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

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Interview statements: Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **42**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Akhirunnesa Mily**: This protocol uses 10-color flow cytometry to follow the immune polarization of human monocyte-derived cells based on the visualization and deep-characterization of green fluorescent protein-labeled Mtb in diverse macrophage subsets **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Akhirunnesa Mily**: This efficient and reproducible method can be used to study the phenotypic and functional responses of M1 and M2 polarized macrophages before and after Mtb infection **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Peripheral Blood Mononuclear Cell (PBMC) Isolation

2.1. To isolate PBMC (P-B-M-C) from human donor buffy coats, use a pipette to slowly overlay 15 milliliters of buffy coat blood down the side of a 50-milliliter tube onto 15 milliliters of density gradient medium **[1-TXT]** and separate the cells by centrifugation **[2-TXT]**.

2.1.1. WIDE: Talent adding sample to tube, with density gradient medium container visible in frame **TEXT: See text for all medium and solution preparation details**

2.1.2. Talent placing tube(s) into centrifuge **TEXT: 25 min, 600 x g, RT, no brake, no acceleration**

2.2. Use a sterile Pasteur pipette to remove the top plasma layer **[1]** and carefully transfer the mononuclear cell layer into a new 50-milliliter tube **[2]**.

2.2.1. Shot of layers, then plasma layer being removed

2.2.2. Mononuclear cell being collected, with new tube visible in frame

2.3. Add serum-free RPMI (R-P-M-eye) medium to the cells to a final volume of 50 milliliters **[1]** and gently invert the tube a few times before centrifuging **[2-TXT]**.

2.3.1. Talent adding medium to tube, with medium container visible in frame

2.3.2. Talent inverting tube before placing in centrifuge **TEXT: 5 min, 500 x g, RT**

2.4. Then resuspend the cells in 20 milliliters of serum-free medium for counting **[1]**.

2.4.1. Shot of pellet if visible, then medium being added to tube, with medium container and hemocytometer visible in frame

2.4.1.2. Added shot: CU of hemocytometer

3. Monocyte-Derived Cell Differentiation and Polarization

3.1. To initiate macrophage differentiation, dilute the cells to a 5 x 10^6 cells/milliliter of serum-free medium concentration **[1]** and seed 2 milliliters of cells into individual wells of a 6-well plate **[2]**.

3.1.1. WIDE: Talent adding medium to cells, with medium container visible in frame *Videographer: Important step*

3.1.2. Talent adding cells to well(s) *Videographer: Important step*

3.2. Place the plate in the cell culture incubator for 2-3 hours to allow the cells to adhere to the well bottoms **[1]**.

3.2.1. Talent placing plate into incubator

3.3. At the end of the incubation, wash the wells three times with 1 milliliter of serum-free medium per wash **[1]** and add 2 milliliters of complete RPMI medium supplemented with 50 nanograms/milliliter of GM-CSF (G-M-C-S-F) or 50 nanograms/milliliter of M-CSF for M1 or M2 differentiation, respectively, to each well of adherent cells **[2-TXT]**.

3.3.1. Talent washing well(s), with serum free medium container visible in frame *Videographer: Important step*

3.3.2. Talent adding medium to well(s), with medium and growth factor containers visible in frame *Videographer: Important step* **TEXT: GM-CSF: granulocyte-macrophage colony-stimulating factor; M-CSF: macrophage colony-stimulating factor**

3.4. Three days after polarization initiation, carefully replace 1 milliliter of supernatant from each well with 1 milliliter of fresh complete medium supplemented with the appropriate growth factors **[1]**.

3.4.1. Medium being added to well(s), with medium and growth factor containers visible in frame

3.5. On day 6 of culture, add 50 nanograms/milliliter of interferon-gamma and 10 nanograms/milliliter of LPS (L-P-S) to the GM-CSF-treated wells **[1~~-TXT~~]** and 20 nanograms/milliliter of IL-4 (eye-L-four) to the M-CSF-treated wells **[2~~-TXT~~]**.

3.5.1. Talent adding IFN-gamma and/or LPS to well(s), with IFN-gamma and LPS containers visible in frame *Videographer: Important/difficult step* ~~**TEXT: LPS: lipopolysaccharide**~~

3.5.2. Talent adding IL-4 to well(s), with IL-4 container visible in frame *Videographer: Important/difficult step* ~~**TEXT: IL: interleukin**~~

3.6. Check the morphology of the monocyte-derived cell cultures regularly by light microscopy to ensure that the smaller monocytes are differentiating into larger macrophage-like cells **[1]**.

3.6.1. ~~LAB MEDIA: Figure 1B Uninfected image~~s Talent at scope

4. *Mycobacterium tuberculosis* (Mtb) Culture

4.1. To set up an Mtb (M-T-B) culture, in a biosafety level 3 facility, mix a 1-milliliter aliquot of thawed bacteria suspension with 9 milliliters of TB (T-B) complete medium in a 50-milliliter filtered cap tube **[1]** for a 24-hour incubation in the tube in the cell culture incubator **[2]**.

