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PhD Thesis

Effect of a good-manufacturing-practice xeno-free medium on mesenchymal stromal cell properties: Multipotentiality, Immunomodulation and Recruitment.

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I’m a great believer in luck.

The harder I work, the more luck I have.

- Coleman Cox -

Acknowledgments

Finally, I am here: Writing the final part of my thesis. It has been a long period, during while I've not only learnt about science, but also about life. It wasn't easy; actually, it was quite a hard time. I've changed my whole life for this project.

Thank you to my supervisors at INEB, Prof Mario Barbosa and Catarina Almeida, for their valuable guidance. Special thanks to Catarina, who, even without sharing lab, was always behind the computer willing to answer my emails. Thank you for all the reviews, support and help. I would also like to thank my supervisor at Grifols, José María Díez, who not only helped me in everything he could in the lab, but also taught me about life in a company and how to build a team. Thank you to Rodrigo Gajardo, Susana Santos, Joao Cortez and Ana Paula Filipe. I would like to thank my colleagues at INEB and, finally, Grifols' team, who made my days happier, sharing beautiful moments in and outside the lab.

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Lo más difícil de esta tesis ha sido estar a 1000 km de ti.

Abstract (ENG)

Human mesenchymal stromal cells (hMSC) are widely tested in clinical trials due to their properties- including their multipotency, tissue-repair involvement, and immunomodulation-, easy obtention and culture. hMSC have shown to be effective to treat myocardial infarction, diabetes, graft versus host disease, osteoarthritis, multiple sclerosis, systemic lupus, Parkinson's disease, brain injury, rheumatoid arthritis, Chron's disease, and others. However, the use of animal components (such as foetal bovine serum, FBS) for their expansion is still a matter of concern that needs to be solved.

In this thesis a human plasma derivative (Supplement for Cell Culture, SCC, Grifols) has been used as a substitute for FBS. SCC is an industrial-grade xeno-free product made following good-manufacturing-practices (GMP) rules. The objective of this thesis was to assess the suitability of SCC to culture and expand hMSC.

After expansion of bone marrow (BM)- , adipose tissue (AT)- and umbilical cord (UC)-derived hMSC on SCC based medium, defining hMSC characteristics were studied. hMSC presented a normal phenotype, as studied by immunostaining, and the ability of hMSC to differentiate into adipocytes, osteoblasts and chondrocytes was maintained. Moreover, the genetic stability of the cultures was checked by classical karyotyping and no chromosomal abnormalities were found.

The immunomodulatory properties of BM-hMSC were studied upon co-culturing hMSC with Mixed Leukocyte Reactions or mitogen stimulated or resting leukocytes. The results obtained showed that BM-hMSC immunomodulation was maintained, acting as immunosuppressor agents in an inflammatory niche, and as immunostimulators in a niche without inflammatory mediators.

Xeno-free expanded BM-hMSC were successfully recruited by macrophages, as seen in transwell assays. Impairment in motility was observed but the addition of FGF successfully increased BM-hMSC motility, as measured with time lapse assays. Flow cytometry, immunostaining and protein inhibition assays were used to study the pathways involved in cell motility. It was found that FGF increased αVβ3 expression, changed cell morphology, which became more similar to FBS-expanded cells, and increased BM-hMSC proliferation rate.

In conclusion, the medium based on a human plasma derivative (SCC) is a good xeno-free alternative to expand hMSC for human cell therapies.

Resumo (PORT)

As células estromais mesenquimatosas humanas (hMSC) estão a ser exploradas em ensaios clínicos devido a propriedades tais como a sua capacidade multipotente, o seu papel em reparação de tecidos e a sua capacidade imunomoduladora, para além de serem de fácil obtenção e cultura. As hMSC são eficientes no tratamento do enfarte do miocárdio, diabetes, doença do enxerto contra hospedeiro, osteoartrite, esclerose múltipla, lupus, doença de Parkinson, danos cerebrais, artrite reumatóide, doença de Crohn, entre outras patologias. No entanto, a utilização de componentes animais para a sua expansão (tais como o soro fetal bovino, FBS) é ainda um motivo de preocupação que necessita de solução.

Nesta tese foi utilizado um produto derivado de plasma humano (*Supplement for Cell Culture,* SCC, Grifols) como substituto para o FBS. O SCC é um produto industrial isento de componentes animais (xeno-free) fabricado segundo boas práticas de fabricação (GMP). O objetivo principal desta tese foi avaliar a adequação do SCC à cultura e expansão das hMSC.

Começou-se por estudar as características de hMSC obtidas a partir de medula óssea (BM), tecido adiposo (AT) e do cordão umbilical (UC) após a sua expansão em meio com SCC. Estas hMSC apresentaram um fenótipo normal, conforme analisado por imunocitoquímica, e a sua capacidade de diferenciação em adipócitos, osteoblastos e condrócitos foi mantida. Além disso, a estabilidade genética das culturas foi testada por cariotipagem clássica e não foram encontradas anomalias cromossómicas.

