Isolation, differentiation and polarization of porcine monocyte derived-macrophages.

Maria Pulido Fresneda (mpulido@ccmijesususon.com)
Stem Cell Therapy Unit, 'Jesús Usón' Minimally Invasive Surgery Centre, Cáceres, Spain
https://orcid.org/0000-0002-9126-7047

Verónica Álvarez Pérez
Stem Cell Therapy Unit, 'Jesús Usón' Minimally Invasive Surgery Centre, Cáceres, Spain
https://orcid.org/0000-0002-7319-6089

María de los Ángeles de Pedro Muñóz
Stem Cell Therapy Unit, 'Jesús Usón' Minimally Invasive Surgery Centre, Cáceres, Spain
https://orcid.org/0000-0002-8989-2468

Ana María Marchena López
Stem Cell Therapy Unit, 'Jesús Usón' Minimally Invasive Surgery Centre, Cáceres, Spain

Esther López Nieto
Stem Cell Therapy Unit, 'Jesús Usón' Minimally Invasive Surgery Centre, Cáceres, Spain
https://orcid.org/0000-0002-5051-5913

Method Article

Keywords: porcine macrophages,isolation, differentiation, polarization.

Posted Date: October 11th, 2022

DOI: https://doi.org/10.21203/rs.3.pex-1948/v1

Abstract

Monocytes can be isolated from the peripheral blood of pigs. The protocol begins with a Ficoll density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMCs) from whole blood, followed by a culture in porcine serum pre-treated plates. Non-adherent cells were washed away after at least 3 h of culture. After 24 h, adherent cells were differentiated to M1- and M2- macrophages adding to the medium hGM-CSF and hM-CSF, respectively. On day 7 of culture, surface receptor expression and gene expression were measured by flow cytometry and qPCR in response to 24 h of LPS + IFNg (M1) or IL-4 (M2) stimulation. Porcine M1-polarized macrophages presented 'fried-egg' like shape morphology, and higher SLA-II and TNFa expression, whereas porcine M2-polarized macrophages displayed the 'spindle' like morphology, higher CD206 expression, and higher IL-10, and Arg-1 expression.

Introduction

Activated macrophages are usually divided into two polarization states based on the expression of surface receptors and the secretion of cytokines: classically activated macrophages that have a pro-inflammatory profile (M1) and alternatively activated macrophages that have an anti-inflammatory profile (M2). Both M1- and M2- macrophages are closely related to inflammatory diseases (1,2). Therefore, improving the inflammatory environment by modulating the activation state of macrophages could be an effective method for the treatment of immune-related diseases.

Although porcine models are increasingly used as models for translational research (3), there is not a specific method to culture and differentiate macrophages from pigs(4–7). For that, we aimed to develop a protocol to use porcine macrophages as a tool to analyze new therapeutic strategies.

Reagents

Reagents:

Phosphate buffer saline (PBS).

Ficoll-Paque PlusTM

EDTA

Fetal bovine serum (FBS)

Porcine serum (PS)

Penicillin-streptomycin

L-glutamine

RPMI (Roswell Park Memorial Institute) 1640 medium

Porcine monoclonal antibodies: CD14, CD16, CD206, CD163, and SLA-II (Swine Leukocyte Antigen class II)

Porcine IFNg

LPS

Porcine IL-4

Human GM-CSF

Human M-CSF

Ethanol 70 % and 100 %

DEPC-treated water

iScript™ cDNA Synthesis Kit (BioRad)

Taq DNA Polymerase Recombinant kit (Invitrogen)

Commercial TaqMan® Gene Expression Assays probes (Thermo-Fisher Scientific Inc., MA, USA) of the following genes: TNFa(Ss03391318_g1), IL-10 (Ss03382372_u1), HPRT1 (Ss03388274_m1), Arg-1(Ss03391394_m1),

PureLink RNA Mini Kit (ThermoFisher Scientific)

Culture media:

Complete RPMI medium: 10 % FBS, 1 % penicillin-streptomycin, and 1 mM L-glutamine in RPMI.

M1 differentiation medium: 50 ng /ml hGM-CSF, 10 % FBS, 5% PS, 1 % penicillin-streptomycin, and 1 mM L-glutamine in RPMI.

M2 differentiation medium: 50 ng /ml hM-CSF, 10 % FBS, 5% PS, 1 % penicillin-streptomycin, and 1 mM L-glutamine in RPMI.

M1 polarization medium: 100 ng/ml LPS, 100 ng/ml IFNg, 10 % FBS, 5% PS, 1 % penicillin-streptomycin, and 1 mM L-glutamine in RPMI.

M2 polarization medium: 100 ng/ml IL-4, 10 % FBS, 5% PS, 1 % penicillin-streptomycin, and 1 mM L-glutamine in RPMI.

Equipment

Equipments:

CO_2 incubator

Laminar flow chamber

FACScalibur cytometer (BD Biosciences)

Optical microscope

QuantStudio™ 3 Real-Time PCR System

UV spectrophometer

Procedure

Protocol workflow (Figure 1)

Isolation and culture of monocytes:

1. Dilute 10 mL of peripheral blood 1:1 in PBS, close the bottle and mix inverting them carefully.

2. Place 8 mL of Ficoll-Paque Plus in 50 mL conical tubes.

3. Cover the Ficoll-Paque with the blood + PBS mixture using a serological pipette as slowly as possible to avoid mixing the Ficoll-Paque and the blood + PBS mixture.

4. Centrifuge the sample at 2,500 rpm and 20 °C for 20 min. The rotor brakes have to be fully deactivated to effectively prevent phase mixing. It is also very important to take into account that temperature differences could change the density rates of the liquids and can have a negative impact on the separation results.

5. Carefully aspirate the middle white layer containing the PBMCs. During this step try to transfer as little Ficoll-Paque as possible.

6. Wash PBMCs two times with PBS 1X (5 min 1500 rpm) and add 20 ml of complete medium.

7. Pre-treated 6-well plates with 2 ml of porcine serum for 1 h at 37 ºC to facilitate adherence.

8. Aspirate the serum and dry completely the plate before culturing 2 ml of PBMCs in RPMI for at least 3 h.

9. After that, wash away the non-adherent cells washing 3 times with fresh RPMI, and add 2 ml of complete RPMI to culture adherent cells.

Monocytes-derived macrophages differentiation and polarization:

1. After 24 h of culture, replace the medium and add 2 ml of M1 differentiation medium or M2 differentiation medium for M1 or M2 differentiation, respectively. For M0 control, add 2 ml of complete RPMI.

2. On day 7 of culture, replace the medium and add 2 ml of M1 polarization medium or M2 polarization medium for M1 or M2 polarization, respectively. For M0 control, add 2 ml of complete RPMI.

3. After, 24 h of culture with polarization medium, observe the cells under the optical microscope. M1- and M2- macrophages, will present morphological differences.

Phenotypic characterization:

1. Aspirate and discard the culture medium, and rinse with PBS. Detach porcine macrophages from culture flasks with PSB-EDTA 5mM pH 7 for 10 min at 37ºC and suspended them in PBS containing 2% FBS.

2. Incubate 2×105 cells for 30 min at 4 °C with appropriate concentrations of the monoclonal antibodies: CD14, CD16, CD163, CD206, and SLA-II (Swine Leukocyte Antigen class II).

3. Wash cells and resuspend in PBS containing 2% FBS.

4. Flow cytometric analysis is performed on a FACScalibur cytometer (BD Biosciences) after the acquisition of 10.000 events. Cells are primarily selected using forward and side scatter characteristics and fluorescence is analyzed using CellQuest software (BD Biosciences).

5. Isotype-matched negative control antibodies should be used in all the experiments.

6. Calculate the % of positive cells for the different markers and the mean relative fluorescence intensity by dividing the mean fluorescent intensity (MFI) by the MFI of its negative control.

Molecular characterization:

1. Aspirate and discard the culture medium, and rinse with PBS. Detach porcine macrophages from culture flasks with PSB-EDTA 5mM pH 7 for 10 min at 37ºC.

2. For isolation RNA, use the PureLink RNA Mini Kit (ThermoFisher Scientific), according to the manufacturer’s instructions.

3. Add 300-600 µL, depending on cell number, of Lysis Buffer with 10% of 2-mercaptoethanol to the cell pellet and transfer the mix to a RNase-free tube. Add one volume 70% ethanol to each volume of cell homogenate and transfer the sample to the Spin Cartridge. Consecutive centrifugations and ethanol washes are performed. Finally, resuspend the pellet in DEPC-treated water.

4. Measure RNA quality and concentration using a UV spectrophotometer, such as NanoPhotometer Implen GMBH NP80 (Biotek, Winooski, VT, USA). Only the RNA samples with a 260/280 nm absorbance ratio between 1.8 and 2.1 and with a 260/230 mm absorbance ratio greater than 2.0 should be retrotranscribed to complementary DNA (cDNA) and amplified by PCR.

5. Synthesize cDNA from 100fg-1ug of RNA in reverse transcription reaction using iScript™ Reverse Transcription, according to manufacturer's instructions.

6. Prepare amplification reactions in triplicate with adequate concentrations of primers, cDNA, DEPC water, and Taq DNA Polymerase Recombinant kit (Invitrogen), according to manufacturer’s instructions. Amplify in a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc.)

7. Gene expression levels are analyzed and normalized with the Thermo Fisher Cloud software (also called Thermo Fisher Connet) using hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) as a reference gene.

Troubleshooting

It is highly recommended to use porcine serum in macrophage culture.

The plate can also be pretreated with 1% porcine gelatin.

Flow cytometry analysis should be performed using porcine-specific reagents.

For total RNA isolation from porcine macrophages, any other guanidinium thiocyanate-phenol-chloroform extraction method or column-based can be used, following the manufacturer’s instructions.

For reverse transcription of the isolated total RNA, any other cDNA synthesis kit can be used, following the manufacturer’s instructions.

For RT-PCR analyses, any other probe-based or dye-based methods can be used, following the manufacturer’s instructions. The use of commercially available primers is suggested over self-designed primers.

RT-PCR analyses should be performed using porcine-specific primers

Time Taken

Isolation of PBMCs from whole blood by Ficoll density gradient, cell adhesion to the plates, and washes take 5 hours.

Phenotypic characterization by flow cytometry can be completed in two hours, but macrophage differentiation and activation take 7 days. On the other hand, PCR requires more steps (RNA isolation, retrotranscription, amplification) than can be extended more than 3 hours.

The estimated time does not include the analysis of results.

Anticipated Results

In morphology M1- polarized macrophages show a fried egg-like shape while M2- polarized macrophages show a spindle-like shape (Figure 2A). In the phenotypic characterization by flow cytometry, M1- polarized macrophages results in a higher expression of SLA-II, while M2-polarized macrophages result in a higher expression of CD206 and CD163. (Figure 2B). In the PCR analysis, M1- polarized macrophages show a higher expression of the pro-inflammatory gene TNFa, while M2- polarized macrophages show a higher expression of anti-inflammatory genes such as IL-10 and Arg-1. (Figure 2C).

References

1. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage Polarization: Different Gene Signatures in M1(LPS+) vs. Classically and M2(LPS–) vs. Alternatively Activated Macrophages. Front Immunol. 24 de mayo de 2019;10:1084.

2. Viola A, Munari F, Sánchez-Rodríguez R, Scolaro T, Castegna A. The Metabolic Signature of Macrophage Responses. Front Immunol [Internet]. 2019 [citado 30 de junio de 2022];10. Disponible en: https://www.frontiersin.org/article/10.3389/fimmu.2019.01462

3. Suzuki Y, Yeung AC, Ikeno F. The Representative Porcine Model for Human Cardiovascular Disease. J Biomed Biotechnol. 2011;2011:195483.

4. Gao J, Scheenstra MR, van Dijk A, Veldhuizen EJA, Haagsman HP. A new and efficient culture method for porcine bone marrow-derived M1- and M2-polarized macrophages. Vet Immunol Immunopathol. junio de 2018;200:7-15.

5. Nielsen MC, Andersen MN, Møller HJ. Monocyte isolation techniques significantly impact the phenotype of both isolated monocytes and derived macrophages in vitro. Immunology. enero de 2020;159(1):63-74.

6. Sautter CA, Auray G, Python S, Liniger M, Summerfield A. Phenotypic and functional modulations of porcine macrophages by interferons and interleukin-4. Dev Comp Immunol. julio de 2018;84:181-92.

7. Singleton H, Graham SP, Bodman-Smith KB, Frossard JP, Steinbach F. Establishing Porcine Monocyte-Derived Macrophage and Dendritic Cell Systems for Studying the Interaction with PRRSV-1. Front Microbiol. 2 de junio de 2016;7:832.

Figures

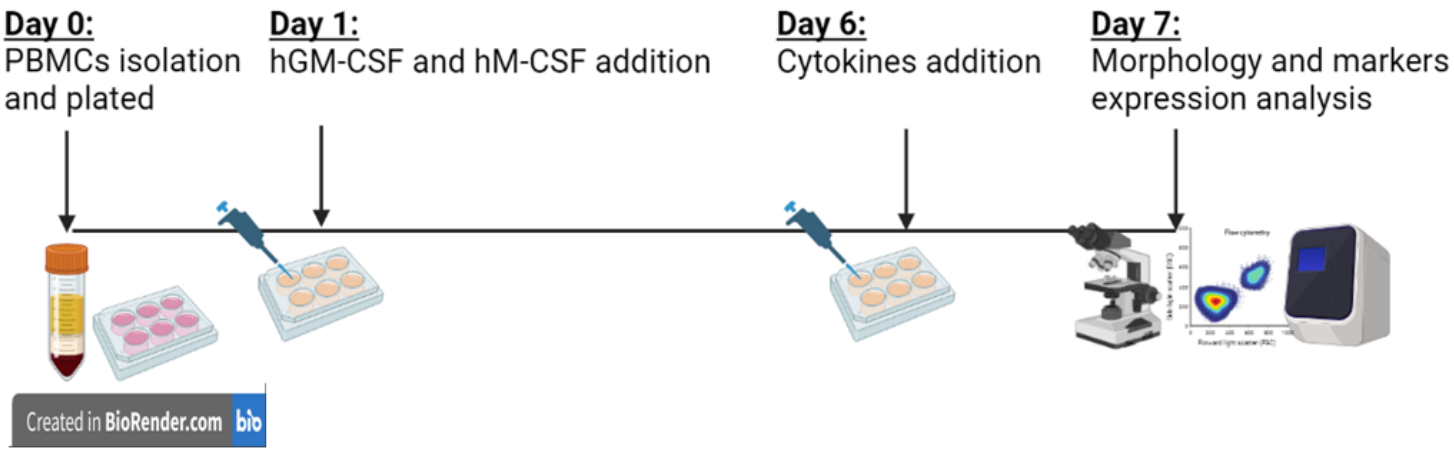

Figure 1

Protocol workflow for isolation and differentiation of porcine macrophages from the peripheral blood.

The protocol begins with a Ficoll density gradient centrifugation to isolate PBMCs from whole blood, followed by a culture in porcine serum pre-treated plates. Non-adherent cells were washed away after at least 3 h of culture. After 24 h of culture, adherent cells were differentiated to M1- and M2- macrophages adding to the medium hGM-CSF and hM-CSF, respectively. On day 7 of culture, surface receptor

expression and gene expression were measured by flow cytometry and qPCR in response to 24 h of LPS + IFNg (M1) or IL-4 (M2) stimulation.

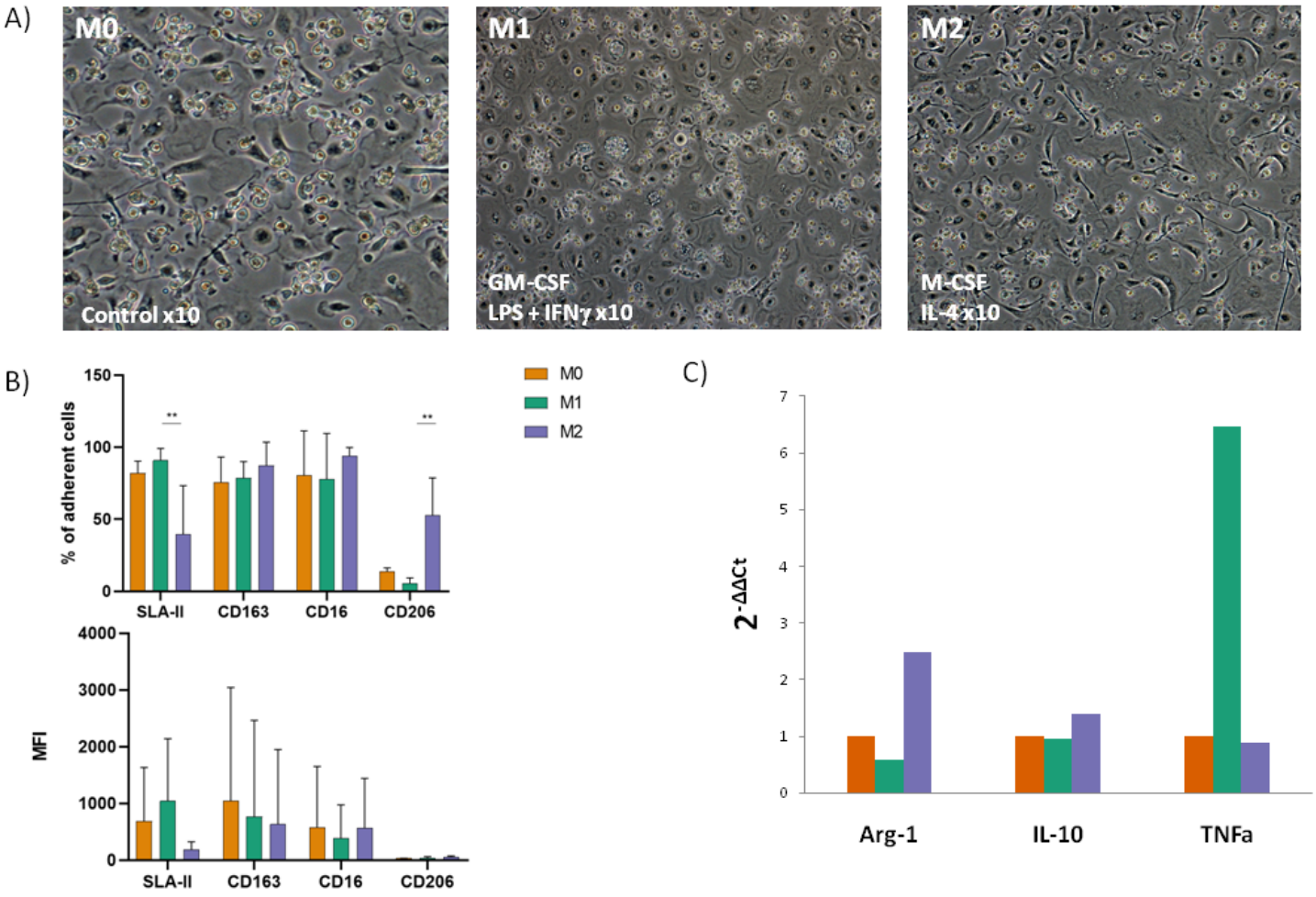

Figure 2

Monocytes were isolated from the blood of healthy pigs by density gradient and cultured with different cytokines for 7 days to differentiate and polarization monocytes to M1- and M2- macrophages.

Figure A shows morphological differences between M1- and M2- polarized macrophages. M1 presents a “fried-egg” like shape whereas M2 presents a “spindle” like shape.

Figure B shows the phenotypic analysis of MF by flow cytometry.

Figure C corresponds to gene expression analysis by qPCR.