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A Simple and Efficient Monocyte Isolation Protocol from Human Peripheral Blood

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Abstract

Monocytes play an important role in immune regulation and are involved in a variety of effector functions. Isolation of human monocytes is essential when studying the functionality of these cells in health and disease. Once isolated, monocytes can then be cultured for functional assays or immunocytochemistry. However, the differentiation and activation state of human monocytes are affected by the culture conditions. Hence to overcome this, freshly isolated monocytes can be studied immediately after the isolation process *via* flow cytometry for immunophenotyping or functional assays acutely.

Our protocol isolates monocytes directly from human peripheral blood by negative selection. The kit used in our protocol removes non-monocytes with antibodies that recognize specific cell surface markers. Monocytes are not labelled with antibody and can easily be collected as a highly enriched population at the interface between the plasma and density gradient medium. Our protocol has a number of advantages: (1) isolated monocytes using our protocol are untouched and free of antibodies and (2) in comparison to other methodology for monocyte isolation, the technique presented in our protocol requires no specific equipment other than centrifuge.

Keywords: Monocyte isolation; Antibodies; Human peripheral blood; Centrifuge

Introduction

Monocytes are phagocytes that originate in the bone marrow from hematopoietic stem cells, and constitute around 10% of the total circulating blood leukocytes [1]. They are characterized by diverse effector functions that include surveillance, activation and propagation of immune responses, as well as phagocytosis [2,3]. Interest in measuring innate immune cell functionality has substantially increased due to emerging literature that suggests the contribution of innate immune cell dysfunction to the pathogenesis of various diseases [4-8]. This growing recognition has fostered the need for isolation techniques to investigate the functionality of innate immune cells *in vitro*. While cell lines are readily available and have extensive proliferative potential [9], the use of primary cells offers additional advantages as they resemble closely to their tissue of origin. Moreover, isolation of distinct blood cell population from patient samples preserves disease-related signatures that could potentially be used as diagnostic or prognostic markers [10].

Monocyte isolation procedures vary widely in terms of purity, yield, cost and volume of peripheral blood required [10-12]. The protocol outlined in this paper describes the isolation of human monocytes by negative selection from small volumes of peripheral blood (~50 mL) with excellent purity (>95%). It is also particularly useful for clinical applications as the method described is well suited to investigations that involve patient populations where a distinct immune cell subset is known to contribute to the pathogenesis or development of the disease.

Materials

Monocyte isolation

Reagents and materials included the following: 5 roller tube roller mixer (Ratek BTR5-12V), 10 mL serological pipettes (Thermo Fisher Scientific Cat# 170356N), 25 mL serological pipettes (Thermo Fisher Scientific Cat# 170357N), bleach (sodium hypochlorite solution), centrifuge 5810 R (Eppendorf Cat# 5810000386), circulated water baths (Thermoline Scientific), Easypet® 3 pipettor (Eppendorf Cat# 4430000018), falcon 50 mL conical centrifuge tubes (Thermo Fisher Scientific Cat# 14-432-22), Lymphopep™ density gradient medium (StemCell Technologies Cat# 07851), RosetteSep™ human monocyte enrichment cocktail (StemCell Technologies Cat# 15068), sterile 1M phosphate-buffered saline (PBS; pH7.4; Thermo Fisher Scientific Cat# 10010023), Sterilin™ plastic transfer pipettes (Thermo Fisher Scientific Cat# 201C), and UltraPure™ 0.5 M ethylenediaminetetraacetic acid (EDTA; pH8.0; Thermo Fisher Scientific Cat# 15575020).

Flow cytometry

Reagents and materials for this aspect of the procedure included: Anti-human CD11b antibody, clone ICRF44 (Stem Cell Technologies Cat# 60040AZ), anti-human CD16 antibody, clone 3G8 (StemCell Technologies Cat# 60041AD), CytoFLEX flow cytometer (Beckman Coulter), DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride; Thermo Fisher Scientific Cat# D1306), eBioscience™ flow cytometry staining buffer (Thermo Fisher Scientific Cat# 00-4222-26), Heraeus™ Pico™ 17 microcentrifuge (Thermo Fisher Scientific Cat# 75002401), human CD68/SR-D1 antibody (R&D Systems Cat# MAB20401),

microcentrifuge tubes (Scientific Specialties Inc. Cat# 1110-00), and PerCP-Cy™5.5 mouse anti-human CD14 (BD Biosciences Cat# 550787).

Cell culture

Reagents and materials for cell culture included the following: 12-well cell culture plates (Sigma-Aldrich Cat# SIAL0512), 18 mm coverslips (SDR technology Cat# 64-0714), 5000units/mL penicillin-streptomycin (Thermo Fisher Scientific Cat# 15070063), Gibco™ 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Cat# 10437028), Gibco™ RPMI 1640 medium (Thermo Fisher Scientific Cat# 11-875-093), Glutamax™ supplement (Thermo Fisher Scientific Cat# 5050061), MCO-170AICUV-PE IncuSafe CO_2 incubator (5%CO_2/95%O_2; PHC Corporation), poly-D-lysine hydrobromide (PDL; Sigma-Aldrich Cat# P6407), recombinant human granulocyte-macrophage colony-stimulating factor protein (GM-CSF; R&D Systems Cat# 7954-GM-010), recombinant human macrophage colony-stimulating factor protein (M-CSF; R&D Systems Cat# 216-MC-005), sterile water, and TC20™ automated cell counter (Bio-Rad Serial No. 508BR02467).

