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Technical Note

A simple and effective method for the isolation and culture of human monocytes from small volumes of peripheral blood

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ABSTRACT

Innate immune cell defects contribute to severe autoimmunity and the pathogenesis of inflammatory disease. Monocyte-derived macrophages typically retain disease related signatures and represent an excellent *in vitro* model to uncover and validate mechanisms contributing to specific pathological states. Monocyte isolation procedures vary widely in terms of purity, yield, cost, degree of technical difficulty and volume of peripheral blood needed. This paper outlines a novel isolation method that yields monocytes through density gradient centrifugation (Ficoll® and hyperosmotic Percoll®). The protocol has been optimised for small volumes of blood (42 ml) and is simple, reproducible and inexpensive compared to other methods. Monocyte recovery is 70% (relative to monocyte numbers within the buffy coat) and the highly functional macrophages produced are characterised by excellent purity (98.6 ± 0.6%) and intact activation and phagocytic capacities. The method is well suited to investigations involving patient populations where a particular subset of immune cells is known to contribute to the pathogenesis of a specific disease or is aberrant as a consequence of that disease.

1. Introduction

Monocytes are phagocytes that originate in bone marrow from myelomonocytic stem cells (Zhou et al., 2012) and constitute ≈ 10% of total leukocytes circulating in peripheral blood. Populations of monocytes are characterised by diverse effector functions that include: a) patrolling of healthy tissues, b) activation of early immune responses, c) phagocytosis of pathogens and foreign substances and d) replenishment of resident macrophages in peripheral tissues following infection, injury or inflammation (Zhao et al., 2018). Growing recognition of the substantial contribution of innate immune cell defects to the pathogenesis of disease and severe autoimmunity has fostered interest in isolation techniques that make it possible to measure the functionality of cells of this important system (Zhou et al., 2012). While cell lines offer the advantages of ready availability and extensive proliferative potential, the use of primary cells adds clinical relevance as such cells exhibit characteristics that closely resemble their tissue of origin. Isolation of distinct blood cell subsets from patient samples confers additional advantages related to the preservation of disease-related signatures and the potential to yield diagnostic or prognostic disease classifiers (Beliakova-Bethell et al., 2014).

While monocyte-derived macrophages are widely accepted as good surrogates of cells infiltrating tissues, not all monocyte isolation methods provide pure cell fractions that adequately reflect the various features of unmanipulated tissue macrophages (Eligini et al., 2013). Purity of cells is an important consideration as mixtures of diverse blood cell types generate multiple gene expression signals that confound interpretation. In addition to purity, factors such as yield, cost, degree of technical difficulty and volume of peripheral blood needed are important considerations. Of the various monocyte isolation methods available, positive selection is associated with lower purity (Mayer et al., 2011), lymphoprep separation is associated with upregulated CD18 and CD11 isotypes (Mayer et al., 2011), and flow cytometry requires specialised equipment and technical expertise (Wahl et al., 2005). In addition, isolation by adherence can result in activation of cells (Wahl et al., 2005), the Rosette-Sep procedure involves high

Abbreviations: DPBS, Dulbecco's phosphate buffered solution; IMDM, Iscove's Modified Dulbecco's Medium containing penicillin, streptomycin and L-glutamine; M-CSF, macrophage colony-stimulating factor; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6

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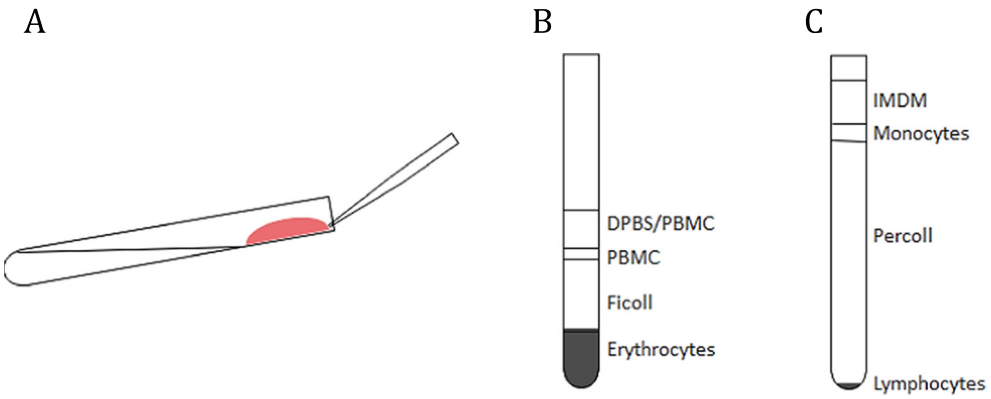

