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Standard Protocols for Generation of Monocyte-derived Cell Types

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Abstract

This document describes standard research protocols used for generation of human monocyte-derived macrophages and DC in the Hutchinson work-group.

Introduction

This document describes standard research protocols used for generation of human monocyte-derived macrophages and DC in the Hutchinson work-group. **Abbreviations** Mreg: Regulatory macrophage DPBS: Dulbecco’s modified phosphate buffered saline M-CSF: Macrophage colony-stimulating factor FBS: Fetal bovine serum HABS: Human AB serum IFN-γ: Interferon-gamma LFH: Laminar flow hood PR: Phenol Red CPDA: Citrate-Phosphate-Dextrose-Adenine solution LRS: Leucocyte Reduction System

Reagents

General reagents RPMI1640 without phenol red \(BE12918F, Lonza-Biozym) Glutamax100X \(35050038, Gibco-life) Pen-Strep 100X \(15140-122, Gibco-life) rhM-CSF \(216-MC-025, R&D) rhIFN-γ \(IF002, Millipore) CD14 microbeads \(130-050-201, Miltenyi) Albunorm 20% - Human Albumin \(Octapharma) Citrate-phosphate-dextrose-adenine solution \(C4431, Sigma) Dulbecco’s Phosphate Buffered Saline without Ca^{2+} or Mg^{2+} \(D8537, Sigma) Biocoll Separating Solution \(L6115, Millipore-Biochrom) Human AB Serum \(ZKT Tübingen) FBS \(10270, Life/Gibco) X-VIVO 10 \(BE04-380Q, Lonza) hrGM-CSF \(300-03, Peprotech) hrTNF-α \(300-01A, Peprotech) hrIL-4 \(200-04, Peprotech) LPS \(L2018, Sigma) Dexamethasone \(D4902, Sigma) **Materials** Stericup+ Steritop 250ml \(SCGPU02RE, Millipore) QuadroMACS Separation Unit \(130-090-976, Miltenyi) Pre-separation Filters \(130-041-407, Miltenyi) LS Columns \(130-042-401, Miltenyi) Sarstedt Cell+ 6-well plates \(833.920.300, Sarstedt)

Procedure

1. PREPARATION OF REAGENTS **1.1 Preparation and storage of rhM-CSF** 1. In a 15 ml Falcon tube, prepare a 7.5% solution of human albumin in DPBS without Ca^{2+}or Mg^{2+}. 2. Remove vial of 25 µg rhM-CSF from -20°C storage. Allow 5 min to equilibrate to RT. 3. Transfer a total of 2.5 ml DBPS without Ca^{2+} or Mg^{2+} into the rhM-CSF vial in fractions to dissolve and wash-out all protein from the vial. 4. Transfer 2.5 ml rhM-CSF solution into a 50 ml Falcon tube. 5. Add 38 µl of human albumin solution from step \(1.1.1). Mix gently. 6. Make 125 µl aliquots \(ie. for 50 ml medium) in Eppendorf cups. 7. Store at -20°C for up to 8 weeks. **1.2 Preparation and storage of rhIFN-γ** 1. In a 15 ml Falcon tube, prepare a 7.5% solution of human albumin in DPBS without Ca^{2+} or Mg^{2+}. 2. Remove vial of 100 µg rhIFN-γ from -20°C storage. Allow 5 min to equilibrate to RT. 3. Transfer a total of 3.8 ml DBPS without Ca^{2+} or Mg^{2+} into the rhIFN-γ vial in fractions to dissolve and wash-out all protein from the vial. 4. Transfer 3.8 ml rhIFN-γ solution into a 50 ml Falcon tube. 5. Add 200 µl of human albumin solution from step \(1.1.1). Mix gently. 6. Make 100 µl aliquots \(ie. for 50 ml medium) in Eppendorf cups. 7. Store at -20°C for up to 8 weeks. **1.3 Heat inactivation and storage of human AB serum and FBS** 1. Thaw 100 ml aliquot of

serum at 4°C overnight. 2. Pre-heat waterbath to 56°C. 3. Submerse serum bottle in 56°C waterbath for 30 min. 4. Transfer serum bottle to ice. Leave for 30 mins to cool. 5. Transfer to LFH and decontaminate. 6. Make 5, 10 or 20 ml aliquots of serum in Falcon tubes. 7. Store aliquots at -20°C for up to 4 weeks. **1.4 Preparation of MACS buffer** CPDA is used in preference to EDTA. Human albumin is used in preference to bovine albumin. 1. In LFH, transfer 90 ml DPBS without Ca^{2+} or Mg^{2+} into a 250 ml Stericup device using a serological pipette 2. Transfer 10 ml CPDA to Stericup 3. Transfer 2.5 ml 20% human serum albumin to Stericup 4. Filter mixture. Store at 4°C. **1.5 Preparation of Mreg culture medium** Protocol to produce 100 ml medium. Scale as required. 1. Assemble medium components in a 250 ml Stericup device in LFH. 2. Transfer 88 ml RPMI-1640 without PR into Stericup using serological pipette. 3. Transfer 1 ml of 200 mM GlutaMax into Stericup using serological pipette. 4. Transfer 1 ml 100 X Pen-Strep into Stericup using serological pipette. 5. Transfer 10 ml heat-inactivated HABS into Stericup using serological pipette. 6. Transfer 250 µl rhM-CSF \(see 1.1) into Stericup using pipette. 7. Filter medium mixture. Medium must be used fresh. **1.6 Preparation of FBS-containing culture medium** Protocol to produce 100 ml medium. Scale as required. 1. Assemble medium components in a 250 ml Stericup device in LFH. 2. Transfer 88 ml RPMI-1640 without PR into Stericup using serological pipette. 3. Transfer 1 ml of 200 mM GlutaMax into Stericup using serological pipette. 4. Transfer 1 ml 100 X Pen-Strep into Stericup using serological pipette. 5. Transfer 10 ml heat-inactivated FBS into Stericup using serological pipette. 6. Transfer 250 µl rhM-CSF \(see 1.1) into Stericup using pipette. 7. Filter medium mixture. Medium must be used fresh. **2. STARTING MATERIAL** **2.1 Obtaining apheresate from LRS chamber** 1. Decontaminate outside of LRS chamber using alcohol wipe. Transfer to LFH. 2. Hold the LRS chamber with the conical point downwards. Clamp the lower tube with sterile surgical forceps. 3. Cut the lower tube with sterile scissors below the clamp. 4. Cut the upper tube using sterile scissors. 5. Hold the LRS chamber over an open 50 ml Falcon tube and remove the clamp. 6. Allow the contents of the LRS chamber to run into the Falcon tube. 7. Perform automated cell count \(differential count including monocytes). 8. Perform flow cytometry analyses \(eg. $CD14^{+}$ monocytes). **2.2 Separation of PBMC by density gradient centrifugation** Ficoll density gradient centrifugation is used to enrich PBMC prior to CD14 selection by MACS. This is necessary when working with apheresates from LRS chambers, buffy coats or whole blood, owing to their high content of neutrophils \(which may be $CD14^{low}$) and erythrocytes. 1. Dilute contents of LRS chamber 1:1 in sterile DBPS without Ca^{2+} or Mg^{2+} at RT. 2. Transfer 20 ml Ficoll solution at RT into a 50 ml Falcon tube. 3. Gently overlay diluted apheresate onto Ficoll using a serological pipette. 4. Place the layered columns into a swing-bucket centrifuge. 5. Centrifuge at RT for 25 min at 850 g with low acceleration and no brake. 6. Remove tubes from centrifuge. Decontaminate with alcohol wipes. Transfer to LFH. 7. Recover mononuclear cells from interface using a 10 ml serological pipette. 8. Transfer mononuclear cells to a 50 ml Falcon tube. 9. Resuspend cells in 50 ml DBPS without Ca^{2+} or Mg^{2+} at RT. 10. Pellet cells by centrifugation at 550 g for 10 mins at RT. 11. Aspirate supernatant using a 50 ml serological pipette. 12. Resuspend cells in 50 ml DBPS without Ca^{2+} or Mg^{2+} at RT. 13. Pellet cells by centrifugation at 300 g for 10 mins at RT. 14. Aspirate supernatant using a 50 ml serological pipette. 15. Resuspend cell pellet in 10 ml DPBS without Ca^{2+} or Mg^{2+} at RT. 16. Perform total and viable cell count using the Trypan Blue method. 17. Transfer

required volume of cell suspension to a 50 ml Falcon tube. **3. MREG CULTURE ** **3.1 Day_0 of culture: Isolation of monocytes \(~90 minutes)** 1. From the mononuclear cell preparation \(see 2.2) take the required volume of PBMC. 2. Transfer to 50 ml Falcon. Pellet cells at 300 g for 6 min. 3. Resuspend cells in MACS buffer: 80 µl per 10^7 total cells. 4. Add 20 µl CD14 Microbeads per 10^7 total cells. Mix by gentle inversion. 5. Incubate for 15 min at 4°C \(in fridge). 6. Add 15 ml MACS buffer to sample. Gentle mixing. 7. Centrifuge for 6 min at 300 g. 8. Place an LS column into magnet. Wash column with 3 ml MACS buffer. 9. Aspirate supernatant using serological pipette. 10. Resuspend cells in 1 ml MACS buffer. 11. Pass cell suspension through a pre-separation filter on column. 12. Wash column with 3 ml MACS buffer 3 times. 13. Remove column from magnet. Apply 5 ml MACS buffer. 14. Insert column plunger and eject column contents into a 50 ml Falcon. 15. Take aliquots of cells for \(i) Trypan Blue count and \(ii) CD14 analysis by flow cytometry. **3.2 Day_0 of culture: Seeding of Mreg cultures: ~30 min** 1. Take the required volume of CD14 monocyte suspension into a 50 ml Falcon. 2. Seed monocytes at 1 x 10^6 monocytes/well \(6-well plate). 3. Centrifuge monocyte suspension at 300 g for 10 min. 4. Aspirate supernatant using a serological pipette. 5. Resuspend cell pellet in Mreg medium at 1 x 10^6 cells/3 ml. 6. Transfer monocyte suspension into well palte: 3 ml cell suspension/well. 7. Place plates into 37°C 5%CO_2 incubator on horizontal shelves. **3.3 Day_6 of culture: IFN-γ stimulation: ~30 min** 1. Remove culture plates from incubator. Transfer to LFH. 2. Thaw aliquots of rhIFN-γ as required. 3. Dilute 1000x rhIFN-γ solution by 1:5 in DPBS . 4. Apply IFN-γ to cultures at 25 ng/ml end concentration \(ie. 15 µl into 3 ml well). 5. Gently mix by swirling. 6. Replace plates into incubator and incubate for a further 18 to 24 hours. **3.4 Day_7 of culture: Cell harvest: ~40 min** 1. Remove culture plates or flask from incubator. 2. Inspect culture plates or flasks using an inverted microscope. Assess morphology and density: a. Cells should be mostly adherent. Cells should have a spreading morphology. Spiky cells forming clusters in suspension are an indication of a poor-quality culture. b. With experience it is possible to judge cell density. Look also for cell debris suggesting death of cells in culture. 3. Transfer to LFH. Discard medium. 4. Wash with DPBS \(1 ml/well or 5 ml/flask). Discard non-adherent cells. 5. Add DPBS \(1 ml/well of 5 ml/flask) 6. Gentle scrape with a soft cell scrapper. 7. Transfer cell solution into a Falcon tube. 8. Centrifuge for 6 min at 300 g. 9. Aspirate supernatant. Resuspend cells in DPBS. 10. Count cells by Trypan Blue method. 11. Assess cell phenotype by flow cytometry. **4. GENERATION OF OTHER MONOCYTE-DERIVED CELL TYPES** **4.1 Generation of monocyte-derived dendritic cells** 1. Isolate $CD14^+$ monocytes as described in Section 2 and 3.1 2. Prepare DC medium: RPMI 1640 containing Pen-Strep, GlutaMAX, 5% HABS, 100 ng/ml rhGM-CSF, 100 ng/ml rhIL-4. 3. Resuspend the required number of $CD14^+$ monocytes in DC medium at 4. 1 x 10^6 cells/3 ml. 5. Seed monocytes at 1 x 10^6 monocytes/well \(6-well plate). 6. Place plates into 37°C 5%CO_2 incubator on horizontal shelves. 7. On day 5 harvest immature mo-DC by pipetting. 8. Transfer cells into a Falcon tube and centrifuge for 6 min at 300 g. 9. Resuspend pellet in X-VIVO 10 medium containing 2mM GlutaMAX and 100 ng/ml rhGM-CSF. 10. Replate immature DCs in 6-well plates at 1 x 10^6 cells/well. 11. For maturation add 50 ng/ml rhTNF-α and culture for 2 more days. 12. Harvest by pipetting. Wash wells with PBS. 13. Transfer cell solution into a Falcon tube. 14. Centrifuge for 6 min at 300 g. 15. Aspirate supernatant. Resuspend cells in DPBS. 16. Count cells by Trypan Blue method. 17. Assess cell phenotype by flow cytometry. **4.2 Generation of polarised

macrophages** 1. Isolate $CD14^+$ monocytes as described in Section 2 and 3.1. 2. Resuspend the necessary amount of monocytes in FBS-containing medium \(Section 1.6) at 1 x 10^6 cells/3 ml. 3. Seed 1 x 10^6 monocytes/well in 6-well. 4. On day 6 stimulate for 18-24h as follows: - Resting Mφ : no stimulation. - IFN-γ-Mφ: 25 ng/mL rhIFN-γ. - IFN-γ+LPS Mφ: 25 ng/mL rhIFN-γ + 100 ng/ml LPS. - IL-4 Mφ: 20 ng/mL rhIL-4. - GC Mφ: 10^{-7} M dexamethasone. 5. On day 7 inspect culture plates using an inverted microscope. 6. Harvest cells by pipetting. 7. Add 1 ml DPBS per well. 8. Gentle scrape with a soft cell scrapper to harvest adherent cells. 9. Transfer cell solution into a Falcon tube. 10. Centrifuge for 6 min at 300 g. 11. Aspirate supernatant. Resuspend cells in DPBS. 12. Count cells by Trypan Blue method. 13. Assess cell phenotype by flow cytometry.

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