Freezing and storage of cells

Reagents and materials included: Centrifuge 5810 R (Eppendorf Cat# 5810000386), Corning® cryogenic vials (Sigma-Aldrich Cat# CLS430659), dimethyl sulfoxide (DMSO; Sigma-Aldrich Cat# D2650), Gibco™ 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Cat# 10437028), graded cryofreezing apparatus, ice, and liquid nitrogen for sample storage.

Methodology

** Caution: Always use personal protective equipment (PPE) and correct waste disposal methods when dealing with human products. **

This research is done in accordance to the World Medical Association Declaration of Helsinki. With human research ethics approval and with informed consent from the participant, 50 mL of blood is taken *via* venepuncture from a peripheral vein (cubital fossa) by a qualified pathologist/clinician. The blood is collected in multiple 10 mL EDTA (ethylenediaminetetraacetic acid) tubes.

Monocyte isolation

The procedure of monocyte isolation is conducted in a PC2 (physical containment level 2) certified tissue culture laboratory in HEPA (high efficiency particulate air) laminar flow tissue culture cabinet (Westinghouse Pty. Ltd.) with appropriate PPE (gown, gloves, and goggles). All individuals are appropriately trained prior to conducting the procedure. The participant undergoes venepuncture by qualified pathologist/clinician. The blood from the venepuncture is collected into EDTA tubes. Immediately after, whole blood is transferred equally (~25 mL per tube) into two 50 mL falcon centrifuge tubes. 0.5 M EDTA is added to whole blood to a final concentration of 1 mM (i.e. 50 µl of 0.5 M EDTA stock solution is added per tube). 1 ml of RosetteSep™ human monocyte enrichment cocktail is added per tube (i.e. 2 mL per buffy coat at 40 µl/mL). Falcon centrifuge tubes containing whole blood (with lid tightly closed) are transferred to a 5-roller tube roller mixer for 30 minutes at room temperature. Thereafter, 1 mM EDTA is added per tube and each tube is topped up to 50 mL with sterile PBS (Figure 1).

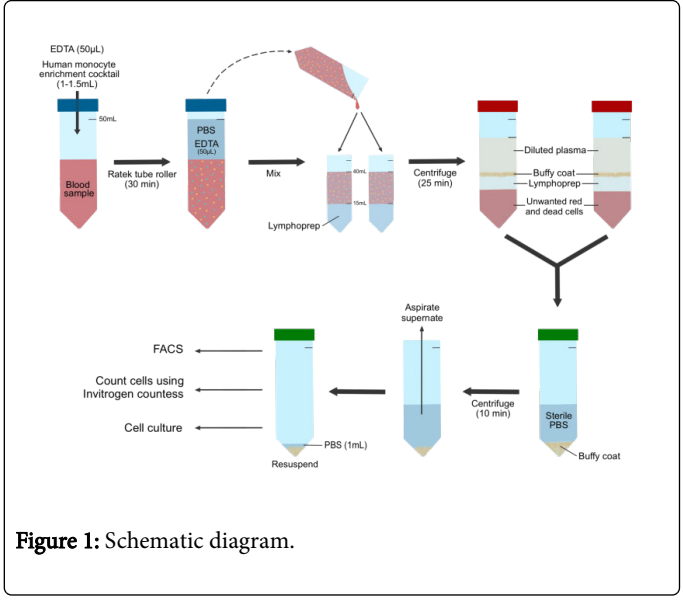

Figure 1: Schematic diagram.

Centrifugation

Centrifugal forces vary between rotors of different centrifuges. Please use the formula below for conversion between relative centrifugal force (RCF) and centrifuge rotor speed (RPM) if required.

$RCF=(1.118 \times 10^{-5}) \times Radius \times (RPM)^2$

Where: RCF=relative centrifugal force in times gravity (g)

RPM=centrifugal speed in revolutions per minute

Radius=radius of rotor in centimeter (cm)

For centrifugation, two new 50 mL falcon centrifuge tubes with 15 mL of lymphopep density gradient medium in each tube are prepared. Using a 25 mL serological pipette, 25 mL of the diluted blood is gently layered on top of lymphopep medium. It is important to ensure not to mix the blood with the medium. The samples are centrifuged for 25 minutes at 2000 rpm (rotations per minute) with break off at room temperature. After centrifugation, the monocytes will appear as a 'buffy' layer at the interface between the lymphopep medium and diluted plasma. This 'buffy coat' which contains the monocytes is collected using a plastic transfer pipette into a new 50 mL falcon centrifuge tube. The 'buffy coat' collected is topped up with sterile PBS to ensure all tubes are of equal volumes (~15 mL) for centrifugation. The 'buffy coat' solution is then centrifuged for 10 minutes at 1600 rpm in centrifuge at room temperature. After centrifugation is completed, supernatant on top of 'buffy coat' pellet is aspirated and disposed as waste (hazardous/potential infectious waste). The remaining 'buffy coat' is re-suspended with 1 mL of sterile PBS.

