The proliferation of MDA-MB-231 cells is repressed by mesenchymal stem cell-mediated macrophage activation conditioned medium through the inhibition of *AKT1* and *YKL-39* genes

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) is characterized by a substantial presence of tumor-associated macrophages (TAMs) exhibiting an M2-like phenotype, which plays a crucial role in promoting tumor cell stemness and invasiveness. Mesenchymal stem cells (MSCs) have the ability to induce the transformation of naive macrophages (M0) into M1-like macrophages. This study delves into the interplay between MSCs and macrophages within the context of breast cancer (BC) progression using a TNBC cell line, as reprogramming of TAMs into M1-like macrophages may emerge as a promising therapeutic strategy for BC. **Methods**: THP-1 cells were induced into M0 macrophages and co-cultured with UC-MSCs, subsequently analyzing CM for M1- and M2-type macrophage-related cytokines. Total RNA from co-cultured cells was used to assess IRF-4 and IRF-5 mRNA gene expression via qRT-PCR. MDA-MB-231 cells were exposed to CM and co-cultured cells to evaluate cell viability through MTT assay over 24, 48, and 72 hours, with qRT-PCR used to examine breast cancer-related gene expression. **Results**: The results indicate that co-culturing M0 macrophages with MSCs promotes M1-like macrophages, as evidenced by upregulated IRF-5 and suppressed M2 macrophage-related genes. Treatment with CM from M0/MSCs co-culture significantly inhibits MDA-MB-231 cell proliferation at 72 hours, accompanied by reduced TNF-α levels. Notably, CM treatment downregulates *AKT1* and *YKL-39* genes in MDA-MB-231 cells, suggesting potential anti-cancer effects. Direct co-culture with M0/MSCs, however, shows no significant impact on TNBC cell growth. **Conclusion**: This study highlights MSCs' ability to induce M0 macrophages to a M1-like phenotype and suggests that CM from M0/MSCs co-culture may contain anti-cancer factors targeting *AKT1* and *YKL-39* genes, underscoring the potential of MSC-mediated macrophage activation as a strategy to enhance BC treatment, especially in the context of TNBC.

Key words: Breast cancer, Mesenchymal stromal/stem cells, Tumor-associated macrophages, Tumor microenvironment, Naïve macrophages, M1 macrophages, M2 macrophages

INTRODUCTION

Breast cancer (BC) is the primary factor responsible for fatalities in women, with approximately 2.3 million newly diagnosed cases and 685,000 reported deaths worldwide in 2020[1]. The mortality rate of BC is significant, contributing to 16% or 1 in every 6 cancer-related deaths in females. The main reason is primarily attributed to the spread of the disease to other parts of the body and the challenge of overcoming systemic treatments[1]. In the beginning, research into BC metastasis and treatment resistance primarily focused on tumor cells alone. Nevertheless, recent years have witnessed thorough investigations into how the tumor microenvironment (TME) contributes to advancing distant metastasis and resistance to therapy[2]. This shift in focus has revealed the significance of non-malignant cells and TME components, such as immune cells and the extracellular matrix, in BC progression[3]. As a result, diverse approaches have undergone investigation to target these non-malignant cells and TME constituents, aiming to disrupt the tumor-promoting environment and enhance treatment outcomes[3]. By understanding the complex interplay between tumor cells and their microenvironment, novel therapeutic approaches can be developed to combat BC metastasis and overcome treatment resistance, offering new hope for patients worldwide.

In normal physiological conditions, tissue-resident macrophages serve as innate immune cells that

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contribute to phagocytic capability, sustaining tissue homeostasis, and defending against pathogens[4]. However, in the context of BC, tumor-associated macrophages (TAMs) heavily infiltrate the TME, comprising over 50% of the cell population within the TME[4]. The TME in BC consists of various components, including adipocytes, fibroblasts, and various categories of leukocytes such as dendritic cells, lymphocytes, and neutrophils[5]. The recruitment of circulating monocytes and resident macrophages sustains the population of TAMs in BC[6]. Upon recruitment, monocytes differentiate into naïve macrophages (M0) under the stimulation of monocyte colony-stimulating factor[6]. Macrophages can polarize into two main phenotypes: M1-like macrophages, also known as classically activated macrophages, and M2-like macrophages, also referred to as alternatively activated macrophages[7]. M1-like macrophages are stimulated by T helper 1-type cytokines such as interferon gamma (IFNγ) or tumor necrosis factor-alpha (TNF-α) and exhibit anti-tumor responses. They secrete pro-inflammatory cytokines like TNF-α and IL-2, along with reactive nitrogen and oxygen intermediates[7]. Conversely, M2-like macrophages are induced by T helper 2-type cytokines such as IL-4, IL-10, and IL-13 and demonstrate pro-tumor responses[7]. Under the influence of specific stimuli, various subtypes of M2a, M2b, M2c, and M2d can be induced from M2-like macrophages[8]. In the TME, cancer cells release cytokines that attract TAMs, which closely resemble M2 macrophages[9]. TAMs, in turn, impede the invasion and role of anti-tumor CD8$^+$ T cells (CTLs), promote tumor angiogenesis, and stimulate tumor cell proliferation and metastasis[9]. The most effective strategy in cancer prevention research has recently shifted from macrophage reduction to TAM re-education[10]. Due to their great ability and flexibility to adjust to external signals, macrophages can have their biological functions taken over and altered in cancer[10]. It is crucial to evaluate TAMs holistically, considering their ontogeny, TME-mediated education, phenotypic diversity, placement within the TME, and tumor-modulating roles[10]. Consequently, targeting the recruitment of TAMs or reconfiguring them into a phenotype competent to eliminate tumor cells has emerged as a potential therapeutic approach in BC.

Mesenchymal stem cells (MSCs) belong to the mesoderm lineage and encompass various multipotent cells, including adipocytes, chondrocytes, fibroblasts, osteoblasts, and smooth muscle cells, among others[11]. MSCs are characterized by the expression of CD73, CD90, and CD105, while lacking hematopoietic lineage markers such as CD14, CD20, CD34, CD45, and HLA-DR[11]. Extensive evidence suggests that MSCs foster the advancement of cancer via numerous avenues, encompassing the stimulation of blood vessel formation, infiltration, proliferation, viability, the creation of carcinoma-associated fibroblasts, and the hindrance of natural and acquired anti-tumor reactions[12]. However, MSCs may even inhibit tumor growth. MSC-CM has been shown to limit breast cancer cell proliferation and make cancer cells more sensitive to radiation by suppressing the STAT3 signaling pathway[13].

The relationship that exists between tissue-draining macrophages and allogeneic MSCs has been explored, in which the macrophages were transformed into regulatory macrophages that inhibited T cell responses *in vivo*[14]. Recent research indicates that the interaction between MSCs and macrophages can promote macrophage polarization to the M2-like phenotype when triggered in the M1 activation stage[15]. MSC-derived soluble factors can convert monocytes or M1 macrophages into M2 cells, with PGE2 playing a significant role[16]. Additionally, a study by Vasandan *et al.* revealed that MSCs induce respiratory burst and nitric oxide-dependent killing mechanisms in macrophages[17]. MSCs altered naïve macrophages towards a pro-inflammatory M1 polarized state, as demonstrated by increased TNFα secretion and decreased levels of DC-SIGN and M2-associated IL-10[17]. Co-culturing naïve primary macrophages with MSCs stimulated CD86 expression, attributing a shift towards M1-like macrophages[17]. Moreover, the study also showed that the co-culture of MSCs with M1-like macrophages led to a reduction in inflammatory M1-like macrophages, accompanied by a shift towards M2-like macrophages[17]. Conversely, the co-culture of MSCs with M2-like macrophages further enhanced the M2 polarization[14]. Vasandan *et al.* concluded that MSC-secreted factors at the MSC-macrophage interface repolarize macrophages by modifying metabolic patterns in variably polarised macrophages[17]. To date, there has been a strong focus on identifying TAMs in the 'either/or' M1 or M2 state, sometimes disregarding other theories[10]. A new study found the expression of both M1 and M2 genes in identical cells[18]. Interestingly, Rabani *et al.* discovered that when MSCs were exposed to M0 macrophages, they produced both M1-like and M2-like macrophage phenotypes simultaneously[18]. From the study, M2-like macrophages showed elongated morphology, were CD163$^+$, had acute phagosomal acidification, low NOX2 expression, and limited phagosomal superoxide production while the

M1-like macrophages produced high levels of phagosomal superoxide but with low or undetectable levels of CD40 [18]. Prostaglandin E2 and phosphatidylinositol 3-kinase were essential for promoting the M1-like macrophages [18]. Because TAMs are highly abundant in tumors, more research is necessary to understand how MSC and macrophages interact to regulate the activation state of macrophages, which will aid in the development of an effective therapeutic strategy for breast cancer.

The present study aims to repolarize M1-like macrophages within the BC microenvironment by co-culturing UC-MSCs with M0 macrophages. Subsequently, the influence of the M0 macrophages co-cultured with UC-MSCs was explored by treating MDA-MB-231 cells with conditioned medium from the co-culture of UC-MSCs with M0 macrophages. In parallel, M0 macrophages treated with MSCs were also cultured directly with MDA-MB-231 to evaluate their effect on TNBC cell growth and their mechanism of action. Repolarizing M1-like macrophages with MSCs may enhance anti-tumor responses and inhibit TAMs, which exhibit pro-tumor responses in the BC microenvironment.

METHODS

Materials

Human umbilical cord mesenchymal stem cells (UC-MSCs), human THP-1 cells, and the MDA-MB-231 cell line were procured from the American Type Culture Collection (ATCC, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), fetal bovine serum (FBS), penicillin and streptomycin (Pen-Strep), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), and phosphate-buffered saline (PBS) were secured from Thermo Fisher Scientific (USA).

Culturing UC-MSCs, THP-1 cells, and MDA-MB-231 cells

UC-MSCs were cultured in DMEM/F12 media containing L-glutamine and sodium bicarbonate, 10% FBS, and 1% Pen-Strep in a 37°C incubator with 5% CO_2. UC-MSCs between passages 7–10 were used for all the experiments. THP-1 cells were grown in RPMI1640 media containing L-glutamine and supplemented with 10% FBS and 1% Pen-Strep. THP-1 cells were incubated with 20 nM PMA for 48 hours to differentiate them into naïve macrophages (M0).