Fig. 1. Representation (not to scale) of the double density monocyte isolation technique. A) Ficoll®-containing centrifuge tubes were held at an angle 10–15 degrees from the horizontal. Diluted buffy coat was layered slowly onto the side of the tube to ensure preservation of the interface. B) The banding patterns of cells following isolation of peripheral blood mononuclear cells on a Ficoll® density gradient. C) The banding patterns of cells following separation of monocytes from lymphocytes on a hyperosmotic Percoll® density gradient.

commercial reagent costs (US$58.27/10 ml of blood) (Zhou et al., 2012), and centrifugal elutriation requires expensive equipment, large volumes of blood and specialised skills (Mayer et al., 2011).

The double density monocyte isolation technique outlined in this paper combines and builds on previous methods (Repnik et al., 2003; Danciger et al., 2004) and is characterised by simplicity, reproducibility and high purity. It provides an excellent yield of monocytes for small quantities of human peripheral blood (42 ml) and is inexpensive compared to other monocyte isolation protocols. The method is practical for clinical applications and is well suited to investigations involving patient populations where a particular subset of immune cells is known to contribute to the development or progression of a specific disease or is aberrant as a consequence of that disease.

2. Materials

2.1. Chemicals and reagents

Ficoll-Paque Premium® 1.073 (GE Healthcare, Sweden), Percoll Plus® (Sigma, USA), Iscove's Modified Dulbecco's Medium (Gibco, USA), 2 mM L-glutamine (Gibco, USA), penicillin-streptomycin (10,000 U/ml, Gibco, USA), interferon gamma (Sigma, USA), *Escherichia coli*-derived lipopolysaccharide (Serotype 0111:B4; Sigma, USA), May-Grunwald stain (Merc Millipore), macrophage colony-stimulating factor (Sigma, USA), phalloidin-tetramethylrhodamine B isothiocyanate (Sigma, USA).

2.2. Preparation of hyperosmotic Percoll®

The Percoll® solution can be prepared ahead of time and stored at 4 °C until use. Hyperosmotic Percoll® solution is prepared by combining Percoll Plus® (endotoxin levels 2 EU/ml; pH 9.4 ± 0.5 at 20 °C; density 1.130 ± 0.005 g/ml at 25 °C) with sterile water and sterile 1.6 M NaCl in a 50.4/39.62/9.98 ratio by volume. The solution should be shaken vigorously and stored at 4 °C overnight. The next day, following gentle inversion of the solution, the density was measured with a refractometer (expected index of refraction, 1.34415 ND).

3. Methods

3.1. Subjects

Male participants aged between 61 and 80 years ($n = 20$) were recruited from the Sunshine Coast, Australia with approval from the University of the Sunshine Coast Human Research Ethics Committee (A/13/473 and A/16/833). All subjects provided written informed consent.

3.2. Detailed monocyte isolation procedure

Whole blood was collected from fasted subjects in EDTA tubes (5 × 6 ml) and serum separator tubes (2 × 6 ml). The EDTA tubes were centrifuged (400 xg, 25 min, 20 °C; brake off), the plasma was removed from each tube and the buffy coat was collected. The buffy coat was divided among three 15 ml centrifuge tubes each containing 8 ml Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate buffered solution and 1 mM EDTA (DPBS/EDTA). Tubes were centrifuged (150 xg, 10 min, 20 °C; brake off) and the supernatants containing platelets were discarded. The wash was repeated twice, and the final spin was followed by removal of the supernatant and resuspension of the cells in each centrifuge tube with 1 ml of DPBS/EDTA.

