**Figure 4B,C**). In agreement to the manual gating, Mtb infection after 24 hours caused a clear downregulation of CD163, CD200R and CD206 on M2 cells and upregulation of CD86 and HLA-DR on M1 cells (**Figures 4B,C**), which suggests that Mtb can modulate macrophage polarization. Subsequent phenograph analysis (**Figures 4D-F**) identified 24 different clusters of different sizes that were uniquely distributed among the M1 and M2 uninfected and Mtb-infected cells as illustrated in the UMAP graphs (**Figure 4D**), pie charts (**Figure 4E**) and heat-maps (**Figure 4F**). Altogether, these results show promising efficiency of this protocol to generate phenotypically and functionally diverse M1 and M2 polarized cells in vitro that are further modulated by Mtb infection.

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Schematic illustration of in vitro differentiation and polarization of human myeloid-derived cells.** (A) M0 (M2-like), M1 (classically activated) and M2 (alternatively activated) cells are depicted. Monocytes obtained from healthy blood donors were polarized with different cytokines as described in the protocol and infected with the GFP-labeled Mtb strain, H37Rv, for 4 hours before analysis with 10-color flow cytometry. M1-polarized cells typically contain fewer bacteria compared to M2-polarized cells. (B) Microscopic images of fully polarized, uninfected M0, M1 and M2 cells in the 6-well plates at day 7, and representative images of M1 cell differentiation from monocytes at day 0, 3 and 7. Magnification is 20x (upper panel) and 10x (lower panel). Note that the M1 cells are more elongated and stretched compared to the more rounded M0 and M2 cells (upper panel).

**Figure 2: Gating strategy of differentially polarized myeloid-derived cells.** Representative dot plots showing (A) Forward scatter (FSC) and side scatter (SSC) properties of uninfected M0 macrophages. The FSC-A/FSC-H plot shows manual gating of single cells proportionate for area and height. The live cell gate excluded cells that were positive for Zombie-UV (viability dye). Intracellular Mtb was detected by GFP-expression in live cells observed in the FITC channel. (B) Gating of M1 and (C) M2 macrophages showing FCS/SSC dot plots of both in uninfected cells and Mtb-infected cells 4 h and 24 h post-infection.

**Figure 3: Efficacy of the in vitro M1/M2 polarization protocol.** Representative dot plots and quadrant gating showing subset frequencies of M1- and M2-polarized cells using CD64 and CD86 (M1) or CD163 and CD200R (M2) in (A) unstained and stained uninfected cells and (B) Mtb-infected stained cells 4 h post-infection. The dot plots in (B) illustrates fluorescence intensity of GFP-expression (heat map) in M1- and M2-polarized macrophages obtained from different sub-gates. (C) Geometric mean of fluorescence intensity (MFI) was shown in histograms from one representative donor after 4 h of Mtb infection. The MFI values in uninfected M1 (light blue) and M2 cells (light purple) are presented in the upper panel and Mtb-infected M1 (deep blue) and M2 cells (deep purple) are presented in the lower panel. (D) Representative confocal images of uninfected and Mtb-infected M1- and M2-polarized cells is shown. M1 and M2 cells were stained for CD64 and CD163 expression, respectively, using immunofluorescence. Positive surface staining is shown in red and GFP-expressing intracellular bacteria is shown in green. DAPI-stained nuclei are shown in blue color. Scale – 10  $\mu$ m. The magnification of images to the right is 350x.

**Figure 4: Dimensionality reduction with Uniform Manifold Approximation and Projection (UMAP) and phenograph analysis of uninfected and Mtb-infected M1 and M2 cells.** (A) UMAP, created by concatenating 11000 live cells from uninfected and Mtb-infected M1 and M2 cell cultures from two representative blood donors, 4 h (left graphs) or 24 h (right graphs) post-infection. The heatmap for GFP-expression (lower panel) indicates uninfected and Mtb-infected cells. (B-C) MFI or markers expressed in uninfected and Mtb-infected M1 and M2 cells 24 h post-infection, shown as (B) heatmap or (C) bar plots. (D-F) Phenograph analysis identified 24 clusters that are differentially distributed among the uninfected and Mtb-infected M1 and M2 cultures. Clusters 8–13 are unique in uninfected M1 cells, clusters 1–7 are unique in Mtb-infected M1 cells, clusters 20–24 are unique in uninfected M2 cells and clusters 14–19 are unique in Mtb-infected M2 cells. The MFI of each marker in each phenograph cluster is shown in (F). The data is

presented as uninfected M1 (light blue) and M2 cells (light purple) and Mtb-infected M1 (deep blue) and M2 cells (deep purple).