The influence of UC-MSCs on M0 macrophage differentiation into M1 and M2 macrophages was assessed by culturing both cell types together in 6-well culture plates (CORNING, USA), physically separated by cell-culture inserts (0.4 microns, CORNING, USA) for 30 hours. 3.5×10^5 THP-1 cells were used for differentiation experiments, and 3.5×10^4 MSCs were seeded on culture inserts in co-culture conditions [17]. The ratio was established by considering the proportions of MSCs inducing immune suppression in MLRs, as previously reported by Vasandan *et al.* [17]. The respective conditions for co-culture of UC-MSCs with M0 macrophages were untreated M0 macrophages, LPS-treated M0 macrophages, UC-MSCs co-cultured with M0 macrophages, and UC-MSCs co-cultured with LPS-treated M0 macrophages for 30 hours. The conditioned medium (CM) from respective treatments was used to treat MDA-MB-231 cells for 72 hours. Meanwhile, the cells from respective treatments were directly co-cultured with MDA-MB-231 cells for 72 hours.

UC-MSCs Immunophenotyping

UC-MSCs and UC-MSCs co-cultured with M0 macrophages for 30 hours with respective treatments were evaluated for their surface markers using the BD Stemflow™ Human MSC Analysis Kit (BD Bioscience, USA) based on the manufacturer's instructions. Briefly, both types of UC-MSCs were suspended in a tube containing at least 1×10^5 cells each in 100 µl of BD Pharmingen™ stain buffer. The cells were mixed with the appropriate monoclonal antibodies for anti-human CD105 (conjugated with PerCP-Cy™5.5), CD73 (conjugated with APC), CD90 (conjugated with FITC), CD19, CD11beta, CD45, CD34, and HLA-DR (all negative markers conjugated with PE) or the respective isotype control. Cells stained with isotype control from the kit and unstained cell suspension were used as controls. The antibodies were included at concentrations recommended by the manufacturers, and the samples were left to incubate away from light for 30 minutes at 2–8 °C. The samples were then washed twice with PBS and analyzed on a NovoCyte Advanteon Flow Cytometer (Agilent, USA), recording at least 10,000 events per sample. The obtained data were further analyzed using NovoExpress software.

Enzyme-Linked Immunosorbent Assay (ELISA)

TNF-α and IL-10 were quantified in the supernatants of untreated and respective treated M0 macrophages

at each treatment time using the TNF-α and IL-10 DuoSet ELISA kit (R&D Systems, USA) according to the guidelines stated by the manufacturer. Firstly, a 96-well ELISA plate was coated with either TNF-α or IL-10 capture antibodies, respectively, and incubated overnight at 4°C. The plate was washed with wash buffer (400 μl) three times. Subsequently, diluent buffer was added to block the plate and incubated at room temperature for one hour. The blocking buffer was pipetted out, and the washing step was repeated as previously. Standards were prepared by diluting them in reagent diluent for a total of seven points, and both samples and diluted standards (100 μl) were added into respective wells of the plate. The plate was sealed properly and incubated overnight at 4°C. The plate was washed with wash buffer three times post-incubation. Next, TNF-α or IL-10 detection antibodies (100 μl) were added and incubated for 2 hours at room temperature. The detection antibodies were then aspirated, and the washing step was repeated. Subsequently, Streptavidin-HRP (100 μl) was added to each well, and the plate was left at room temperature for a 20-minute incubation period. The aspiration and washing steps were repeated. Finally, substrate solution (100 μl) was added to each well and then incubated for 20 minutes at room temperature. The reaction was terminated by adding stop solution (50 μl) and the optical density of each well was evaluated immediately using a FLUOstar Omega microplate reader (BMG Labtech, Germany) set to 450 nm and 570 nm for wavelength correction.

MTT Assay

The anti-cancer activity of both direct cell-cell interaction and CM of M0 macrophages co-cultured with UC-MSCs (M0/MSCs) on MDA-MB-231 cells was determined by the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay. For the effect of CM, MDA-MB-231 cells (5×10^3/well) were plated in 96-well plates 24 hours before treatment. During treatment, the old medium from the wells was removed and replaced with CM from M0/MSCs (100 μl). For direct cell-cell effect, MDA-MB-231 cells (5×10^3/well) and M0/MSCs cells (5×10^3/well) were seeded together in the well. Both MDA-MB-231 cells for either CM or direct cell-cell treatment were incubated at 24, 48, and 72 hours. Post-treatment, the old media was removed and new media (90 μl) and MTT (10 μl) were added into each well. The MTT assay (Invitrogen, USA) was initially prepared by dissolving in PBS at 5 mg/ml. The plates were incubated at 37°C with 5% CO_2 for 3 hours and the medium was then replaced with 100 μL DMSO. The plates were then incubated for 10 minutes at 37°C with 5% CO_2 and the absorbance for each well was measured at 570 nm using an ELISA microplate reader.

Total RNA Extraction and cDNA synthesis

Total RNA was extracted from MDA-MB-231 cells cultured with CM of M0/MSCs (in a ratio of 1:1 with fresh culture media) and MDA-MB-231 cells that directly co-cultured with M0/MSCs for 72 hours using TRIsure^{TM} (Bioline, UK) following the instructions stated by the manufacturer. NanoDrop (Thermo Fisher Scientific, USA) was used to evaluate the concentration and purity of RNA with an A260:A280 ratio of 1.7–1.9 obtained, demonstrating isolated samples were high-quality RNA with low levels of protein contamination. RNA integrity was assessed by conducting agarose gel electrophoresis with the presence of 28S and 18S rRNA bands. Then, RNA (2 μg) was reverse transcribed into cDNA using the Tetro cDNA Synthesis kit (Bioline, UK) based on instructions stated by the manufacturer. Briefly, the reaction conditions were as follows: 2 μg of RNA, 4 μL of 5x RT buffer, 1 μL of Oligo (dT)18 primer mix, 1 μL of dNTP mix 10 nM, 1 μL of RNase inhibitor, 1 μL of (200 U/μL) reverse transcriptase, and DEPC water to a final volume of 20 μL. Samples were mixed and placed at 45°C for 30 minutes incubation, followed by incubating at 85°C for 5 minutes to terminate the reaction and chilled on ice.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Relative mRNA expression levels were detected via qPCR assays on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). All PCR reactions were carried out in triplicates using a SensiFAST SYBR Hi-Rox Kit (Bioline, UK). Reactions were set up in a final volume of 20 μL containing: 10 μL 2x SensiFAST SYBR® Hi-ROX Mix, 0.8 μL of each primer pair mixture [10 μM of each primer], 2 μL of cDNA and 6.4 μL of nuclease-free water to make up the total volume. All primer sequences are displayed in **Table 1**. The three-step cycling conditions included an initial polymerase activation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 5 seconds, annealing, and extension at 60°C for 10 seconds and 75°C for 5 seconds, respectively. The assessment of mRNA expression levels relative to GAPDH, used as the reference gene, was executed through the $\Delta\Delta$Ct method. To achieve relative quantification, we employed the $2^{-\Delta\Delta Ct}$ method.

Table 1: Sequences of the primers used for q-PCR analyses

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
CD80	CACTTCTGTTCAGGTGTTATCC	GGTGTAGGGAAGTCAGCTTTG
CD163	ACATAGATCATGCATCTGTCATTTG	CATTCTCCTTGGAATCTCACTTCTA
IRF-5	TTCTCTCCTGGGCTGTCTCTG	CTATACAGCTAGGCCCCAGGG
IRF-4	GCTGATCGACCAGATCGACAG	CGGTTGTAGTCCTGCTTGC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-10	TACGGCGCTCGTCATCGATTT	TAGAGTCGCCACCCTGATGT
Akt1	GAAGGACGGGAGCAGGCGGC	CCTCCTCCAGGCAGCCCT
mTOR	AGTGGACCAGTGGAAACAGG	TTCAGCGATGTCTTGTGAGG
YKL-39	AAGATGACCTTGCTGCCT	TGATCTAAGAGGAAGTCAGG
P53	CCCCTCCATCCTTTCTTCTC	ATGAGCCAGATCAGGGACTG

Statistical Analysis

For all conducted experiments, the reported values are the means derived from a minimum of three distinct trials, along with their corresponding standard deviations (SD), unless specified differently. Every experiment was replicated in triplicate. To compare data among different treatment conditions and test samples, we employed a Student's independent t-test. In each experiment, pairwise comparisons were conducted between two groups at a time. Statistical significance was determined at *P < 0.05, **P < 0.01, and ***P < 0.001.

RESULTS

UC-MSCs co-cultured with M0 macrophages and LPS-treated macrophages displayed a low percentage of CD105 expression

UC-MSCs showed strong expression of CD90 (> 98%) and CD73 (> 98%) but low expression of CD105 and no expression of any negative MSC markers when co-cultured with M0 macrophages, LPS-treated M0 macrophages, and UC-MSCs alone (**Figure 1** A-C).

UC-MSCs and LPS transform M0 macrophages into an elongated shape

M0 macrophages adhered to the cell culture plate and had outstretched parapodia (**Figure 2A**), while LPS-treated M0 macrophages acquired a rounded and flat shape phenotype (**Figure 2B**). M0 macrophages and LPS-treated M0 macrophages transformed their round shape to an elongated shape when co-cultured with UC-MSCs, respectively (**Figure 2** C-D).