3.3. Peripheral blood mononuclear cells were isolated by density gradient centrifugation

The resuspended cells were combined into a single 15 ml centrifuge tube and made up to 8 ml with DPBS/EDTA. Ficoll-Paque Premium® 1.073 (2 ml) was added to the bottom of each of four 15 ml centrifuge tubes. The Ficoll®-containing tubes were held at an angle 10–15 degrees from the horizontal and the cell suspension (2 ml) was layered to the surface of the Ficoll® in 1 ml aliquots (Fig. 1A). The preparation was centrifuged (400 xg, 30 min, 18 °C; brake off) and cells within a dense white mononuclear band (Fig. 1B) were collected and diluted with 5 ml DPBS/EDTA. The cells were pelleted by centrifugation (500 xg, 10 min, 20 °C) and the supernatant was discarded.

3.4. Monocyte purification was achieved by density centrifugation using hyperosmotic Percoll Plus® at a density of 1.058 g/ml

Pelleted cells were resuspended in 1 ml serum-free Iscove's Modified Dulbecco's Medium supplemented with 1000 U/ml penicillin, 1000 µg/ml streptomycin and 2 mM L-glutamine (IMDM). A 10 ml aliquot of room temperature hyperosmotic Percoll® solution was added to a 15 ml centrifuge tube. The tube was held upright, and the cell suspension was layered onto the Percoll® solution in 200 µl aliquots at a rate of approximately 200 µl/60 s using a 1–200 µl gel-loading tip. The preparation was centrifuged (580 xg, 15 min, 21 °C; brake off) and monocytes within a band located at the interface of the IMDM and Percoll® solution (Fig. 1C) were collected and diluted with 4 ml DPBS/EDTA. The cells were pelleted by centrifugation (550 xg, 10 min, 21 °C) and the supernatant was discarded. An aliquot of the monocyte cell pellet (1 µl) was applied to a glass slide and stained with May-Grunwald stain to confirm the presence of monocytes. Monocytes were resuspended in 1 ml IMDM and cell number was determined using a haemocytometer.