#### **Table 1: List of antibodies used for flow cytometry**

#### **DISCUSSION:**

This experimental protocol describes effective polarization of myeloid-derived cells into M1 or M2 phenotypes including assessment with a 10-color flow cytometry panel that allows visualization and deep-characterization of GFP-labeled Mtb in diverse macrophages subsets. Although TB is an ancient human disease, there is currently no golden standard model to study Mtb-macrophage interactions, and multi-color flow cytometry of macrophages could be complicated as compared to analyses of lymphocyte responses. Few available protocols for in vitro differentiation of human monocytes to macrophages present deep knowledge of the type of macrophages generated. A basic protocol for macrophage polarization and flow cytometric assessment of macrophage activation using a solid panel of markers can likely facilitate such characterization and offer opportunities to explore additional features of polarized cells treated under different conditions. This includes analyses of cells cultured in vitro as well as analyses of cells in vivo in clinical samples, i.e., both PBMC and single-cell suspensions from body fluids (i.e., bronchoalveolar lavage) or homogenized tissue. Accordingly, differentiation and/or activation status of monocytes and macrophages obtained from patients could be related to disease outcome. Expansion of CD16<sup>+</sup>CD163<sup>+</sup> monocytes in peripheral blood have been reported in pulmonary TB patients<sup>30</sup>. An increased frequency of CD163<sup>+</sup> cells was also detected in the inflamed skin of atopic dermatitis patients<sup>31</sup>. Similarly, CD206<sup>+</sup> M2-like macrophages have been shown to inhibit proliferation and differentiation of cells in the microenvironment of adipocyte tissue<sup>32</sup> and to be enriched in bone marrow samples from patients with acute myeloid leukemia (AML)<sup>29</sup>. An elevated ratio of CD64 (M1) to CD163 (M2) cells in whole blood of patients with osteoarthritis was found to be associated to disease severity<sup>33</sup>. Another study used CD86 (M1) and CD163 (M2) to demonstrate that high M1 expression in tissue correlated to worse outcome in a subgroup of malignant brain tumors<sup>34</sup>.

There are several significant advantages of this experimental M1/M2 flow cytometry protocol. This model provides the opportunity to study innate immune responses to virulent Mtb infection and can be developed to contain studies of adaptive immune responses by adding autologous T cells together with M1 or M2 macrophages in mixed-lymphocyte reactions (MLRs). The protocol is also suitable for drug screening and testing of different immunomodulatory and antimicrobial compounds. Here, we have previously studied the effects of vitamin D and the histone deacetylase inhibitor phenylbutyrate on myeloid-derived cells after Mtb infection<sup>25,35</sup>. M1/M2 flow cytometry could also be used to assess macrophage activation after conditioning with cell culture supernatants or patient plasma. While in vivo studies of TB co-infection with HIV or helminths or TB-diabetes co-morbidity could be challenging, the less complex M1/M2 model may facilitate studies of co-morbidities in vitro. Likewise, the protocol could be exploited for transmission studies to examine the Mtb infectivity of cells or to investigate phagocytic as well as antigen presentation capacity of individual M1/M2 cells. M1/M2 flow cytometry is also attractive for use in biomarker and vaccine studies, to follow disease prognosis during treatment

and to test therapies targeting myeloid-derived cells. Importantly, a number of different methods could be applied in parallel to flow cytometry for simultaneous assessment of macrophage polarization phenotypes and functional responses using confocal microscopy (**Figure 3D**), real-time PCR, western blot, multiplex assays and ELISA of soluble factors in the culture supernatant as well as assessment of intracellular bacterial infectivity and growth using GFP-expression (flow cytometry and confocal microscopy) and colony forming units (CFU). Infection of M1 or M2 cells with Mtb-GFP bacteria also enables sorting the uninfected and Mtb-infected cells from the same sample for single cell-RNA sequencing analysis.

The described protocol has also some limitations including both technical and scientific disadvantages. The drawback using monocyte-derived macrophages from human blood donors is that the donor variability often is high and the fact that the cells are not polarized in the physiological environment of human tissues. Large variability in M1/M2 polarization efficacy or Mtb-infectivity between donors may result in problems with both intra- and interexperimental variations, low statistical power, and a need to include many donors to obtain reliable results. In addition, plastic adherence of monocytes from PBMCs result in a donor-dependent number of monocytes/well that may eventually provide an arbitrary MOI that could impact the macrophage polarization and cell viability after Mtb infection. Critical steps in the protocol involves proper washing to prevent other cells types to contaminate the cell cultures that could also affect macrophage polarization. While a too low MOI may mimic latent TB infection, a too high MOI will kill the cells, highlighting the importance of using an appropriate MOI. Furthermore, it could be difficult to retrieve firmly adherent cells upon detachment, which may result in a biased representation of certain macrophages subsets used for flow cytometry analyses. A crucial step in flow cytometry analysis involves proper use of beads compensation matrix and negative controls such as unstained cells or FMO (Fluorescence Minus One) controls to ensure correct manual gating.