M0 macrophages co-cultured with UC-MSCs exhibited lower expression of *CD163, TNF-α*, and *IL-10* genes but induced *IRF-5* gene expression

LPS-treated M0 macrophages co-cultured with UC-MSCs significantly increased CD80 gene expression compared to untreated M0 macrophages (P < 0.05) (**Figure 3A**). M0 macrophages co-cultured with UC-MSCs significantly inhibited *CD163* gene expression compared to untreated M0 macrophages (P < 0.001) (**Figure 3B**). Similarly, M0 macrophages and LPS-treated M0 macrophages co-cultured with UC-MSCs inhibited *TNF-α* gene expression compared to untreated M0 macrophages (P < 0.01 and P < 0.001, respectively). In addition, LPS-treated M0 macrophages co-cultured with UC-MSCs also suppressed *TNF-α* gene expression compared to LPS-treated M0 macrophages (P < 0.01) (**Figure 3C**). Furthermore, M0 macrophages co-cultured with UC-MSCs reduced *IL-10* gene expression compared to untreated M0 macrophages (P < 0.001) (**Figure 3D**). On the other hand, LPS-treated M0 macrophages co-cultured with UC-MSCs significantly increased *IRF-4* gene expression when compared with untreated M0 macrophages and LPS-treated M0 macrophages (P < 0.05) (**Figure 3 E**). However, only M0 macrophages co-cultured with UC-MSCs significantly induced *IRF-5* gene expression compared to untreated M0 macrophages (P < 0.05) (**Figure 3 F**).
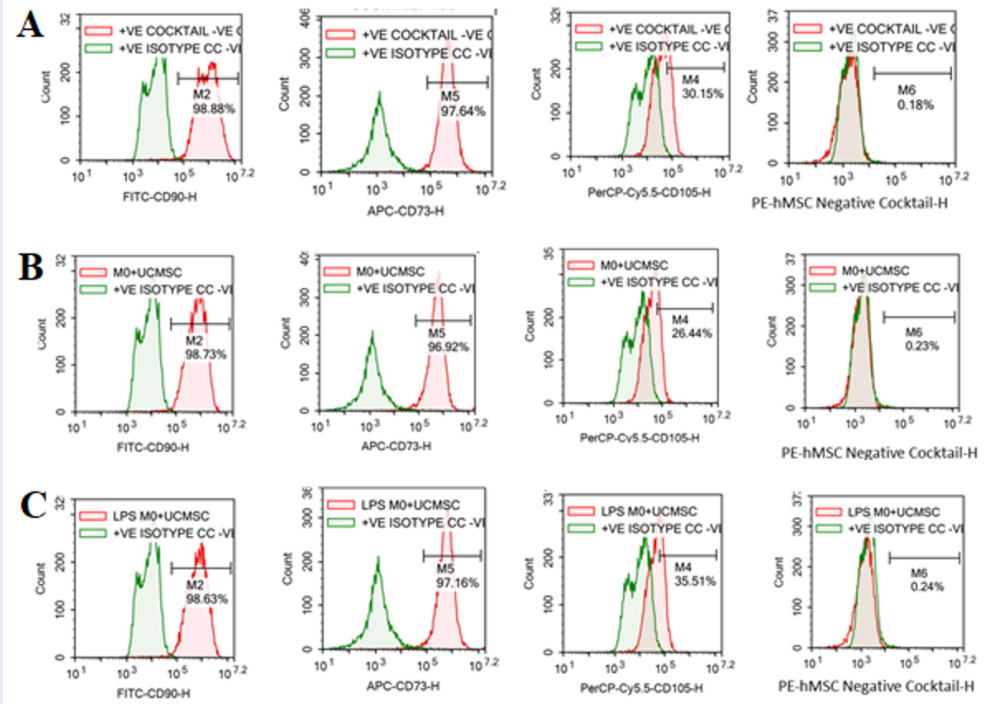
Figure 1: **Representative flow cytometry analysis of UC-MSCs phenotypes based on minimal criteria to define human MSCs proposed by International society for cell and gene therapy (ISCT)**. Respective UC-MSCs were stained with FITC-CD90, APC-CD73, PercP-Cy5.5-CD105 and PE human MSC negative cocktail based on the concentration as suggested by manufacturers, incubated and analysed using flow cytometer. **A**) Untreated UC-MSCs, **B**) UC-MSCs co-cultured with M0 macrophages for 30 hours and **C**) UC-MSCs co-cultured with LPS-treated M0 macrophages for 30 hours. **Abbreviations**: **MSC**: Mesenchymal stem cell, **LPS**: Lipopolysaccharide, **UC-MSC**: Umbilical cord derived mesenchymal stem cell

CM from UC-MSCs co-cultured with M0 macrophages and LPS-treated macrophages did not contain TNF-α

CM from UC-MSCs co-cultured with M0 macrophages and LPS-treated macrophages did not contain TNF-α (**Figure 4A**). However, CM from LPS-treated M0 macrophages significantly highly secreted TNF-α compared to CM from M0 macrophages (P < 0.001) (**Figure 4A**). Meanwhile, CM from LPS-treated M0 macrophages and CM from UC-MSCs co-cultured with M0 macrophages and LPS-treated macrophages highly secreted IL-10 compared to CM from M0 macrophages, but not statistically significantly (**Figure 4 B**).

The proliferation of MDA-MB-231 cells was suppressed when cultured with CM from M0 macrophages co-cultured with UC-MSCs through the inhibition of *AKT1* and *YKL-39* genes

CM from M0 macrophages, CM from M0 macrophages co-cultured with UC-MSCs, and CM from LPS-treated M0 macrophages co-cultured with UC-MSCs significantly inhibited the proliferation of MDA-MB-231 cells at 72 hours compared to 24 hours (P < 0.05) (**Figure 5A**). The expression of the *AKT1* gene was reduced in MDA-MB-231 cultured with CM from M0 macrophages and CM from M0 macrophages co-cultured with UC-MSCs compared to untreated MDA-MB-231 (control) (P < 0.001 and P < 0.05 respectively) (**Figure 5B**). However, the expression level of *AKT1* was increased in MDA-MB-231 cultured with CM from M0 macrophages co-cultured with UC-MSCs, CM from LPS-treated M0 macrophages, and CM from LPS-treated M0 macrophages co-cultured with
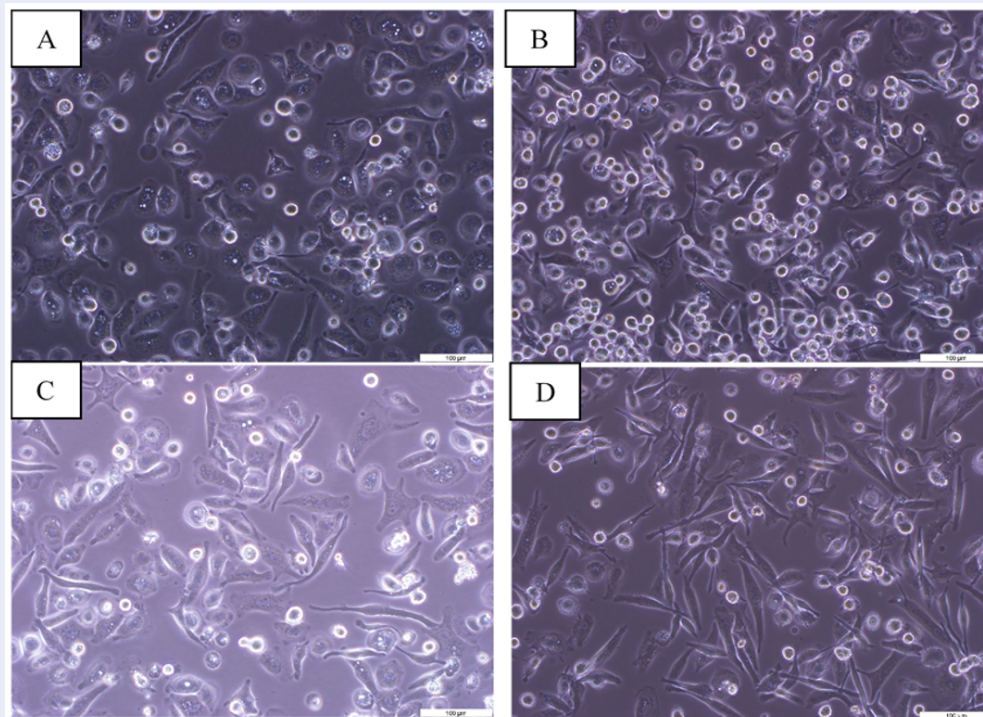
Figure 2: **Evaluation of macrophages polarization**. Images of post 30 hours of (**A**) untreated M0 macrophages, (**B**) LPS-treated M0 macrophages, (**C**) M0 macrophages co-cultured with UC-MSCs and (**D**) LPS-treated M0 macrophages co-cultured with UC-MSCs. All images were visualised under 200x magnification. (Scale bar= 100 μm). **Abbreviations**: **LPS**: Lipopolysaccharide, **UC-MSC**: Umbilical cord derived mesenchymal stem cell

UC-MSCs compared to MDA-MB-231 cultured with CM from M0 macrophages (P < 0.01 and P < 0.05 respectively) (**Figure 5B**). Furthermore, the *p53* gene expression level was significantly decreased in MDA-MB-231 cultured with CM from M0 macrophages (P < 0.001) (**Figure 5C**). Meanwhile, MDA-MB-231 cultured with CM from M0 macrophages co-cultured with UC-MSCs and CM from LPS-treated M0 macrophages displayed a significantly higher level of *p53* gene expression compared to MDA-MB-231 cultured with CM from M0 macrophages (P < 0.01) (**Figure 5C**). On the other hand, there are no significant differences in *mTOR* gene expression in MDA-MB-231 cultured with CM from all respective treatments (**Figure 5 D**). In addition, the expression level of the *YKL-39* gene was reduced in MDA-MB-231 cultured with CM from M0 macrophages and CM from M0 macrophages co-cultured with UC-MSCs compared to untreated MDA-MB-231 (control) (P < 0.001 and P < 0.01) (**Figure 5 E**).