3.5. Autologous serum preparation

Whole blood, collected in serum separator tubes (2 × 6 ml), was

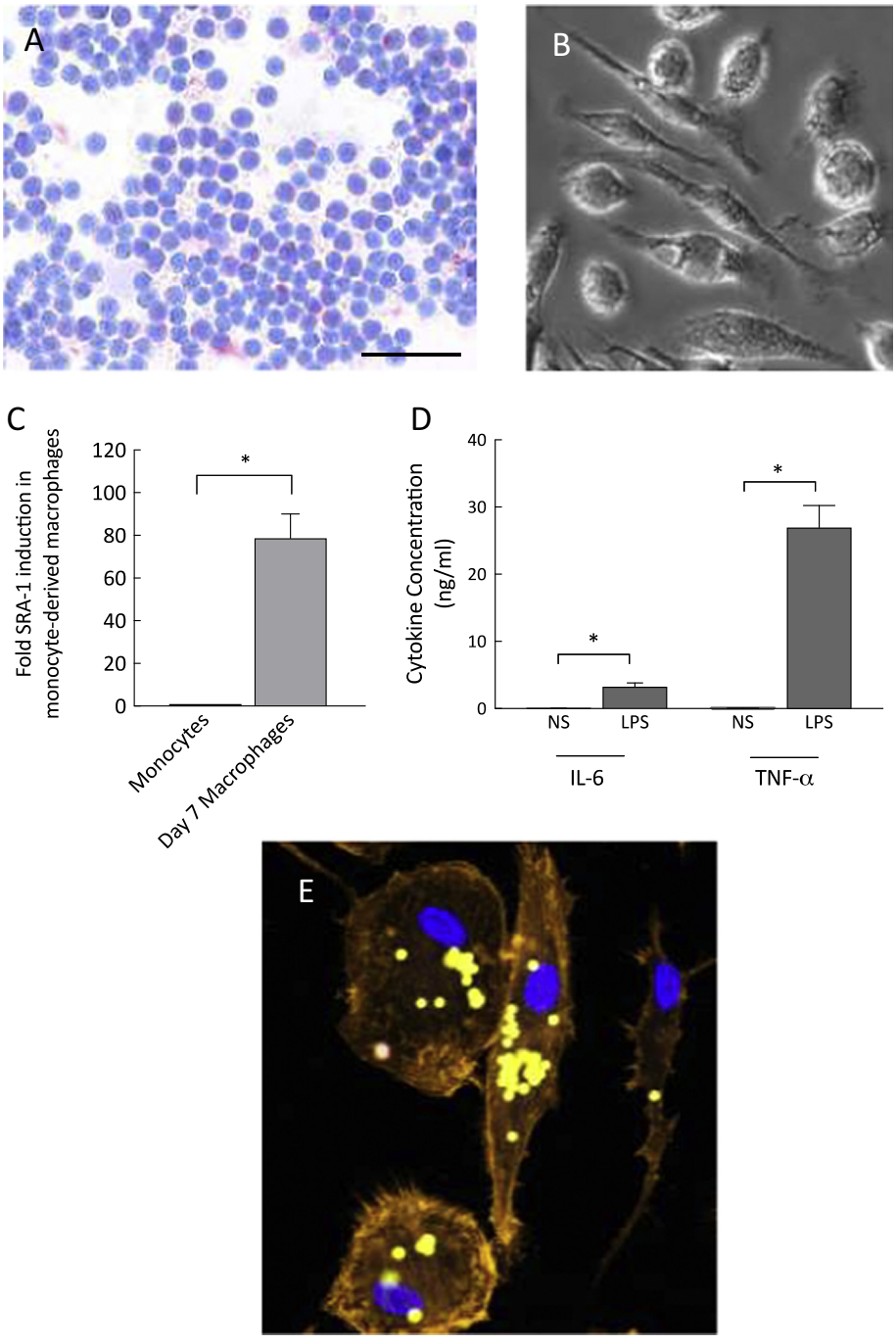

Fig. 2. Morphological and functional characteristics of human monocyte-derived macrophages. Human monocytes obtained after Percoll® separation and stained with May-Grunwald Stain (A, scale 30 μm). Phase contrast image of spontaneously differentiated human monocyte-derived macrophages (B). Composition of cell isolates, calculated at Day 7 of cell culture, was 98.6 ± 0.6% macrophages and 1.4 ± 0.6% lymphocytes (n = 7; 16 fields of view per participant). Comparison of relative levels of SRA-1 transcripts, normalised to GAPDH, in freshly isolated monocytes and cultured macrophages, measured by real time qPCR (C). The concentration of macrophage supernatant TNF-α and IL-6 concentrations were increased after exposure of cells to 0.1 μg/ml lipopolysaccharide (LPS; D). Macrophages were observed to phagocytose opsonised latex beads conjugated to a fluorophore (E, confocal image, blue - DAPI, yellow – latex beads, F-actin (phalloidin) - orange). Data are expressed as mean ± SEM, * $p < .05$. NS, non-stimulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

allowed to clot at 22 °C for a minimum of 30 min prior to centrifugation (1500 xg, 15 min, 15 °C). The serum was collected, centrifuged (4000 xg, 5 min, 4 °C) and the supernatant was stored at −80 °C for use as an autologous serum supplement in cell cultures of isolated monocytes.

3.6. Monocyte culture

Monocytes were seeded onto 24 well plastic multiwell culture plates (Corning Costar, USA) at a density of 3×10^5 cells/well (final volume 600 μl/well). Plated cells were transferred to a 37 °C humidified 5% CO_2 incubator for a period of 2 h, at which time the supernatant containing non-adherent cells was removed following gentle flushing and the medium was replaced with IMDM containing 5% autologous serum and 50 ng/ml macrophage colony-stimulating factor (M-CSF). Further media changes occurred on days 1 and 4 and, at these time points, removal of the supernatant was also accompanied by gentle flushing of the cells with a Gilson pipette.

3.7. Macrophage maturation, purity estimation and activation

Monocytes were allowed to mature into a morphologically heterogeneous, adherent macrophage population over the course of 7 days. On day 7, cells were counted (n = 7; 16 fields of view per participant) using a Nikon digital sight DS-QilMc monochrome camera (square pixel CCD, 1.5 megapixels) attached to a Nikon Eclipse T*i* microscope. For macrophage activation experiments, the medium was replaced with IMDM containing 5% serum and the adherent macrophages were exposed to 20 ng/ml interferon gamma (IFN-γ) and 0.1 μg/ml *Escherichia coli*-derived lipopolysaccharide (LPS) for 24 h. All supernatants were collected and centrifuged (10,000 xg, 5 min, 4 °C). Supernatants were

stored at −80 °C until use.