4.1.1. WIDE: Talent adding bacteria to tube, with bacteria vial and medium container visible in frame

4.1.2. Talent placing tube into incubator

4.2. The next day, collect the bacteria by centrifugation **[1-TXT]** and resuspend the pellet in 15-20 milliliters of fresh TB complete medium in a new 50-milliliter filtered cap culture tube **[2]** before returning the bacteria to the cell culture incubator **[3-TXT]**.

4.2.1. Talent placing tube(s) into centrifuge **TETX: 10 min, 2300 x g, RT**

4.2.2. Talent adding medium to tube(s), with medium container visible in frame

4.2.3. Talent placing tube into incubator **TEXT: Mix bacteria every 2-3 d**

4.3. After 7-10 days of culture, wash the bacteria two times in 35-40 milliliters of sterile wash buffer per wash **[1]**, resuspending the bacteria in 1 milliliter of serum-free RPMI medium after the second wash **[2]**.

4.3.1. Talent adding buffer to tube, with buffer container visible in frame

4.3.2. Shot of pellet, then medium being added to tube, with medium container visible in frame Use as 4.4.1.

4.4. Add another 9 milliliters of serum-free RPMI medium to the bacteria **[1]** and sonicate the bacteria in a water bath sonicator in class two biosafety cabinet for 5 minutes at 37 degrees Celsius **[2]**.

4.4.1. Talent adding medium to bacteria

4.4.2. Talent sonicating bacteria

4.5. Then measure the optical density of 1 milliliter of bacterial suspension at a 600-nanometer wavelength in a spectrophotometer placed inside the biosafety cabinet **[1]**.

4.5.1. Talent adding sample to spectrophotometer

5. Monocyte-Derived Cell Mtb Infection

5.1. To infect the monocyte-derived cells, dilute the bacteria to a 5 x 10^6 colony forming units/milliliter concentration in serum-free medium **[1]** and replace the supernatant in each well of the 6-well cell culture plate with 1 milliliter of fresh serum-free medium **[2]** and 1 milliliter of bacteria suspension per well to obtain a multiplicity of infection of 5 **[3]**.

5.1.1. WIDE: Talent adding medium to bacteria, with medium container visible in *Videographer: Important step* frame

5.1.2. Talent adding medium to well(s), with medium container visible in frame *Videographer: Important step*

5.1.3. Talent adding bacteria to well(s), with bacteria container visible in frame *Videographer: Important step*

5.2. After a 4-hour incubation in the cell culture incubator, wash each well three times with 1 milliliter of sterile wash buffer per wash **[1]**, tilting the plate carefully to remove the entire volume of buffer from the corners of the wells after each wash **[2]**. Note: 5.2.1. and 5.2.2. are merged

5.2.1. Talent washing well, with buffer container visible in frame

5.2.2. Shot of tilted plate and buffer being removed

5.3. Then resuspend the Mtb-infected monocyte-derived cells in 2 milliliters of complete medium without antibiotics **[1]**.

5.3.1. Talent adding medium to well(s), with medium container visible in frame

6. Flow Cytometry Staining and Analysis

6.1. To stain the cells for flow cytometry, incubate the infected cells with 1 milliliter of FACS buffer supplemented with 0.5-millimolar EDTA per well for at least 30 minutes **[1-TXT]**. Note: 6.1.1. and extra shot 6.1.2. are merged

6.1.1. WIDE: Talent adding buffer to well(s), with buffer container visible in frame **TEXT: FACS: fluorescence activated cell sorting**

6.2. At the end of the incubation, collect the detached cells with gentle pipetting **[1-TXT]** and transfer the cells into individual screw capped microcentrifuge tubes for centrifugation **[2-TXT]**.

6.2.1. Talent pipetting buffer **TEXT: Confirm detachment by light microscopy**

6.2.2. Talent adding cells to tube(s) **TEXT: 5 min, 200 x g, RT**

6.2.2.1. Extra shot (labeled as 6.2.2. in another version of script)

6.3. Resuspend the cells in approximately 50 microliters of the fluorochrome-conjugated anti-human antibody cocktail and viability dye of interest **[1-TXT]** and incubate the cells for 30 minutes at 4 degrees Celsius protected from light **[2]**.

6.3.1. Talent adding antibod(ies) to tube, with antibody and viability dye containers visible in frame *Videographer: Important step* **TEXT: See text for all Ab and viability dye details**

6.3.2. Talent covering cells/placing cells at 4 °C *Videographer: Important step*

6.3.3. Extra shot: placing cells at 4 °C

6.4. After washing, fix the cells with 200 microliters of freshly prepared fixation buffer for 30 minutes at room temperature protected from light **[1].**

6.4.1. Talent adding buffer to tube(s), with buffer container visible in frame

6.5. After the fixation and washing, resuspend the cells in 400 microliters of FACS buffer **[1]** and transfer the cells to 1-milliliter microcentrifuge tubes **[2]**.

6.5.1. Talent adding buffer to tube(s), with buffer container visible in frame

6.5.2. Talent adding cells to tube(s)

6.6. To analyze the cells, use compensation beads to compensate the fluorescent signal for each fluorochrome-conjugated antibody in the cocktail **[1]** and use unstained cells to set the gate for the negative cell population **[2]**.