MDA-MB-231 cells directly co-cultured with M0 macrophages co-cultured with UC-MSCs displayed a consistent increase in cell proliferation

The results of the cell viability study indicated that MDA-MB-231 co-cultured with M0 macrophages, M0 macrophages co-cultured with UC-MSCs, LPS-treated M0 macrophages, and LPS-treated M0 macrophages co-cultured with UC-MSCs consistently increase in cell proliferation compared to the control group at both 24 and 48 hours (**Figure 6A**). However, as the study progresses to the 72-hour mark, a slight deviation from this trend is observed when MDA-MB-231 is co-cultured with LPS-treated M0 macrophages and LPS-treated M0 macrophages co-cultured with UC-MSCs (P < 0.05) (**Figure 6A**). However, the treatments still maintain a relatively high level of cell viability, with only a minor decrease of approximately 5% below the baseline 100% viability observed when MDA-MB-231 is co-cultured with LPS-treated M0 macrophages co-cultured with UC-MSCs (**Figure 6A**). At 72 hours, the expression level of the *AKT1* gene was significantly reduced
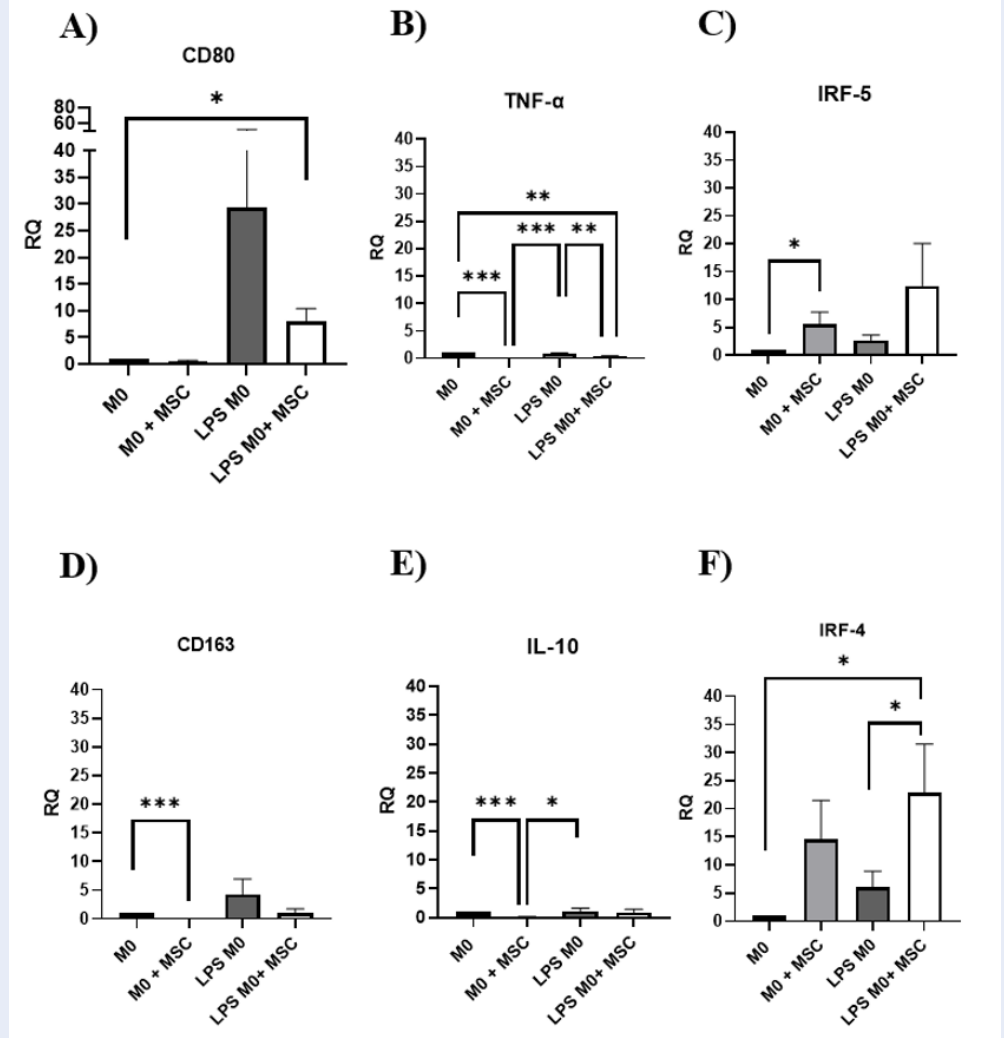
Figure 3: **Expression of M1/M2 related genes following M0 macrophages co-cultured with UC-MSCs for 30 hours**. M0 macrophages were cultured respective treatment for 30 hours and were extracted total RNA and synthesized into cDNA. Relative quantification of M1/M2 related gene expression were determined by qRT-PCR and normalized with GAPDH. Values are expressed as mean $\pm$ SD from three independent experiments (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. **Abbreviations**: **MSC**: Mesenchymal stem cell, **LPS**: Lipopolysaccharide, **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction, **SD:** Standard Deviation; **UC-MSC**: Umbilical cord derived mesenchymal stem cell

in MDA-MB-231 co-cultured with LPS-treated M0 macrophages co-cultured with UC-MSCs compared to untreated MDA-MB-231 (control) (P < 0.01) (**Figure 6B**). There are no significant differences in *p53* gene expression in all MDA-MB-231 treated groups (**Figure 6C**). Meanwhile, MDA-MB-231 co-cultured with M0 macrophages, M0 macrophages co-cultured with UC-MSCs, LPS-treated M0 macrophages, and LPS-treated M0 macrophages co-cultured with UC-MSCs had a significantly lower expression of the *mTOR* gene compared to untreated

MDA-MB-231 (control) (P < 0.05, P < 0.001, P < 0.01) (**Figure 6D**). In addition, the expression level of the *YKL-39* gene was significantly reduced in MDA-MB-231 co-cultured with M0 macrophages and M0 macrophages co-cultured with UC-MSCs compared to untreated MDA-MB-231 (control) (P < 0.05 and P < 0.01) (**Figure 6 E**). In contrast, MDA-MB-231 co-cultured with LPS-treated M0 macrophages co-cultured with UC-MSCs had a significantly higher expression of the *YKL-39* gene compared to MDA-MB-231 co-cultured with M0
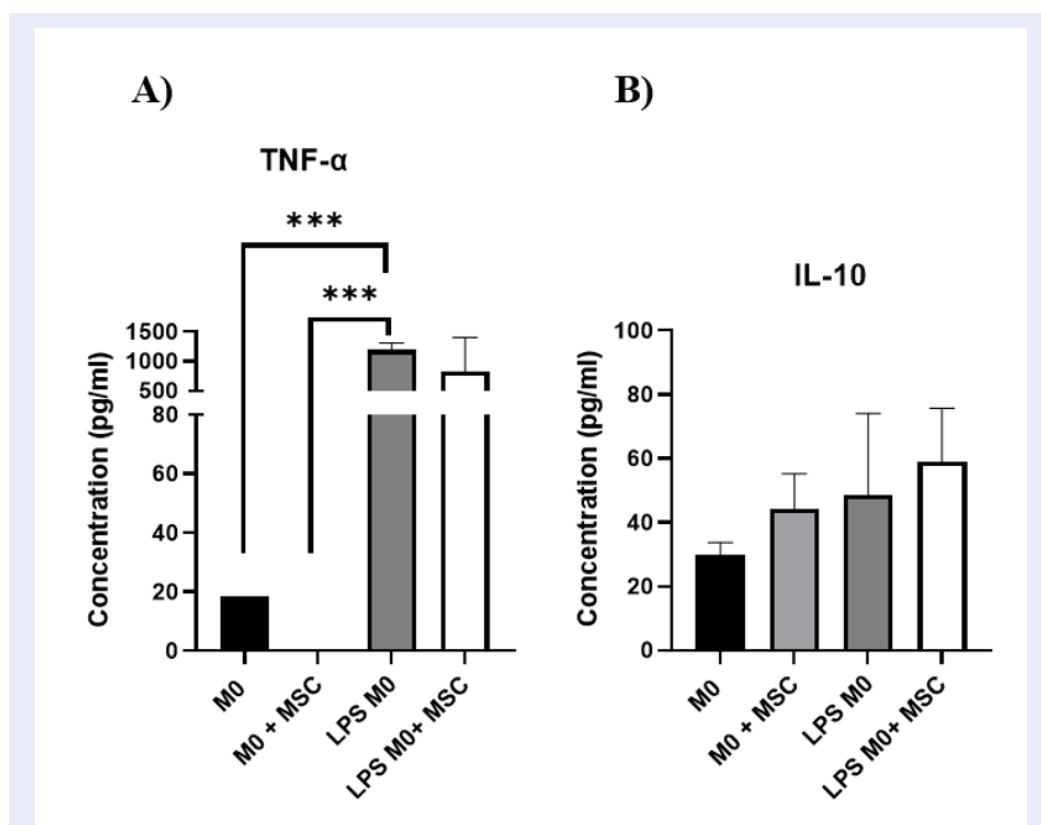
Figure 4: **CM from UC-MSCs co-cultured with M0 macrophages and LPS-treated macrophages did not secreted TNF-α and IL-10**. The level of TNF-α (**A**) and IL-10 (**B**) secreted in the CM of untreated M0 macrophages, LPS-treated M0 macrophages and CM from UC-MSCs co-cultured with M0 macrophage and LPS-treated macrophages after at 30 hours were measured using ELISA kit. Values are expressed as mean $\pm$ SD from three independent experiments (n = 3). ***P < 0.001. **Abbreviations**: **ELISA**: Enzyme-Linked Immunosorbent Assay, **IL**: Interleukin, **MSC**: Mesenchymal stem cell, **LPS**: Lipopolysaccharide, **qRT-PCR**: Quantitative Real-Time Polymerase Chain Reaction, **SD**: Standard Deviation, **UC-MSC**: Umbilical cord derived mesenchymal stem cell, **TNF-α:** Tumor Necrosis Factor-alpha

macrophages (P < 0.05) (**Figure 6 E**).

DISCUSSION

Mesenchymal stem cells, also known as multipotent stromal cells (MSCs), represent a subset of non-hematopoietic adult stem cells that can be found in a wide range of tissues throughout the body[19]. They play a crucial role as resident tissue reservoirs for precursor cells, facilitating tissue replacement and repair through their unique capacity for differentiation and their ability to influence the adjacent surroundings by secreting trophic factors[20]. The phenotype of the UC-MSCs employed in our current study was characterized by positive expression of CD73, CD90, and CD105, while being negative for CD34, CD45, CD11b, CD14, CD19, CD79α, and HLA-DR. These findings are consistent with previous observations, even when these cells were co-cultured with

M0 macrophages and LPS-treated M0 macrophages for a duration of 30 hours. This characterization aligns with the guidelines established by the International Society for Cellular Therapy (ISCT)[21]. However, it is worth noting that the percentage of CD105-positive cells ranged from 26% to 35%. While CD105 is commonly considered a signature MSC marker, research addressing its applicability is not entirely consistent. For instance, Cleary *et al.* demonstrated that the chondrogenic differentiation potential of bone marrow-derived MSCs was not dependent on their CD105 surface expression[22]. Dizaji *et al.* reported that 76% of amniotic membrane-derived MSCs were CD105-positive, while 92% of adipose tissue-derived MSCs exhibited this marker[23]. Lee *et al.* demonstrated that the culture conditions may control the expression of CD105 in MSCs generated from bone marrow, with alginate and transforming growth fac-
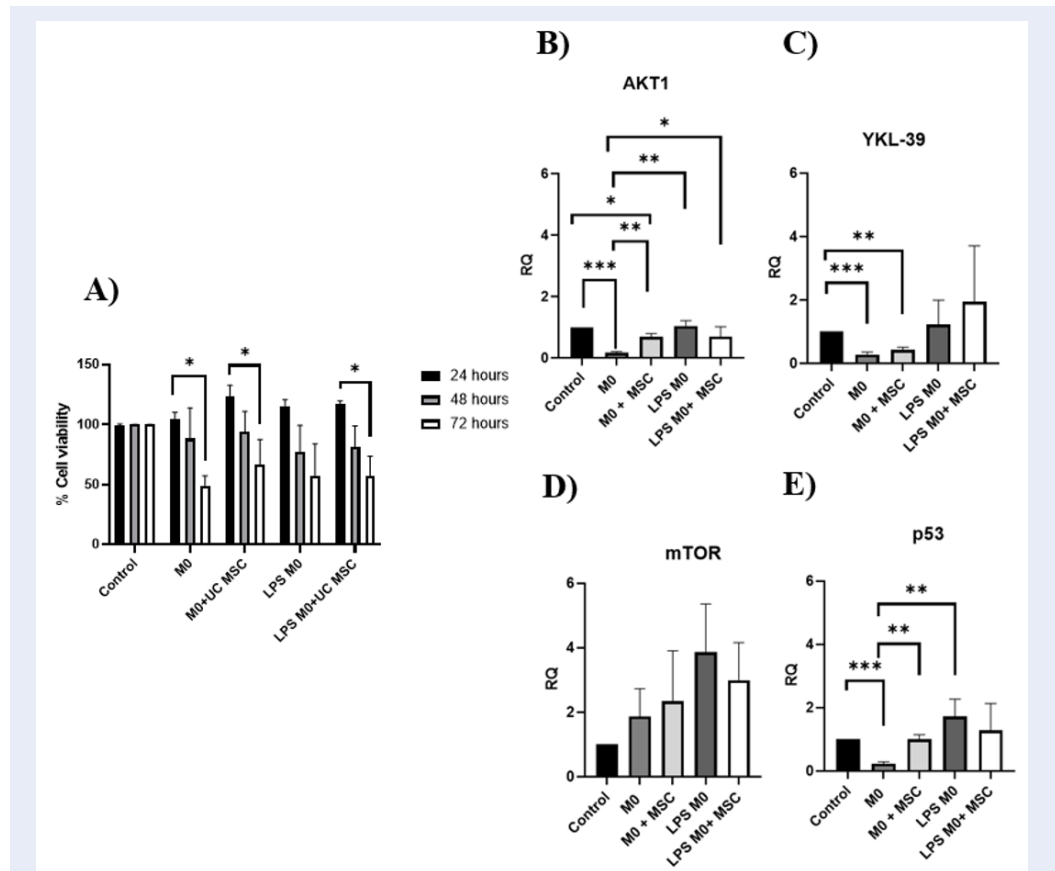
Figure 5: **Cell viability measurement when MDA-MB-231 cultured with CM from M0 macrophages co-cultured with UC-MSCs and selected breast cancer gene expression**. MDA-MB-231 were cultured with CM of M0 macrophages, CM of M0 macrophages co-cultured with UC-MSCs, CM of LPs-treated M0 macrophages and CM of LPS-treated M0 macrophages co-cultured with UC-MSCs for 24, 48 and 72 hours and measured their cells proliferation by MTT assay (**A**). Subsequently, MDA-MB-231 cultured with aforementioned CM of M0 macrophages for 72 hours were extracted total RNA and synthesised into cDNA. Breast cancer related genes expression were measured by using qRT-PCR and normalised against GAPDH (**B-E**). Values are expressed as mean $\pm$ SD from three independent experiments (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001. **Abbreviations**: **CM:** Conditioned Medium, **MSC:** Mesenchymal stem cell, **LPS**: Lipopolysaccharide, **SD:** Standard Deviation, **MTT:** 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, **UC-MSC**: Umbilical cord derived mesenchymal stem cell

tor beta-3 significantly lowering its expression [24]. As a result, the origin of the UC-MSCs, the number of passages, and the particular culture conditions used could all have an impact on the percentage of CD105 surface expression (< 90%) for our study.

The TME in breast cancer consists of a population of tumor cells that interact with a variety of immune cells and MSCs, creating a complex network driving tumor development [25,26]. Apart from their direct interaction with tumor cells, MSCs regulate different types of immune cells in the TME, such as neutrophils, macrophages, and natural killer cells, which eventually affects the growth of the tumor. Macrophages are heterogeneous cells that can be classified as either M1-like or M2-like macrophages based on the

particular stimuli present at the time of activation. Notably, TAMs isolated from metastatic tumors often display a suppressive M2-like phenotype. To close the existing gaps in our understanding of the mechanisms at the MSC-macrophage interface in breast cancer, this current work aims to analyze the interactions between MSCs and macrophages at different activation states. THP-1 monocytes, once differentiated, are often used as an in vitro model for human macrophages. From these THP-1 cells, M1-like and M2-like macrophage populations were generated through co-culturing with UC-MSCs. Microscopic examination revealed that PMA-treated THP-1 cells (M0 macrophages), LPS-treated M0 macrophages, M0 macrophages co-cultured with UC-MSCs, and
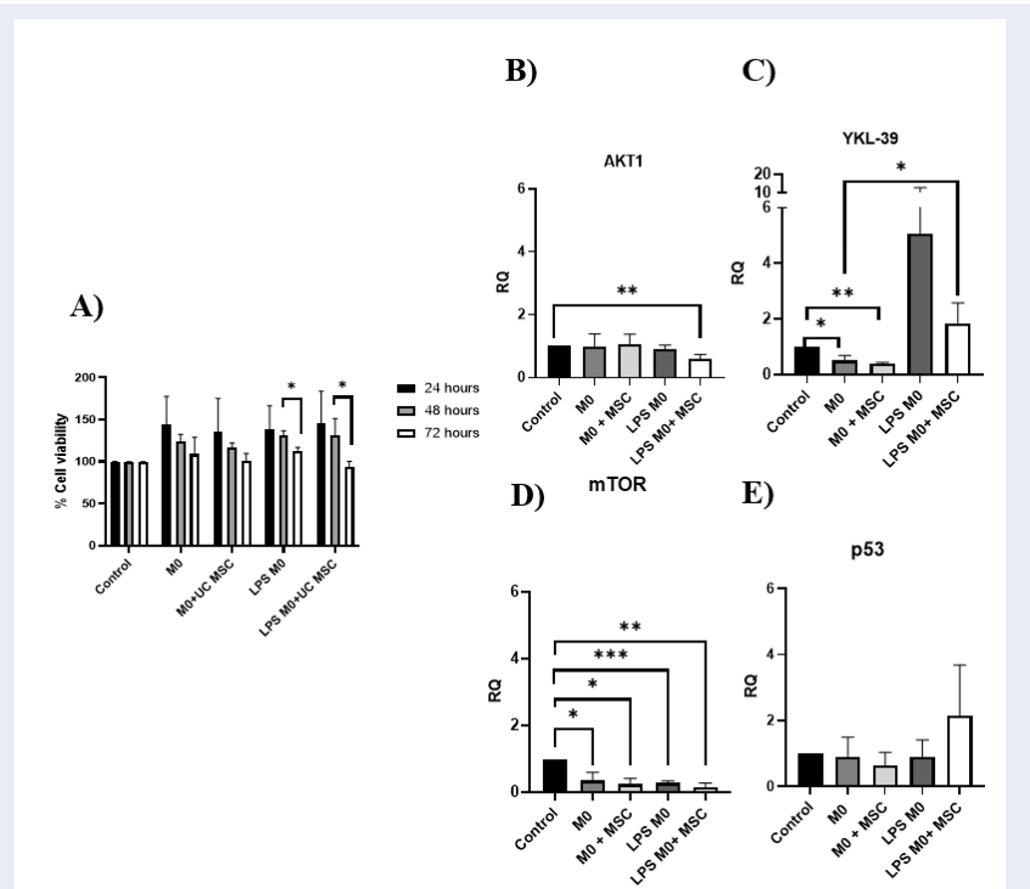
Figure 6: **Cell viability measurement of MDA-MB-231 when directly cultured with M0 macrophages co-cultured with UC-MCSs and selected breast cancer gene expression**. MDA-MB-231 were co-cultured with M0 macrophages, M0 macrophages co-cultured with UC-MSCs, LPs-treated M0 macrophages and LPS-treated M0 macrophages co-cultured with UC-MSCs for 24, 48 and 72 hours and measured their cells proliferation by MTT assay (**A**). Subsequently, MDA-MB-231 co-cultured with aforementioned M0 macrophages for 72 hours were extracted total RNA and synthesised into cDNA. Breast cancer related genes expression were measured by using qRT-PCR and normalised against GAPDH (**B-E**). Values are expressed as mean $\pm$ SD from three independent experiments (n = 3). *P < 0.05, **P<0.01, ***P < 0.001. **Abbreviations**: **MSC:** Mesenchymal stem cell, **LPS**: Lipopolysaccharide, **qRT-PCR:** Quantitative Real-Time Polymerase Chain Reaction, **SD:** Standard Deviation, **MTT:** 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, **UC-MSC**: Umbilical cord derived mesenchymal stem cell

LPS-treated M0 macrophages co-cultured with UC-MSCs all exhibited adhesion characteristics, displaying the typical flattened, amoeboid-like, elongated, and branching macrophage morphology. The assessment of M0 macrophages co-cultured with M0 macrophages were treated with UC-MSCs in the absence of M1 or M2 stimuli (IFN-γ and IL-4, respectively) to observe if they could shift towards an M1 or M2-like phenotype. LPS without IFN-γ has been found to bind Toll-like receptor 4 on the surface of M0 macrophages, facilitating their transition to an M1-like phenotype [27]. Peshkova *et al.* previously selected UC-MSCs for their study due to high levels of cy-

tokines identified in their conditioned medium compared to MSCs from adipose tissue, bone marrow, gingival tissue, and placenta [28]. Pro-inflammatory cytokines such as IFNγ, TNF-α, IL-1β, and IL-6 made up the majority of the cytokines detected in the UC-MSCs conditioned media from the study, whereas anti-inflammatory cytokines such as IL-1RA, IL-10, and IL-13 were found at lower quantities [28]. It is well established that MSCs have an immunomodulatory influence on macrophage polarization away from traditional pro-inflammatory M1-like macrophages and toward alternatively activated anti-inflammatory M2-like macrophages. However, the mechanism of

MSC-mediated macrophage phenotypic regulation is currently still being investigated[28]. Our work indicated a substantial increase in IRF-4 expression in LPS-treated macrophages co-cultured with UC-MSCs compared to those without UC-MSCs. This is consistent with earlier studies because IRF-4 is a transcription factor that drives M2 activation[29]. In naive or M0 macrophages, UC-MSCs skewed the macrophages toward an M1-like phenotype by enhancing the expression of the IRF-5 gene while suppressing the CD163 and IL-10 genes, both of which are associated with M2-like macrophages. UC-MSCs also inhibited the expression of the TNF-α gene, a marker of M1-like macrophages, in both M0 macrophages and LPS-treated M0 macrophages. This finding aligns with previous research demonstrating the suppression of TNF-α secretion by MSC-conditioned media in M1-like macrophages[30,31]. As a result, our findings demonstrate that UC-MSCs can exert immunomodulatory effects via soluble factors, effectively converting M0 macrophages to an M1-like phenotype.

MSCs produced from umbilical cord/cord blood (UC-MSCs) have been shown in studies to have better anti-cancer capability, inhibiting the growth of solid tumor cell lines such as breast, liver, prostate, and bladder cancer. BM-MSCs mostly promote tumor growth, while some studies have found anti-proliferative effects[32]. We then investigated the interaction between M0 macrophages co-cultured with UC-MSCs and MDA-MB-231 cells, a TNBC cell line. To investigate this interaction, we treated MDA-MB-231 cells both directly via cell-to-cell contact and indirectly utilizing conditioned media (CM) from M0 macrophages co-cultured with UC-MSCs (M0/MSCs). A study investigated the effects of CM from THP-1 macrophages differentiated with PMA and human M1 macrophages on the colon cancer cell lines HT29 and CACO-2[33]. The study found G0/G1 and G2/M cell cycle arrest, but the influence of TNF-α and CXCL9 generated by M1-like macrophages on HT-29 cell proliferation appeared insignificant. This suggests the participation of other macrophage-released mediators in lowering cancer cell proliferation[33]. Another study by Song *et al.* reported that 25% CM from naive macrophages could restrain the proliferation of both MCF-7 and MDA-MB-231 breast cancer cell lines. Inflammatory cytokines, particularly IL-6 and IL-8, were implicated in limiting breast cancer cell growth rather than promoting it[34]. In our investigation, CM from M0 macrophages, along with CM from M0 macrophages co-cultured with UC-MSCs and CM from LPS-treated M0 macrophages co-cultured with UC-MSCs,

significantly inhibited MDA-MB-231 cell proliferation at 72 hours compared to their respective treatments at 24 hours. Similar inhibitory patterns were observed in MDA-MB-231 cell proliferation when co-cultured with LPS-treated macrophages, although statistical significance was not achieved. Additionally, CM from human bone marrow MSCs (BM-MSCs) previously inhibited the proliferation of CAL-27 and FaDu squamous cell carcinoma cell lines by lowering the expression of PCNA (a proliferation marker) and BCL-2 (an anti-apoptotic protein) in both cell lines[35]. CM from Wharton's jelly-derived MSCs exhibited potent inhibition of proliferation in T47D and MCF-7 breast cancer cell lines[36]. Khalil *et al.* also demonstrated the anti-proliferative and anti-apoptotic effects of MSCs from adipose tissue, bone marrow, and umbilical cord on ovarian cancer cell lines, leading to a significant reduction in cancer markers and cell proliferation[37]. CM from M0/UC-MSCs with or without LPS may contain multiple secretomes, which can either positively or negatively affect cell behavior[38]. Interferon-β (IFN-β) secretion by UC-MSCs has been demonstrated to effectively impede MDA-MB-231 cell proliferation by triggering apoptosis[39]. Furthermore, it is possible that UC-MSCs may secrete exosomes within the CM of M0/UC-MSCs. Exosomes are nanosized membrane vesicles ranging from 40 to 160 nm in diameter, which can be released by various cell types[40]. These exosomes contain a complex cargo, including nucleic acids such as DNA, mRNAs, and noncoding RNAs, lipids, and a variety of proteins[41]. Remarkably, multiple studies have highlighted the central role of MSC-derived exosomes in modulating TME and influencing the development of various human cancers[42]. Moreover, MSC-derived exosomes have demonstrated anti-angiogenic properties. In a breast cancer model, Lee *et al.* demonstrated that exosomes derived from MSCs could suppress angiogenesis by downregulating the expression of VEGF[43]. Zhou *et al.* have provided evidence that exosomes secreted from bone marrow-derived MSCs can accelerate anti-tumoral macrophage polarization and facilitate the engagement of cytotoxic lymphocytes, thereby improving immune-based treatment for pancreatic cancer within a living organism[44]. As a result, it is possible that unidentified pro-inflammatory substances, such as cytokines or exosomes from M0 macrophages, may become more abundant after co-culture with UC-MSCs, leading to their anti-cancer action.

Regarding direct interactions between M0 macrophages and MDA-MB-231 cells, noteworthy inhibitory effects were primarily observed in

LPS-treated macrophages, both with and without co-culture alongside UC-MSCs, at the 72-hour time point in comparison to their 48-hour counterparts. However, it's important to highlight that the percentages of cell viability for both cell types when co-cultured directly with MDA-MB-231 cells mostly remained higher compared to the viability of the control group. In the control group, MDA-MB-231 cells were cultured alone without any treatment for 24 hours, 48 hours, and 72 hours, respectively. This suggests that direct cell-to-cell contact may potentially influence macrophages to support the TNBC cell line growth rather than inhibiting it, which contrasts with the outcomes observed when using CM. Interestingly, a previous study demonstrated that direct mixed co-cultures of M1 and M2 macrophages could enhance the proliferation of T98G cells, a human glioblastoma cell line, with M2 macrophages promoting significantly higher proliferation rates[45]. Direct physical contact between macrophages and tumor cells also provided protection to the tumor cells against apoptosis induced by chemotherapy drugs, whereas interactions between these cells without direct contact did not yield the same effect[46]. Furthermore, this direct co-culture could result in greater STAT3 activation in tumor cells when compared to indirect co-culture when physically separated using a transwell co-culture system[47]. An earlier study indicated that the activation of STAT3 in ovarian and kidney cancer cells significantly increased when these cells were directly co-cultured with macrophages[48,49]. Macrophages may have a supporting role in promoting cancer cell proliferation and survival in patients with malignant tumors, as STAT3 activation is intimately associated with these two outcomes[50]. Macrophages and cancer cells' direct interaction promote the growth of the cancer cells, possibly regulated by STAT3.

The following intriguing question concerned the genes involved in regulating the reduction in MDA-MB-231 cell proliferation when co-cultured with CM generated from M0/MSCs. Notable effects were only observed on the expression of *AKT1* and *YKL-39* genes in MDA-MB-231 cells when treated with CM from M0 macrophages co-cultured with UC-MSCs, as compared to the control group. The role of AKT1 genes in breast cancer involves promoting cell survival and proliferation through its involvement in the PI3K-AKT-mTOR signaling pathway[51]. In a study by Choi *et al.* the anti-cancer effects of compound K (CK) were examined in SKBR3 and MDA-MB-231

cells, revealing that CK hinders cancer cell proliferation and induces apoptosis by targeting AKT1[51]. Additionally, AKT activation has been reported to trigger resistance to tamoxifen in breast cancer cells[52,53]. Arranz and colleagues have highlighted the significant involvement of AKT isoforms in macrophage polarization in which the absence of the AKT1 isoform promotes the M1-like phenotype, whereas the loss of the AKT2 isoform leads to the development of the M2-like phenotype[54]. This observation suggests that the CM from M0/MSCs may contain a high level of M1-like macrophage-associated secretomes, which are responsible for suppressing *AKT1* gene expression. On the other hand, YKL-39, a closely related homolog of YKL-40, was initially identified as a highly secreted protein in primary cultures of human articular chondrocytes[55]. Initially, YKL-39 was proposed as a biomarker for chondrocyte activation and the progression of osteoarthritis in humans[56]. The only reported study investigating the link between *YKL-39* and cancer was conducted by Kavsan *et al.*, who revealed heightened *CHI3L2* gene expression in glioblastoma[57]. Subsequently, Liu *et al.* conducted a study that revealed *YKL-39* expression in TAMs but not in human breast cancer cells[58]. Therefore, the suppression of MDA-MB-231 cell growth following culture with CM from M0/MSCs may result in a reduced TAMs population, leading to the inhibition of the *YKL-39* gene.

Our current work will undoubtedly benefit from a number of improvements that could improve understanding of the MSC-macrophage-breast cancer cell interaction and assist to design more effective therapeutic options targeting the TME. Our study employed two-dimensional (2D) cell culture which is the most common research model utilized since the early 1900s. Future study can take advantage of the 3D cell culture technology, which has recently gained popularity for various applications, particularly in cancer research, stem cell research, pharmaceutical development, and disease investigations[59]. When it comes to simulating the biological behavior of tumor cells, particularly the mechanisms that lead to therapeutic escape and drug resistance, the 2D culture technique is appearing less appropriate than the 3D cell culture approach[60]. However, although these 3D models are promising, their application still presents several obstacles. The selection of the most appropriate 3D model to the question posed is also based on the analytical processes that will be done[60]. With 3D culture, there is still the need to break up spheroids into a single-cell suspension prior to subsequent assay analysis, as well as a limitation due to the automation

of liquid handling when using viscous liquids such as collagen- and Matrigel-based hydrogels [59]. Since our *in vitro* study exposed macrophages to MSCs for 30 hours, which may not precisely reflect interactions *in vivo*, more analysis is required to understand the timing of the effect [18]. Moreover, MSCs from other sources, such as bone marrow or adipose tissue, may exhibit different properties. Additionally, MSCs can vary between donors, potentially influencing their interactions with tumor cells and their immunomodulatory effects. The current study employs CM to investigate indirect cell interactions but does not fully characterize the composition of these media. Identifying and quantifying the various factors present, such as cytokines, chemokines, growth factors, and exosomes, is crucial for elucidating the mechanisms driving the observed effects. Furthermore, the current research was based on analyses at specific time points, which might not capture the full dynamics of cell interactions and gene expression changes. More frequent sampling could provide insight into transient or early signaling events. The reliance on gene expression data alone to infer functional outcomes might overlook important aspects of cellular responses. Study on protein expression levels and conducting functional assays such as migration and invasion assays for cancer cells or phagocytosis assays for macrophage would offer a more holistic view of cellular activities. Lastly, while the study identifies gene expression changes linked to MSC-macrophage interactions and their impact on breast cancer cells, it lacks a thorough investigation of the underlying signaling pathways and molecular mechanisms. A deeper exploration into these aspects in future study could enhance our understanding of the MSC-macrophage-breast cancer cell interactions and contribute to the development of more effective TME-targeted therapies.

CONCLUSIONS

Our research reveals that UC-MSCs are capable of inducing an M1-like phenotype in M0 macrophages. The CM from these M0 macrophages, which have been influenced by MSCs, may contain anti-tumor factors derived from both M1 macrophages and MSCs. These factors have the potential to inhibit the growth of a TNBC cell line by targeting key molecules such as *AKT1* and *YKL-39*. This study highlights the need for further investigation into the interactions between MSCs, macrophages, and BC cells. Such research could lead to the development of more effective therapeutic strategies aimed at targeting the TME to enhance TNBC treatment outcomes.

ABBREVIATIONS

AKT1 - A serine/threonine-specific protein kinase, **BC** - Breast Cancer, **BCL-2** - B-cell lymphoma 2, **BM-MSCs** - Bone Marrow Mesenchymal Stem Cells, **CD** - Cluster of Differentiation, **CM** - Conditioned Medium, **CTLs** - Cytotoxic T Lymphocytes, **CXCL9** - Chemokine (C-X-C motif) ligand 9, **DEPC** - Diethylpyrocarbonate, **DMEM** - Dulbecco's Modified Eagle Medium, **ELISA** - Enzyme-Linked Immunosorbent Assay, **FBS** - Fetal Bovine Serum, **HLA-DR** - Human Leukocyte Antigen - DR isotype, **IFNγ** - Interferon gamma, **IL** - Interleukin, **ISCT** - International Society for Cellular Therapy, **IRF** - Interferon Regulatory Factor, **LPS** - Lipopolysaccharide, **M0** - Naïve macrophages, **M1** - Classically activated macrophages, **M2** - Alternatively activated macrophages, **MDA-MB-231** - A type of human breast cancer cell line, **MSC** - Mesenchymal Stem Cells, **MTT** - 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide, **mTOR** - Mammalian Target of Rapamycin, **NOX2** - NADPH oxidase 2, **PBS** - Phosphate-Buffered Saline, **PCNA** - Proliferating Cell Nuclear Antigen, **PGE2** - Prostaglandin E2, **PMA** - Phorbol 12-myristate 13-acetate, **qRT-PCR** - Quantitative Real-Time Polymerase Chain Reaction, **RPMI** - Roswell Park Memorial Institute Medium, **SD** - Standard Deviation, **STAT3** - Signal Transducer and Activator of Transcription 3, **TAM** - Tumor-Associated Macrophages, **TME** - Tumor Microenvironment, **TNBC** - Triple-Negative Breast Cancer, **TNF-α** - Tumor Necrosis Factor-alpha, **UC-MSCs** - Umbilical Cord Mesenchymal Stem Cells, **VEGF** - Vascular Endothelial Growth Factor, **YKL-39** - A gene/protein sometimes associated with inflammation

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AUTHOR'S CONTRIBUTIONS

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

1. Arnold M, Morgan E, Rumgay H, Mafra A, Singh D, Laversanne M. Current and future burden of breast cancer: global statistics for 2020 and 2040. The Breast. 2022;66:15–23. Available from: https://doi.org/10.1016/j.breast.2022.08.010.

2. Arneth B. Tumor microenvironment. Medicina (Kaunas, Lithuania). 2019;56(1):15. Available from: https://doi.org/10.3390/medicina56010015.

3. Belli C, Trapani D, Viale G, D'Amico P, Duso BA, Vigna PD. Targeting the microenvironment in solid tumors. Cancer Treatment Reviews. 2018;65:22–32. Available from: https://doi.org/10.1016/j.ctrv.2018.02.004.

4. Qiu SQ, Waaijer SJ, Zwager MC, de Vries EG, van der Vegt B, Schröder CP. Tumor-associated macrophages in breast cancer: innocent bystander or important player? Cancer Treatment Reviews. 2018;70:178–89. Available from: https://doi.org/10.1016/j.ctrv.2018.08.010.

5. Gao Y, Bado I, Wang H, Zhang W, Rosen JM, Zhang XH. Metastasis organotropism: redefining the congenial soil. Developmental Cell. 2019;49(3):375–91. Available from: https://doi.org/10.1016/j.devcel.2019.04.012.

6. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. Nature Reviews Immunology. 2019;19(6):369–82. Available from: https://doi.org/10.1038/s41577-019-0127-6.

7. Hourani T, Holden JA, Li W, Lenzo JC, Hadjigol S, O'Brien-Simpson NM. Tumor associated macrophages: Origin, recruitment, phenotypic diversity, and targeting. Frontiers in Oncology. 2021;11:788365. Available from: https://doi.org/10.3389/fonc.2021.788365.

8. Lu J, Ma L. The role of tumor-associated macrophages in the development, metastasis and treatment of breast cancer. Pathology-Research and Practice. 2020;216(9):153085. Available from: https://doi.org/10.1016/j.prp.2020.153085.

9. Chen Y, Song Y, Du W, Gong L, Chang H, Zou Z. Tumor-associated macrophages: an accomplice in solid tumor progression. Journal of Biomedical Science. 2019;26(1):78. Available from: https://doi.org/10.1186/s12929-019-0568-z.

10. Kowal J, Kornete M, Joyce JA. Re-education of macrophages as a therapeutic strategy in cancer. Immunotherapy. 2019;11(8):677–89. Available from: https://doi.org/10.2217/imt-2018-0156.

11. Hmadcha A, Martin-Montalvo A, Gauthier BR, Soria B, Capilla-Gonzalez V. Therapeutic potential of mesenchymal stem cells for cancer therapy. Frontiers in Bioengineering and Biotechnology. 2020;8:43. Available from: https://doi.org/10.3389/fbioe.2020.00043.

12. Xuan X, Tian C, Zhao M, Sun Y, Huang C. Mesenchymal stem cells in cancer progression and anticancer therapeutic resistance. Cancer Cell International. 2021;21(1):595. Available from: https://doi.org/10.1186/s12935-021-02300-4.

13. He N, Kong Y, Lei X, Liu Y, Wang J, Xu C. MSCs inhibit tumor progression and enhance radiosensitivity of breast cancer cells by down-regulating Stat3 signaling pathway. Cell Death {&}amp; Disease. 2018;9(10):1026. Available from: https://doi.org/10.1038/s41419-018-0949-3.

14. Eggenhofer E, Luk F, Dahlke MH, Hoogduijn MJ. The life and fate of mesenchymal stem cells. Frontiers in Immunology. 2014;5:148. Available from: https://doi.org/10.3389/fimmu.2014.00148.

15. Jumat NR, Yunus MA, Yahaya BH, Mohamed R. Reprogramming macrophages toward M1-like phenotypes in the breast cancer microenvironment using mesenchymal stromal/stem cells: A review. Biomedical Research and Therapy. 2022;9(12):5418–36. Available from: https://doi.org/10.15419/bmrat.v9i12.781.

16. Carty F, Mahon BP, English K. The influence of macrophages on mesenchymal stromal cell therapy: passive or aggressive agents? Clinical and Experimental Immunology. 2017;188(1):1–11. Available from: https://doi.org/10.1111/cei.12929.

17. Vasandan AB, Jahnavi S, Shashank C, Prasad P, Kumar A, Prasanna SJ. Human Mesenchymal stem cells program macrophage plasticity by altering their metabolic status via a PGE2-dependent mechanism. Scientific Reports. 2016;6(1):38308. Available from: https://doi.org/10.1038/srep38308.

18. Rabani R, Volchuk A, Jerkic M, Ormesher L, Garces-Ramirez L, Canton J, et al. Mesenchymal stem cells enhance NOX2-dependent reactive oxygen species production and bacterial killing in macrophages during sepsis. The European Respiratory Journal. 2018;51(4):1702021. PMID: 29519920. Available from: https://doi.org/10.1183/13993003.02021-2017.

19. Ullah I, Subbarao RB, Rho GJ. Human mesenchymal stem cells - current trends and future prospective. Bioscience Reports. 2015;35(2):e00191. Available from: https://doi.org/10.1042/BSR20150025.

20. Xi J, Yan X, Zhou J, Yue W, Pei X. Mesenchymal stem cells in tissue repairing and regeneration: progress and future. Burns and Trauma. 2013;1(1):13–20. Available from: https://doi.org/10.4103/2321-3868.113330.

21. Lv FJ, Tuan RS, Cheung KM, Leung VY. Concise review: the surface markers and identity of human mesenchymal stem cells. Stem Cells (Dayton, Ohio). 2014;32(6):1408–19. Available from: https://doi.org/10.1002/stem.1681.

22. Cleary MA, Narcisi R, Focke K, van der Linden R, Brama PA, van Osch GJ. Expression of CD105 on expanded mesenchymal stem cells does not predict their chondrogenic potential. Osteoarthritis and Cartilage. 2016;24(5):868–72. Available from: https://doi.org/10.1016/j.joca.2015.11.018.

23. Asl KD, Shafaei H, Rad JS, Nozad HO. Comparison of characteristics of human amniotic membrane and human adipose tissue derived mesenchymal stem cells. World Journal of Plastic Surgery. 2017;6:33–9.

24. Lee HJ, Choi BH, Min BH, Park SR. Changes in surface markers of human mesenchymal stem cells during the chondrogenic differentiation and dedifferentiation processes in vitro. Arthritis and Rheumatism. 2009;60(8):2325–32. Available from: https://doi.org/10.1002/art.24786.

25. Mantovani A. MSCs, macrophages, and cancer: a dangerous ménage-à-trois. Cell Stem Cell. 2012;11(6):730–2. Available from: https://doi.org/10.1016/j.stem.2012.11.016.

26. Ren G, Zhao X, Wang Y, Zhang X, Chen X, Xu C. CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFα. Cell Stem Cell. 2012;11(6):812–24. Available from: https://doi.org/10.1016/j.stem.2012.08.013.

27. Parisi L, Gini E, Baci D, Tremolati M, Fanuli M, Bassani B, et al. Macrophage polarization in chronic inflammatory dis-

eases: killers or builders? Journal of Immunology Research. 2018;2018:8917804. Available from: https://doi.org/10.1155/2018/8917804.

28. Peshkova M, Korneev A, Suleimanov S, Vlasova II, Svistunov A, Kosheleva N, et al. MSCs' conditioned media cytokine and growth factor profiles and their impact on macrophage polarization. Stem Cell Research & Therapy. 2023;14(1):142. Available from: https://doi.org/10.1186/s13287-023-03381-w.

29. Huang SC, Smith AM, Everts B, Colonna M, Pearce EL, Schilling JD. Metabolic reprogramming mediated by the mTORC2-IRF4 signaling axis Is essential for macrophage alternative activation. Immunity. 2016;45(4):817–30. Available from: https://doi.org/10.1016/j.immuni.2016.09.016.

30. Prasanna SJ, Gopalakrishnan D, Shankar SR, Vasandan AB. Pro-inflammatory cytokines, IFNgamma and TNFalpha, influence immune properties of human bone marrow and Wharton jelly mesenchymal stem cells differentially. PLoS One. 2010;5(2):e9016. Available from: https://doi.org/10.1371/journal.pone.0009016.

31. Ißleib C, Kurz S, Scholl S, Amberg B, Spohn J. Plasticity of proinflammatory macrophages depends on their polarization stage during human MSC immunomodulation - an in vitro study using THP-1 and human primary macrophages. Immuno. 2021;1(4):518–28. Available from: https://doi.org/10.3390/immuno1040036.

32. Hill BS, Pelagalli A, Passaro N, Zannetti A. Tumor-educated mesenchymal stem cells promote pro-metastatic phenotype. Oncotarget. 2017;8(42):73296–73311. Available from: https://doi.org/10.18632/oncotarget.20265.

33. Engström A, Erlandsson A, Delbro D, Wijkander J. Conditioned media from macrophages of M1, but not M2 phenotype, inhibit the proliferation of the colon cancer cell lines HT-29 and CACO-2. International Journal of Oncology. 2014;44(2):385–92. Available from: https://doi.org/10.3892/ijo.2013.2203.

34. Song W, Thakor P, Vesey DA, Gobe GC, Morais C. Conditioned medium from stimulated macrophages inhibits growth but induces an inflammatory phenotype in breast cancer cells. Biomedicine and Pharmacotherapy. 2018;106:247–54. Available from: https://doi.org/10.1016/j.biopha.2018.06.126.

35. Bagheri R, Bitazar R, Talebi S, Alaeddini M, Etemad-Moghadam S, Eini L. Conditioned media derived from mesenchymal stem cells induces apoptosis and decreases cell viability and proliferation in squamous carcinoma cell lines. Gene. 2021;782:145542. Available from: https://doi.org/10.1016/j.gene.2021.145542.

36. Widowati W, Murti H, Widyastuti H, Laksmitawati DR, Rizal R, Kusuma HSW. Decreased inhibition of proliferation and induction of apoptosis in breast cancer cell lines (T47D and MCF7) from treatment with conditioned medium derived from hypoxia-treated Wharton's Jelly MSCs compared with normoxia-treated MSCs. International Journal of Hematology-Oncology and Stem Cell Research. 2021;15:77–89. Available from: https://doi.org/10.18502/ijhoscr.v15i2.6038.

37. Khalil C, Moussa M, Azar A, Tawk J, Habbouche J, Salameh R. Anti-proliferative effects of mesenchymal stem cells (MSCs) derived from multiple sources on ovarian cancer cell lines: an in-vitro experimental study. Journal of Ovarian Research. 2019;12(1):70. Available from: https://doi.org/10.1186/s13048-019-0546-9.

38. Vis MA, Ito K, Hofmann S. Impact of culture medium on cellular interactions in in vitro co-culture systems. Frontiers in Bioengineering and Biotechnology. 2020;8:911. Available from: https://doi.org/10.3389/fbioe.2020.00911.

39. Rachakatla RS, Pyle MM, Ayuzawa R, Edwards SM, Marini FC, Weiss ML. Combination treatment of human umbilical cord matrix stem cell-based interferon-beta gene therapy and 5-fluorouracil significantly reduces growth of metastatic human breast cancer in SCID mouse lungs. Cancer Investigation. 2008;26(7):662–70. Available from: https://doi.org/10.1080/07357900701871134.

40. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Science. 2020;367(6478):eaau6977. Available from: https://doi.org/10.1126/science.aau6977.

41. Keerthikumar S, Chisanga D, Ariyaratne D, Saffar HA, Anand S, Zhao K. ExoCarta: A web-based compendium of exosomal cargo. Journal of Molecular Biology. 2016;428(4):688–92. Available from: https://doi.org/10.1016/j.jmb.2015.09.019.

42. Vakhshiteh F, Atyabi F, Ostad SN. Mesenchymal stem cell exosomes: a two-edged sword in cancer therapy. International Journal of Nanomedicine. 2019;14:2847–59. Available from: https://doi.org/10.2147/IJN.S200036.

43. Lee JK, Park SR, Jung BK, Jeon YK, Lee YS, Kim MK, et al. Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. PLoS One. 2013;8(12):e84256. Available from: https://doi.org/10.1371/journal.pone.0084256.

44. Zhou W, Zhou Y, Chen X, Ning T, Chen H, Guo Q, et al. Pancreatic cancer-targeting exosomes for enhancing immunotherapy and reprogramming tumor microenvironment. Biomaterials. 2021;268:120546. Available from: https://doi.org/10.1016/j.biomaterials.2020.120546.

45. Komohara Y, Horlad H, Ohnishi K, Fujiwara Y, Bai B, Nakagawa T. Importance of direct macrophage-tumor cell interaction on progression of human glioma. Cancer Science. 2012;103(12):2165–72. Available from: https://doi.org/10.1111/cas.12015.

46. Zheng Y, Cai Z, Wang S, Zhang X, Qian J, Hong S. Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis. Blood. 2009;114(17):3625–8. Available from: https://doi.org/10.1182/blood-2009-05-220285.

47. Bogdanowicz DR, Lu HH. Multifunction co-culture model for evaluating cell-cell interactions. Methods in Molecular Biology (Clifton, NJ). 2014;1202:29–36. Available from: https://doi.org/10.1007/7651_2013_62.

48. Komohara Y, Hasita H, Ohnishi K, Fujiwara Y, Suzu S, Eto M. Macrophage infiltration and its prognostic relevance in clear cell renal cell carcinoma. Cancer Science. 2011;102(7):1424–31. Available from: https://doi.org/10.1111/j.1349-7006.2011.01945.x.

49. Takaishi K, Komohara Y, Tashiro H, Ohtake H, Nakagawa T, Katabuchi H. Involvement of M2-polarised macrophages in the ascites from advanced epithelial ovarian carcinoma in tumor progression via Stat3 activation. Cancer Science. 2010;101(10):2128–36. Available from: https://doi.org/10.1111/j.1349-7006.2010.01652.x.

50. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nature Reviews Immunology. 2007;7(1):41–51. Available from: https://doi.org/10.1038/nri1995.

51. Hinz N, Jücker M. Distinct functions of AKT isoforms in breast cancer: a comprehensive review. Cell Communication and Signaling. 2019;17(1):154. Available from: https://doi.org/10.1186/s12964-019-0450-3.

52. Choi E, Kim E, Kim JH, Yoon K, Kim S, Lee J. AKT1-targeted proapoptotic activity of compound K in human breast cancer cells. Journal of Ginseng Research. 2019;43(4):692–8. Available from: https://doi.org/10.1016/j.jgr.2019.07.001.

53. deGraffenried LA, Friedrichs WE, Russell DH, Donzis EJ, Middleton AK, Silva JM. Inhibition of mTOR activity restores tamoxifen response in breast cancer cells with aberrant Akt activity. Clinical Cancer Research. 2004;10(23):8059–67. Available from: https://doi.org/10.1158/1078-0432.CCR-04-0035.

54. Arranz A, Doxaki C, Vergadi E, de la Torre YM, Vaporidi K, Lagoudaki ED. Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(24):9517–22. Available from: https://doi.org/10.1073/pnas.1119038109.

55. Hu B, Trinh K, Figueira WF, Price PA. Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. The Journal of Bi-

ological Chemistry. 1996;271(32):19415–20. Available from: https://doi.org/10.1074/jbc.271.32.19415.

56. Tsuruha JI, Masuko-Hongo K, Kato T, Sakata M, Nakamura H, Sekine T. Autoimmunity against YKL-39, a human cartilage derived protein, in patients with osteoarthritis. The Journal of Rheumatology. 2002;29:1459–66.

57. Kavsan V, Dmitrenko V, Boyko O, Filonenko V, Avdeev S, Areshkov P. Overexpression of YKL-39 gene in glial brain tumors. Scholarly Research Exchange. 2008;2008:1–8. Available from: https://doi.org/10.3814/2008/814849.

58. Liu T, Larionova I, Litviakov N, Riabov V, Zavyalova M, Tsyganov M, et al. Tumor-associated macrophages in human breast cancer produce new monocyte attracting and pro-angiogenic factor YKL-39 indicative for increased metastasis after neoadjuvant chemotherapy. OncoImmunology. 2018;7(6):e1436922. Available from: https://doi.org/10.1080/2162402X.2018.1436922.

59. Jensen C, Teng Y. Is It Time to Start Transitioning From 2D to 3D Cell Culture? Frontiers in molecular biosciences. 2020;7:33. Available from: https://doi.org/10.3389/fmolb.2020.00033.

60. Jubelin C, Muñoz-Garcia J, Griscom L, et al. Three-dimensional in vitro culture models in oncology research. Cell & Bioscience. 2022;12(1):155. Available from: https://doi.org/10.1186/s13578-022-00887-3.