Flow cytometry

The freshly isolated monocytes (before tissue culture plating) can also be immunophenotyped further using flow cytometry. 50 µl of 'buffy coat' solution per microcentrifuge tube (~200,000 cells per tube) is distributed. The following antibodies are added to each tube at a concentration of 1:200 – 1:400: (1) anti-CD14, anti-CD16 or anti-CD11b antibody could be used to identify monocytes and (2) anti-CD68 antibody for macrophages. After addition of antibodies, 360 µl flow cytometry staining buffer is added to each tube and incubated in

fridge (13°C) for 30 minutes. After incubation, samples are centrifuged for 5 minutes at 4500 rpm in microcentrifuge in room temperature. Thereafter, the liquid on top of the samples is disposed as waste. 360 µl of flow cytometry staining buffer is added to each tube again. It is important to mix each sample using a pipette to ensure all pellets are dislodged. To identify live cells, DAPI is added to a final concentration of 5µM per tube. The number of live monocytes expressing various markers can be analyzed using FlowJo software.

Cell culture

The cells are counted using automated cell counter. One million cells per well are plated in 12-well cell culture plate with pre-warmed RPMI 1640 medium (37 Celsius) enriched with heat-inactivated FBS (10%), Glutamax supplement and 5000 units/mL penicillin-streptomycin (1%). Each well of 12-well plate must be pre-loaded with PDL coated 18 mm coverslips. Each coverslip is coated with 300 µl of PDL hydrobromide stock solution diluted with 50 ml of sterile water. In order to preserve monocyte viability beyond 24 hours and also to induce M1/M2 differentiation, the cells are treated with either GMCSF (5000 units/mL) or MCSF (10,000 units/mL). The monocytes are incubated in a 37 Celsius CO_2 incubator for up to a week while functional assays or other techniques (i.e. immunocytochemistry) are performed.

** Human waste solution should be collected and treated with 1 part sodium hypochlorite (10.5-13.5%) to 9 parts solution for 10 minutes prior to disposal. **

Freezing and storage of cells

The freshly isolated monocytes can also be frozen in gas phase of liquid nitrogen for future use. The freezing medium is prepared by combining 10% DMSO and 90% heat-inactivated FBS on ice. A cell suspension of >1 million cells is centrifuged at 1500 rpm for 5 minutes in room temperature. The pellet is re-suspended in cold freezing medium at a density of 1 million cells per mL. Thereafter, the cell suspension is aliquoted into cryogenic vials and frozen slowly using a cryofreezing apparatus (graded temperature decrease of 1 Celsius per minute). The cryofreezing container is then placed in a -80 Celsius freezer overnight. Cryogenic vials containing the frozen cells are transferred to liquid nitrogen and stored for future use.

Results and Discussion

Accumulating literature on the association of innate immune defects and the pathogenesis of many diseases, particularly autoimmunities has prompted the development of various immune cell isolation procedures. However, the results from investigations using these isolated cells depend on the isolation protocol [13].

The procedure outlined in this paper involves obtaining human peripheral blood (under ethics approval from an institutional review board) and mixing it with a cocktail of antibodies that will bind to markers absent on monocytes, but present on other mononuclear cells (e.g. T lymphocytes, erythrocytes) in peripheral blood. The RosetteSep ™ antibody cocktail used crosslinks unwanted cells to multiple erythrocytes, forming immunerosettes. Due to having different densities, the unwanted (rosette) cells and monocytes (which are not labelled with antibody) are easily separated when centrifuged over a density gradient medium. Monocytes are collected as a highly enriched population at the interface between the plasma and density gradient medium. Isolated monocytes are immediately available for downstream experiments such as flow cytometry, cell culture and functional assays. To preserve monocytes for future applications, they could either be frozen in liquid nitrogen (gas phase) or incubated as cell cultures for 1-2 weeks.

It is important to note that different isolation procedures could result in possible alterations in cell phenotype. The isolation protocol using RosetteSep ™ antibody cocktail has both benefits and disadvantages. Investigators of innate cell functions should be aware that the optimum isolation procedure depends on the aim of their study and the assays to be used. Taken together, the isolation protocol using RosetteSep™ antibody cocktail offers many advantages; it is simple, relatively inexpensive and has excellent purity.

Conclusion

The monocyte isolation protocol outlined in this paper is simple, reproducible and practical with a high purity. The monocytes isolated are free of antibodies and in comparison to other protocols, it does not require specific equipment other than centrifuge. Similar antibody cocktails are also available for enrichment of other cell populations, such as natural killer cells [14].

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