3.8. Cytokine assays

The cytokines interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α) were measured in monocyte-derived macrophage supernatants using commercially available enzyme immunoassay kits (Affymetrix eBioscience, San Diego, USA) in accordance with manufacturer's instructions.

3.9. RNA isolation and real-time quantitative PCR

Freshly isolated monocytes (10 μl) and 7-day cultured macrophages were transferred to RNA*Later* RNA Stabilisation Reagent (Qiagen) and cooled at 4 °C for 24 h followed by transfer to −20 °C for storage until analysis. Total RNA was extracted from samples using the Isolate II RNA mini kit (Bioline) prior to generation of cDNA using the SensiFAST cDNA synthesis kit (Bioline) and amplification of samples against transcripts by real-time quantitative PCR using the SensiMix SYBR No-Rox kit (Bioline) and a Rotor-Gene Q thermal cycler (Qiagen). Primers for SRA-1 and GAPDH were as per Danciger et al., 2004 and Long et al., 2009, respectively. No template controls were run in each reaction and no contamination was observed. Normalisation points included a) spectrophotometric RNA quantification (Nanodrop, ND100), b) inclusion of a reference sample in each run and c) determination of mRNA levels of an internal control (GAPDH) for all samples. Quantitation of relative gene expression was based on the relative standard curve method with raw data normalised to GAPDH.

3.10. Phagocytosis assay

Human monocytes (1.3×10^5 cells/well) were seeded onto 8-well chamber slides (Sarstedt, Germany) and maintained in culture as described above. On day 8 of culture, carboxylate-modified red fluorescent latex beads (diameter 2 μM; Sigma Aldrich) were opsonised in 40% serum (10% goat serum, 10% foetal calf serum, 10% horse serum, 10% human serum) with IMDM for 30 min at 37 °C. Latex beads were washed three times by centrifugation (3000 x*g*, 2 min, RT) using DPBS/EDTA. Serum-starved cells were incubated with latex beads and DAPI (Sigma, 2 μg/ml) for 4 h at 37 °C. Cells were labelled with phalloidin-tetramethylrhodamine B isothiocyanate (5 μg/ml), as per Bürgin-Maunder et al., 2013. Cells were placed on ice and washed twice with cold DPBS/EDTA to remove unbound beads. Cells were mounted with glycerol and immobilised beneath glass coverslips. Confocal images were acquired using a Nikon digital sight DS-QilMc monochrome camera (square pixel CCD, 1.5 megapixels) attached to a Nikon Eclipse T*i* microscope.

4. Results and discussion

The number of monocytes in the buffy coat obtained from 30 ml of whole blood was $5.86 \pm 0.67 \times 10^6$. The number of monocytes isolated after Percoll® centrifugation was $4.05 \pm 0.45 \times 10^6$ providing a monocyte yield of 69.6 ± 1.9% ($n = 7$). Macrophage differentiation was confirmed by a) the presence of significantly enlarged cytoplasmic volumes compared to monocytes adhered for 2 h (Fig. 2A), b) the occurrence of morphological changes from round cells to either spindle-shaped or oblong-shaped cells, both of which included filopodia (Fig. 2B) and c) significant increases in transcript levels of macrophage scavenger receptor class A type 1 (SRA-1) in cells cultured for 7 days (Fig. 2C). Human monocytes express low levels of SRA-1 mRNA and substantial upregulation occurs following differentiation to macrophages. In this study, SRA-1 mRNA showed a 79-fold increase in 7-day macrophages compared to freshly isolated monocytes ($p < .001$). Exposure of these monocyte-derived macrophages to LPS produced a 500-fold increase in TNF-α (Fig. 2D, $n = 17$) and a 75-fold increase in IL-6 concentration (Fig. 2D, $n = 14$) compared to non-stimulated cells and suggested high cell viability and functionality. In addition, monocyte-derived macrophages were shown to retain the capacity to phagocytose opsonised particles (Fig. 2E), a finding that confirmed the preservation of functionality in these cells.

To conclude, the monocyte isolation method outlined in this paper is inexpensive, simple, reproducible and practical for clinical environments. The cell fraction produced is characterised by high purity and an ability to differentiate into viable, highly functional macrophages with intact activation and phagocytic capacities.

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