6.6.1. Talent loading beads onto cytometer, with bead container(s) visible in frame *Videographer: Important step*

6.6.2. Talent at cytometer, setting gate for negative population, with monitor visible in frame *Videographer: Important step*

6.6.3. Extra shot CU

6.7. Then acquire a minimum of 50,000 cells/sample **[1]**.

6.7.1. Talent loading cells onto cytometer

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
3.1., 3.3., 3.5., 5.1., 6.3., 6.6.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
3.3., 3.5. Avoid repeated freeze-thaw cycles of growth factors.
3.6. To generate fully polarized M1/M2 cells is the most difficult aspect of this protocol. Monitoring cultured cells at a regular basis with microscopy is essential to follow cell morphology and differentiation.

Results

7. Results: Representative Phenotypic M1 and M2 Cell Characterization Before and After Mtb Infection

7.1. Both M1 and M2 monocyte-derived cells display a vertical shift to a higher granularity and reduced cell size upon Mtb infection **[1]**.

7.1.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize both Mtb-infected cells Live/Dead Stain vs SSC-A plots*

7.2. An enhanced cell death is also observed in Mtb-infected M1 and M2 cells at a multiplicity of infection of 5 **[1]** compared to uninfected M0 cells **[2]**.

7.2.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize cells outside of gate in both Mtb-infected cells Live/Dead vs SSC-A plots*
7.2.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize cells outside of gate in Uninfected cells Live/Dead stain vs SSC-A*

7.3. Of note, a substantially higher Mtb-GFP (G-F-P) expression is observed in M2 cells after 4 hours of infection **[1-TXT]** compared to M1 cells **[2]**.

7.3.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize cells in gate in M2 H37Rv-GFP 4h plot* **TEXT: GFP: green fluorescent protein**
7.3.2. LAB MEDIA: Figure 2 *Video Editor: please emphasize cells in gate in M1 H37Rv-GFP 4h plot*

7.4. Uninfected M1 and M2 cells **[1]** can be characterized by their CD64 (C-D-sixty-four) and CD86 **[2]** or CD200 (C-D-two hundred) receptor and CD163 (C-D-one-sixty-three) co-expression, respectively **[3]**.

7.4.1. LAB MEDIA: Figure 3A
7.4.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize M1 row*
7.4.3. LAB MEDIA: Figure 3A *Video Editor: please emphasize M2 row*

7.5. After 4 hours of Mtb infection, an increased frequency of CD200 receptor-positive cells is observed in the Mtb-GFP-positive M1 polarized cells **[1]**, while CD163 expression is reduced in M2 cells **[2]**.

7.5.1. LAB MEIDA: Figure 3B *Video Editor: please emphasize cells in top left quadrant in M1 CD163 vs CD200R plot*

7.5.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize cells in top right quadrant in M2 CD163 vs CD200R plot*

7.6. Mtb-GFP bacteria can also be observed in CD64-positive M1 cells **[1]** and CD163-positive M2 cells by confocal microscopy **[2]**.

7.6.1. LAB MEDIA: Figure 3D *Video Editor: please emphasize green signal in top right image*
7.6.2. LAB MEDIA: Figure 3D *Video Editor: please emphasize green signal in bottom right image*

7.7. UMAP (U-map) analysis **[1-TXT]** reveals that 4 hours of Mtb infection is not sufficient to affect macrophage polarization **[2]**, while 24 hours of infection results in clearly separated clusters of uninfected and infected M1 and M2 cells **[3]** that express distinct surface marker expression profiles **[4]**.

7.7.1. LAB MEDIA: Figure 4A **TEXT: UMAP: Uniform Manifold Approximation and Projection**
7.7.2. LAB MEDIA: Figure 4A *Video Editor: please emphasize 4h infection column*
7.7.3. LAB MEDIA: Figure 4A *Video Editor: please emphasize 24h infection column*
7.7.4. LAB MEDIA: Figures 4B and 4C

7.8. Further, phenograph analysis shows that 24 different clusters of different sizes **[1]** are uniquely distributed among the M1 and M2 uninfected and Mtb-infected cells **[1]**.

7.8.1. LAB MEDIA: Figures 4D-4F *Video Editor: please emphasize uninfected M1 and M2 plots and pie graphs*
7.8.2. LAB MEDIA: Figures 4D-4F *Video Editor: please emphasize infected M1 and M2 plots and pie graphs*

Conclusion

8. Conclusion Interview Statements

8.1. **Akhirunnesa Mily**: The efficacy of M1/M2 polarization and/or Mtb-infectivity can vary substantially between human donors. Therefore, the inclusion of at least two donors in each experiment is recommended to reduce experimental variations **[1]**

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

8.2. **Akhirunnesa Mily**: This model allows the simultaneous assessment of human macrophages using flow cytometry, confocal microscopy, mRNA expression analysis, multiplex assays of soluble factors, and bacterial quantification using GFP-expression or colony forming units **[1]**

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera