**Universidade de Aveiro** Departamento de Química
**2014/2015**

**ANA MARGARIDA FERREIRA CAMPOS**

# ANÁLISE LIPIDÓMICA DE CÉLULAS MESENQUIMAIS CANDIDATAS A TERAPIA CELULAR

# LIPIDOMIC ANALYSIS OF MESENCHYMAL CELLS CANDIDATES FOR CELL THERAPY

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# LIPIDOMIC ANALYSIS OF MESENCHYMAL CELLS CANDIDATES FOR CELL THERAPY

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica com especialização em Métodos Biomoleculares, realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, Professora Auxiliar com Agregação do Departamento de Química da Universidade de Aveiro e do Doutor Francisco Santos, Diretor Científico da empresa Cell2B

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Dedico este trabalho aos meus Pais e ao Tiago por todo o apoio e por terem sempre acreditado em mim

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Professor Adjunto Equiparado da Escola Superior de Tecnologia da Saúde de Coimbra da Universidade de Coimbra

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**Palavras-chave** Células mesenquimais do estroma, lipidómica, resposta imune, doenças auto-imunes, doenças inflamatórias, espectometria de masssa.

**Resumo** As células mesenquimais do estroma são células estaminais adultas que apresentam propriedades imunossupressoras e têm sido aplicadas como terapia clínica em vários estudos clínicos relativos a doenças autoimunes. Apesar do vasto número de estudos clínicos que utilizam estas células, ainda não se conhece o mecanismo de ação das mesmas, nem foram ainda identificados marcadores permitam avaliar o seu potencial imunomodulador. A lipidómica poderá dar algumas respostas a estas questões uma vez que os lípidos são importantes componentes das células, desempenhando um papel na sinalização celular. No presente trabalho estudou-se o lipidoma das células mesenquimais e avaliou-se a sua variação consoante o meio de cultura e a presença de estímulos pró-inflamatórios, mimetizando as condições fisiológicas em que as células são utilizadas. Este foi o primeiro estudo que analisou as diferenças no perfil fosfolípidico entre células mesenquimais do estroma e avaliou a variação do lipidoma destas células após a sua estimulação por mediadores pró-inflamatórios. Este estudo foi conduzido num primeiro conjunto de células cultivado num meio padrão suplementado com soro animal e num segundo conjunto de células cultivado num meio sintético. Nas células cultivadas no meio padrão, observou-se uma diminuição nas espécies moleculares de fosfatidilcolina (PC) com cadeias de ácidos gordos (FAs) após estímulos pro-inflamatórios. A quantidade de PC(40:6) também diminuiu, relacionando-se com o aumento expressão de lisoPC (LPC)(18:0) – LPC anti-inflamatória – em células estimuladas. Simultaneamente, a quantidade relativa de PC(36:1) e PC(38:4) aumentou. TNF-α and IFN-γ também levou ao aumento dos níveis de fosfatidiletanolamina PE(40:6) e diminuiu os níveis de PE(38:6). Também se verificou um aumento da expressão de fosfatidilserina PS(36:1) e esfingomielina (SM)(34:0), bem como a diminuição na expressão de PS(38:6). Contudo, em células mesenquimais cultivadas em meio sintético, com TNF-α and IFN-γ apenas aumentaram os níveis de PS(36:1). Estes resultados indicam que o metabolismo dos lípidos é modulado durante a ação imunossupressora das células.

**Key words** Mesenchymal stromal cells, lipidomics, immune response, autoimmune diseases, inflammatory diseases, mass spectrometry.

**Abstract** Mesenchymal stromal cells are adult stem cells found mostly in the bone marrow. They have immunosuppressive properties and they have been successfully applied as biological therapy in several clinical trials regarding autoimmune diseases. Despite the great number of clinical trials, MSCs' action is not fully understand and there are no identified markers that correlate themselves with the immunomodulatory power. A lipidomic approach can solve some of these problems once lipids are one of the major cells' components. Therefore, in this study cells' lipidome was analysed and its deviations were evaluated according to the medium of culture and to the presence of pro-inflammatory stimuli, mimicking physiological conditions in which these cells are used. This was the first study ever made that aimed to analyse the differences in the phospholipid profile between mesenchymal stromal cells non-stimulated and stimulated with proinflammatory stimulus. This analysis was conducted in both cells cultured in medium supplemented with animal serum and in cells cultured in a synthetic medium. In cells cultured in the standard medium the levels of phosphatidylcholine (PC) species with shorter fatty acids (FAs) acyl chains decreased under pro-inflammatory stimuli. The level of PC(40:6) also decreased, which may be correlated with enhanced levels of lysoPC (LPC)(18:0) - an anti-inflammatory LPC - observed in cells subjected to TNF-α and IFN-γ. Simultaneously, the relative amounts of PC(36:1) and PC(38:4) increased. TNF-α and IFN-γ also enhanced the levels of phosphatidylethanolamine PE(40:6) and decreased the levels of PE(38:6). Higher expression of phosphatidylserine PS(36:1) and sphingomyelin SM(34:0) along with a decrease in PS(38:6) levels were observed. However, in cells cultured in a synthetic medium, TNF-α and IFN-γ only enhanced the levels of PS(36:1). These results indicate that lipid metabolism and signaling is modulated during mesenchymal stromal cells action.

# Abbreviations

Ag antigen
APC antigen presentig cell
BAFF B cell-activating factor
BM bone marrow
CD cluster of differentiation
CD Crohn's disease
CFU-F colony-forming unit fibroblasts
CL cardiolipin
CNS central nervous system
CTL cytotoxic T lymphocytes
DC dendritic cell
Dkk-1 dickkopf-1
DMEM Dulbecco's modified Eagle Medium
EAE experimental autoimmune encephalomyelitis
ELOVL6 elongation of very long chain family member 6
ESI electrospray ionization
FA fatty acid
FAME fatty acid methyl ester
FBS fetal bovine serum
FOXP3 forkhead box P3
Gal-9 galactin-9
GC gas chromatography
GPL glycerophospholipid
GvHD Graft versus Host Disease
HB-EGF heparin-binding epidermal growth factor
hBM human bone marrow
HER-1 epidermal growth factor receptor-1
HLA human leukocyte antigen
HLA-DR human leukocyte antigen-D related
HPLC high-performance liquid chromatography
ICAM-1 intercellular adhesion molecule
iDC immature dendritic cell
IDO indoleamine 2,3-dioxygenase
IFN-γ type I interferon gamma
Ig immunoglobulin
IL interleukine
IP3 inositol 1,4,5-trisphosphate
IS immune system
KIR killer immunoglobulin-like receptors
LC liquid chromatography
LFA-1 lymphocyte function-associated antigen 1
LysoPC lysophosphatidylcholine
mDC mature dendritic cell
MHC major histocompatibility complex
MNC mononuclear cells
MSC mesenchymal stromal cell
NF-κB kappa-light-chain-enhancer of activated B cells
NK natural killer

NO nitric oxide

PA phosphatidic acid

PBL peripheral blood lymphocytes

PC phosphatidylcholine

PD-1 programmed death-1 receptor

PE phosphatidylethanolamine

PG phosphatidylglycerol

$PGE_2$ prostaglandin 2

PHA phytohemagglutinin

PI phosphatidylinositol

$PLA_1$ phospholipase $A_1$

$PLA_2$ phospholipase $A_2$

PLP proteolipid protein

PRR pattern recognition receptor

PS phosphatidylserine

PUFA polyunsaturated fatty acid

regDC regulator dendritic cells

rhHGF recombinant human hepatocyte growth factor

SFM serum-free media

SM sphingomyelin

TCR T cell receptor

$T_H$ cell T helper cell

TLC thin layer chromatography

TLR toll-like receptor

TNF-α tumor necrosis factor

TNFR1 tumor necrosis factor receptor 1

Treg regulatory T cell

VCAM-1 vascular adhesion molecule 1

VEGF vascular endothelial growth factor

# Table of contents

# I. INTRODUCTION

# Introduction

## 1. Mesenchymal Stromal Cells

In the last years, mesenchymal stromal cells (MSCs) have been successfully applied in many clinical trials (1). MSCs are primitive cells with origin in the mesodermal germ layer. They are considered adult stem cells, once these cells are characterized as capable of self-renewal and directed differentiation. In fact, they are able to originate connective tissues, skeletal muscle cells and vascular system cells (1).

MSCs show some characteristics, namely ease of accessibility for isolation, enormous expansion potential in culture without losing the ability to differentiate, presumptive plasticity, immunosuppressive properties, use in allogeneic transplantation, paracrine-mediated effects, homing and migratory behaviour to sites of tissue injury that support their use as a biologic therapeutic for several clinical applications (1) (Figure 1). All this topics will be addressed further in this work.

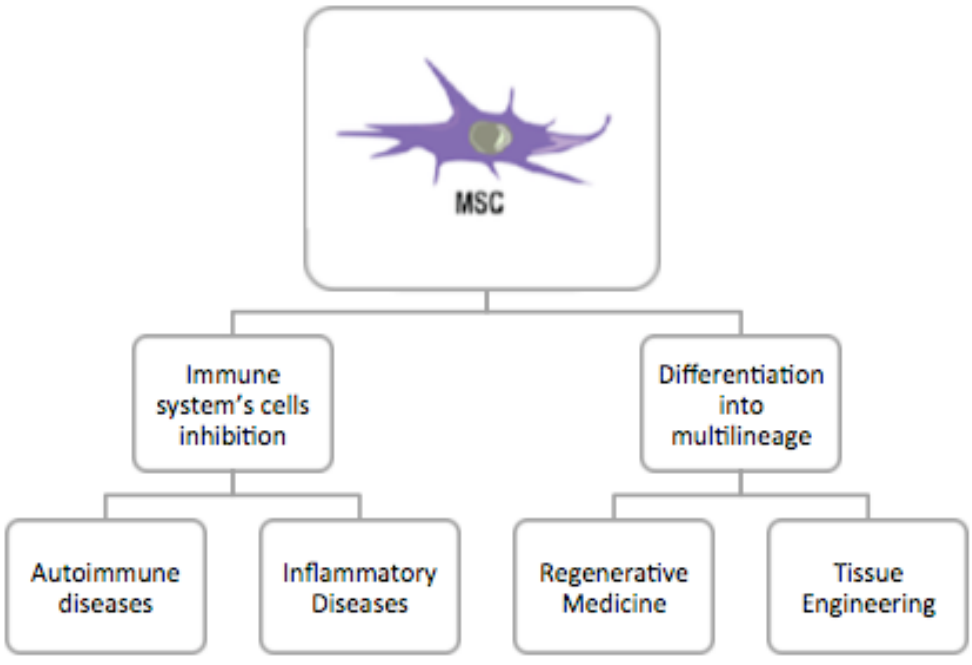


**Figure 1. Schematic representation of several current applications of mesenchymal stem cells**

*1.1 Location and differentiation of MSCs*

Adult stem cells can be found in different adult tissues, such as brain, heart, lungs, kidneys and spleen but display different characteristics depending on the source. However, adult stem cells from bone marrow (BM) are the best characterized ones (1). In BM it is possible to find a wide diversity of cells, including hematopoietic stem cells, macrophages, erythrocytes, fibroblasts, adipocytes and endothelial cells. In the heterogeneous cell population found in BM, there is a particular subset of nonhematopoietic stem cells with the ability to differentiate in cells from different lineages. These cells are MSCs. MSCs are not capable to produce blood cells because they are not hematopoietic stem cells. Therefore MSCs are classified as nonhematopoietic, multipotential stem cells capable of originating both mesenchymal and nonmesenchymal cell lineages (1–3).

Ernest A. McCulloch and James E. Till were the first to discover MSCs in the 1960s and named them as clonal bone marrow stromal cells (3). Later in the 1970s Friedenstein and his research group performed further studies and characterized these cells as fibroblast-like that can be obtained from bone marrow due to their intrinsic plasticity in culture. He distinguished these cells as multipotential stromal precursor cells, spindle-shaped and clonogenic in culture, naming the stromal cells colony-forming unit fibroblasts (CFU-F) (Figure 2) (4,5).

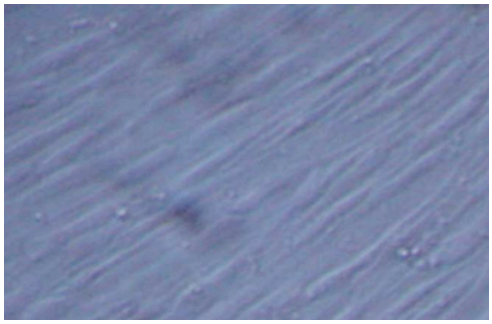

**Figure 2. MSCs developed in culture.** Appearence of MSCs after second passage under inverted microscope (100x) (adapted from (6)).

These cells were capable of making bone in subcutaneous transplants. Furthermore, Friedenstein was able to prove the CFU-F's self-renewal potential because he demonstrated MSCs were able to regenerate heterotopic bone tissue in several transplants. Several research groups have then focused their work in expanding these findings and came across with results that revealed CFU-F's capacity

to differentiate into a variety of mesenchymal lineages' cells such as osteoblasts, chondrocytes, adipocytes and myoblasts both *in vitro* and *in vivo* (7–11). Apart from this discovery, it became obvious the possibility to use these cells in clinical applications and they have been applied as cellular sources to regenerate bone, cartilage and adipose tissues (2)**.** Later in 2007 these cells were renamed "mesenchymal stromal cells" or MSCs by Caplan and colleagues (8). Further studies reported MSCs as capable of originating cardiomyocytes, neurons and astrocytes *in vitro* and *in vivo* (1). However, MSCs power of differentiation depends on the age of MSCs donors (12).

Nevertheless scientific community is not sure about MSCs' true nature and identity, comprising location, origin and multipotential capacity. MSCs can be obtained from several different tissues, including adipose tissue, liver, muscle, amniotic fluid, placenta, umbilical cord blood and dental pulp, however the main source is BM (1,3,12). Although, only between approximately 0,01 and 0,001% of BM aspirates are MSCs. Even though this very low percentage, there is great interest in these cells once they have a great expansion rate in culture and they can also be easily isolated from small aspirates that are also easily obtained (1).

*1.2 Culture and Expansion of MSCs*

In order to clinically apply MSCs, it is necessary to have a great amount of cells. So it becomes necessary to expand these cells *ex vivo*. Despite stem cells huge ability to continuously regenerate and expand *in vivo*, they have limited proliferation rate in *ex vivo* settings. Moreover, MSCs from the same donor can have different capability to expand. There are several parameters that influence MSCs' expand rate. Culture conditions are within these parameters. In fact, nutritional level, cell confluence, oxygen level, number of passages and plastic surface quality interfere with MSCs growth (13). Cell-seeding density and media used for cell propagation also change MSCs functional properties. The most common media used to culture MSCs is Dulbecco's modified Eagle Medium (DMEM) supplemented with fetal bovine serum (FBS). However, the use of a serum from animal origin gathers several concerns regarding variability and immunological reactions originated by reactions between bovine sialic acid and protein derivatives in MSCs. For this reason, xeno-free serum is a really positive alternative for culturing cells.

MSCs can also be developed in serum-free media (SFM) becoming possible to simultaneously overcome the regulatory requirements of these cells and improve the efficiency of production systems. SFM also selectively isolate MSCs and enrich their characteristics. Data gathered also demonstrated MSCs culture in synthetic media have powerful immunomodulatory abilities.

According to the media or condition in which MSCs are cultured, they present different characteristics and properties. However, few studies have characterized MSCs grown in SFM. Properly characterize cells cultured in this media will allow a wider use of MSCs cultured in SFM and, consequently, concerns regarding bovine serum will be avoided.

*1.3 Growth and Phenotypic characteristics of MSCs*

According to kinetic studies previous conducted, MSCs growth has 3 different phases, namely (1) initial lag phase lasting 3 to 5 days, (2) fast expansion and (3) stationary phase. In the end of the lag phase, cells begin to secrete dickkopf-1 (Dkk-1) that inhibits the Wnt signalling cascade. As consequence, the levels of β-catenin drop and cell proliferation stops. At the same time, epidermal growth factor receptor-1 (HER-1) interacts with heparin-binding epidermal growth factor (HB-EGF), inducing MSCs proliferation and inhibiting differentiation (13).

MSCs do not have a unique specific cell surface marker and if they are obtained from different sources they show different proliferative and multilineage potential. To clarify how to identify MSCs, the International Society for Cellular Therapy has established minimum criteria (14):

1) plastic-adherent under standard culture conditions;
2) positive expression of cluster of differentiation (CD) 105, CD73 and CD90, plus absent expression of hematopoietic cell surface markers CD34, CD45, CD11a, CD19 and human leukocyte antigen-D related (HLA-DR);
3) under specific stimulus, cells should differentiate into osteocytes, adipocytes and chondrocytes *in vitro*;

However, many other markers can be analysed (absence or presence) in order to characterize MSCs. Among the huge diversity of markers, it is possible to find proteins with different functions, such as cellular adhesion molecules, integrins,

selectins, chemokine receptors or membrane-bound receptors involved in apoptosis or necrosis. The markers analysed are proteins naturally expressed by several different cells, namely stem cell precursor cells, endothelial or epithelial cells, T-lymphocytes, B-lymphocytes, natural killer (NK) cells, macrophages/monocytes, granulocytes, dendritic cells (DCs), platelets and erythrocytes. MSCs surface marker expression profiles can vary depending on their source, isolation procedure, *in vitro* senescence and detections methods (15).

Finding unique markers to identify and characterize MSCs is particularly important, once this information will allow to obtain and to expand more homogeneous MSCs populations. Furthermore, it will allow developing therapeutic MSCs for a specific disease indication with improved disease-specific efficiency. However, despite some of this information remains unknown, MSCs have already been successfully applied in many pathologies.

## 2. Animal and Clinical Application of MSCs

MSCs have the ability to repair or regenerate damaged tissues and thus can be used to treat some human diseases. MSCs can be isolated and expanded from different sources and their use to tissue engineering and cell therapy has been exploited (15). As a matter of fact, the initial therapeutic interest in MSCs was based in their ability to differentiate into several multilineages and in applying this ability to regenerate tissues and organs. MSCs have already been applied in bone and cartilage repair. For example, MSCs were successfully used to improve bone healing (16) and in osteogenesis imperfect (17,18). Also in bone reconstruction (19) and in vascular wall restoration (20) have MSCs revealed reliable.

Nevertheless, MSCs possess other characteristics that have drawn the interest of researchers. In fact, as referred above, MSCs interfere with immune cells and inhibit the proliferation of activated allogeneic lymphocytes either *in vitro* as *in vivo*, crossing histocompability barriers. Moreover, MSCs home towards damaged tissue through several growth factors, cytokines and chemokines, enhancing the efficiency of their repair and immunomodulatory mechanisms (15). MSCs also act upon most major components of the innate and adaptive immune system and they show proangiogenic, cytoprotective and antifibrotic effects. In addition, MSCs have proven capable of migrating towards tissues experiencing inflammatory events and they are "licensed" by inflammatory cytokines and pattern recognition receptor (PRR) ligands

to become more powerful (21). For these reasons, most recently there has been a rising interest in applying these cells as a treatment for inflammatory and autoimmune diseases (21). Up to date, a total of 432 studies are registered in the ClinicalTrial.gov database. Some of these studies focus in therapeutically applying MSCs as modulatory mediators in models of inflammatory and autoimmune diseases, such as Graft versus Host Disease (GvHD), multiple sclerosis and Crohn's disease (CD). Some of the performed studies and their results are briefly addressed below.

*2.1 MSCs application in Graft versus Host Disease*

GvHD is among the pathologies in which MSCs immunomodulatory characteristics are more often therapeutically applied. GvHD is a mortal complication characterized by donor's T cells aggression to host's tissue. Steroids are the most common form of treatment, however some patients are refractory and no effective therapy exists. Back in 2004, Le Blanc successfully infused haplo-identical MSCs in a patient suffering from grade IV acute GvHD. The patient received two infusions of MSCs and no toxicity events were detected. Upon the second infusion, the patient showed a complete response and no minimal residual disease was found either in blood or in BM (22). In further phase II clinical trials, haplo-identical MSCs were infused in 18 patients, while 79 patients received MSCs from third-party HLA-mismatched donors. Surprisingly, 30 patients responded completely and 9 showed some improvements. Once again, no patient developed any kind of adverse effects (23).

In another phase II clinical trial (24), allogeneic MSCs were infused in 31 patients suffering from acute GvHD. Of the treated patients, none developed toxic events and 94% developed an initial response, 77% responded fully and 17% showed a partial response. In a larger phase III clinical trial, from 260 steroid-resistant GvHD patients, interesting results were obtained. In this study, patients with GvHD present in the liver and in the gastrointestinal tract showed the best improvement after being infused with allogeneic MSCs, indicating some tissues experiencing GvHD are more susceptible to MSCs action than others (25).

*2.2 MSCs application in Multiple Sclerosis*

MSCs application is of interest also in patients suffering from multiple sclerosis. Multiple sclerosis is a chronic autoimmune inflammatory disease in which

nerve cells from the brain and spinal cord suffer demyelination. In a pilot clinical study, 5 patients with advanced multiple sclerosis showed great improvements after administration of *ex vivo*-expanded autologous MSCs. This trial was very important to prove MSCs safety and feasibility as a therapy for patients with multiple sclerosis (26). In another phase II study concerning 15 patients, the previous results were confirmed. Moreover, more regulatory T cells were found, along with weaker proliferative responses of lymphocytes and activation markers from DCs (27). In general, MSCs decline the progression of general disability in patients suffering from multiple sclerosis (28).

Studies conducted in experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis, in which rats were injected with MSCs, showed MSCs transplantation weakened the clinical severity of EAE, along with central nervous system (CNS) inflammation and demyelination. It is supposed this happens because MSCs engraft in lymphoid organs where they induce T-cell aenergy (29). Another studied proved MSCs are capable of supressing proteolipid protein (PLP)-induced EAE when administrated by iv in rats (30).

*2.3 MSCs application in Crohn's Disease*

CD is a pathology whose origin is still not clear. CD can affect the entire gastrointestinal system with continuous transmural inflammation and fistulization. Not many studies using MSCs as a biological therapy have been performed. However, the results obtained from these studies were very promising. As a matter of fact, in a phase I clinical trial, 6 out of 8 patients suffering from CD and locally injected with MSCs showed healed fistulas after the injection and no controversial effects were registered. This results were proved in the subsequent phase II trial (31).

Further and more extensive applications of MSCs in other inflammatory and autoimmune diseases require a deeper knowledge about MSCs' behaviour, phenotypic characteristics and mechanisms. Lipidomics approach can provide some of the needed information.

## 3. The main targets of MSCs' immunomodulatory effects

MSCs could be successfully applied in all the previous pathologies due to their effects upon the immune system (IS) and its cells. IS is the system used to protect the host from pathogens and it distinguishes different pathogens, acting

accordingly in a process known as immune response. Monocytes, DCs, T and B cells are some of the cells responsible for the referred immune response (32).

IS can be subdivided in two systems dependent of each other: innate and adaptive immune systems. The innate immune system acts upon pathogens that break physical barriers like the skin. Leucocytes have PRRs that identify and bind to pathogen-associated molecular patterns from the pathogen, inducing pathogen apoptosis or recruiting inflammatory mediators (32).

The adaptive immune system uses antibodies and T and B cells that act upon specific pathogens. This originates memory of the pathogens and consequently promotes a faster response from IS the next time T and B cells are exposed to the pathogen (32).

In both immune systems, IS triggers inflammatory pathways evolving a complex process network composed by inflammatory cells and mediators. Once these cells and mediators are activated, they initiate and later stop inflammation acute phase. This process occurs only at the infected tissue, resulting in angiogenesis and renewed tissue. It is possible to distinguish two types of inflammatory mediators: mediators of cell adhesion and chemotactic mediators. Intercellular Adhesion Molecule (ICAM-1) also known as Cluster of Differentiation 54 (CD54) and Vascular Adhesion Molecule 1 (VCAM-1) also known as Cluster of Differentiation 106 (CD106) are mediators of cell adhesion and they are involved in the leucocytes interactions, as well as in the interaction between leucocytes and endothelial or target cells. Among chemotactic mediators are cytokines, interleukins, chemokines and bioactive lipids that are responsible for leucocytes chemotactic migration and are involved in angiogenesis, collagen production and proliferation of hematopoietic precursors (32).

### *3.1 Dendritic cells*

DCs are the main antigen presenting cells (APCs) and they are specialized in uptaking, transporting and presenting antigens (Ags). These cells also stimulate naïve and memory T cells and interact with B cells and NK cells (33).

Immature DCs (iDCs) are found in peripheral tissues, where they capture and process Ags. However, iDCs do not have the capacity to stimulate T cells. Locally produced inflammatory cytokines are responsible for DCs maturation, in a process characterized by upregulation of MHC and costimulatory molecules, production of

interleukine (IL)-12 and migration towards lymphoid tissue (33,34). Therefore, mature DCs (mDCs) show lower capacity to uptake Ags, but acquire the ability to stimulate T cells (34). In fact, only mDCs are capable of inducing immunogenic T cell responses without inducing tolerance (33,34).

*3.2 T cells*

T cells, also known as T lymphocytes, are a type of lymphocyte characterized by the presence of a T cell receptor (TCR) on its cell surface. T cells function is to destroy pathogens and viruses that replicate themselves inside cells and, consequently are not recognized by antibodies present in the blood or in extracellular spaces (35).

T lymphocytes need to interact directly with the cells bearing the antigen they recognize, in order trigger an immune response. Cytotoxic T cells are the ones that act more directly. Infected cells display in their surface antigens derived from the pathogen. These antigens are recognized by cytotoxic T cells that act accordingly and kill the infected cells. Usually, cytotoxic T cells display CD8 on their surfaces (35).

Other T lymphocytes display in their surfaces CD4 marker and are called helper T, or $T_H$ cells. However, CD4 T cells can be divided into two subsets with different roles in defending the body. The first subset of CD4 T cells - $T_H1$ cells - controls intracellular bacterial infections, activating macrophages and inducing other phagocytic antibacterial mechanisms. $T_H1$ cells also release cytokines and chemokines that direct macrophages towards the inflammation site (35).

T cells are also important in killing extracellular pathogens by activating B cells. This function is up to the second subset of CD4 T cells - $T_H2$ cells. Only a limited number of antigens are capable of directly activating B cells. Most of them require $T_H2$ cells to stimulate B lymphocytes proliferation and differentiation into antibody secreting cells (35).

*3.3 B cells*

B cells, often called B lymphocytes, are responsible for producing antibodies, present pathogens and release cytokines. These cells flow in the bloodstream, where they recognized pathogens relying upon their antigen specific receptors: membrane-type surface immunoglobulins (36).

When B cells get across with pathogens flowing in the bloodstream, they capture the foreign body and are activated through B cell receptor pathways. As a

result, the expression of MHC class II antigens and co-stimulatory signals, as for example CD80/86 molecules, is upregulated. Subsequently, the stimulated B cells go to the spleen and expose T cells to pathogen' specific antigens, greatly activating $CD4^+$ T cells (36).

B cells can also be involved in aggravating autoimmunity and controlling infection propagation. When healthy cell are injured, they release autoantigens that are captured by autoreactive B cells. Once again, B cells present the autoantigens to autoreactive T cells from the spleen, enhancing specific T-cell mediated autoimmunity. As a result, pathogenic T cells receptors spread and B cells secret greater amounts of antibodies. B cells can also produce pathogenic cytokines, enhancing autoreactive T cells proliferation and activation and leading to T cell mediated autoreactivity. All together, this leads to more deterioration of target cells. All this together forms a vicious cycle of amplified autoreactivity and target damaged cells ate the site of autoimmunity (36).

*3.4 Natural Killer cells*

NK cells are important effector cells of innate immune system and migrate towards peripheral tissues driven by chemokines and type I interferones (IFNs) upon inflammation. NK cells express at their surface receptors specific for the chemokines usually secreted during inflammatory events by macrophages, neutrophils, DCs and endothelial cells (37).

NK cells also display intense cytotolic activity against cells infected by a virus or a tumour. NK cells show several surface receptors involved whether in inhibitory and activation pathways. The main inhibitory receptors are killer immunoglobulin-like receptors (KIRs) that bind specifically MHC class I molecules. Under normal condition, autologous cell express these molecules, preventing NK cells activation. However, tumour- or virus-infected cells downregulate the expression of MHC class I molecules at the cell surface, resulting in few inhibitory interactions and consequently in activation of NK cells. Once NK cells are activated, infected cells become susceptible to killing mediated by NK cells (37,38).

However, iDCs are very susceptible to NK cells-mediated lysis, despite expressing MHC class I molecules. It is supposed this mechanism serves to control the quality of DCs undergoing maturation. NK cells also eliminate DCs that would not efficiently present Ags and prime T cells (37).

## 4. Immunomodulatory effects of MSCs

As previous referred, MSCs have the ability to modulate immune responses. In fact, MSCs can supress secondary alloimmune responses *in vitro* and primary alloimmune responses (39). MSCs also manage to induce lymphocyte proliferation whether they are autologous or allogeneic to the tissue, indicating MSCs exert their immunomodulatory effects independently of MHC (40). Nevertheless, MSCs act upon most cells of the immune system, namely T and B cells, DCs and NK cells (Figure 3).

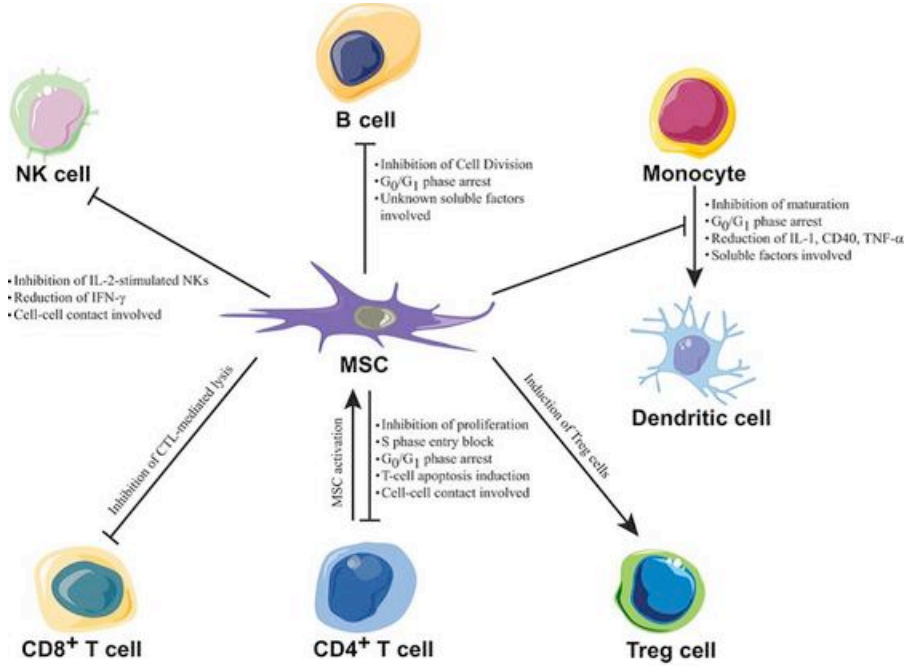


**Figure 3. Immunomodulatory effects of MSCs over immune system's cells.** Schematic and simplified representation of MSCs effects over NK cells, DCs, T and B cells.

### *4.1 Immunomodulatory Effects of MSCs on DCs*

The induction of immune response depends on both T cells and interactions between DCs and T cells. MSCs modulate the expression and antigen-presentation capacity of DCs before lymphocyte activation and proliferation. Jiang and colleagues performed one of the earliest studies ever regarding MSCs immunomodulation over DCs. Jiang demonstrated monocytes cultured with MSCs fail to express either immature as mature DCs markers, i.e., in the presence of MSCs monocytes do not differentiate into DCs (41). More specifically, MSCs stop DCs generation by

inhibiting precursor cells ($CD34^+$ and monocytes) differentiation into DCs (33). MSCs mediate their effect on DCs differentiation mainly through prostaglandin 2 ($PGE_2$) (supresses T cells receptor signalling) during the early stages, i.e. during cytokine-induced monocytes differentiation into iDCs (42). However, primed MSCs also secrete IL-6 and vascular endothelial growth factor (VEGF). VEGF is involved in MSCs immunosuppressive effect and IL-6 is an important cytokine involved in the immune regulatory mechanism employed by MSCs. Furthermore, IL-6 impairs monocytes differentiation into DCs when monocytes are cultured with MSCs (43). However, other studies stated MSCs and DCs interact directly with each other during DCs activation using adherent junctions (44).

Later studies concerning MSCs-DCs' phenotype found results slightly different from the ones obtained by Jiang. In fact, MSCs-DCs display mature DCs (maDCs) phenotype and normal IL-12 (T cells stimulating factor) and IL-10 (anti-inflammatory cytokine) production, although they also maintain some characteristics of iDCs, namely their structure resembling a podosome and endocytic activity and not being capable to form active immune synapses with lymphocytes. MSCs alter DCs function by interfering with DCs activation through an alteration of the cytoskeleton organization in a long-lasting effect (44). As a result, DCs become regulatory DCs capable of both inhibiting alloreactivity of T cells and inducing the generation of alloantigen-specific regulatory T cells in a manner independent of IL-10 secretion (45).

MSCs also interfere directly with iDCs and mDCs. When MSCs act upon iDCs, these either convert to monocytes or do not enter maturation, failing to induce T cells activation (33,41). IL-12 production is detrimental to DCs maturation and function but in the presence of MSCs, IL-12 production is greatly reduced (41). MSCs induce mDCs proliferation and differentiation into a different DC population - regulator DCs (regDCs) population - and LPS stimulation is not capable of reversing this trend. This novel regDCs population supress T cells (46).

Although several studies have reported MSCs effect in DCs maturation, only in 2013 one mechanism was proposed regarding how this process is controlled. MSCs or DCs in monoculture release low amounts of IL-10. IL-10 is released in considerable amounts by monocyte-derived iDCs and by stimulated mDCs; it is known IL-10 inhibits DCs differentiation and function. However, when MSCs and DCs are co-cultured IL-10 combines itself with IL-10 receptor, activating JAK-STAT

signal transduction pathway. JAK-STAT signalling pathway transmits chemical information from outside the cell into gene promoters on the DNA, leading to DNA transcription. When MSCs and DCs are co-cultured, this pathway is activated and IL-12 secretion is inhibited, leading to impaired maturation of DCs, increased number of iDCs and decreased number of mDCs (47).

*4.2 Immunomodulatory effects of MSCs on T cells*

MSCs have a rather unique immunophenotype profile characterized by low expression of costimulatory molecules and absence of human leukocyte antigen (HLA). This profile provides MSCs the ability to actively modulate T-cell proliferation. Di Nicola and colleagues were able to prove that T cell proliferation stimulated by allogeneic peripheral blood lymphocytes (PBL) and DCs or mitogen as phytohemagglutinin (PHA) or IL-2 is inhibited by MSCs in a dose dependent manner (48). Interestingly, this effect still occurs when MSCs' source is different from both the T cells and the stimulators molecules source (49). Probably because MSCs do not express MHC class I and II molecules (50). Cytokines released by MSCs, such as recombinant human hepatocyte growth factor (rhHGF) and/or recombinant human hepatocyte growth factor β1 (rhTGF-β1) mediate the inhibitory effects. Finally, Di Nicola and colleagues have also demonstrated T lymphocytes inhibited by MSCs do not enter apoptosis; in fact, when restimulated with cellular or humoral activators if MSCs are not present, these cells are still able to proliferate (48,49). However, MSCs need to be induced in order to act as an immunoregulator and this process is called "priming". Once MSCs are primed by proinflammatory cytokines, they perform all the effects referred above and also decrease IFN-γ (macrophages activator) and IL-2 secretion by activated T cells (49). However these cells exert their immunoregulatory activity in culture and in contact with T-cells, i.e., MSCs mediate their immunoregulatory activity in a cell-contact dependent manner (51).

Interestingly, despite MSCs specifically but reversibly inhibit T cells proliferation, they do not completely supress T cells activation. In fact, MSCs stop cell division at $G_0/G_1$ phase of the cell cycle inhibiting T-cell proliferation, but T cells' surface continues to show activators molecules. MSCs are able to stop the cell cycle because they block cyclin D2 induction. Cyclin D2 induction is important for cell cycle G1/S transition. Also MSCs inhibit T-cell effector function because the number of IFN-γ

producing cells are reduced in T cells cultured with MSCs (52,53). T cells co-cultured with MSCs secrete no cytokines besides IFN-γ and tumor necrosis factor α (TNF-α). TNF-α stimulates acute phase reaction in response to inflammation. IFN-γ induces the expression of the protein B7-H1 on MSCs, triggering MSCs' immunosuppressive effects (54). It is important to refer that B7-H1 induces T cell proliferation and IL-10 secretion. IFN-γ in addition with other cytokines, such as TNF-α, IL-1α or IL-1βs (proinflammatory cytokines) can also be used to prime MSCs. TNF-α and IL-1α induce nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. This pathway induces some cell responses to several stimuli, such as cytokines and it also regulates immune response to infection. NF-κB activation by TNF-α leads to the activation of MSCs regulatory function. According to Dorronsoro, once TCR is stimulated, T cells begin to clonal expanding and to secrete cytokines, such as TNF-α. TNF-α stimulates NF-κB pathway, via tumor necrosis factor receptor 1 (TNFR1), in MSCs present in inflammatory environment, triggering MSCs immunosuppression (55). NF-κB pathway is also activated by the ligation between toll-like receptors (TLRs) 3 and 4 present in MSCs and their respective ligands. TLRs are widely distributed on immune system's cells and their activation is essential for inducing immune response and enhancing adaptive immunity against pathogens (56).

In APCs expressing indoleamine 2,3-dioxygenase (IDO) is a very important effector pathway that immunosuppresses T-cell responses to autoantigens and fetal alloantigens *in vivo*. This pathway is induced by IFN-γ and other proinflammatory cytokines; IDO catalyzes tryptophan conversion into kynurenine. Despite MSCs do not intrinsically express IDO, IFN-γ induces the production of considerable amounts of IDO by primed MSCs in a dose-dependent manner. Tryptophan depletion by IDO is a T-cell inhibitory effector mechanism employed by MSCs (57).

Nitric oxide (NO) is known to inhibit T cell proliferation and it was reported MSCs produce NO when they differentiate into chondrocytes. Furthermore, NO mediates T-cell suppression by MSCs and also acts inhibiting Stat5 phosphorylation (Stat5 is required for T cell division) (58).

Curiously, MSCs immunomodulation does not act only upon T cell proliferation and activation. According to a study conducted by Rasmusson, MSCs loaded with MHC class I peptide epitopes are resistant to lysis by cytotoxic T lymphocytes

(CTLs) and the interaction between MSCs and CTLs does not induce the production of proinflammatory cytokines by CTL (59).

A new subset of $T_H$ cells was discovered and denominated $T_H17$. $T_H17$ cells, a population of $CD4^+$ cells, play an important role protecting the host against extracellular pathogens and in murine models of inflammatory disease. MSCs affect $T_H17$ lineage differentiation and inhibit the production of proinflammatory cytokines, while simultaneously increase the production of IL-10 and the expression of forkhead box P3 (FOXP3). FOXP3 is one of the main regulators of regulatory T cells (Tregs) development and function. MSCs induces $T_H17$ cells to develop a phenotype similar to one found in Tregs (60). Tregs are known for their immunosuppressive effect. Even though these cells release soluble factors, it is documented that they also act in a cell-cell contact manner in order to perform their immunosuppressive effect and programmed death-1 receptor (PD-1) is known to be one of the molecules involved. PD-1 prevents T cells activation, decreasing autoimmunity and enhacing self-tolerance. MSCs increase IL-10 and TGF-β1 secretion in cocultures - TGF-β1 regulates the growth, proliferation, differentiation of cells and apoptosis - and upregulate PD-1 expression on Tregs cell surface, enhancing Tregs' immunoregulatory capacity (61).

*4.3 Immunomodulatory effects of MSCs on B cells*

B cells development occurs in BM and it depends on the production of cytokines capable of supporting B cells survival and proliferation. Corcione studied if BM-derived MSCs affect the function of mature B cells and concluded MSCs are capable of inhibiting B cells proliferation, differentiation into antibody-secreting cells and chemotaxis in a dose dependent manner. They also demonstrated that when B cells interact with MSCs, the former do not enter apoptosis but stop at the $G_0$-$G_1$ phases of the cell cycle. Corcione has also proved MSCs exert their effect by releasing soluble factors after direct cross-talk with B cells. In fact, data acquired by Corcione suggested B cells release activation signals that stimulate MSCs suppressive action. However, MSCs do not affect B cells APC function (62).

It is also known MSCs stimulate immunoglobulin (Ig) secretion by B cells in a dependent-contact manner. However, upon B cells activation MSCs reduce Ig

production, indicating that in an ongoing infection B cells antibody production may be decreased by great amounts of MSCs (63).

However, one study reported that primed MSCs increase the expression of B cell activation factor (64). In fact, MSCs need to be stimulated by IFN-γ, TNF-α and TLR3 in order to perform their immunosuppressive functions on T lymphocytes. TLR2, TLR3 and TLR4 are the most expressed in MSCs and when TLR agonists, namely TLR4 activates MSCs, they increase B lymphocytes proliferation. B cell-activating factor (BAFF) is crucial to B lymphocytes generation, differentiation and maturation. It is known that TLR4-priming in MSCs has great impact in BAFF expression and, consequently regulates B lymphocyte proliferation (64).

*4.4 Immunomodulatory effects of MSCs on NK cells*

Upon interaction with MSCs, NK cells begin to express the activation Ag CD69 and to release IFN-γ and TNF-α. So NK cells activation by MSCs is a process involving lymphocyte function-associated antigen 1 (LFA-1)/ICAM-1 interaction and the NKp30 receptor. LFA-1 is found in cells from the immune system and recruits these cells to the site of infection. LFA1 binds to ICAM-1 on APCs, acting as an adhesion molecule and promoting T cells differentiation. Newsworthy, NK cells in different activation status use different triggering receptors, like NKp30 or NKG2D, to interact with MSCs (65). As referred previously, MSCs can also supress the generation of cytolytic activity from DCs and, consequently the production of cytokines. However, IL-2 – activated NK cells can kill both allogeneic and autologous MSCs (42).

Interestingly, NK cells can lyse MSCs depending on which ligand connects to the NK receptors (38). Actually, NK cells effector functions are regulated by the presentation of receptors in the cell surface that can either activate or inactivate these cells. Usually when NK cells are exposed to IL-2, they begin to express activating NK receptors that are correlated with NK cells function. Resting NK cells do not proliferate in response to IL-2 or IL-15 in the presence of MSCs. IL-2 and IL-15 induce NK cells proliferation. However, MSCs show little effect in activated NK cells proliferation (38).

MSCs mediate their actions upon NK cells through the release of IDO and $PGE_2$ that act synergistically. More specifically, MSCs through IDO and $PGE_2$

inactivate surface expression of both receptors involved in NK cells activation and molecular markers of activated NK cells (42).

Some of the information referred above was obtained due to studies that addressed MSCs' immunosuppressive mechanism. These studies were important not only to understand the mechanisms employed by these cells, but were also important to identify which proteins characterize MSCs. Consequently, new targets for therapeutics intervention were discovered. Many proteomics studies have been conducted in turn of MSCs. These studies are important to identify new proteins that are important to characterize MSCs, as well as new targets for therapeutics intervention. Moreover, proteomics provided some information regarding which mechanisms and molecules are employed by MSCs during its differentiation into new tissues or when they are exerting their immunomodulatory effects. However, further data concerning MSCs immunomodulatory mechanisms and differentiation pathways is necessary to obtain, before these cells became completely accepted by the scientific community as a safe and completely reliable biological therapy. Lipidomics can provide some of the remain unknown information, once lipids are one of the main components of the cells and their metabolism is highly regulated and changes according to cells' different stages. Moreover, lipidomics can provide reliable markers to identify MSCs and to assess their differentiation state and their immunomodulatory capacity.

## 5. Lipidomics

Due to improvements in genomics and proteomics, it became possible to sequence genes and identify several proteins and their functions. In the last few years, a new metabolomics field called Lipidomics has been explored (66,67). Each lipid class has specific functions and take part in different metabolic and signalling processes, it is important to analyse differences of specific lipid species in different pathways. This is one of the reasons that justifies the exponential interest in lipidomics. Lipidomics refers to 'the systems-level analysis of lipids and their interacting moieties'. However other definitions claim that lipidomics goes beyond lipids characterization, involving also understanding how lipids interfere in all cellular pathways (Figure 4) (68).

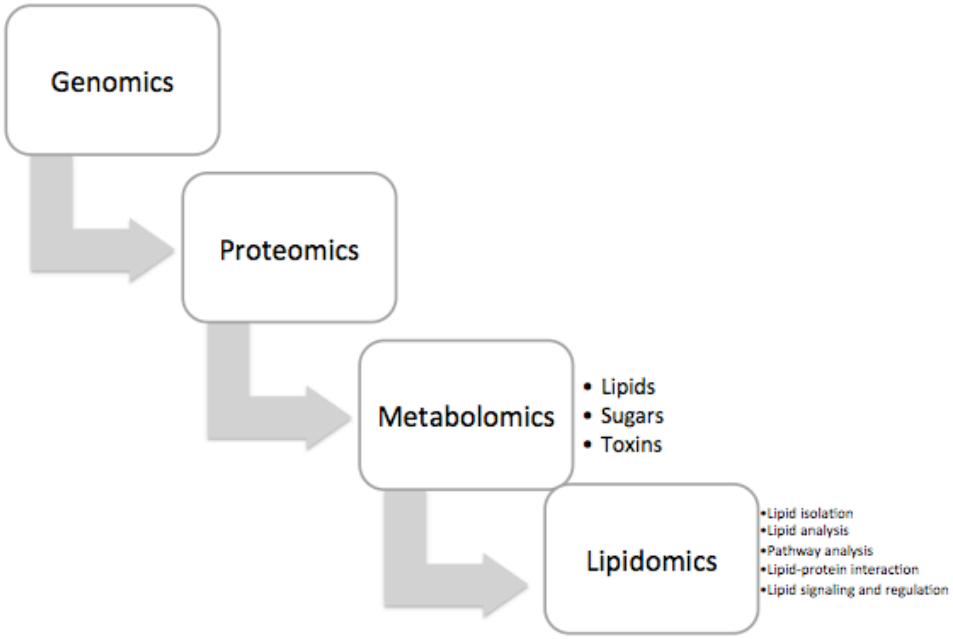


**Figure 4. Lipidomics – different lipids analysis.** A cell's genome encodes for proteins that along with environmental factors account for the different metabolites found in a cell. Due to important advances, it is possible to study these different scales both qualitatively and quantitatively.

Initial lipidomic analyses relied in analytical techniques with low resolution and sensitivity, such as thin-layer chromatography. The advances regarding lipidomics studies appeared later because only recently researchers have realized lipids can be a part of the etiology of some diseases. Furthermore, researchers merely now recognize the importance of studying the metabolism of molecular lipids and lipid classes, in order to complete understand a specific pathway involved in cell or tissues homeostasis (67,68). This field of research has also improved due to novel advances achieved regarding new mass spectrometric techniques and established theories on the structural and functional organization of biological membranes (67). These recent advances have provided us with the possibility to study lipids at the molecular level, becoming possible to study complex lipid mixtures in a high-throughput manner and pathways involving lipids. These developments also allow us to analyse how lipids interfere in cell signalling and how a cell's lipidome changes according to different cell stages. Therefore, lipidomics analysis can be applied to obtain biomarkers capable of acting as quality control markers (66).

However, performing and applying a lipidomic approach is not simple because biological lipids are a very diverse group of compounds (Figure 5) and cell's lipidome is very complex. Lipids are a very wide group of molecules. They are one of the main

constituents of cells membranes and organelles and they regulate many biological processes. The majority of lipids are found in biological membranes and different lipid compositions leads to membranes with different properties. This wide group of molecules also form the ideal media for the implementation of membrane proteins and the interaction, in the membranes, between protein and lipids defines the fluidity of biological membranes. The pathways that control membranes' lipid composition are tightly regulated by different extracellular receptor-regulated pathways according to the cells' state (68). Moreover cells exert great efforts to maintain lipid homeostasis, once lipids are not equally distributed among organelles and even the two leaflets of biological membranes have different relative amounts of each lipid class (69).

Lipids' most defining characteristic is their insolubility in water. However, some lipid classes, as glycosphingolipids are soluble both in water and organic solvents, becoming necessary to find another criteria besides their solubility to define them. Back in 2003, the LIPID MAPS consortium has defined as hydrophobic or amphiphilic small molecules that arise from complete or partial condensation of ketoacyl or isoprene subunits (69).

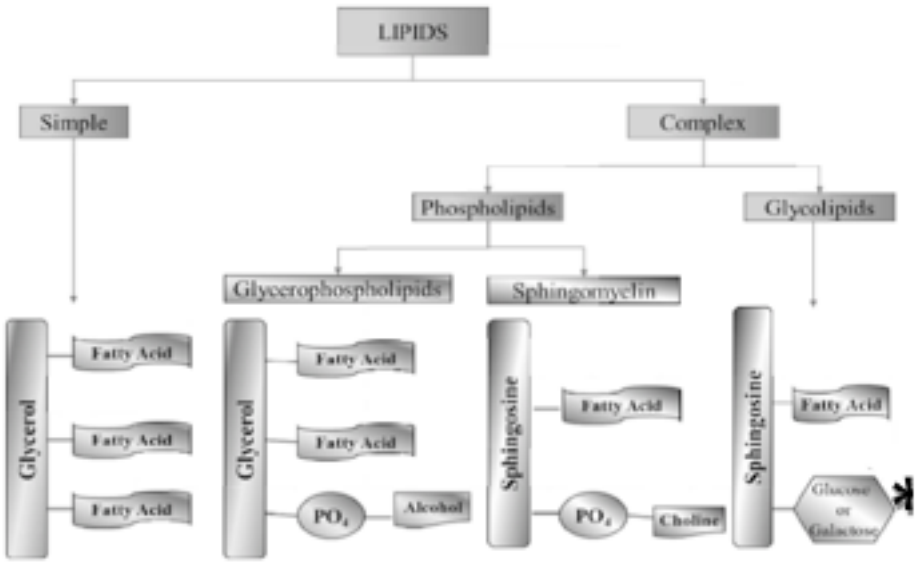


**Figure 5. Schematic representation of the main lipids found in cells.** (Adapted from (66)). * One or more saccharide groups.

In mammalian cells, it is possible to find between 30 to 60 different fatty acids attached to the lipids. The fatty acyl chains have between 12 and 26 carbons, with up to six double bonds and they can also have hydroxyl groups attached. Once fatty acids can have chains with variable length, different number and position of double bonds, they account for the huge diversity found among lipids (69).

According to their structure, lipids perform different functions. For example, fats and oils are mainly used to store energy, while phospholipids and sterols are the principal structural elements in biologic membranes. Other lipids act as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors for proteins, "chaperones", emulsifying agents, hormones and intracellular messengers (70). Despite lipids represent a very structurally diverse class of molecules, their defying characteristic comes from one structural effect all lipids share, namely a main domain of hydrophobic molecules. Usually this domain corresponds to long hydrocarbon chains or multiple linked rings (66).

Glycerophospholipids are the main component of biological membranes and in a lipid extract can exist up to approximately 10 000 different molecular glycerophospholipids species (69). Structurally, they are composed by two fatty acids ester-linked to a glycerol's first and second carbon and glycerol's third carbon is attached through a phosphodiester linkage to a polar or charged group. All glycerophospholipids are formed from phosphatidic acid (PA). Structurally, PAs have a glycerol blackbone and, usually, at glycerol's first carbon is attached a saturated fatty acid, at its second carbon is bonded an unsaturated fatty acid and the third carbon has a phosphate group attached. PA is a minor component of the cells and it is present mostly in the inner leaflet of biological membranes. PA also interferes with membrane curvature and it acts as a signalling lipid, recruiting the appropriate cytosolics proteins according to the biological membrane (70). According to the polar group, also called, polar head group attached to the glycerol, glycerophospholipids form different classes with different functions (66).

Phosphatidylglycerols (PGs) are one of the main phospholipid classes of the lung surfactant. However, PGs are present in mammalian tissues mainly because they are cardiolipins (CLs) precursors (70). In mammalian cells CLs are found mostly in the inner leaflet of mitochondrial membrane, where they are involved in the generation of cellular energy in the form of ATP (69,71).

Phosphatidylcholines (PCs) contain a choline molecule attached to glycerol's third carbon and are the main phospholipids of biological membranes in most animal tissues. PCs compose almost totally the outer leaflet of the plasma membrane. PCs act as precursor for sphingomyelin, PA, lysophosphatidylcholine and platelet-activating factor (66). Phosphatidylethanolamines (PEs) represent the second most abundant phospholipid class found in biological membrane from mammalian cells. PEs are in charge of fusion and maintenance of the functional structure of integral membrane proteins. PEs also interfere with membrane proteins conformation and functions (66). Phosphatidylserines (PSs) are present in lower amounts in biological membrane from mammalian cells. Along with phosphatidylinositol (PI) and PE, PSs are in the inner leaflet of the plasma membrane. PSs act as a signalling molecule because when they are found in the outer leaflet, apoptotic pathways are activated. PSs are also an essential cofactor and activator of protein kinase C (66). PIs are a minor class of the membrane glycerophospholipids and are only found in eukaryotes. They act as precursors of secondary messengers (66). Moreover, PI and its phosphorylated derivates interfere in the cell structure and metabolism. PI 4,5-bisphosphate saves messenger molecules that will be release inside the cell as a response to extracellular signals. When PI 4,5-bisphosphate is hydrolysed, it originates two intracellular messengers: inositol 1,4,5-trisphophate ($IP_3$) that is soluble in water and diacylglycerol that remains in the plasma membrane. $IP_3$ acts upon endoplasmic reticulum and triggers $Ca^{2+}$ release to the cytosol. High levels of $Ca^{2+}$ associated with diacylglycerol, activate protein kinase C (70).

Lysophosphatidylcholines (LysoPCs) result from the action of the enzyme phospholipase A2 upon PCs. LysoPCs activate the expression of many genes involved in inflammatory responses, for example in endothelial and T cells. LysoPCs are found in cell's plasma and help transporting polyunsaturated fatty acids and cholines to tissues. However, if LysoPCs levels get out of control pathological conditions, such as ischemia and atherosclerosis can arise (66).

Sphingolipids are another of the main lipids classes. Unlike glycerophospholipids, sphingolipids have a sphingosine backbone and no glycerol. The amino group at sphingolipids' second carbon is bonded to a fatty acid in amine linkage. Sphingolipids differ from each other according to the polar head group attached to the first carbon, either by a glycosidic linkage or a phosphodiester. Sphingolipids are the fourth larger class of membrane lipids (70).

Sphingomyelins (SMs) are one of the three subclasses of sphingolipids and contain phosphocoline or phosphoethanolamine as polar head group. Due to SMs polar head, they are also considered phospholipids, along with glycerophospholipids. Structurally and physiologically, SMs resemble PCs. SMs are also present in animal biological membranes, especially in the outer leaflet. SMs have high affinity towards cholesterol and usually are located together in specific sub-domains or lipid rafts of membranes. Moreover, it seems SMs control cholesterol distribution in membranes (70).

In animals, SMs are the major source of ceramides, which are necessary to trigger apoptosis, along with other metabolic pathways. Sphingomyelins also seem to be involved in the activity of some membrane-bound proteins, including those of some ion channels and receptors (70). SM and ceramides found in membranes are powerful regulators of protein kinases (70).

## 6. Lipidomic Approach

Cell's lipidome is very complex and can encompass approximately between 180 000 and 200 000 different lipid species. For this reason, it is necessary very precise steps in order to be correctly analysed (69). Thus **lipid isolation** from tissue or cells is the first procedure to be executed. Lipid isolation methods rely on lipids high solubility in organic solvents. **Analysis of lipids** previously extracted is the following method to be applied. This can be made directly, using mass spectrometry (MS), or after chromatographic separation. Using phosphorous assay that measures inorganic phosphorous and/or additional analysis by MS, it becomes possible to identify and quantify each phospholipid species present in the sample after phospholipids classes separation usually by thin layer chromatography (TLC). These steps provide a 'lipid profile' of the cell, i.e. an inventory of the lipids present in the cell or tissue under investigation. Different functional states of the cells or tissue under investigation are reflected in different 'lipid profile' obtained after applying this steps, providing us with information regarding which pathways are affected. This step is known as **pathways analysis** (67) (Figure 6). Therefore, according to the main goals of this master thesis, we hypothesize MSCs subjected to different stimuli will produce different 'lipid profiles' and this information may be useful to understand which metabolic pathways are affected.

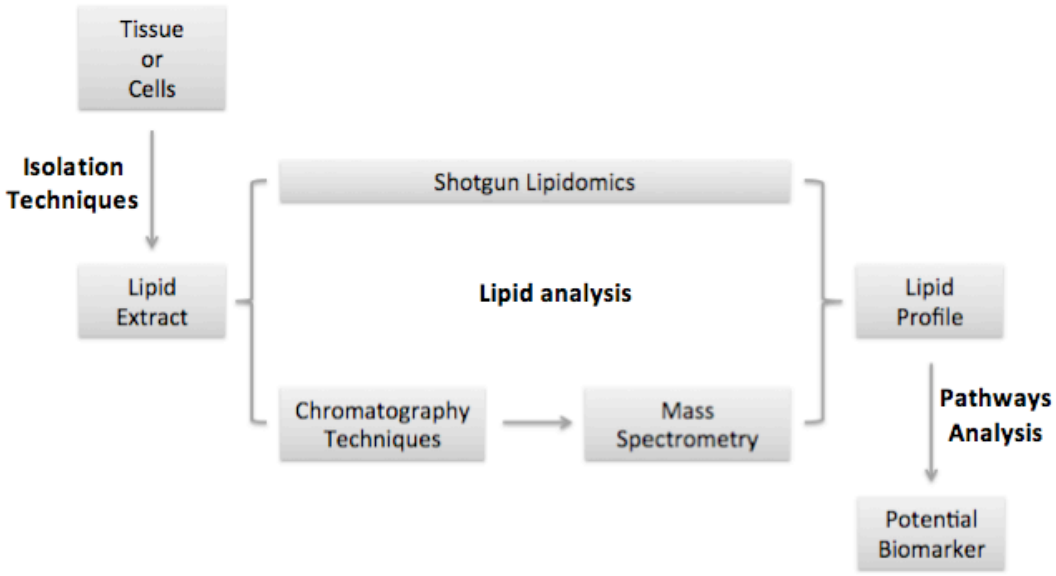


**Figure 6. Schematic representation of several steps during lipidomic approach**

*6.1 Lipid Isolation*

Lipid isolation from tissues is the first step to accomplish before further analysis. The lipid extract must be free of non-lipid contaminants (72). The solvent or mixture of solvents used must be quite polar, in order to break the interaction between lipids and cell membranes or lipoproteins. Usually these interactions are weak and hydrophobic or are Van der Waals forces. However, the solvent can not be totally polar to prevent chemical reactions with lipids. In resume, the solvent must simultaneously interfere with both the hydrophobic and polar interactions (72).

The first lipid extraction methods were developed by Folch in 1957 (73) and later in 1959 by Bligh & Dyer (74). These are capable of isolating both neutral and polar constituents of lipids and modified forms of these methods are still widely used nowadays. These methods are based on a mixture of chloroform and methanol (2:1) (v/v) and following wash with salt solution or adjusting the amount of water (75,76). Most of the contaminating material can be removed using this mixture. Once the ratio of chloroform-methanol to tissue is higher than 17:1, the water manages to remain in an independent phase. Yet, the solution of chloroform and methanol is toxic, irritant to the skin and neither one of the solvents are totally stable. Many solvents or solvents combinations can be used. However, they must ensure that there is no enzymatic or lipolytic activities (72). Despite the technique chosen, all ensure the risk of hydrolysis or autoxidation of lipids is minimized (72).

The extraction method is chosen according to the nature of the tissue matrix, its nature (animal, plant or microbial origin) and the analytical method used. In this study, a modified version of the method developed by Bligh & Dyer will be used. In this modified version, the pellet is ressuspended in a solution of chloroform and methanol (1:2) (v/v) and the mixture is left in ice for 30 minutes. After this time, chloroform is added, followed by extra pure water that will induce the separation between the aqueous and the organic phases. The lipids will be dissolved in the organic phase (bottom) due to their polarity. The polarity of lipids is controlled by their hydrophobic hydrocarbon chains of fatty acids and any possible hydrophilic groups, namely phosphate or sugar residues (72).

*6.2 Lipidomic Analysis*

After applying the most adequate extraction method, it remains a lipid extract from cells, tissues or body fluids. Chromatographic separation on silica thin-layer plates (77) or silica columns can be applied to fractionate lipids extracts before further and more thorough analysis (78). After applying separations techniques, it is possible further identification and quantification of each molecular species (67).

There are several chromatography techniques that can be used for the analysis of lipids: TLC, gas chromatography (GC), liquid chromatography (LC) and high-performance liquid chromatography (HPLC). These techniques can be coupled with MS and act as pre-separation techniques (67). In this study, HPLC will be the technique employed. This technique allows the separation of different lipids classes as PEs, PGs, PCs, among others (78). After the different lipid classes have been chromatographically separated, the lipid extract is introduced to MS for further analysis. This approach is known as targeted lipidomics because the aim is to quantify lipids from a specific class (78).

When performing MS of lipids samples, the most common used ionization technique is electrospray ionization (ESI). ESI is a soft, non-destructive technique that generates positive and negative molecular lipid ions with minimal in-source fragmentation (79). ESI can be used in its positive mode (ESI+) to analyse major phospholipids and sphingolipids classes. However, some phospholipids classes, such as PIs, PSs and PAs are properly analysed using the negative ion mode (ESI-) (80). Tandem mass spectrometry analysis allows the confirmation of the structural details of each molecular species, such as information regarding polar head group and fatty

acid substitution. It is also aimed to study the profile of fatty acids. To do so, fatty acids will be assessed after transmethylation, followed by analysis of fatty acids' methyl esters by gas chromatography coupled with mass spectrometry and gas chromatography coupled with a flame ionization detector.

**7. Lipidomics of Mesenchymal Stromal Cells**

As referred above, lipids are one of the main constituents of mammalian cells. Most lipids are found in biological membranes, so biological membranes' characteristics and properties depend on the different lipids that compose the membranes. The metabolism that controls the different lipids found in membranes is highly regulated and it depends on the cell's state. In fact, changes in the lipids present in the membranes regulate enzymatic activity, channels and transport of proteins (68). In addition, lipidomics profiles provide information regarding the global lipid concentration of cells, tissues or body fluids during specific physiological states (78).

For these reasons, several studies concerning cell's lipidome have aroused in order to gain better insight over different diseases and cells markers and also correlated with cells of the immune system. In fact, lipidomic approach was used to characterize the molecular diversity of the main classes of phospholipids and sphingolipids found in DCs and to identify changes in DCs' lipidome during lipopolysaccharide-induced maturation (68). A lipidomic approach was also performed to identify the main lipid classes and their species in circulating blood cells. Performing this analysis in cells from healthy donors allowed to identify specific characteristics of individual blood cells. Moreover, detailed knowledge of lipid profiles of circulating blood cells provided important new information regarding lipid abnormalities on diseases and perception about how these pathologies are affected by lipids (81).

Up to date many few studies were conducted regarding stem cells' membrane lipids and alterations in these cells' lipids profile have not been thoroughly analysed. Despite glycosphingolipids and signalling lipids have been analysed, glycerophospholipids have not received much attention (82). Concerning MSCs, few studies have been conducted. However these studies can be very helpful since characterization of these type of cells is required for their application as biological therapy.

When culturing MSCs to apply them as a biological therapy, it remains unknown how many cell doublings can these cells undergo before they became malignant or lose their differentiation ability. Kilpinen was the first to address this question and did so performing a lipidomic study regarding MSCs (82). More specifically, Kilpinen's aim was to compare glycerophospholipids profile between MSCs from young and old donors and how sequential expansion of the cells affected these profiles. In order to accomplish these objectives, MSCs were cultured in a medium supplemented with FBS and MSCs' total lipids were extracted using Folch extraction method. According to this study, there are no significant differences between the percentages of phospholipids classes present in MSCs from young or old donors. Indeed, in average PC is the most abundant class (41-46%), followed by PE (34-38%), PS (5-8%) and PI (4-8%). Interestingly, the average amount of PC, PS and PI resembles the values usually found in human fibroblasts. The relative amount of PE found was much higher than what is usually found in human fibroblast or mammalian cells. However, the percentages of both PC alkyl and PE alkenyl were significantly different in MSCs from late passages when comparing MSCs from young and old donors. Besides, the ration PI/PS was higher in MSCs from late passages than in MSCs from previous passages (82).

Kilpinen also studied the most common species within the different phospholipids classes. Regarding the most abundant PC species, in descending order, they are 34:1, 36:2, 36:1, 38:4; in the case of PE class, the most abundant classes, in descending order, they are 38:4, 36:1, 36:2, 40:5, 38:5 and 34:1. PS species 36:1, 40:5 and 40:6 are, in descending order, the most abundant. 38:4 represents almost 50% of the total PI species existent. In this study, Kilpinen has also studied changes in individual lipid species among MSCs from different passages. Among PC diacyl species, the molar percentage of 38:4, 36:4 and 36:1 is higher in MSCs from later MSCs, while the opposite occurrs for the species 34:1, 34:2 and 36:2. The PE diacyl species profiles have higher amounts of 38:4 and 36:4 species and fewer amounts of monounsaturated species, such as 34:1 and 36:1 in MSCs from later passages. In general, the levels of PC 34:1 and PE 36:1 are enhanced in MSCs from young donors compared with MSCs from old donors. Kilpinen was able to conclude that MSCs that experience more expansions in culture suffer harmful changes in the phospholipid composition of their membranes, which affects lipid signalling and it can lead to

impaired functionality. Therefore biosynthetic precursors in membranes can be used as indicators of cell functionality (82).

Even though this work portrays great advances regarding MSCs' lipidome, it did not provide specific biological markers capable of identifying MSCs neither provided further insight regarding MSCs' mechanism. Furthermore, these studies have focused in MSCs cultured in media supplemented with FSB and there is no study that characterizes MSCs cultured in xeno-free media. Characterization of xeno-free media-cultured MSCs will allow applying these cells in many therapies without arising concerns regarding patient's safety. Thus, future work is needed to fulfil these gaps in literature.

## 8. Objectives

In order to gain new insights regarding MSCs' lipidome, the main goals of the present thesis are:

a) To study MSCs' lipid profile
b) To identify the variation in MSCs' lipidome when
    a. MSCs are cultured in two different medias and when
    b. MSCs are subjected to pro-inflammatory stimulus.
c) Besides, it is also aimed to better understand how MSCs exert their immunomodulatory ability and which biological markers may possibly be analysed in order to ensure only powerful immunomodulatory MSCs are being collected and employed as a biological therapy in inflammatory diseases.

To achieve these aims, MSCs will be cultured in both a media containing an animal serum and in a media xeno-free, i.e. a synthetic media. MSCs from both cultures were further subjected to pro-inflammatory studies. MSCs were provided by CELL2B.

Lipidomic analysis will be performed using HPLC-MS and MS/MS of the total lipid extracts, along with GC-MS and GC-FID analysis of the fatty acid profile. This approach will allow identifying which phospholipids classes and species were present in MSCs from all conditions. With this data, it becomes possible to understand how the relative amounts of phospholipids classes and species change according to the culture conditions and can be used to molecular phenotyping these type of cells.

# II. Material and Methods

# Material and Methods

*1. Chemicals*

Phospholipid internal standards 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dMPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (dMPE), 1,2-dimyristoyl-*sn*-glycero-3-phospho-(1-rac-glycerol) (dMPG), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (dMPS), tetramyristoylcardiolipin (TMCL), 1,2-dipalmitoyl -*sn*-glycero-3-phosphatidylinositol (dPPI), 1,2-dimyristoyl--*sn*-glycero-3-phosphate (dMPA), 1-heptadecanoyl-2-hydroxy-sn-glycero-3phosphocholine (LPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Chloroform, methanol and hexane from Fisher scientific (Leicestershire, UK); all were of high HPLC grade and were used without further purification. All the other reagents and chemicals used were of the highest grade of purity commercially available. The water was of miliQ purity filtered through a 0.22-mm filter (Millipore, USA).

*2. Collection of Bone Marrow*

Human bone marrow (hBM) aspirates were either commercially obtained from (Lonza) or from donations from "Instituto Português de Oncologia (IPO) de Lisboa, Francisco Gentil, EPE or IPO-Porto". Samples were obtained from healthy donors (age 20-40 years old) after informed consent.

*3. Isolation of hMSCs from Bone Marrow*

BM mononuclear cells (MNC) were isolated using Sepax S-100 system (Biosafe), according to manufacturer's instructions. The Sepax cell processing system uses a rotating syringe technology that provides both separation through rotation of the syringe chamber (centrifugation) and component transfer through displacement of the syringe piston. The Sepax system allows the automated processing of blood components in a functionally closed and sterile environment. The system uses Ficoll (GE Healthcare) to separate the low density BM-MNC, and saline solution, NaCl 0.9% (Labesfal), to wash the cells. BM-MNCs were then plated in Corning® Polystyrene Tissue Culture Flasks, using Dulbecco's Modified Essential Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum MSC qualified (FBS,

Gibco) or with xeno-free medium at a density of 600 000 MNC/cm$^2$. Cell counting was performed using trypan blue (Gibco) exclusion assay. Culture medium was changed every 3/4 days and cells were passaged when reached a confluence of 70-80%.

*4. Cell cryopreservation*

After harvesting and counting, cells were centrifuged and resuspended in defined cryopreservation medium, Synth-a-Freeze™ (Gibco). The cell suspension was transferred to cryovials and maintained at -80ºC storage freezer. In case of long-term cryopreservation, cells were transferred to liquid nitrogen.

*5. Cell Thawing and Seeding*

Cryoval containing MSCs were thawed in a 37 ºC water bath. The vial content was trnsfered to a conical tube previously filled with medium DMEM (Gibco) supplemented with FBS (Sigma-Aldrich) 10% (v/v) or with xeno-free medium in 1:10 dilution. After centrifugation at 1250 rpm for 7 min, the supernant was discarded and the cell pellet ressuspended in culture fresh medium. From the homogeneous cell suspension, 10 μL were removed to perform cell count and viability analysis, using the Trypan Blue exclusion Method (83). Briefly, 10 μL of cell suspension were placed in a microplate and 10 μL of Trypan Blue Stain (Gibco) were added and mixed very well. Then 10 μL of the previous mix were applied into a hemacytometer. Using a microscope the viable cells (unstained) and nonviable cells (stained blue) were count separately in the hemacytometer. Cells were seeded at 3000 cells/cm$^2$ density in tissue culture flasks. Afterwards the cell vessels were incubated in a $CO_2$ incubator at 37 ºC, 5% $CO_2$. The culture medium were changed every 3 to 4 days until MSCs cultured reached 70-80% confluency. At this point MSCs cultured were either stimulated with IFN-γ (R&D System) and TNF-α (Sigma-Aldrich) or harvested.

*6. MSCs culture stimulation*

Once MSCs cultures reached 70-80% confluency, 500 U/mL of IFN-γ (R&D System) and 10 ng/mL of TNF-α (Sigma-Aldrich) were added to the cells cultures. Then the cultures were incubated with the cytokines for 48 hours at 37 ºC, 5% $CO_2$ and harvested.

*7. MSCs harvesting*

The medium from the culture vessel was discarded and the flask washed with 10 mL DPBS. TrypLE™ (Gibco) were added to detach cells from culture dish and dissociate cell aggregates. Then the culture vessel was incubated in $CO_2$ incubator at 37 ºC for 7 min. Once it was confirmed cells detachment from the surface, culture medium were added, in order to inactivate the enzyme and the mixture were transferred to a conical tube. Afterward the mix was centrifuged at 1250 rpm for 7 min and the supernant discarded. The cell pellet was ressuspended in culture fresh medium and 10 μL were removed to perform cell count and viability analysis using Trypan Blue exclusion Method. The cell suspension was again centrifuged at 1250 rpm for 7 min, the supernant discarded and the cells stored at -80 ºC until further analysis.

*8. Phospholipid Extraction*

Cell pellets from MSCs cultured in DMEM or xeno-free medium with no stimulus or cultured under TNF-α and IFN-γ were ressuspended in 1 mL miliQ water. Phospholipids were extracted from all cell pellets by the Blight and Dyer method (74). Briefly, 2.50 mL of MeOH were added to the homogenized tissues and well vortexed. Subsequently 1.25 mL $CHCl_3$ were added to previous mix and incubated in ice for 30 min. Afterward 1.25 mL of $CHCl_3$ and 1.25 mL of $H_2O$ were added and mixed well. Then the suspension was centrifuged at 1,000 x g, 5 min, at room temperature, using a centrifuge Mixtasel Centrifuge (Selecta), and two phases were obtained: the aqueous phase on top and the organic phase below, where lipids were retrieved. The extracts were dried under nitrogen stream and stored at -20ºC.

*9. Separation of phospholipids classes by TLC*

PL classes were separated from total lipid extract by TLC using a plate of silica gel 60 with concentration zone 2.5x20cm. Previous to TLC separation, the plate were washed in $CHCL_3$:MeOH (1:1 v/v) and treated with boric acid in ethanol (2.3% w/v). The plate with spots containing about 30 μg of sample were developed in a mixture of solvents $CHCl_3$:MeOH:$H_2O$:tN($CH_2CH_3$) (30:35:7:35 v/v/v/v). The phospholipid spots were revealed sprinkling the TLC plate with a primuline solution

(50 μg/100 mL acetone: water, 80/20 v/v) and visualized with UV lamp (λ=254 nm). Phospholipid spots were identified after comparison with phospholipid standards. Then the phospholipid spots were scraped from the plate and quantified using the phosphorous assay.

*10. Quantification of Phospholipids classes using phosphorous assay*

In order to determine the amount of phospholipid in each extract and the amount of phospholipid class separated by thin layer chromatography (TLC), a phosphorous quantification method was performed according to the protocol of Bartlett and Lewis (84). Briefly, 125 μL of percloric acid (70% m/v) were added to the samples, which were incubated 60 min at 180 ºC in a heating block (Stuart, U.K.). 825 μL of miliQ $H_2O$ and 125 μL of ammonium molybdate (2.5 g ammonium molybdate/ 100 mL $H_2O$) and 125 μL of ascorbic acid (10 g ascorbic acid/100 mL $H_2O$) were added to all samples and vortexed following the addition of each solution. Then the samples were incubated in a bath at 100 ºC for 10 min. Additionally standards were prepared using 0.1 to 2 μg of phosphate, which suffered the same treatment as the samples. The absorbance of samples and standards were measured at 793 nm, in a microplate reader (Multiscan 90, ThermoScientific).

*11. GC-FID conditions*

Fatty acid methyl esters (FAMEs) were obtained upon transesterification of lipid extracts according to the procedure described by Aued-Pimentel et al. (85). Briefly, dried lipid extracts (30 μg of total phospholipid) were ressuspended in 1 mL n-hexane. A KOH solution in methanol (2 M) was added (200 μL), followed by 1-2 min of intense vortex-mixing. A saturated NaCl solution (2 mL) was then added. After centrifugation at 2000 rpm for 5 min (centrifuge Mixtasel Centrifuge (Selecta)), the organic phase – upper phase - was collected and dried under a nitrogen stream. The resulting FAMEs were dissolved in n-hexane and volumes of 5 μL were analyzed by gas chromatography (GC) on a PerkinElmer Clarus 400 gas chromatograph (Waltham, MA) equipped with a flame ionization detector (FID) and a DB-1 column with 30 m of length, 0.25 mm of internal diameter, and 0.1 μm of film thickness (J&W Scientific, Folsom, CA). The oven temperature was programmed from an initial temperature of 50 ºC, and hold time of 3 min; a linear increase at 25 ºC/min to

180 ºC, and hold time of 6 min; followed by linear increase at 40 ºC/min to 260ºC, and hold time of 3 min. The injector and detector temperatures were 220 and 230 °C, respectively. The carrier gas was hydrogen and the flow rate was of 1.7 mL/min. The peak identification was made on the retention times of each 34 FAME standards (C6-C24, Supelco 37 Component Fame Mix).

Relative abundance of each FAME identified in both conditions was determined upon integration of each peak using the equipment's software. This analysis was carried out in triplicate for each sample and three different samples were analyzed for each experimental group.

*12. GC-MS conditions*

FAMEs were obtained after transesterification of lipid extracts according to the method described by Aued-Pimentel et al. (85). Briefly, dried lipid extracts (30 μg of total phospholipid) were dissolved in 1 mL n-hexane. A methanolic KOH solution (2 M) was added (200 μL), followed by intense vortex-mixing for 1-2 min. Saturated NaCl solution (2 mL) was added. After centrifugation at 2000 rpm for 5 min, the organic phase was collected and dried under a nitrogen stream. The resulting FAMEs were dissolved in n-hexane and volumes of 2.0 μL were analyzed by gas chromatography–mass spectrometry (GC–MS) on an Agilent Technologies 6890 N Network (Santa Clara, CA) equipped with a DB-FFAP column with 60 m of length, 0.25 mm of internal diameter, and 0.25 μm of film thickness (J&W Scientific, Folsom, CA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 50–550 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 90 °C, a linear increase to 220 °C at 14.4 °C $min^{-1}$, followed by linear increase at 10 °C $min^{-1}$ to 240 °C, then at 5 °C $min^{-1}$ to 250 °C. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 mL $min^{-1}$. The relative amounts of FAs were calculated by the percent area method with proper normalization considering the sum of all areas of the identified FAs.

*13. HPLC - Electrospray mass spectrometry conditions*

To identify the molecular phospholipid species and their changes in MSCs under pro-inflammatory stimulus, phospholipid classes were separated by hydrophilic

interaction liquid chromatography (HILIC)-MS, using an HPLC system (Waters Alliance 2690) coupled with and electrospray (ESI) linear ion trap mass spectrometer (ThermoFinningan, San Jose, CA, USA). The mobile phase A consisted of 55% (v/v) acetonitrile, 25% (v/v) of methanol and 25% water with 10 mM ammonium acetate. The mobile phase B consisted of acetonitrile 60%, methanol 40% with 10 mM ammonium acetate. 25 μg of total lipid extract were diluted in 90μL of mobile phase B and the reaction mixture was introduced into Ascentis Si HPLC Pore column (15 cm x 1.0mm, 3 μm) (Sigma-Aldrich). The solvent gradient was set as followed: gradient began with 0% of A and linear increase of 100% A during 20 min, and held isocratically for 35 min, returning to the initial set up after 5 min. The follow rate through the column was 16 μL/min, obtained using a pre-column split (Acurate, LC Packings, USA), and it was redirected to a LXQ linear ion trap mass spectrometer (ThermoFinningan, San Jose, CA, USA) by a capillary (0.350 × 0.150 mm) of 70 cm length. The LXQ linear ion trap mass spectrometer was operated in negative-ion mode. Typical ESI conditions were as follows: electrospray voltage, 4.7 kV; capillary temperature, 275 °C; and sheath gas flow, 25 units. To obtain the product-ion spectra of the major components during LC experiments, cycles consisting of one full scan mass spectrum and three data-dependent MS/MS scans were repeated continuously throughout the experiments with the following dynamic exclusion settings: repeat count 3; repeat duration 30s; exclusion duration 45s. LC-MS was performed with internal standards to confirm and quantify the ion differences present in the spectrum according to the Lipid Maps methods (86). The PL standards chosen were PC (dMPC 14:0/14:0), PS (dMPS 14:0/14:0), PI (dPPI 16:0/16:0), PE (dMPE 14:0/14:0), phosphatidic acid (PA) (dMPA 14:0/14:0), cardiolipin (CL) (14:0/14:0/14:0/14:0), LPC (19:0) and phosphatidylglycerol (PG) (dMPG 14:0/14:0).

Data acquisition and treatment of results was done with Xcalibur data system (V2.0) (87). Relative quantitation of individual phospholipid species was determined by he ratio between the area of reconstructed ion chromatogram of a given m/z value against the area of the reconstructed ion chromatogram of the respective class (88).

*14. Statistical Analysis*

Results were represented as means ± standard error (SD). One-way analysis of variance (ANOVA) with the Bonferroni post-hoc test was used to determine significant differences among samples. A value of $p<0.05$ was considered significant. Statistical analysis was performed using GraphPad Prism 5 for Mac OS X (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

# III. Results

# Results

## 1. MSCs cultured in DMEM

### *1.1 Effects of TNF-α and IFN-γ stimuli in fatty acid profile of MSCs*

In order to evaluate alterations in the FA profile of MSCs after pro-inflammatory stimulus, FAMES obtained after transesterification of the total lipid extracts from both MSCs cultured in DMEM with no stimuli (MSCs_DMEM nstim) and MSCs cultured in DMEM under TNF-α and IFN-γ (MSCs_DMEM TNF+IFN) were separated and quantified by GC-FID. This approach allowed the identifying 9 most abundant fatty acids in both samples: myristic acid (C14:0), palmitoleic acid (C16:1), palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1 Δ9 cis), elaidic acid (C18:1 Δ9 trans), stearic acid (C18:0), arachidonic acid (C20:4 Δ6) and dihomo-γ-linoleic acid (C20:3) (Figure 7).

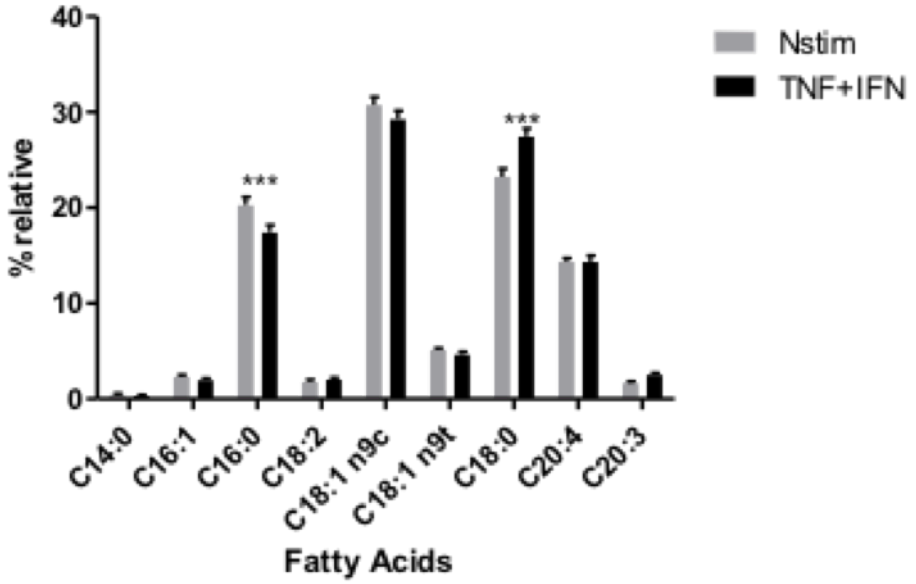


**Figure 7. Fatty acid profile of total lipid extracts from MSCs cultured in DMEM with no stimuli (Nstim) and MSCs cultured in DMEM subjected to TNF-α and IFN-γ (TNF+IFN).** Relative content of the major fatty acids analysed by GC-FID. Values are means ± standard deviation of three independent experiments. ***, significantly different from Nstim (P<0.001).

The most abundant FAME species in MSCs from both conditions were C16:0, C18:1 Δ9 cis, C18:0 and C20:4 Δ6 (Figure 7). Significant differences in the relative amount of C16:0 and C18:0 in MSCs fatty acid profile under pro-inflammatory stimuli were observed (Figure 7). The presence of TNF-α and IFN-γ caused a significant increase in C18:0 levels, while C16:0 levels decreased (Figure 7). No significant changes were detected in the levels of the remainder FAME between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN.

*1.2 Effects of TNF-α and IFN-γ in phospholipid profile of MSCs*

In order to evaluate alterations in the PL profile of MSCs_DMEM TNF+IFN, the total lipid extracts of MSCs of both experimental groups were fractionated by TLC. Each class was identified by comparison with pure phospholipid standards applied in the same TLC plate and relative amount of each PL class observed in TLC plate was determined by quantification of phosphate content in each PL spot. This approach allowed the separation of six phospholipid classes: sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE), and lysoPC (LPC) (Figure 8, A.1 and B.1). The most abundant PL class was PC, followed by PE, SM, PS, PI and LPC (Figure 8, A.2 and B.2). No significant differences in PLs class content were observed between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN.

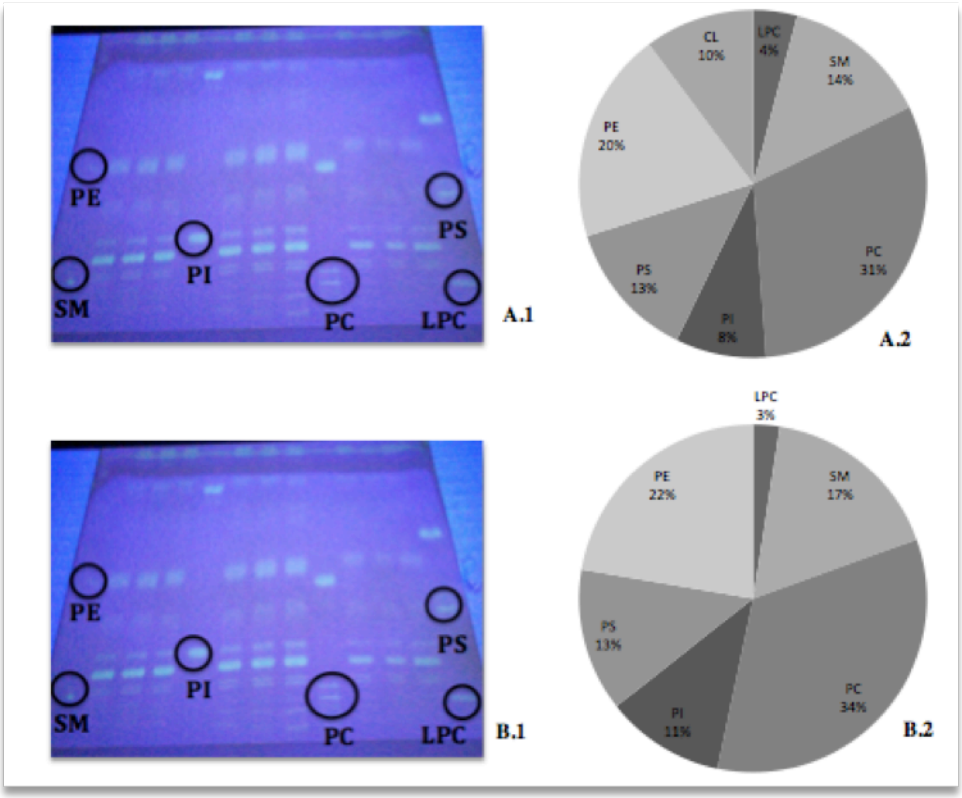


**Figure 8. Thin-layer chromatography of total lipid extract obtained from MSCs cultured in DMEM with no stimuli (A.1) and MSCs cultured in DMEM subjected to TNF-α and IFN-γ (B.1).** Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM) - Sphingomyelin; (PI) - Phosphatidylinositol; (LPC) - Lysophosphatidylcholine. The phospholipid classes were separated by thin-layer chromatography and the phosphorous content of each spot was calculated taking in account the amount of phosphorous in the total lipid extract. Relative content of phospholipid classes in MSCs cultured in DMEM with no stimuli (A.2) and MSCs cultured in DMEM subjected to TNF-α and IFN-γ (B.2) in total lipid extract.

### *1.3 Analysis of phospholipid molecular profiling in MSCs after TNF-α and IFN-γ stimuli by HILIC-MS*

In order to evaluate alterations in the molecular composition of each PL class, phospholipid molecular species were identified by HILIC-MS and LC-MS/MS in negative mode(89–91). For all six identified PL classes, a phospholipid molecular profile was obtained. This analysis was carried out once for each sample and three different samples were analysed for each experimental group. PL classes were analysed by LC and MS/MS in negative mode. MS/MS interpretation for each ion allowed identification of PL molecular species.

PC and LPC were identified as $[M+CH_3COO]^-$ in LC-MS spectra. In both conditions the most abundant PC molecular specie was PC(34:1) at *m/z* 818.2 for $[M+CH_3COO]^-$ ion (Figure 9, A). Also MSCs' PC molecular profile was much similar to one previous reported (82). Significant alterations in MSCs' PC molecular profile were observed comparing MSCs from experimental and control groups. In fact, a significant decrease of PC(34:1) at *m/z* 818.2 specie, along with PC(32:1) at *m/*z 790.2, PC(32:0) at *m/z* 792.4 and PC(40:6) at *m/z* 892.3 were found. However, in MSCs_DMEM TNF+IFN, a small increase of some other molecular species were also observed: PC(36:1) at *m/z* 846.2 and PC(38:4) at *m/z* 868.2 (Figure 9, A). PC is the major PL class in eukaryotic cells and it is not only the main structural component of biological membranes, but it is also involved in signal transduction being precursor of signalling molecules (92).

In the LC-MS negative mode spectra obtained from both MSCs_DMEM nstim and MSCs_DMEM TNF+IFN, three $[M+CH_3COO]^-$ ions attributed to LPC were identified (Figure 9, B). In both conditions the most abundant LPC molecular specie was LPC(18:0) at *m/z* 582.3. Comparing LPC profile of both MSCs_DMEM nstim and MSCs_DMEM TNF+IFN, a significant increase of LPC(18:0) specie was observed (Figure 9, B). LPC is formed in physiological and pathological conditions and can be involved in many cellular processes (93). LPC induces secondary messengers activation and it interferes with their pathways (94,95) and it is also a bioactive lipid that plays a role in inflammatory conditions (96).

SM were identified as $[M+CH_3COO]^-$ ions in LC-MS spectra (Figure 9, C). In both conditions the most abundant SM molecular specie was SM(34:1) at *m/z* 761.3. In MSCs_DMEN TNF+IFN a significant increase of an sphingomyelin molecular specie – SM(34:0) at *m/z* 763.1 – was identified (Figure 9, C). SM's assymetric molecular structure and hydrogen-bonding properties are essential to its role as structural compounds in biological membranes. Moreover, due to SM great ability to bind itself to cholesterol, indicates SM's role as cholesterol distribution modulator (97).

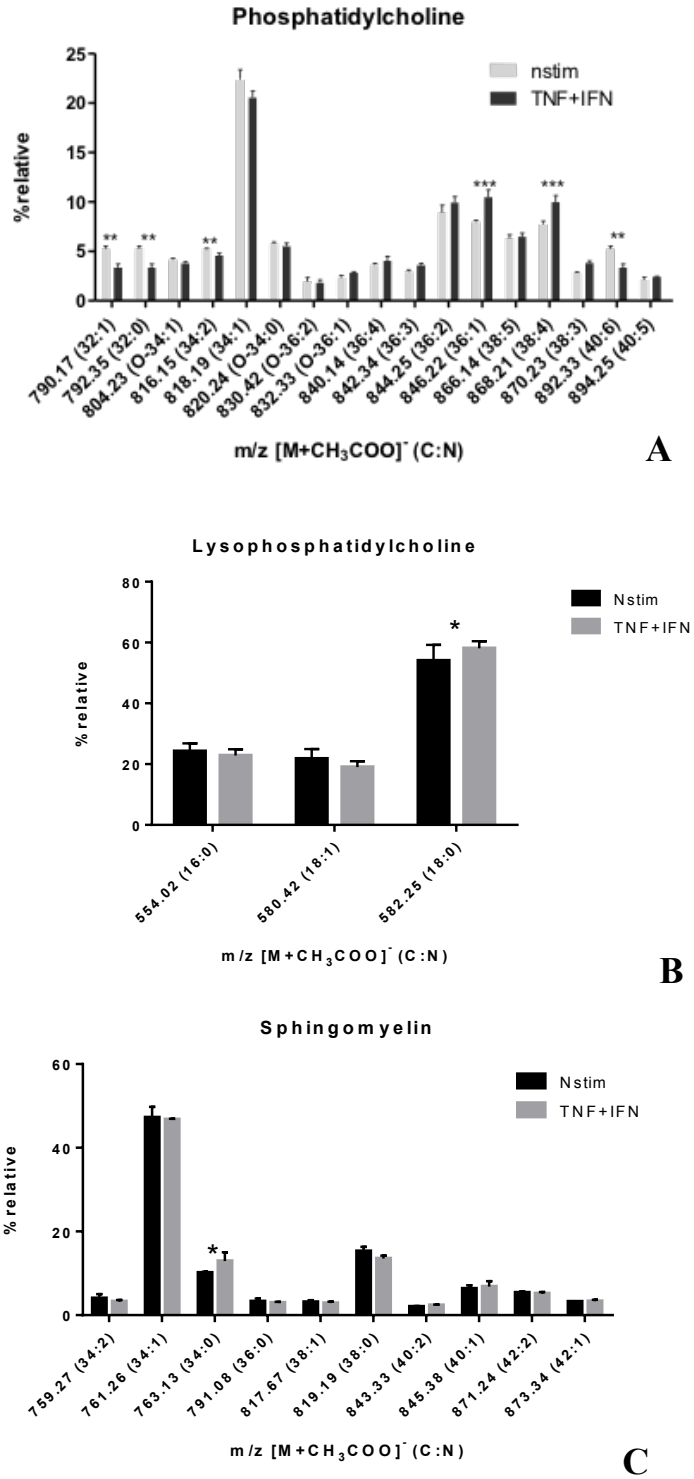


**Figure 9. Percentage of the major phosphatidylcholine (PC) (A), lysoPC (LPC) (B) and sphingomyelin (SM) (C) molecular species identified in MSCs cultured in DMEM after LC-MS and MS/MS analysis.** The results were expressed as percentage obtained by dividing the ratio between the peak areas of each molecular species and the respective internal standards and the total of all ratios. Values are means ± standard deviation of three independent experiments. ***, significantly different from Nstim (P<0.001). **, significantly different from Nstim (P<0.01). *, significantly different from Nstim (P<0.05).

PE were identified as $[M-H]^-$ ions in LC-MS spectra. The most abundant PE species previous reported by Kilpinen (82) were also observed in this study. However, we also identified two more PE species: PE(36:0) and PE(O-36:5). Significant alterations in MSCs' PE molecular profile were observed comparing MSCs_DMEM nstim and MSCs_DMEM TNF+IFN (Figure 10, A). In fact, a decrease of PE(O-38:6) at *m/z* 748.3 and a increase of PE(40:6) at *m/z* 790.3 were observed in MSCs_DMEM TNF+IFN (Figure 10, A). PE comprises about 15-25% of the total biological membrane phospholipids. Similar to PC, PE also acts as a structural component of membranes. However, PE is also substrate for other metabolic pathways originating PS in the reticulum endoplasmic (92,98).

PS was identified as $[M-H]^-$ and the most abundant PS in both conditions was PS(36:1) at *m/z* 788.3 (Figure 10, B). Significant alterations in PS molecular profile were observed between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN. For MSCs_DMEM TNF+IFN a decrease of PS(40:4) at *m/z* 838.3 was observed while levels of PS(36:1) at *m/z* 788.3 increased (Figure 10, B). Also in PS profile, PS(36:0) was identified, which has not been previously reported. Nevertheless PS most abundant species reported by Kilpinen were also observed in this study. PS is present in small amounts in cells but is an important signal molecule both inside and outside of the cell. In fact, when PS is present in apoptotic cells' outer leaflet of biological membranes, it acts as a signal for phagocytosis (92,98). Moreover, PS has a role in targeting intracellular proteins and in maintaining their function (98).

PI molecular species were identified as $[M-H]^-$ ions (Figure 10, C). Three PI species – PI(36:0), PI(O-38:6) and PI(40:6) - that have not identified by Kilpinen (82) were identified in this study. In both experimental conditions the most abundant PI molecular specie was PI(38:4) at *m/z* 885.3. No significant alterations in MSCs PI molecular profile were observed between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN (Figure 10, C). PI comprises about 10-15% of cellular phospholipids and has an important role as precursor for secondary messengers, namely PIPs. Glycosylphosphatidylinositol also acts as an anchor for soluble proteins (92).

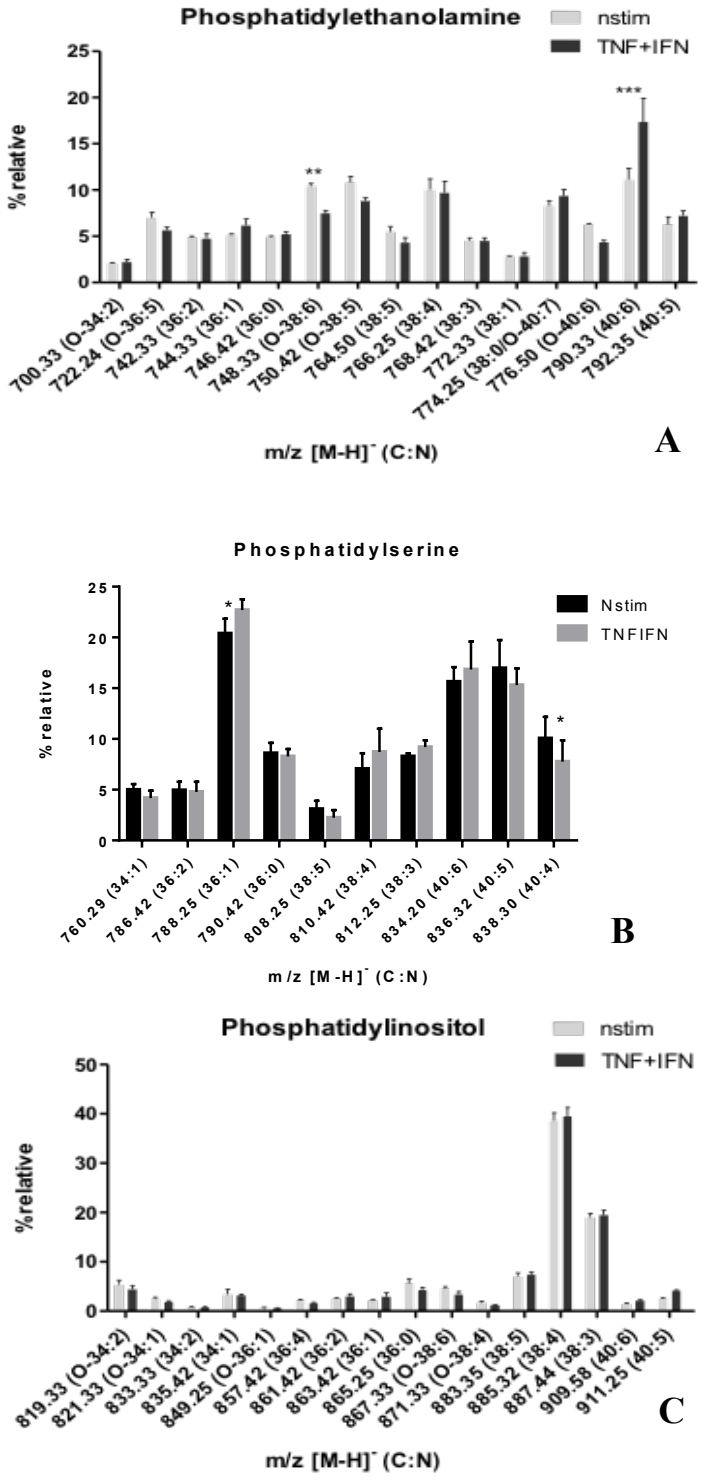


**Figure 10. Percentage of the major phosphatidylethanolamine (PE) (A), phosphatidylserine (PS) (B) and phosphatidylinositol (PI) (C) molecular species identified in MSCs cultured in DMEM after LC-MS and MS/MS analysis.** The results were expressed as percentage obtained by dividing the ratio between the peak areas of each molecular species and the respective internal standards and the total of all ratios. Values are means ± standard deviation of three independent experiments. ***, significantly different from nstim (P<0.001). **, significantly different from nstim (P<0.01). *, significantly different from nstim (P<0.05).

In resume, the same molecular species were found for each PL class either in MSCs_DMEM nstim and MSCs_DMEM TNF+IFN. However, significant differences in the relative amount of some molecular species were found for all PL classes - except for PI - between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN (Table 1). Table 1 summarizes which PL molecular species' levels vary in MSCs cultured in DMEM (MSCs_DMEM) under pro-inflammatory environment.

**Table 1 -** Summary of all identified PL molecular species whose amounts were significantly different in MSCs cultured in DMEM subjected to TNF-α and IFN-γ (MSCs_DMEM TNF+IFN) in comparison with MSCs cultured with no stimuli.

| **MSCs_DMEM TNF+IFN** | |
|---|---|
| **↑%relative** | **↓%relative** |
| **C:N (m/z)** | **C:N (m/z)** |
| PC(36:1) | PC(32:1) |
| PC(38:4) | PC(32:0) |
| SM(34:0) | PC(34:1) |
| PE(40:6) | PC(40:6) |
| PS(36:1) | PE(38:6) |
| LPC(18:0) | PS(40:4) |

**2. MSCs cultured in xeno-free media**

The lipid profile of MSCs cultured in xeno-free media (MSCs_XF) was obtained and compared with the previous one obtained for MSCs cultured in DMEM (MSCs_DMEM). Furthermore, the effects of pro-inflammatory stimuli in MSCs_XF were evaluated, in the same manner as what was described previously in this work for MSCs_DMEM.

*2.1 Effect of culture medium in the profile of fatty acids and phospholipid in MSCs*

MSCs_DMEM and MSCs_XF expressed the same FAs (Figure 11). Interestingly no differences were detected in FA profile after pro-inflammatory profile. In fact, neither in MSCs_DMEM or MSCs_XF TNF-α and IFN-γ induced differences in the levels of FAs (Figure 11).

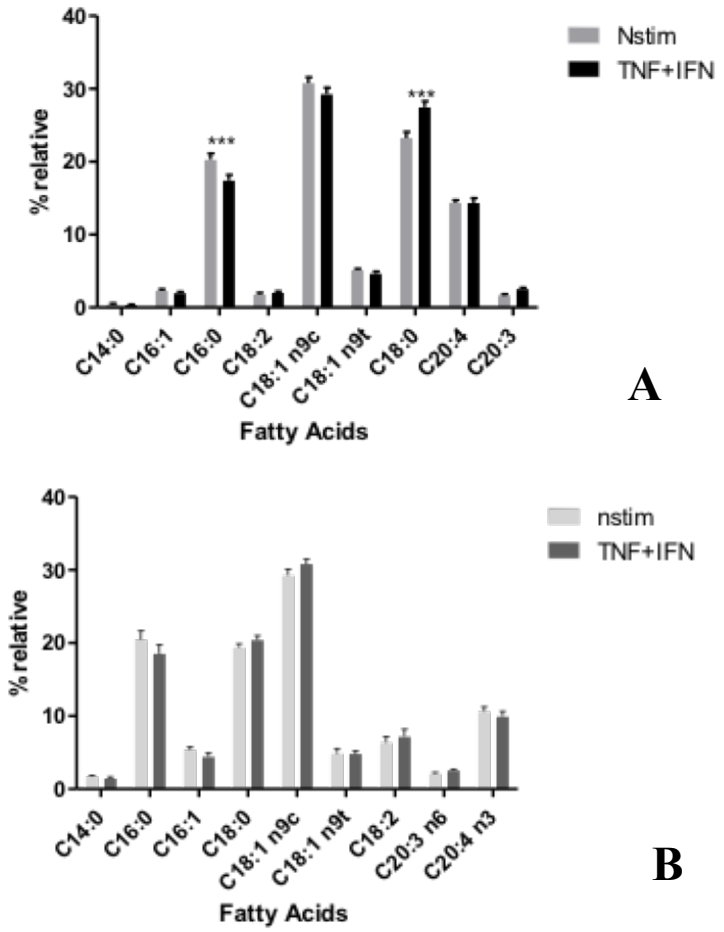


**Figure 11. Fatty acid profile of total lipid extracts from MSCs cultured in DMEM (A) and in xeno-free media (B) with no stimuli (Nstim) and MSCs cultured in xeno-free media subjected to TNF-α and IFN-γ (TNF+IFN).** Relative content of the major fatty acids analysed by GC-MS. Values are means ± standard deviation of three independent experiments.

However, some differences in the levels of specific fatty acids were observed between MSCs_DMEM and MSCs_XF (Figure 12, A and B). In fact, both in MSCs_XF cultured with no stimuli (MSCs_XF nstim) and MSCs_XF subjected to TNF-α and IFN-γ (MSCs_XF TNF+IFN) we observed higher levels of C18:2, along with lower levels of C18:0 and C20:4 Δ6 (Figure 12, A and B). In MSCs_XF nstim, enhanced expression of C18:1 n9cis was also observed (Figure 12, A). No further deviations on the FA profile of MSCs were observed.

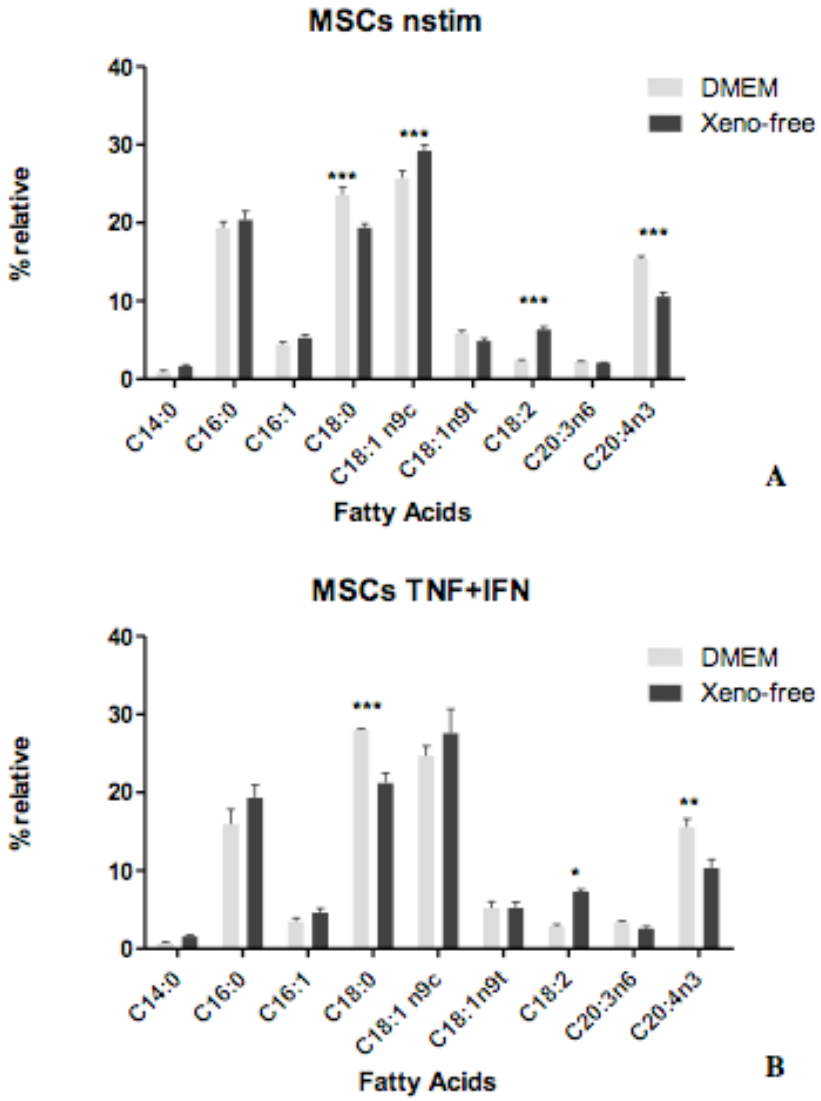


**Figure 12. Fatty acid profile of total lipid extracts from both MSCs cultured in DMEM and xeno-free media with no stimuli (A) and subjected to TNF-α and IFN-γ (B).** Relative content of the major fatty acids analysed by GC-MS. Values are means ± standard deviation of three independent experiments. ***, significantly different from DMEM (P<0.001). **, significantly different from DMEM (P<0.01). *, significantly different from DMEM (P<0.05).

MSCs_DMEM and MSCs_XF also expressed the same PL classes (Figure 13, A.1 and B.1). In the same manner, MSCs_DMEM and MSCs_XF expressed similar amounts of the six identified PL classes when cultured with no stimulus (Figure 14, A). However, MSCs_XF TNF+IFN expressed significant lower amounts of SM than MSCs_DMEM TNF+IFN (Figure 14, B).

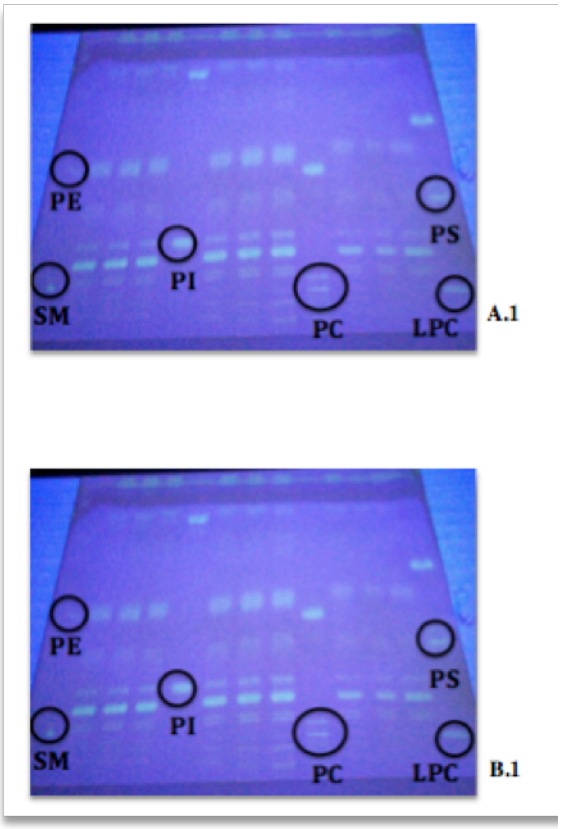


**Figure 13. Thin-layer chromatography of total lipid extract obtained from MSCs cultured in DMEM with no stimuli (A.1) and MSCs cultured in xeno-free media with no stimuli (B.1).** Phospholipid standards were also applied: (PC) - Phosphatidylcholine; (PS) - Phosphatidylserine; (PE) - Phosphatidylethanolamine; (SM) - Sphingomyelin; (PI) - Phosphatidylinositol; (LPC) - Lysophosphatidylcholine.

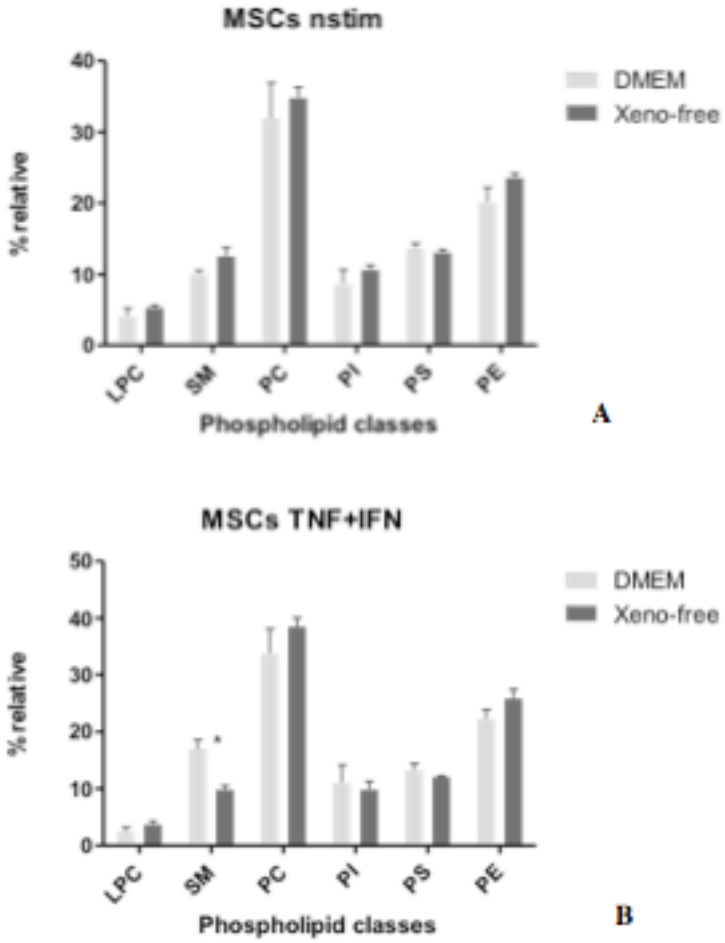


**Figure 14. Phospholipid profile of MSCs cultured in DMEM or xeno-free media subjected to no stimuli (MSCs nstim) (A) and MSCs subjected to TNF-α and IFN-γ (MSCs TNF+IFN) (B)**. *, significantly different from DMEM ($P<0.05$)

Besides the fact that both MSCs_DMEM and MSCs_XF expressed the same FAs species and the same PL classes, both sets of cells expressed the same PL molecular species and similar PL profiles. The molecular profiles of MSCs_XF are represented in figures 15 and 16.

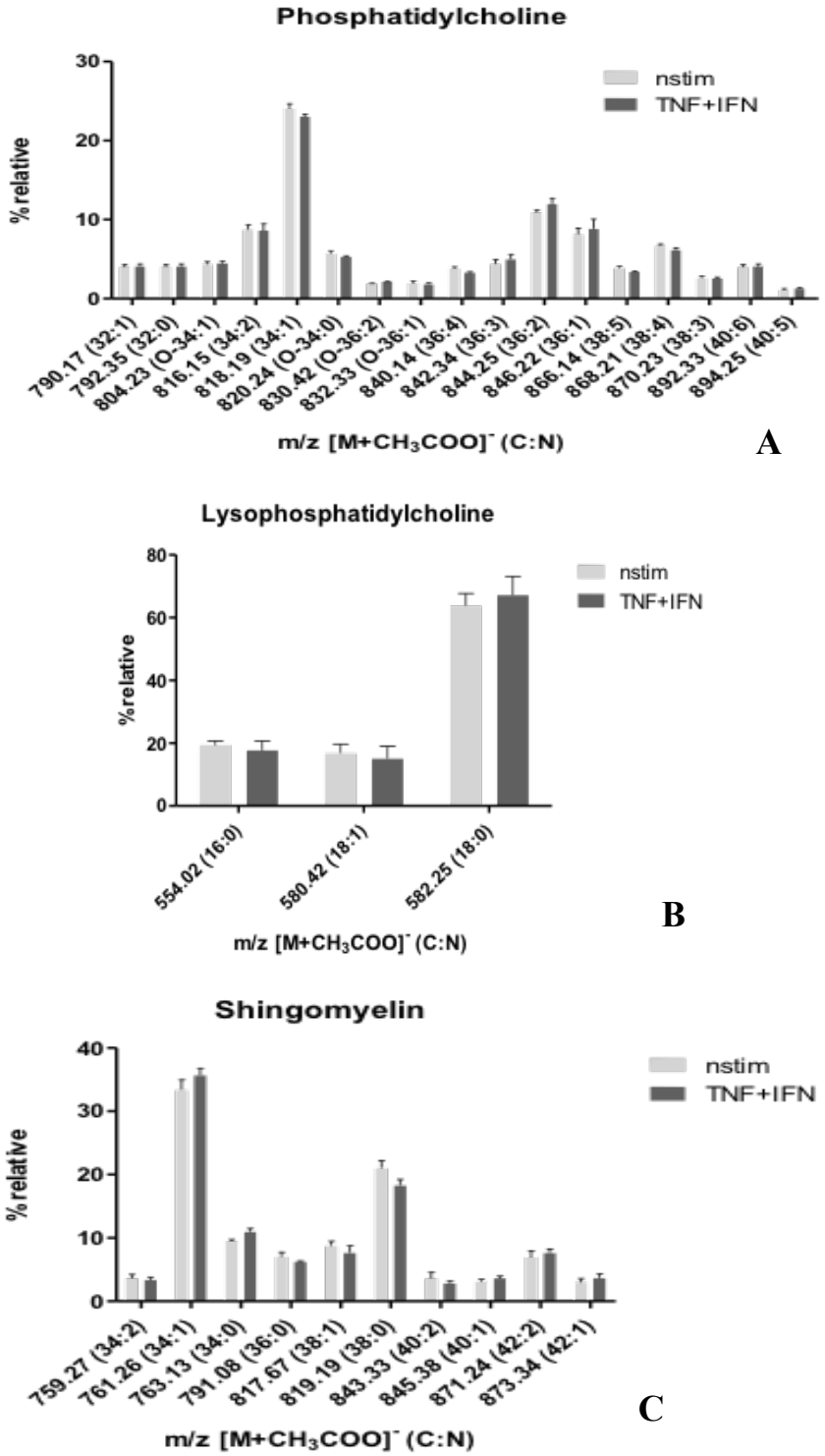


**Figure 15. Percentage of the major phosphatidylcholine (PC) (A), lysoPC (LPC) (B) and sphingomyelin (SM) (C) molecular species identified in MSCs cultured in xeno-free media after LC-MS and MS/MS analysis.** The results were expressed as percentage obtained by dividing the ratio between the peak areas of each molecular species and the respective internal standards and the total of all ratios. Values are means ± standard deviation of three independent experiments**.**

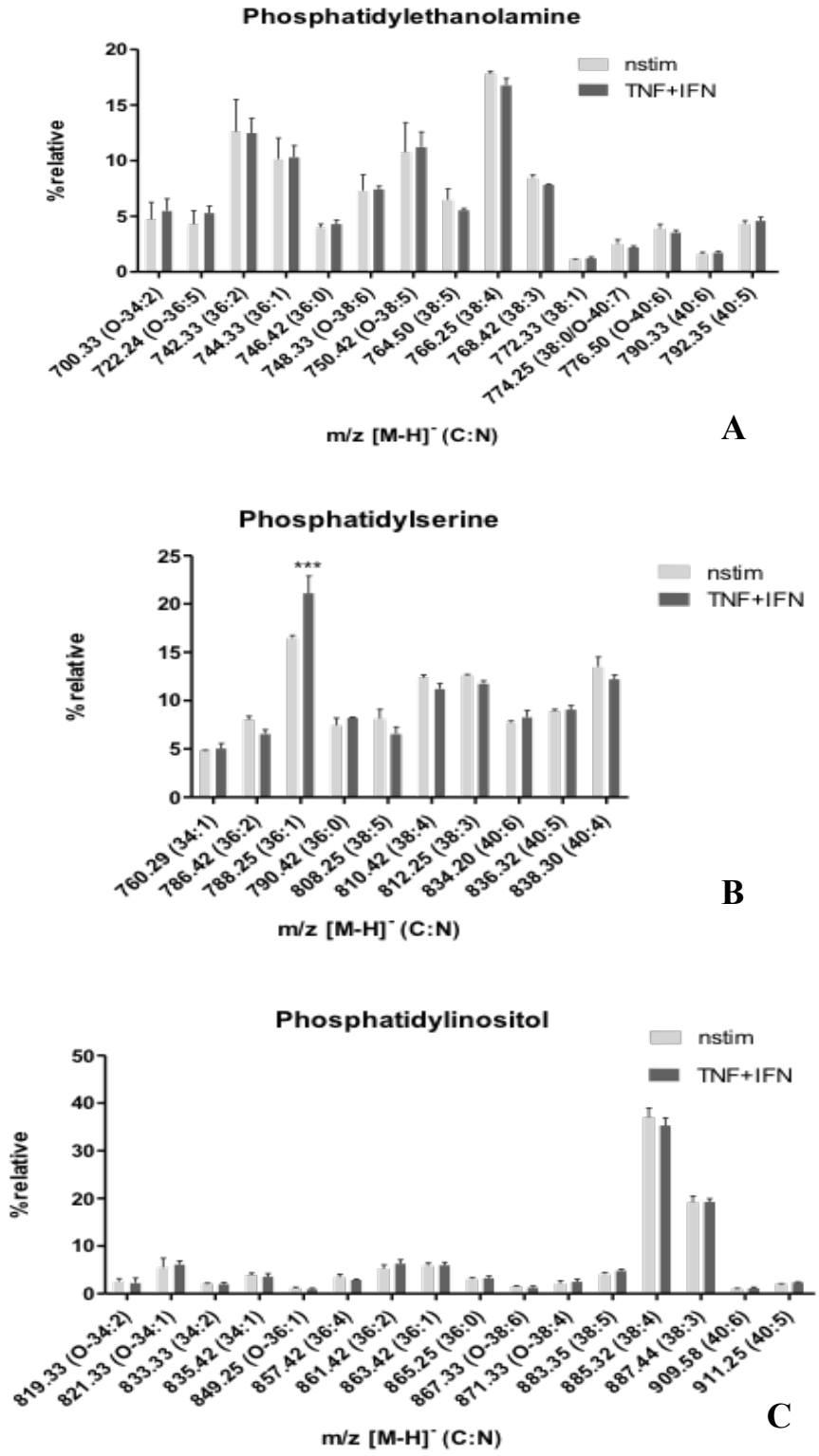


**Figure 16. Percentage of the major phosphatidylethanolamine (PE) (A), phosphatidylserine (PS) (B) and phosphatidylinositol (PI) (C) molecular species identified in MSCs cultured in xeno-free media after LC-MS and MS/MS analysis.** The results were expressed as percentage obtained by dividing the ratio between the peak areas of each molecular species and the respective internal standards and the total of all ratios. Values are means ± standard deviation of three independent experiments. ***, significantly different from nstim ($P<0.001$).

As stated above, both MSCs_DMEM and MSCs_XF expressed the same PL molecular species and the PL molecular profiles expressed by MSCs_XF are similar to the previous ones observed in MSCs_DMEM, except for PE. In PE profile some differences were observed between MSCs_DMEM and MSCs_XF (Figure 17). In fact, in the case of MSCs_DMEM the most abundant PE specie was PE(40:6) at *m/z* 790.3 while PE(38:4) at *m/z* 766.3, PE(O-38:5) at *m/z* 750.4 and PE(O-38:6) at *m/z* 748.3 were expressed in similar amounts (Figure 17, A). However, in MSCs_XF the most abundant PE specie was PE(38:4) at *m/z* 766.3 and PE(36:1) at *m/z* 744.3, PE(36:2) at *m/z* 742.3 and PE(O-38:5) at *m/z* 750.4 were expressed in lower amounts (Figure 17, B).

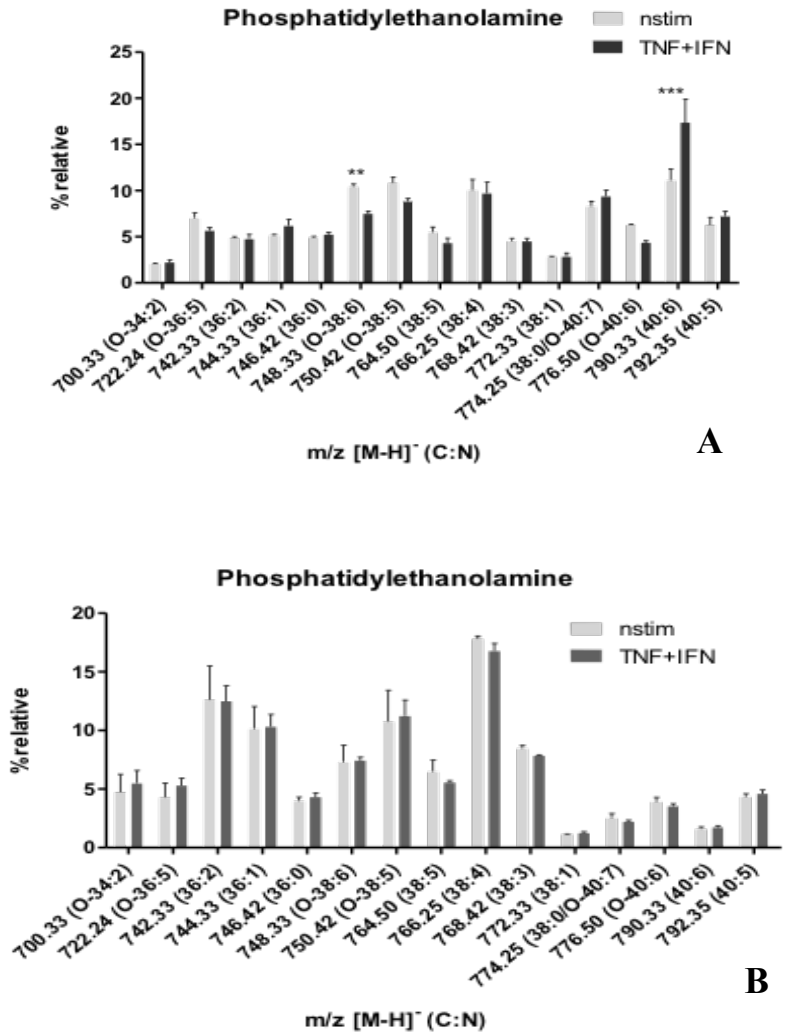


**Figure 17. Percentage of the major phosphatidylethanolamine (PE) molecular species of MSCs cultured in DMEM (A) and of MSCs cultured in xeno-free media identified after LC-MS and MS/MS analysis.** The results were expressed as percentage obtained by dividing the ratio between the peak areas of each molecular species and the respective internal standards and the total of all ratios. Values are means ± standard deviation of three independent experiments. ***, significantly different from nstim (P<0.001). **, significantly different from nstim (P<0.01).

*2.2 Effects of TNF-α and IFN-γ in phospholipid profile of MSCs cultured in xeno-free media*

As described previously, TNF-α and IFN-γ induced several differences in the levels of specific PL molecular species from MSCs_DMEM. In fact, TNF-α and IFN-γ led to deviations in the levels of some PC, PE, SM, PS and LPC species (Figures 9 and 10).

Similar to what was observed for MSCs_DMEM, TNF-α and IFN-γ led to no deviations in the levels of PI species (Figure 15, C) of MSCs_XF. However, in contrast with MSCs_DMEM TNF+IFN, TNF-α and IFN-γ effects in MSCs_XF only led to an enhancement on the amount of PS(36:1) at *m/z* 788.3 (Figure 15, B). Pro-inflammatory stimuli induced higher expression of PS(36:1) in MSCs_XF, in accordance with MSCs_DMEM (Figure 10, B).

# IV. Discussion

# Discussion

This study unveiled the deviations of MSCs lipidome due to pro-inflammatory stimuli. MSCs_DMEM lipid profile showed six PL classes: SM, PC, PI, PS, PE and LPC (Figure 8, A.1 and A.2). The profile of PC, PI, PS, PE, LPC classes was similar to the ones reported in a previous published work (82), but we were able to identify the presence of SM in MSCs that was not referenced previously (82). The exposure of MSCs_DMEM to TNF-α and IFN-γ caused no significant differences in PLs class levels but in contrast some variations were found in specific molecular species within all classes, except PI. These variations can be correlated with MSCs' immunomodulatory properties.

Our study demonstrated that MSCs_DMEM TNF+IFN expressed higher levels of PC molecular species with longer FAs chains - PC species (36:1 and 38:4) – along with decreased levels of PC molecular species with smaller FAs chains (Figure 9, A). It was reported that senescence MSCs, which have less immunosuppressive capacity, showed the same pattern of deviation regarding PC species with shorter FAs chains. However MSCs with weaker immunosuppressive capacity did not enhance PC(36:1) levels (82). PC comprises about 50% of total cellular lipids and it is the main constituent of eukaryotic cells, where it plays a structural role (92,99,100). Thus it is not expected that PC molecular species contribute directly to the MSCs' immunosuppressive mechanism. Hence we suppose that these changes reflect adaptations occurring in MSCs_DMEM when subjected to inflammatory stress. Biological membranes have many functions in a cell, which depend on the physical state of the bilayer: the bilayer must be fluid in order to assure biological membrane's function (101). Many factors interfere with membrane fluidity, namely lipid composition. In fact, biological membrane with higher levels of long-chain, saturated fatty acids is less fluid because those fatty acids are more firmly packed and form stronger van der Walls interactions with each other (102,103). MSCs_DMEM TNF+IFN expressed higher levels of stearic acid, while the levels of palmitic acid were diminished. These results may help to explain the observed changes in PC molecular profile of MSCs_DMEM TNF+IFN but also suggests that TNF-α and IFN-γ stimuli enhanced the activity of elongation of very long chain family member 6

(ELOVL6) enzyme. ELOVL6 is the only enzyme capable of elongating C16:0 into C18:0 (104). Thereafter, it is probable that MSCs biological membrane becomes less fluid when these cells are under pro-inflammatory stimuli. Changes in the membrane fluidity can interfere with both the activity of membrane-bond enzymes and the function of membrane-bond receptors, affecting signal transduction (105,106).

Along with PC species composed of shorter FAs chains, we found that TNF-α and IFN-γ led to decreased expression of PC(40:6) in MSCs_DMEM (Figure 9, A) assigned by MS/MS as PC 18:0/22:6n-3 (Supplemental Material, Table S1). Because higher levels of LPC(18:0) were also observed in MSCs_DMEM TNF+IFN (Figure 9, B) and the activity of phospholipase $A_2$ ($PLA_2$) is usually upregulated in inflammatory environments, we suppose $PLA_2$ acts upon PC(40:6) releasing LPC(18:0) and the fatty acid C22:6n-3. Interestingly, stearoyl lysophosphatidylcholine (LPC(18:0)) is one of the main anti-inflammatory LPC molecular species, increasing the resistance to LPS-induced mortality and diminishing LPS-induced levels of pro-inflammatory cytokines in mice (107). Moreover, LPC(18:0) administration also decreased high mobility group box 1 – cytokine mediator of inflammation - secretion by macrophages (108). In humans LPC(18:0) inhibited LPS-induced tissue factor activity and mRNA expression by human monocytes and simultaneously increased monocytes levels of cyclic adenosine monophosphate (109). Thereafter we are led to believe LPC(18:0) is a key player in the immunomodulator mechanism employed by MSCs. However, while MSCs with diminished immunosuppressive capacity showed higher levels of LPC (82), we did not observe significant differences in LPC levels as result of TNF-α and IFN-γ stimuli (Figure 8, A.2 and B.2). As stated above $PLA_2$ activity upon PCC(40:6) leads to the release of C22:6n-3, which is precursor of protectins, resolvin ds and maresins – new anti-inflammatory, pro-resolving families of mediators in inflammation (110,111).

PE molecular profile is slightly different between MSCs_DMEM TNF+IFN and MSCs_DMEM nstim (Figure 10, A). TNF-α and IFN-γ led to enhanced expression of PE(40:6) and to declined expression of PE(O-38:6) (Figure 10, A). These results are in opposition with the previous reported ones for senescence and less active MSCs (82), which indicates that these PE species have important roles in the immunosuppressive mechanisms of MSCs. Even though PE acts as a structural component in biological membranes, it is also a precursor of other lipids and a

subtract for other pathways (92,98). The evidence that levels of some PE molecular species are susceptible to TNF-α and IFN-γ is rather interesting because previous studies demonstrated PE is capable of bounding hyaluronic acid, inhibiting pro-inflammatory effects of IFN-γ and lipopolysaccharide in cultured endothelial and renal proximal tubular cells, lymphocyte reaction and after mitogen stimulation (112).

In opposition to Kilpinen's report in senescence MSCs (82), we found no significant differences in the levels of PS or PI (Figure 8, A.2 and B.2) or in the ratio PI/PS between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN. We also did not identify differences in the molecular profile of PI between MSCs_DMEM nstim and MSCs_DMEM TNF+IFN (Figure 10, C). However, we verified TNF-α and IFN-γ caused both enrichment of PS(36:1) and impoverishment of PS(40:4) (Figure 10, B). Interestingly PS has been recently highlighted for its immunomodulatory abilities (113,114). In a study conducted by Hoffman and colleagues, results showed PS inhibited T and B cells responses in spleen and lymph node cells, indicating PS acts by regulating inflammation in tissue (113). In a more recent study, investigators came to the conclusion that PS interferes with DC cells maturation and T cell activity by increasing the production of anti-inflammatory cytokines and simultaneously decreasing the production of pro-inflammatory cytokines (114).

In the only previous study concerning MSCs' lipidome, SM molecular profile of MSCs was not reported. However, in our study we identified SM as the third most abundant PL class. Besides, an increase in the levels of SM(34:0) in MSCs_DMEM subjected to TNF-α and IFN-γ was also observed (Figure 9, C). Sphingolipids are one of the essential components of lipid membrane and lipid rafts. Therefore, changes in sphingolipids' metabolism can result in lipid membranes and lipid rafts with different characteristics, interfering with receptor clustering. Some of sphingolipids' metabolites are bioactive and mediate essential cell functions (115). Actually one of those bioactive metabolites – sphingosine-1-phosphate - is capable of down regulate mast cells activation. Mast cells secret compounds that trigger allergic responses (116). The same metabolite also interferes with T-cell exit from thymus (117) and stops macrophages and T cells from enter apoptosis (118,119). Interestingly, despite MSCs action over immune system cells, evidences suggest they do not induce apoptosis. Moreover some sphingolipids can inhibit T helper cells proliferation (120). Curiously, MSCs also act upon T helper cells (60). In a previous work about stem cell

homing and mobilization, it was showed that the migration of hematopoietic stem progenitor cells is directed by sphingosine-1-phosphate (121). As referred above, MSCs are capable of migrating towards inflammation sites (21).

From the deviations stated above, the only deviation resultant from TNF-α and IFN-γ in MSCs_XF TNF+IFN was assigned as higher levels of PS(36:1). Because pro-inflammatory stimuli led to no differences in the levels of PC species, we suppose MSCs_XF do not need to make structural adaptations in order to perform their mechanisms. However, comparing MSCs_DMEM and MSCs_XF further differences were found. In fact, MSCs_XF TNF+IFN express significant lower amounts of SM and a slightly different PE molecular profile. Moreover, MSCs_XF present enhanced amounts of FA C18:2, along with reduced levels of FAs C18:0 and C20:4n-6.

# V. Conclusion

# Conclusion

This study evidences that MSCs phospholipid profile changes when these cells are subjected to pro-inflammatory stimulus and these differences are more evident in MSCs cultured in a medium supplemented with biological serum. The differences were identified only in the level of specific PL molecular species thus suggesting that each PL molecular specie could play a role in MSCs' immunological functionality. However, MSCs' lipidome is far more stable in cells cultured in a synthetic and fully controlled medium. In order to fully understand how each one of PL molecular species interfere with mechanism of MSCs, further studies are needed. Once levels of PS(36:1) were the only ones affected in MSCs from both media by TNF-α and IFN-γ and PS is known recognized for its immunomodulatory and anti-inflammatory properties abilities, we believe this PL specie is one of the major key players in MSCs' immunosuppressive action. The study of cells' lipidome is important either to understand the MSCs' immunomodulator mechanism or to be used as a biomarker for cells' functionality.

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# Supplemental Material

# Supplemental Material

**Table S1.** Major PC molecular species from MSCs cultured in DMEM with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturation bonds

| [M+CH3COO]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| **790.17 (32:1)** | 14:0/18:1 and 14:1/18:0 and 16:0/16:1 |
| **792.35 (32:0)** | 16:0/16:0 |
| **804.23 (34:1)** | O-16:0/18:1 |
| **816.15 (34:2)** | 14:1/20:1 and 16:0/18:2 and 16:1/18:1 |
| **818.19 (34:1)** | 14:0/20:1 and 16:0/18:1 and 18:0/16:1 and 18:1/16:0 |
| **820.24 (34:0)** | 16:0/18:0 |
| **830.42 (36:2)** | Plasmenyl specie |
| **832.33 (36:1)** | Plasmenyl specie |
| **840.14 (36:4)** | 16:0/20:4 and 18:0/18:4 |
| **842.34 (36:3)** | 16:0/20:3 |
| **844.25 (36:2)** | 14:1/22:1 and 18:0/18:2 and 18:1/18:1 |
| **846.22 (36:1)** | 14:0/22:1 and 16:0/20:1 and 18:0/18:1 |
| **866.14 (38:5)** | 16:0/22:5 and 18:0/20:5 and 18:1/20:4 and 18:2/20:3 |
| **868.21 (38:4)** | 18:1/20:3 |
| **870.23 (38:3)** | ------ |
| **892.33 (40:6)** | 18:0/22:6 and 18:1/22:5 and 20:1/20:5 and 20:2/20:4 |
| **894.25 (40:5)** | 16:0/24:5 and 18:0/22:5 and 18:1/22:4 and 20:0/20:5 and 20:1/20:4 |

**Table S2.** Major PI molecular species from MSCs cultured in DMEM with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M-H]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| **819.33 (34:2)** | O-16:0/18:2 and O-16:1/18:1 |
| **821.33 (34:1)** | Plasmenyl specie |
| **833.33 (34:2)** | ------ |
| **835.42 (34:1)** | 16:0/18:1 and 16:1/18:0 |
| **849.25 (36:1)** | Plasmenyl specie |
| **857.42 (36:4)** | ------ |
| **861.42 (36:2)** | 16:0/20:2 and 16:1/20:1 and 18:0/18:2 and 18:1/18:1 |
| **863.42 (36:1)** | 16:0/20:1 and 18:0/18:1 |
| **865.25 (36:0)** | 16:0/20:0 and 18:0/18:0 |
| **867.33 (38:6)** | O-16:0/22:6 and O-16:1/22:5 |
| **871.33 (38:4)** | Plasmenyl specie |
| **883.35 (38:5)** | 16:0/22:5 and 18:0/20:5 and 18:1/20:4 |
| **885.32 (38:4)** | 16:0/22:4 and 18:0/20:4 and 18:1/20:3 |
| **887.44 (38:3)** | 18:0/20:3 and 18:1/20:2 |
| **909.58 (40:6)** | ------ |
| **911.25 (40:5)** | 18:0/22:5 and 18:1/22:4 and 20:1/20:4 and 20:2/20:3 |

**Table S3.** Major PE molecular species from MSCs cultured in DMEM with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M-H]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| **700.33 (34:2)** | |
| **722.24 (36:5)** | O-16:0/20:5 and O-16:1/20:4 |
| **742.33 (36:2)** | 16:0/20:2 and 16:1/20:1 and 18:0/18:2 and 18:1/18:1 |
| **744.33 (36:1)** | 16:0/20:1 and 18:0/18:1 |
| **746.42 (36:0)** | 18:0/18:0 |
| **748.33 (38:6)** | O-16:0/22:6 and O-16:1/22:5 and O-18:1/20:5 and O-18:1/20:4 |
| **750.42 (38:5)** | O-16:0/22:5 and O-16:1/22:4 and O-18:0/20:5 and O-18:1/20:4 |
| **764.50 (38:5)** | 16:0/22:5 and 16:1/22:4 and 18:0/22:5 and 18:1/20:4 and 18:2/20:3 |
| **766.25 (38:4)** | 16:0/22:4 and 16:1/22:3 and 18:0/20:4 and 18:1/20:3 |
| **768.42 (38:3)** | ------ |
| **772.33 (38:1)** | 18:0/20:1 |
| **774.25 (38:0)** | 18:0/20:0 |
| **774.25 (40:7)** | O-18:1/22:6 and O-18:2/22:5 |
| **776.50 (40:6)** | O-18:0/22:6 and O-18:1/22:5 and O-18:2/22:4 |
| **790.33 (40:6)** | 18:0/22:6 and 18:1/22:5 and 18:2/22:4 and 20:1/20:5 and 20:2/20:4 and 20:3/20:3 |
| **792.35 (40:5)** | 18:0/22:5 and 18:1/22:4 and 20:0/20:5 and 20:1/20:4 |

**Table S4.** Major PS molecular species from MSCs cultured in DMEM with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M-H]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| **760.29 (34:1)** | 14:0/20:1 and 16:0/18:1 and 16:1/18:0 |
| **786.42 (36:2)** | 16:1/20:1 and 18:0/18:2 and 18:1/18:1 |
| **788.25 (36:1)** | 16:0/20:1 and 16:1/20:0 and 18:0/18:1 |
| **790.42 (36:0)** | 16:0/20:0 and 18:0/18:0 |
| **808.25 (38:5)** | ------ |
| **810.42 (38:4)** | 16:0/22:4 and 18:0/20:4 and 18:1/20:3 |
| **812.25 (38:3)** | 18:0/20:3 |
| **834.20 (40:6)** | 18:0/22:6 and 18:1/22:5 and 20:1/20:5 and 20:2/20:4 and 20:3/20:3 |
| **836.32 (40:5)** | 18:0/22:5 and 18:1/22:4 and 20:0/20:5 and 20:1/20:4 |
| **838.30 (40:4)** | 18:0/22:4 |

**Table S5.** Major LPC molecular species from MSCs cultured in DMEM with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M+$CH_3COO$]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 554.02 (16:0) | 16:0 |
| 580.42 (18:1) | 18:1 |
| 582.25 (18:0) | 18:0 |

**Table S6.** Major SM molecular species from MSCs cultured in DMEM with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M+$CH_3COO$]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 759.27 (34:2) | Sphingosine d18:1/16:1 |
| 761.26 (34:1) | Sphingosine d18:1/16:0 |
| 763.13 (34:0) | Sphinganine d18:0/16:0 |
| 791.08 (36:0) | Sphinganine d18:0/18:0 |
| 817.67 (38:1) | Sphingosine d18:1/20:0 |
| 819.19 (38:0) | Sphinganine d18:0/20:0 |
| 843.33 (40:2) | Sphingosine d18:1/22:1 |
| 845.38 (40:1) | Sphingosine d18:1/22:0 |
| 871.24 (42:2) | Sphingosine d18:1/24:1 |
| 873.34 (42:1) | Sphinganine d18:1/24:0 |

**Table S7.** Major PC molecular species from MSCs cultured in ImmuneSafe® with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M+$CH_3COO$]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 790.17 (32:1) | 14:0/18:1 and 16:0/16:1 |
| 792.35 (32:0) | 16:0/16:0 |
| 804.23 (34:1) | O-16:0/18:1 |
| 816.15 (34:2) | 14:1/20:1 and 16:0/18:2 and 16:1/18:1 |
| 818.19 (34:1) | 14:0/20:1 and 16:0/18:1 and 18:0/16:1 |
| 820.24 (34:0) | 16:0/18:0 |
| 830,42 (36:2) | Plasmenyl specie |
| 832,33 (36:1) | Plasmenyl specie |
| 840.14 (36:4) | 16:0/20:4 |
| 842.34 (36:3) | 16:0/20:3 |
| 844.25 (36:2) | 14:1/22:1 and 16:0/20:2 and 16:1/20:1 and 18:0/18:2 and 18:1/18:1 |
| 846.22 (36:1) | 16:0/20:1 and 18:0/18:1 |
| 866.14 (38:5) | 18:1/20:4 |
| 868.21 (38:4) | 18:1/20:3 |
| 870.23 (38:3) | ------- |
| 892.33 (40:6) | 18:1/22:5 and 20:2/20:4 |
| 894.25 (40:5) | 16:0/24:5 and 18:0/22:5 and 18:1/22:4 and 20:1/20:4 |

**Table S8.** Major PI molecular species from MSCs cultured in ImmuneSafe® with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M-H]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 819.33 (34:2) | O-16:0/18:2 and O-16:1/18:1 |
| 821.33 (34:1) | Plasmenyl specie |
| 833.33 (34:2) | ------- |
| 835.42 (34:1) | 16:0/18:1 and 16:1/18:0 |
| 849.25 (36:1) | Plasmenyl specie |
| 857.42 (36:4) | ------- |
| 861.42 (36:2) | 16:0/20:2 and 16:1/20:1 and 18:0/18:2 and 18:1/18:1 |
| 863.42 (36:1) | 16:0/20:1 and 18:0/18:1 |
| 865.25 (36:0) | 16:0/20:0 and 18:0/18:0 |
| 867.33 (38:6) | O-16:0/22:6 and O-16:1/22:5 |
| 871.33 (38:4) | Plasmenyl specie |
| 883.35 (38:5) | 16:0/22:5 and 18:1/20:4 |
| 885.32 (38:4) | 16:0/22:4 and 18:0/20:4 and 18:1/20:3 |
| 887.44 (38:3) | 18:0/20:3 and 18:1/20:2 |
| 909.58 (40:6) | ------- |
| 911.25 (40:5) | 18:0/22:5 and 18:1/22:4 and 20:1/20:4 and 20:2/20:3 |

**Table S9.** Major PE molecular species from MSCs cultured in ImmuneSafe® with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M-H]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 700.33 (34:2) | |
| 722.24 (36:5) | O-16:0/20:5 and O-16:1/20:4 |
| 742.33 (36:2) | 16:0/20:2 and 16:1/20:1 and 18:0/18:2 and 18:1/18:1 |
| 744.33 (36:1) | 16:0/20:1 and 18:0/18:1 |
| 746.42 (36:0) | 18:0/18:0 |
| 748.33 (38:6) | O-16:0/22:6 and O-16:1/22:5 and O-18:1/20:5 and O-18:1/20:4 |
| 750.42 (38:5) | O-16:0/22:5 and O-16:1/22:4 and O-18:0/20:5 and O-18:1/20:4 and O-18:2/20:3 |
| 764.50 (38:5) | 16:0/22:5 and 16:1/22:4 and 18:0/22:5 and 18:1/20:4 and 18:2/20:3 |
| 766.25 (38:4) | 16:0/22:4 and 16:1/22:3 and 18:0/20:4 and 18:1/20:3 |
| 768.42 (38:3) | ------- |
| 772.33 (38:1) | 18:0/20:1 |
| 774.25 (38:0) | 18:0/20:0 |
| 774.25 (40:7) | O-18:1/22:6 and O-18:2/22:5 |
| 776.50 (40:6) | O-18:0/22:6 and O-18:1/22:5 and O-18:2/22:4 |
| 790.33 (40:6) | 18:0/22:6 and 18:1/22:5 and 18:2/22:4 and 20:1/20:5 and 20:2/20:4 and 20:3/20:3 |
| 792.35 (40:5) | 16:1/24:4 and 18:0/22:5 and 18:1/22:4 and 18:2/22:3 and 20:0/20:5 and 20:1/20:4 and 20:2/20:3 |

**Table S10.** Major PS molecular species from MSCs cultured in ImmuneSafe® with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M-H]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 760.29 (34:1) | 16:0/18:1 and 16:1/18:0 |
| 786.42 (36:2) | 18:0/18:2 and 18:1/18:1 |
| 788.25 (36:1) | 16:0/20:1 and 16:1/20:0 and 18:0/18:1 |
| 790.42 (36:0) | 16:0/20:0 and 18:0/18:0 |
| 808.25 (38:5) | ------- |
| 810.42 (38:4) | 16:0/22:4 and 18:0/20:4 and 18:1/20:3 and 18:2/20:2 |
| 812.25 (38:3) | 16:1/22:2 and 18:0/20:3 and 18:1/20:2 |
| 834.20 (40:6) | 18:0/22:6 and 18:1/22:5 and 20:1/20:5 and 20:2/20:4 and 20:3/20:3 |
| 836.32 (40:5) | 18:0/22:5 and 18:1/22:4 and 20:1/20:4 |
| 838.30 (40:4) | 18:0/22:4 and 18:4/22:0 and 18:1/22:3 and 20:0/20:4 and 20:1/20:3 |

**Table S11.** Major LPC molecular species from MSCs cultured in ImmuneSafe® with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M+$CH_3COO$]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 554.02 (16:0) | 16:0 |
| 580.42 (18:1) | 18:1 |
| 582.25 (18:0) | 18:0 |

**Table S12.** Major SM molecular species from MSCs cultured in ImmuneSafe® with no stimuli and MSCs subjected to TNF-α and IFN-γ. (C:N) – C indicates the sum of the number of carbons and N corresponds to the sum of unsaturations bonds

| [M+$CH_3COO$]- m/z (C:N) | Fatty Acid Composition |
|---|---|
| 759.27 (34:2) | Sphingosine d18:1/16:1 |
| 761.26 (34:1) | Sphingosine d18:1/16:0 |
| 763.13 (34:0) | Sphinganine d18:0/16:0 |
| 791.08 (36:0) | Sphinganine d18:0/18:0 |
| 817.67 (38:1) | Sphingosine d18:1/20:0 |
| 819.19 (38:0) | Sphinganine d18:0/20:0 |
| 843.33 (40:2) | Sphingosine d18:1/22:1 |
| 845.38 (40:1) | Sphingosine d18:1/22:0 |
| 871.24 (42:2) | Sphingosine d18:1/24:1 |
| 873.34 (42:1) | Sphinganine d18:1/24:0 |