Another limitation involves polarization of monocytes derived from blood and not from the local tissue environment. The hallmark of human TB is formation of granulomas in Mtb-infected tissues and thus, immunopathology in TB should preferentially be studied at the local tissue site. However, monocytes are recruited to the lung from peripheral blood upon inflammation/infection, where cells can differentiate into macrophages in the presence of inflammatory cytokines such as GM-CSF<sup>12</sup>. Importantly, in the physiological milieu of tissue in vivo, there is likely a large heterogeneity of macrophage polarization including a mixture and different ratios of diverse M1- and M2-like macrophage populations that contribute to the fate of TB infection<sup>36</sup>. We have previously developed a human organotypic lung tissue model which enables 3D-studies of macrophage-mediated granuloma formation in TB<sup>37</sup>. It could be interesting to exploit the current M1/M2 polarization protocol in combination with the lung tissue model to further study TB granuloma formation, effector functions and M1/M2 ratio in experimental tissue.

This M1/M2 flowcytometry protocol could readily be adapted to include an extended panel of myeloid markers useful for assessment of features associated with inhibitory as well as inflammatory responses. There is a great research interest in inhibitory immune checkpoint



molecules such as PD-1, SIRP- $\alpha$ , IDO and arginases that could modulate macrophage responses<sup>38</sup>. In this context, polarization of myeloid cells could also involve other stimuli that promotes immunoregulatory macrophages (Mreg) or myeloid-derived suppressor cells (MDSC) that has been shown to be involved in several diseases including TB<sup>38</sup>. More advanced flow cytometry panels of M1/M2/Mreg macrophage subsets may also include intracellular staining of cytokines/chemokines IL-1 $\beta$ , TNF- $\alpha$ , IL-10 and MCP-1 or other soluble factors or effector molecules such as inducible nitric oxide (iNOS) and antimicrobial peptides. This could enhance the possibilities to study polyfunctional macrophage responses, similar to what has been extensively described for T cells<sup>39</sup>.

Currently, flow cytometry staining panels can include up to 30-40 colors, which provides the ability to immunophenotype multiple cell subsets and molecules simultaneously. The basic experimental set up of this M1/M2 flow cytometry protocol can be used as a backbone that is compatible with most old as well as new flow cytometers and can be built upon and tailored according to individual needs including the challenges posed by work with virulent Mtb in a BSL-3 environment. Nowadays, dimensionality reduction techniques such as UMAP are available in the new versions of flow cytometry software, which enables analysis of large number of parameters generated in single-cell studies that is essential for improved visualization and interpretation of high-dimensional data<sup>40</sup>. The constant technological improvements in flow cytometry will likely continue in the coming years including the combination of multi-parametric phenotyping together with modern cell sorting capabilities, where this protocol could prove useful in several macrophage based Mtb infection assays.

#### **ACKNOWLEDGMENTS:**

We thank our colleagues at the Public Health Agency of Sweden, Matilda Svensson and Solomon Ghebremichael for assistance in the BSL-3 laboratory.

This work was supported by grants from the Swedish Heart and Lung Foundation (HLF) (2019-0299 and 2019-0302 to SB), the Swedish Research Council (VR) (2019-01744 and 2019-04720 to SB), the Foundation to Prevent Antibiotic Resistance (Resist), Karolinska Institutet Foundations and KID to SB (partial financing of doctoral education for Marco Loreti) from the Karolinska Institutet. ML was supported from the Swedish Children's Cancer Foundation (TJ2018-0128 and PR2019-0100).

#### **DISCLOSURES:**

The authors have nothing to disclose.

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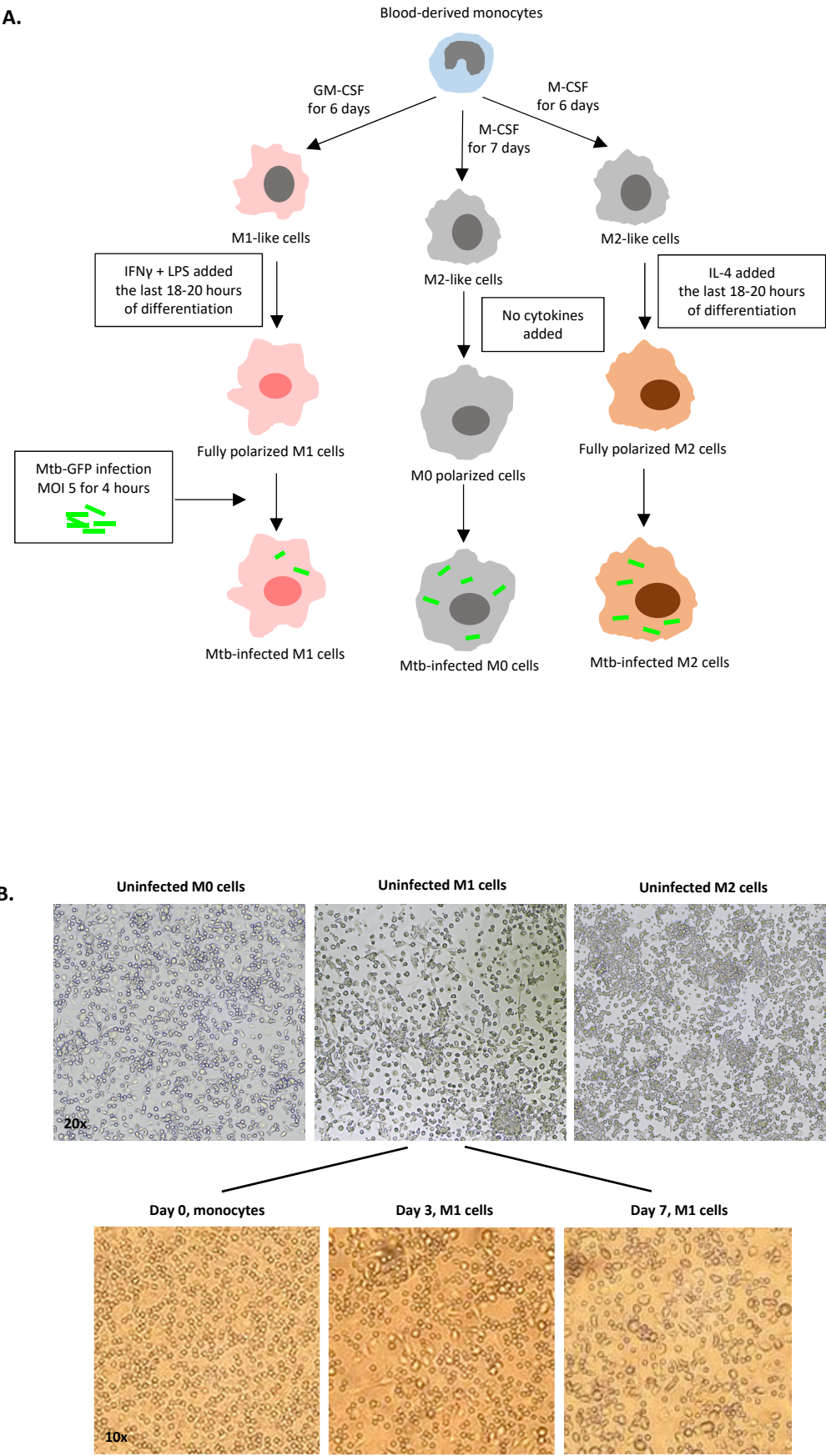
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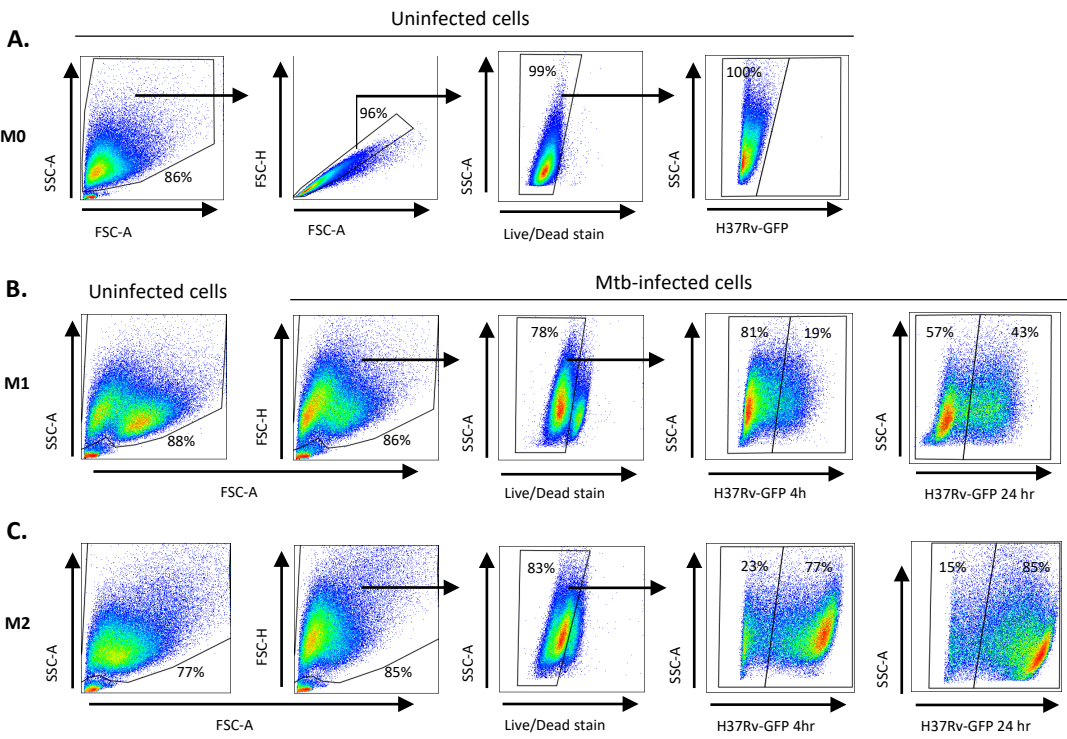
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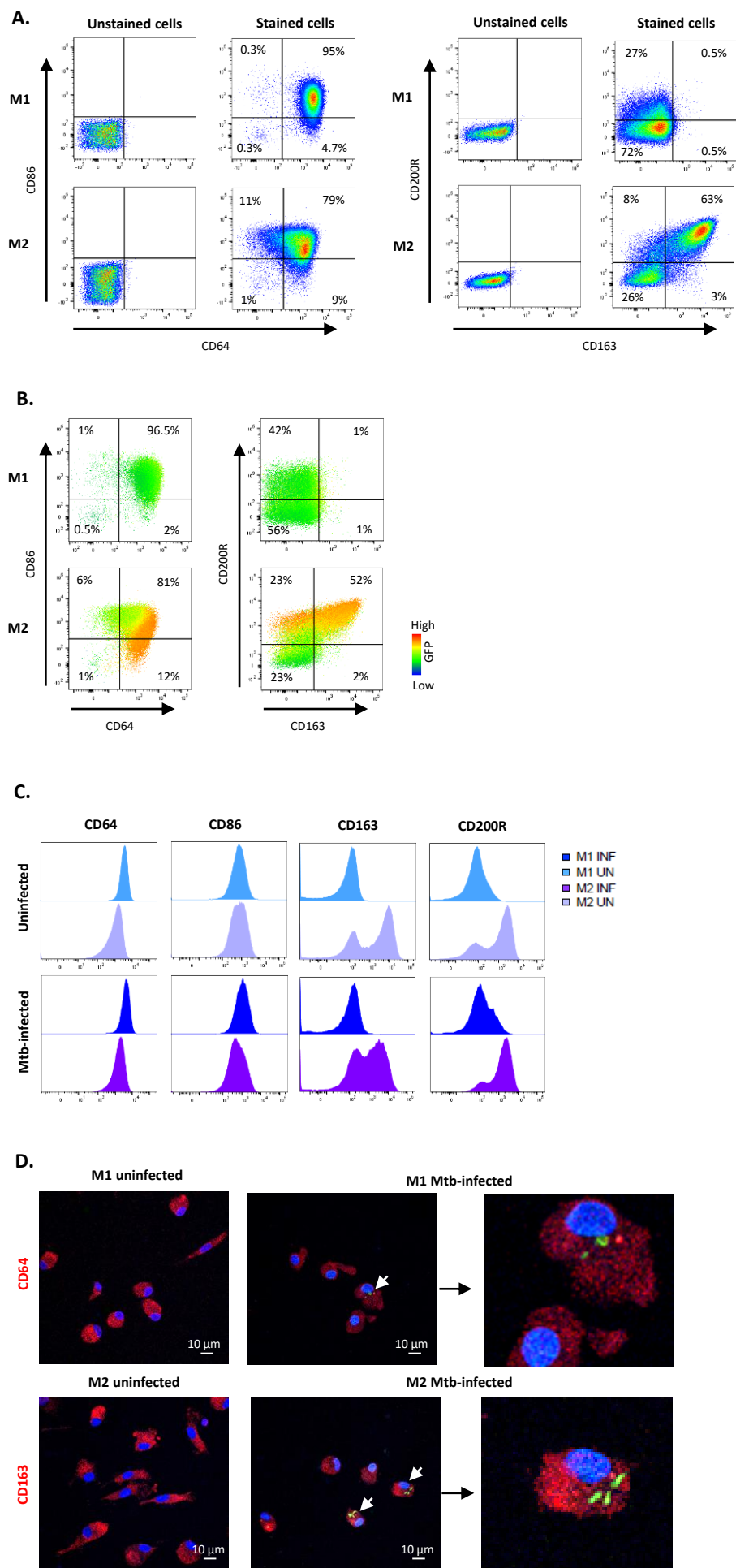
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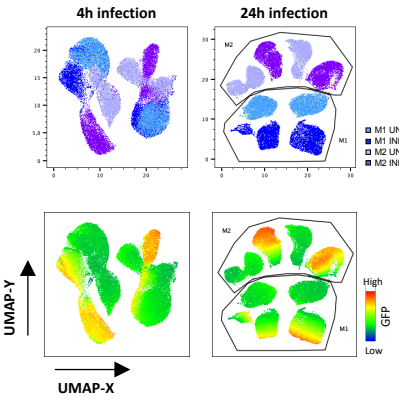
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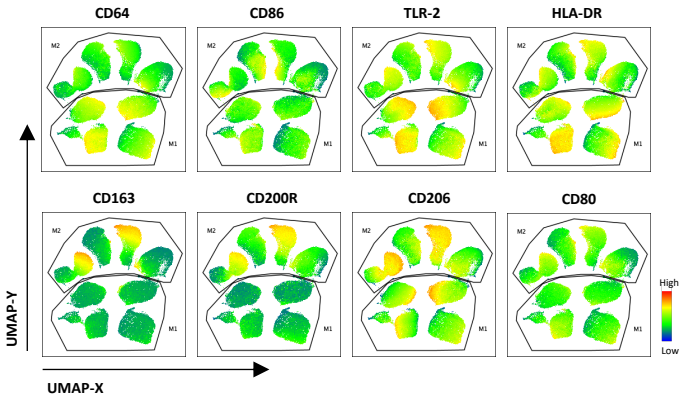




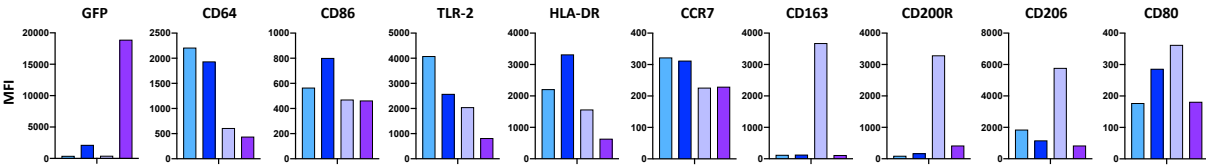
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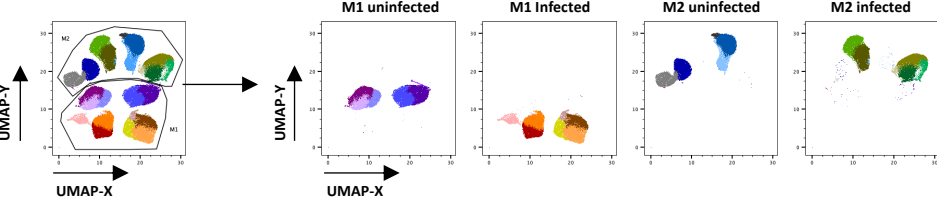
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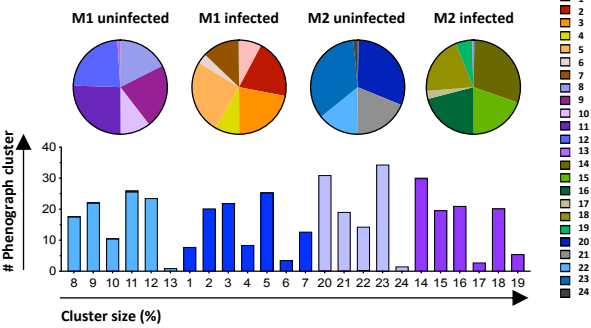
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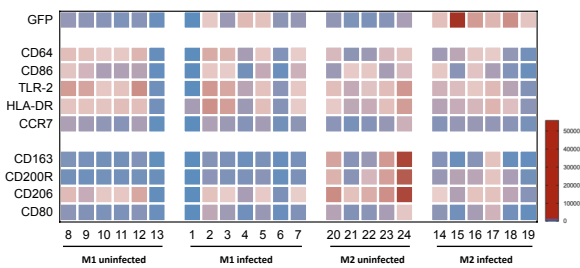
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F.





Laser	Filter	Fluorochrome	Phenotype	Function	Clone	Catalog no.	Company
639	670/30	AF647	TLR2	Pathogen recognition receptor	TL2.1	309714	BioLegend
639	780/60	APC-Cy7	CD206	Mannose receptor	15-2	321120	BioLegend
405	610/20	BV605	CD163	Scavenger receptor	GHI/61	333616	BioLegend
405	670/30	BV650	CD80	Co-stimulatory molecule	2D10	305227	BioLegend
405	710/50	BV711	CCR7	Complement receptor	G043H7	353228	BioLegend
405	780/60	BV785	CD86	Co-stimulatory molecule	IT2.2	305442	BioLegend
488	530/30	GFP	Mtb	Intracellular bacteria			
561	586/15	PE	CD200R	Inhibitory receptor	OX-108	329306	BioLegend
561	620/14	PE/DAZZLE 594	CD64	Fc gamma receptor-I of IgG	Y1/82A	305032	BioLegend
561	661/20	PE-Cy5 (PC5)	HLA-DR	MHC class II molecule	L243	307608	BioLegend
355	450/50	BUV395	Viability Dye	Live/dead cell marker	Zombie UV	423108	Invitrogen

Name of Material/Equipment	Company	Catalog Number	Comments/Description
8-well chamber slides	Lab-Tek	154534	
BD Comp bead plus	BD	560497	
Bovine Serum Albumin	Sigma-Aldrich	A7906	
DAPI Mounting media	Vector Laboratories	H-1200-10	
EDTA (0.5 M)	Karolinska University hospital, Huddinge	N/A	
Falcon 6-well Flat Bottom plates	Corning Life Sciences	353046	
Fetal bovine serum (FBS)	Sigma-Aldrich	F7524	
Formaldehyde	Sigma-Aldrich	F8775	
Glycerol (70%)	Karolinska University hospital, Huddinge	N/A	
GM-CSF	Peprtech	300-03	
Goat anti-mouse IgG Alexa Fluor 594 secondary antibody	Invitrogen	R37121	Secondary antibody for CD64
Goat anti-Rabbit IgG Alexa Fluor 594 secondary antibody	Invitrogen	A-11037	Secondary antibody for CD163
HEPES	GE Healthcare Life Sciences	SH30237.01	
IFN- $\gamma$	Peprtech	300-02	
IL-4	Peprtech	200-04	
L-Glutamine	GE Healthcare Life Sciences	SH30034.01	
LPS (Escherichia coli O55:B5)	Sigma-Aldrich	L6529	
Lymphoprep	Alere Technologies AS	11508545	
M-CSF	Peprtech	300-25	
Middle Brook 7H10 agar plates	Karolinska University hospital, Huddinge	N/A	
Middle Brook 7H9 media	Karolinska University hospital, Huddinge	N/A	
Mouse anti-human CD64 primary antibody	Bio-Rad	MCA756G	Clone: 10.1
Na-pyruvate	GE Healthcare Life Sciences	SH300239.01	
Normal goat serum	Jackson ImmunoResearch	005-000-121	

Rabbit anti-human CD163 primary antibody	GeneTex	GTX81526	Polyclonal
RPMI 1640	Life Technologies Corporation	SH30096.01	
Triton X-100	Sigma-Aldrich	X-100	
TubeSpin bioreactor tubes	TPP Techno Plastic Products AG	87050	
Tween-20	Sigma-Aldrich	P9416	
Tween-80	Sigma-Aldrich	P4780	

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**Point-by-point referee reply:**

Please, note that all changes in the text of the manuscript are marked using track changes in a compare copy (**Marked revision**) of the manuscript file.

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

**Response:** The document is now thoroughly checked for spelling or grammatical issues.

2. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

**Response:** Thank you, we have added more information to the Protocol as requested in the comments below.

3. 2.4: How much RPMI is used here?

**Response:** Now added to the Protocol section 2.4: Add serum-free RPMI medium to the PBMC pellet to obtain a final volume of 50 mL and mix carefully by inverting the tube a few times before centrifugation at 500xg for 5 min at RT.

4. 2.6: How much volume of serum free RPMI is used for the wash?

**Response:** Now added to the Protocol section 2.6: To remove ficoll contamination from the PBMCs, wash the cells 2-3 times with serum-free RPMI to obtain a final volume of 50 mL before centrifugation at 500xg for 5 min at RT. Wash until the cell supernatant becomes transparent.

5. 3.3: Please specify the concentration/composition of the cytokine working solution. Also in a 5% CO<sub>2</sub> incubator?

**Response:** We have rewritten this sentence to clarify how the working solution was mixed and the conditions for culturing the cells in the CO<sub>2</sub> incubator.

6. Please specify the volume and concentrations of all solution used.

**Response:** The volumes and concentrations of all the solutions used are now provided in the Protocol (pages 2-8).

7. 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

**Response:** We have now highlighted the sections in the Protocol that will be used for the video.

8. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

**Response:** Thank you, we have tried to prepare for the 3 pages Protocol for the video in a logical flow.

9. Please spell out all journal titles in the references.

**Response:** The reference list has now been changed to include full journal names.

---

**Reviewers' comments:**

*Reviewer #1:*

Manuscript Summary:

This is an elegant well detailed protocol that will be useful for the scientific community working on host-pathogen interactions

Minor Concerns:

The authors wrote in the Abstract section (line 41-44) " We have established an experimental protocol for immune polarization of myeloid-derived cells into M1 (classically activated) or M2 (alternatively activated) macrophage-like cells including assessment with a 10-color flow cytometry panel that allows visualization and deep-characterization of green-fluorescent-protein (GFP)-labelled Mtb in diverse macrophages subsets " .

The indicated macrophage polarization protocol has been previously used by several teams. The novelty of the protocol is the following by flow cytometry of the polarized Mtb-infected and non-infected cells.

They should change by " We have established an experimental protocol to follow the immune polarization of myeloid-derived cells into M1 (classically activated) or M2 (alternatively activated) macrophage-like cells through the assessment with a 10-color flow cytometry panel that allows visualization and deep-characterization of green-fluorescent-protein (GFP)-labelled Mtb in diverse macrophages subsets"

**Response:** We agree with the reviewer that it is more appropriate to rephrase the sentence according to the reviewer's suggestion. The change has now been included in the Abstract section.

*Reviewer #2:*

Manuscript Summary:

This manuscript by Brighenti et al. describes a method to differentiate macrophages into M1 and M2 subtypes and the appropriate techniques to assess the differentiation process in the absence or presence of M. tuberculosis infection. These methods will be of great assistance for scientists interested in the field. It is easy to follow and to understand. Still, the authors need to address some minor concerns to make it entirely accessible for others.

Minor Concerns:

ABSTRACT

1.-Line 50. Jargon: acquisition is performed using a Flow cytometer, and analysis is performed in FlowJo.

**Response:** Thank you, this sentence is now corrected.

PROTOCOL

1.-TB or RPMI "media." Media is plural of medium. Are the authors sure the plural is their intended form of the word? Please, revise the whole document.

**Response:** We thank the reviewer for observing this mistake. The term "Medium" is now used throughout the manuscript.

2.-Step 4, page 4. "On day 3, remove 1 ml of the cell culture media carefully from the top layer of each well and supplement the cell cultures with 1 mL of fresh RPMI complete media containing a double concentration (1:2) of M-CSF or GM-CSF to receive 50 ng/mL final

concentration in the wells. Add the growth factors in a pre-made working solution of 100 ng/mL/well." Although the authors clarify the final concentrations of the cytokines, the indication 1:2 is confusing. It gives the idea of a dilution instead of a double concentration.

**Response:** The ratio (1:2) of growth factors is removed from the Protocol now, to avoid confusion.

3.-Point 5, lines 220-221, are these concentrations final or stock? The concentration of LPS is extremely low. Lines 221-222. The last sentence is incomplete.

**Response:** To avoid confusion, we have rewritten section 3.5 in the Protocol: On day 6, different stimuli are added for the last 18-20 hours of cell differentiation to obtain fully polarized and mature M1 (interferon- $\gamma$ ; IFN- $\gamma$ , and lipopolysaccharide; LPS (E. coli O55:B5)) or M2 (interleukin 4; IL-4) macrophages. For M1 polarization, prepare IFN- $\gamma$  and LPS in RPMI complete medium and add 50  $\mu$ l per well to obtain a final concentration of 50 ng/mL IFN- $\gamma$  and 10 ng/mL LPS in the cell cultures. For M2 polarization, prepare IL-4 in RPMI complete medium and add 50  $\mu$ l per well to obtain a final concentration of 20 ng/mL in the cell cultures.

4.-Lines 252, 256. The proper name is Biosafety Cabinet (BSC), not "hood." The name was adequately used on line 263.

**Response:** Line 252 and 256 are now corrected according to the reviewer's suggestion.

5.-Staining of infected MDMs. Step 6. The same reason for using a blocking buffer for the chamber slides staining stands for the flow cytometry assays (macrophages express high levels of Fc $\gamma$ R). Why did the authors not use a blocking step before staining here?

**Response:** We have used the protocol with (IVIG) and without a blocking step, but as we did not observe any major changes in the staining with the different primary antibodies, we decided to omit this step. This way, we reduce the work time in the BSL-3 laboratory, which is desirable.

6.-Step 8. Immunofluorescence. Does the fixation buffer contain paraformaldehyde? It would be the right thing to do. I assumed that is what PFA stands for. Why did the authors use formaldehyde for the flow cytometry assays? Please, revise the buffer contents or the reagent table if necessary.

**Response:** We apologize for this mistake, we used the same fixation buffer in flow cytometry and immunofluorescence, which is 4% formaldehyde, mixed from a stock solution of 37% formaldehyde. We have now corrected this mistake in the Protocol section 8.1 and also added which stock is used in the Protocol section 1.5.

7.-Step 7. Acquisition. How long for the acquisition? Immediately after staining? Less than 20 h after staining? The authors have three tandem fluorochromes. Those are prone to dissociation in the presence of paraformaldehyde. Thus, the acquisition must be performed within 20h.

**Response:** This is a valid comment and we have now added to the Protocol section 7: To avoid problems with cell clumping and dissociation of fluorochromes after cell fixation, sample acquisition of both Mtb-infected and uninfected cells was performed within 4-10 hours after primary antibody staining.

We have also added to the Protocol, end of section 6: (store the stained cells in +4°C until sample acquisition)

8.-Step 8. It would be helpful if the storage conditions of the slides are specified.

**Response:** Now added to the Protocol section 8.1: Fixed slides are stored in the freezer at -20 °C until further analyses.

## REPRESENTATIVE RESULTS

1.-Figure 1. It would be helpful to have a photograph of the wells with each of the three macrophages, so the readers know what to expect should they decide to replicate the experiments. I find it difficult to imagine that around 1 million monocytes, or less, would grow successfully in such a vast area (I believe 6-well plates better support 3 million monocytes while differentiating into macrophages).

**Response:** We agree with the reviewer that it could be helpful for the readers to see how the cell cultures look like and therefore we have now included representative images of fully differentiated M0, M1 and M2 cells as well as representative images of M1 differentiation from monocytes at Day 0, 3 and 7. Please, see the new Figure 1B.

Please, also note that our group has set up this macrophage differentiation protocol by culturing 1 million monocytes per well in 6-well plates as published previously this year in *Frontiers Immunology* (Rao Muvva et al 2020; PMID: 32038652). The same differentiation protocol has been used in this manuscript.

2.-Figure 2. It would be helpful to show the negative controls used to set the quadrants on the dot plots.

**Response:** We assume the reviewer suggests adding unstained/negative controls in the dot plots in Figure 3. The quadrant graphs of the unstained negative controls have now been added in Figure 3A.





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Author(s):	Akhirunnesa Mily, Sadaf Kalsum, Marco Giulio Loreti, Rokeya Sultana Rekha, Jagadeeswara Rao Muvva, Magda Lourda and Susanna Brighenti

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