As propriedades imunomoduladoras das BM-hMSC foram analisadas após co-cultura das BM-hMSC com Reações entre Leucócitos (mixed leukocyte reactions) ou com leucócitos estimulados por um agente mitogénio ou ainda em repouso. Os resultados obtidos mostraram que as BM-hMSC mantêm a sua capacidade de imunomodulação, atuando como agentes imunossupressores

quando em nichos inflamatórios, ou como imunoestimuladoras em nichos sem mediadores inflamatórios.

As BM-hMSC expandidas em meio sem componentes animais continuaram a ser recrutadas por macrófagos, de acordo com ensaios feitos com transwells. Foi observada uma deficiência na motilidade das células, mas a adição de FGF levou a um aumento dessa motilidade, tal como quantificado com ensaios de microscopia.

As vias se sinalização envolvidas na motilidade destas células foram analisadas por citometria de fluxo, imunocitoquímica e ensaios com inibidores de proteínas de sinalização. Verificou-se que a adição de FGF levou a um aumentou na expressão de αVβ3, alterações na morfologia celular, que se tornou mais semelhante às células expandidas por FBS, e um aumento da taxa de proliferação de BM-hMSC.

Em conclusão, o meio que contém o derivado de plasma humano SCC é uma boa alternativa para expandir hMSC para terapias celulares em humanos.

Resumen (ESP)

Actualmente se están llevando a cabo numerosos estudios con células madre mesenquimales humanas (hMSC). El interés en estas células es debido a sus propiedades – incluyendo su multipotencialidad, su papel en la reparación de tejidos y sus capacidades inmunomoduladoras-, y adicionalmente por su fácil obtención y cultivo. Diferentes estudios han demostrado que las hMSC son efectivas para tratar infartos de miocardio, diabetes, enfermedad de injerto contra huésped, osteoartritis, esclerosis múltiple, lupus sistémico, párkinson, daño cerebral, artritis reumatoide, enfermedad de Crohn, etc. Sin embargo, el uso de componentes derivados de animales para su expansión (tales como el suero fetal bovino, FBS) sigue siendo un problema a resolver.

En la presente tesis se ha usado un derivado de plasma humano (Suplemento de Cultivo Celular, SCC, Grifols) para sustituir el FBS. El SCC es un producto *xeno-free* (sin componentes animales no humanos) producido a gran escala siguiendo la normativa de buenas prácticas de manufacturación. El objetivo principal de esta tesis es estudiar si el SCC puede usarse para cultivar y expandir hMSC.

hMSC derivadas de médula ósea (BM-hMSC), tejido adiposo (AT-hMSC) y cordón umbilical (UC-hMSC) fueron expandidas en medio suplementado con SCC. Posteriormente se estudiaron las principales características que definen las hMSC. Las hMSC presentaron un fenotipo normal, el cual fue estudiado mediante inmunotinción. Además, las células se diferenciaron correctamente en adipocitos, osteoblastos y condrocitos. Mediante cariotipado clásico se estudió la estabilidad genética de los cultivos, y no se encontró ninguna aberración cromosómica.

Las propiedades inmunomoduladoras de las BM-hMSC fueron estudiadas co-cultivando BM-hMSC con un “Cultivo mixto de leucocitos” (MLR) o leucocitos estimulados o no con mitógenos. Los resultados obtenidos mostraron que las propiedades inmunomoduladoras de las BM-hMSC se mantenían después de

cultivarlas en medio suplementado con SCC. Las BM-hMSC actuaron como inmunosupresoras en ambientes inflamatorios, y como inmunoestimuladoras en ambientes sin agentes inflamatorios.

Las propiedades quimiotácticas de las BM-hMSC fueron estudiadas mediante el denominado "ensayo de *transwell"*. Las BM-hMSC expandidas en medio *xeno-free* fueron correctamente atraídas por macrófagos. Sin embargo, la movilidad de las BM-hMSC se vio afectada. La suplementación del medio *xeno-free* con FGF aumentó la movilidad celular, tal y como se vio en ensayos de *time lapse*.

Para estudiar las vías relacionadas con la movilidad de las BM-hMSC se usó citometría de flujo, inmunotinción y ensayos de inhibición de proteínas. FGF aumentó la expresión de αVβ3, cambió la morfología celular, siendo más similar a la observada en células expandidas en medio suplementado con FBS, y aumentó la proliferación de las BM-hMSC.

En conclusión, el medio basado en un derivado de plasma humano (SCC) es una buena opción *xeno-free* para expandir hMSC para su uso en terapia celular humana.

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List of abbreviations

Ang-1: Angiopoietin-1.

AT: Adipose tissue.

BM: Bone marrow.

CCLX: Chemokine ligand.

CD: Cluster of differentiation.

CHO: Chinese hamster ovarian cells.

CXCR: Chemokine receptor.

DAPI: 4’,6-diamidino-2-phenylindole.

DC: Dendritic cell.

DMEM: Dulbecco's Modified Eagle's Medium.

DMSO: Dimethyl sulfoxide.

EGF: Epidermal growth factor.

ERK: Extracellular signal–regulated kinases

ESC: Embryonic stem cell.

FasL: Fas ligand.

FBS: Fetal bovine serum.

FDA: Food and drug administration.

FGF: Fibroblast growth factor.

Flt-1: Vascular endothelial growth factor receptor 1.

GM-CSF: Granulocyte-macrophage colony-stimulating factor.

GMP: Good manufacturing practices.

GvHd: Graft-versus-host-disease.

HGF: Hepatocyte growth factor.

HLA-X: Human leukocyte antigen.

hMSC: Human mesenchymal stromal cells.

HSC: Hematopoietic stem cell

HSCT: Hematopoietic stem cell transplant

ICAM-1: Inter-cellular adhesion molecule-1.

IDO: Indoleamine 2,3-dioxygenase.

IFN: Interferon.

IGF-1: Insulin growth factor-1.

IL: Interleukin.

iNOS: Inducible nitric oxidase synthase .

iPSC: Induced pluripotent stem cell.

ISCT: International Society of Cellular Therapy.

ISE: Insulin, Selenium, Ethanolamine

KGF: Keratinocyte growth factor.

LIF: Leukaemia inhibitory factor.

LPS: Lipopolysaccharides.

MAPK: Mitogen-Activated Protein Kinases.

M-CSF: Macrophage colony-stimulating factor.

MFI: Mean fluorescent intensity.

MHC: Major histocompatibility complex.

MIF: Macrophage migration inhibitory factor.

MLR: Mixed Leukocyte Reaction.

MMP: Matrix metalloproteinase

MSC: Mesenchymal stromal cells.

NK: Natural killer cell.

NO: Nitric oxide

PAMP: Pathogen-associated molecular pattern.

PBMC: Peripheral blood mononuclear cells.

PBS: Phosphate buffer solution.

PDGF: Platelet-derived growth factor.

PD-L1: Programmed death-ligand 1.

PDT: Population doubling time.

PFA: Paraformaldehyde.

PGE2: Prostaglandin-E2.

PHA: Phytohemagglutinin (mitogen).

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase.

PL: Platelet lysate.

PRC: Proliferative Responder Cells.

R: Responder cells/PBMCs

RT: Room temperature.

S: Stimulator cells/PBMCs

SC: Stem Cell.

SCC: Supplement for Cell Culture [product].

SCF: Stem cell factor.

SDF-1: Stromal cell-derived factor.

TGF: Transforming growth factor.

TLR: Toll-like receptor.

TNF-α: Tumor necrosis factor alpha.

UC: Umbilical cord.

VCAM-1: Vascular cell adhesion molecule-1.

VEGF: Vascular endothelial growth factor.

XF: Xeno-free.

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Objectives and structure of the thesis

This project started with the desire of bringing academia and industry closer. In this project participated different organisms from Portugal and Spain. I have worked at INEB (Porto) and Grifols (Spain), having the opportunity to work both, in an academic and industrial environment.

The main objective of this project was to study the effect that a human plasma derivative used as supplement for cell culture (SCC) has on human mesenchymal stem/stromal cells (hMSC) properties, with the final goal of developing a xeno-free medium for the safe expansion of these cells. hMSC immunomodulatory and chemotactic properties were our main focus.

The following questions were addressed during the thesis project:

1. Can hMSC be expanded in a SCC based xeno-free medium?
    a. Can hMSC from different origins and sources be expanded in SCC containing medium?
    b. Are hMSC characteristics maintained?
        i. Do they adhere to plastic?
        ii. Is hMSC phenotype maintained?
        iii. Are hMSC able to differentiate into adipocytes, osteoblasts and chondrocytes?
    c. Which is the proliferation rate?
    d. Are hMSC genetically stable?
2. Do xeno-free expanded hMSC maintain their immunomodulatory properties?
    a. Can hMSC suppress a Mixed Leukocyte Reaction?

b. Can hMSC inhibit proliferation of mitogen stimulated lymphocytes?

c. What happens when hMSC are co-cultured with non-activated lymphocytes? Do they act as immunostimulators?

3. Do xeno-free expanded hMSC maintain their chemotactic properties?

    a. Are hMSC attracted by macrophages?

    b. Is hMSC motility impaired?

        i. What can be done to improve motility?

        ii. Which pathways are affected?

This thesis is divided in five chapters. In Chapter 1 it is given a broad introduction of MSC: their characteristics, functions, interesting properties for clinical applications, and how are they expanded in the laboratory. Chapter 2 is an adaptation of the studies performed at Grifols and published at Stem Cell Research & Therapy journal. In this chapter defining hMSC characteristics were studied after hMSC expansion in xeno-free medium, in order to determine if cells maintained their hMSC phenotype and genetic stability. In Chapter 3, the immunomodulatory and chemotactic properties of xeno-free-expanded hMSC were tested. This chapter is an adaptation of the article published at Stem Cells International journal, which contains part of the work performed at INEB. In Chapter 4, the effect of different supplements on the motility of xeno-free expanded hMSC is studied. This chapter is an adaptation of an article submitted to Tissue Engineering. Finally, in Chapter 5 it is given a broad discussion of the results obtained, and a final conclusion is made.

Chapter 1. State of the art

1. MSC definition and properties

1.1. MSC Definition

Mesenchymal stem/stromal cells (MSCs) are a promising tool in regenerative medicine and as a treatment for immune-mediated diseases, with immunoregulatory and paracrine properties [Ucceli et al, 2008]. MSCs are a subset of non-hematopoietic adult multipotent cells originated from the mesoderm that can be isolated from almost all tissues and expanded *in vitro* (Figure 1)[Singer et al, 2011]. MSCs are located close to blood vessels and its physiological functions are still under study. Bone marrow (BM) MSC are the precursors of some non-haematopoietic components of the BM, such as osteoblasts, adipocytes and fibroblastic reticular cells, thus, MSC are related to the maintenance of cellular homeostasis and tissue repair [Reviewed in Nombela Arrieta et al, 2012]. These cells have a fibroblast shape and have been defined by the International Society of Cellular Therapy (ISCT) as cells [Horwitz et al, 2005]:

(1) Able to adhere to plastic;

(2) Expressing the immunophenotypic markers CD73, CD90 and CD105;

(3) Not expressing the hematopoietic markers CD14 or CD11b, CD19 or CD79α, CD34 and CD45, neither class-II major histocompatibility complex (MHC HLA II) molecules;

(4) Able to differentiate into adipocytes, osteoblasts and chondrocytes *in vitro*.

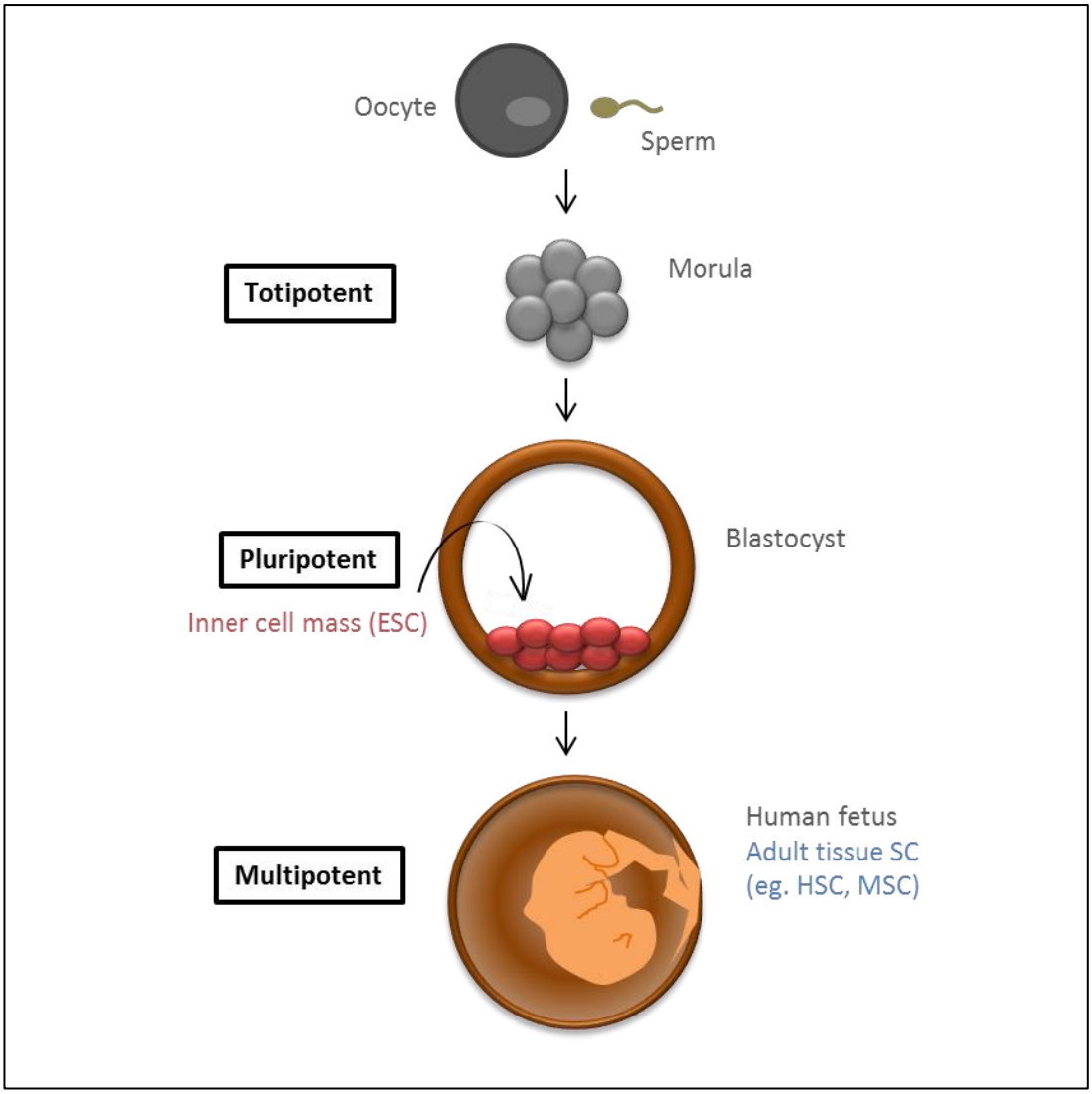

Figure 1. Schematic illustration of the potency of the different stem cells. The zygote is defined as a **totipotent** cell, as it can turn into all the mature cell types of the body, as well as embryonic components required for development, such as the placenta. A **pluripotent** cell has the potential to originate all the mature cell types of an organism, but not a new organism, to differentiate in the 3 germ layers (endoderm, mesoderm and ectoderm). These cells can be obtained from the blastocyst (embryonic stem cell (ESC). A **multipotent** cell only has the potential to originate cells of the tissue/organs where they are nested. Progenitor cells are multipotent cells that can be found in many adult tissues (adipose tissue, bone marrow, etc.). Haematopoietic stem cells (HSC) or Mesenchymal stem/stromal cells (MSC) are multipotent Singec et al, 2007 [Adaptation from Avasthi et al, 2008].

Other markers used are: CD13, CD44, CD54, CD63, CD166, which must be positive, and CD31 and CD133 which must be negative [Dominici et al, 2006]. It has been proposed that some MSC can also differentiate into multiple cell types of mesodermal origin as well as transdifferentiate in cells of non-mesodermal origin, such as neural cells, endothelial cells, cardiomyocytes and hepatocytes (Figure 2) [Arthur et al, 2008; Kaseem et al, 2006; Oswald et al, 2004; Makino et al, 1999; Snykers et al, 2009].

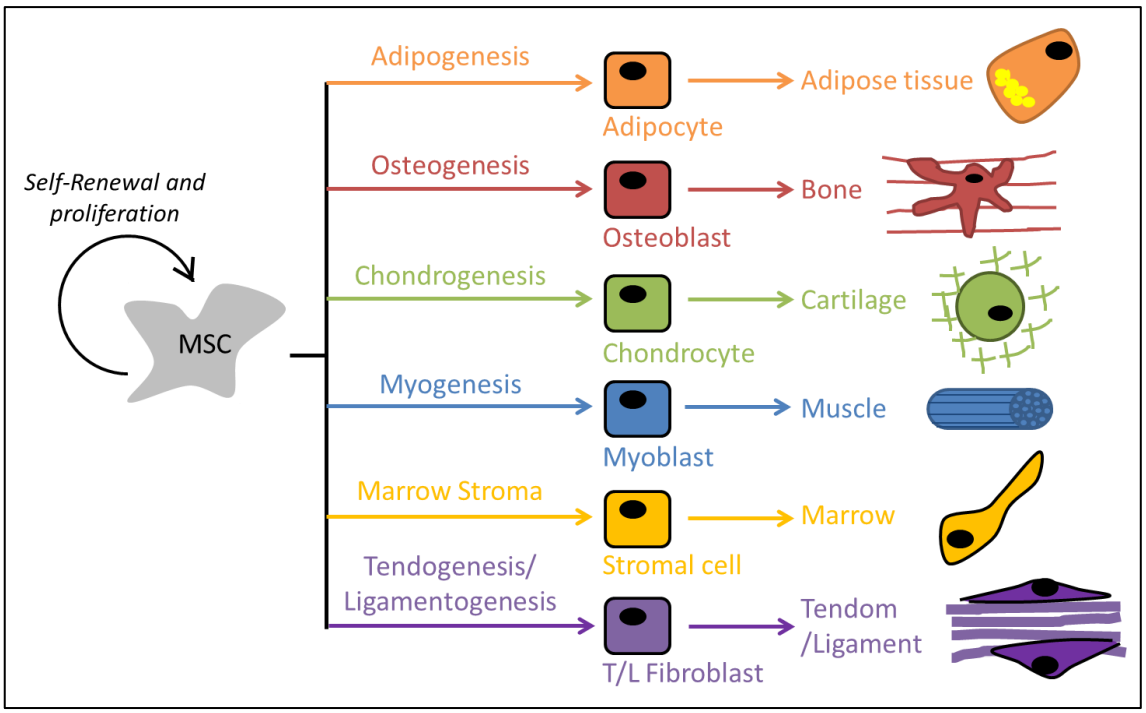

Figure 2. Hypothesis of the mesengenic process that hMSCs undergo *in vitro* and *in vivo*. Human MSC (hMSCs) can develop into bone, cartilage, muscle, marrow, tendon, adipose tissue or other connective tissue, depending on which stimuli they receive in vitro [Adapted from Caplan 2009].

However, these properties do not define the real *in vivo* stemness of the cells, which is why some scientists prefer to call MSCs as 'multipotent stromal cells' [Dominici et al, 2006], 'mesenchymal stromal cells' [Horwitz et al, 2005], or 'medicinal signalling cells' [Caplan & Correa, 2011]. These authors use the acronym 'MSC' to describe a type of cell in culture, defined by *in vitro* stablished characteristics, not specific of stem cells [Dominici et al, 2006]. Moreover, as MSCs need to be reprogrammed to become

pluripotent and stem cells do not, the term 'stem' seems not the most appropriate [Bianco et al, 2013, Bianco 2014]. From here on the acronym 'MSC' will be used to mean 'Mesenchymal stromal cells'.

It is also important to consider that MSC represent a mixture of diverse cell types, with different phenotypes and functions. For example, different studies have shown that MSC colonies derived from a single cell contain, at least, three morphologically different cell types: small MSC, which are rapidly self-renewing cells, fibroblast-like MSCs, and the large, cuboidal and slowly replicating cells [Colter et al, 2001; Prockop et al, 2001]. Differences in protein expression have been also shown (e.g. C-X-C chemokine receptors, platelet-derived growth factor receptor (PDGFR), among others) [Iinuma et al, 2015; Seeger et al, 2009].

1.2. MSC reparative functions

Endogenous MSCs are important in repairing damaged tissues (Figure 3). Upon tissue injury, apoptotic and necrotic cells, as well as damaged microvasculature, lead to recruitment and activation of cells of the immune system (macrophages, neutrophils, CD4+ T cells, CD8+ T cells and B cells)[Eming et al, 2007; Luster et al, 2005]. Moreover, damaged cells and spilled contents promote activation of phagocytes, which start secreting inflammatory mediators (tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), chemokines, free radicals, leukotrienes). These inflammatory molecules and immune cells, together with fibroblasts and endothelial cells, produce changes in the microenvironment, which recruit MSCs [Ma et al, 2014].

The cytokines present in the site of injury (TNF-α, IL-1, Interferon gamma (IFNγ)), together with hypoxia and/or toxins of infectious agents, activate MSC secretion of different growth factors (Table 1), which promote activation of tissue progenitor cells, fibroblasts and endothelial cells, responsible for tissue

regeneration and repair [Shi et al, 2012; Ma et al, 2014; Aguilar et al, 2009; Hung et al, 2013]. More specifically, MSCs promote angiogenesis by regulating proliferation and permeability of endothelial cells, avoiding their interaction with leukocytes and increasing extracellular matrix production [Giacca et al, 2012; Lee et al, 2011]. Moreover, wound healing is promoted by fibroblasts, which secrete extracellular matrix and matrix metalloproteinases [Chen et al, 2008; Timmers et al, 2011; Gnecchi et al, 2005].

MSCs are also involved in the long-term functional recovery of an injured tissue. Engrafted stem cells can differentiate into tissue cells or produce growth factors (stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), stromal cell-derived factor (SDF-1), Leukaemia inhibitory factor (LIF), Angiopoietin-1 (Ang-1)) and chemokines that promote tissue repair [Shi et al, 2012].

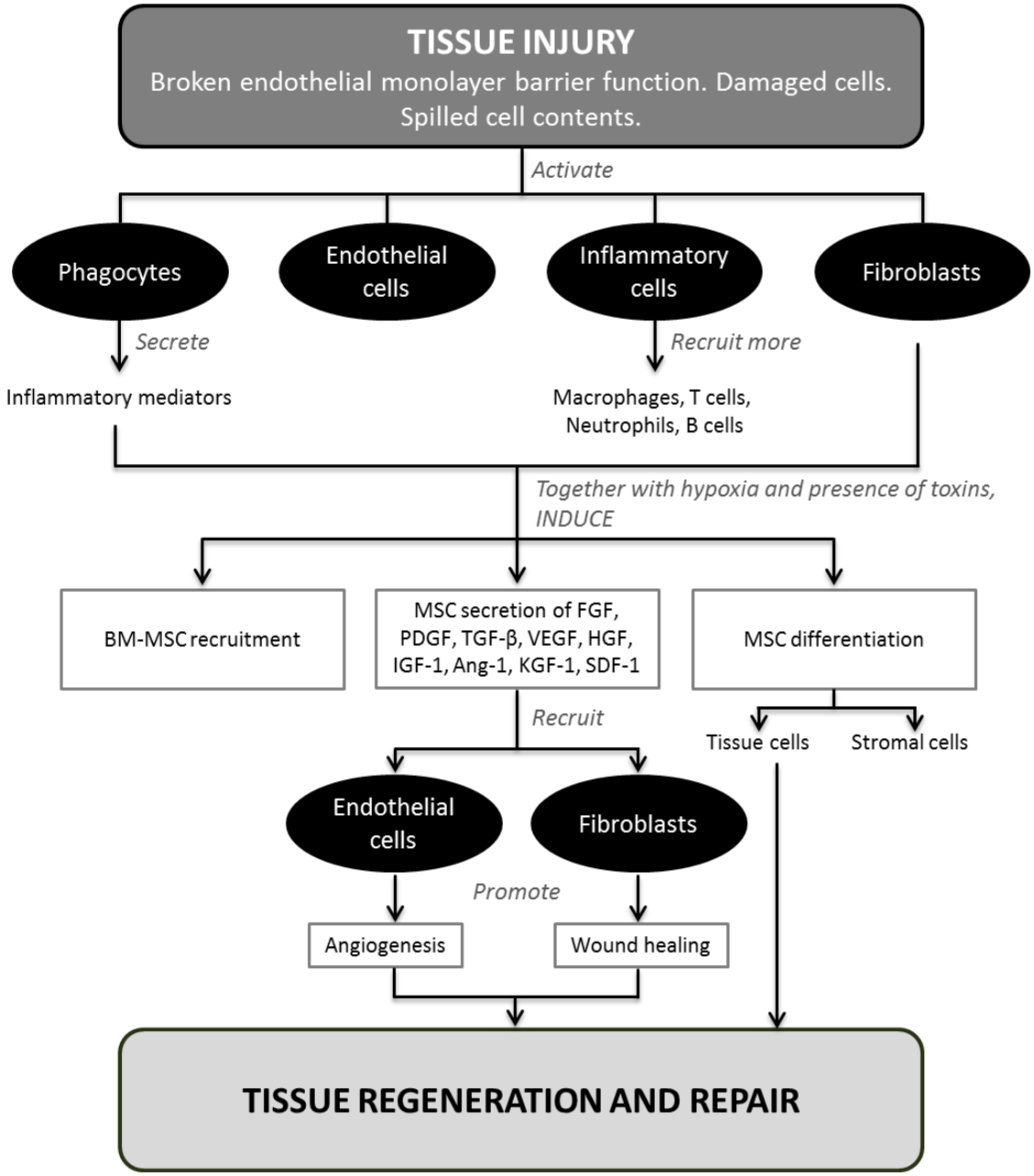

Figure 3. Reparative functions of MSCs, communication between MSCs and damaged tissues.

Table 1. Growth factors secreted by MSCs. Adaptation from Ma et al, 2014

GROWTH FACTOR	ABBREVIATION	FUNCTION
Epidermal growth factor	EGF	Wound healing Kim et al, 2012 Tissue regeneration Khalili et al, 2012; Yang et al, 2011 Neurogenesis Park et al, 2012
Platelet-derived growth factor	PDGF	Tissue repair Chung et al, 2009
Fibroblast growth factor	FGF	Tissue repair Kim et al, 2012 Intrinsic stem cell survival Houchen et al, 1999 Regeneration Houchen et al, 1999
Transforming growth factor beta	TGF-β	Wound healing Yoon et al, 2012; Hayashi et al, 2008
Vascular endothelial growth factor	VEGF	Angiogenesis Hayashi et al, 2008; Beckermann et al, 2008; Wu et al, 2007 Wound healing Hayashi et al, 2008; Beckermann et al, 2008; Wu et al, 2007
Hepatocyte growth factor	HGF	Vasculogenesis Kilroy et al, 2007 Intrinsic neural cell regeneration Bai et al, 2012
Insulin growth factor-1	IGF-1	Wound healing Kim et al, 2012 Neurogenesis Wakabayashi et al, 2010
Keratinocyte growth factor	KGF	Wound healing Kwon et al, 2008
Angiopoietin-1	Ang-1	Angiogenesis Wu et al, 2007 Tissue repair Wu et al, 2007
Stem cell-derived factor-1	SDF-1	Neuroprotective effect Wang F et al, 2010 Wound healing Landry et al, 2010; Li et al, 2009

1.3. MSC immunomodulatory properties

In addition to reparative functions, MSCs exert immunomodulatory effects on cells of the adaptive and innate immunity, such as NK cells, dendritic cells, T cells, B cells, macrophages and neutrophils. This property depends on the source of MSCs, the specific niche where MSCs are, and, if cells are expanded *in vitro*, the number of passages in culture Ma et al, 2014. The mechanism of action is

not clear yet, but they are likely mediated by soluble factors and cell-cell contact with immune cells [Gao et al, 2016]. It has been observed that the balance of the stimuli received by the MSCs determines the acquisition of an immunosuppressive or immunostimulatory behaviour. In the early phase of inflammation, the pro-inflammatory properties of MSC may be beneficial to induce a proper immune response while in the later phases it might be more beneficial to dampen the immune response, avoiding chronic inflammation and subsequent fibrosis [Bernardo et al, 2013].

MSC phenotypes – Toll-like receptors

Toll-like receptors (TLRs) link the innate and the adaptive immune systems. TLRs recognize pathogen-associated molecular patterns (PAMPs) and molecules derived from cell death. They are located on the cell surface or in intracellular membranes of innate effector cells, such as macrophages and neutrophils, and also of MSCs [Gordon, 2011; Waterman, 2010]. Their expression can be regulated by different factors, affecting MSCs properties [Kanzler et al, 2007]. TLR expression and function varies depending on the MSC source and species [Raicevic et al, 2011; DeLaRosa et al, 2012]. The main TLRs expressed by MSCs, as well as some of their functions, are shown in Table 2.

Table 2. Main TLRs expressed by hMSCs and their functions on hMSCs.

TOLL-LIKE RECEPTOR	FUNCTION ON hMSC
TLR 2	Induce IL-6 production [Pevsner-Fisher et al 2007] Induce MSC proliferation [Pevsner-Fisher et al 2007] Enhance hMSC osteogenic differentiation [DeLaRosa et al 2012] Enhance UC-hMSC chondrogenic differentiation [Kim et al, 2010]
TLR 3	Induce BM-hMSC migration [Tomchuck et al, 2008] Induce the production of mostly anti-inflammatory molecules (IL-4, IDO, PGE2) [Waterman et al, 2010] Enhance hMSC osteogenic differentiation [DeLaRosa et al 2012]
TLR 4	Induce the production of mostly pro-inflammatory molecules (IL-6, IL-8) [Waterman et al, 2010] Enhance hMSC osteogenic differentiation [DeLaRosa et al 2012]
TLR9	Inhibit hMSC osteogenic differentiation [DeLaRosa et al 2012] Inhibit AT-hMSC proliferation [Cho et al, 2006]

TLR3 and TLR4 are important for the immunological properties of human MSCs. The binding of poly(I:C) from viral dsRNA to TLR3 and lipopolysaccharide (LPS) from Gram-negative bacteria to TLR4 lead to down-regulation of Jagged1 expression on the surface of MSCs, decreasing the interaction between MSCs and T cells (Jagged1 binds Notch1 on T cell surface), and thus reducing the immunosuppressive activity of MSC [Liotta et al, 2008]. Additionally, activation of TLR3 and TLR4 on MSC induces secretion of pro-inflammatory molecules (IL-1, IL-6, IL-8, TNF- related apoptosis –inducing ligand and CCL5) and inducible nitric oxidase synthase (iNOS) activity, enhancing the inflammatory response against pathogens [Romieu-Mourez et al, 2009]. But on the other hand, *in vitro* pre-treatment of MSC with LPS or poly (I:C) before an immunosuppressive assay enhances the immunosuppressive effects of MSC [Opitz et al, 2009]. Waterman and colleagues (2010) proposed a model based on the macrophage M1/M2 phenotypes. The model states that MSC can present two phenotypes: MSC1, which mostly produce pro-inflammatory mediators, or MSC2, which express mostly immunosuppressive ones. Chemokines secreted by MSC1 recruit T cells. On the other hand, in an inflammatory niche, or upon binding of dsRNA from viruses to TLR3, the presence of high levels of IFN-γ and TNF-α prime MSC, adopting an immunosuppressive phenotype (MSC2). MSC2 secrete high levels of soluble factors that suppress T cell proliferation. Moreover, MSC secretion of TGF-β (constitutively expressed) favours the presence of regulatory T cells (Tregs). (Figure 4) [Waterman et al, 2010].

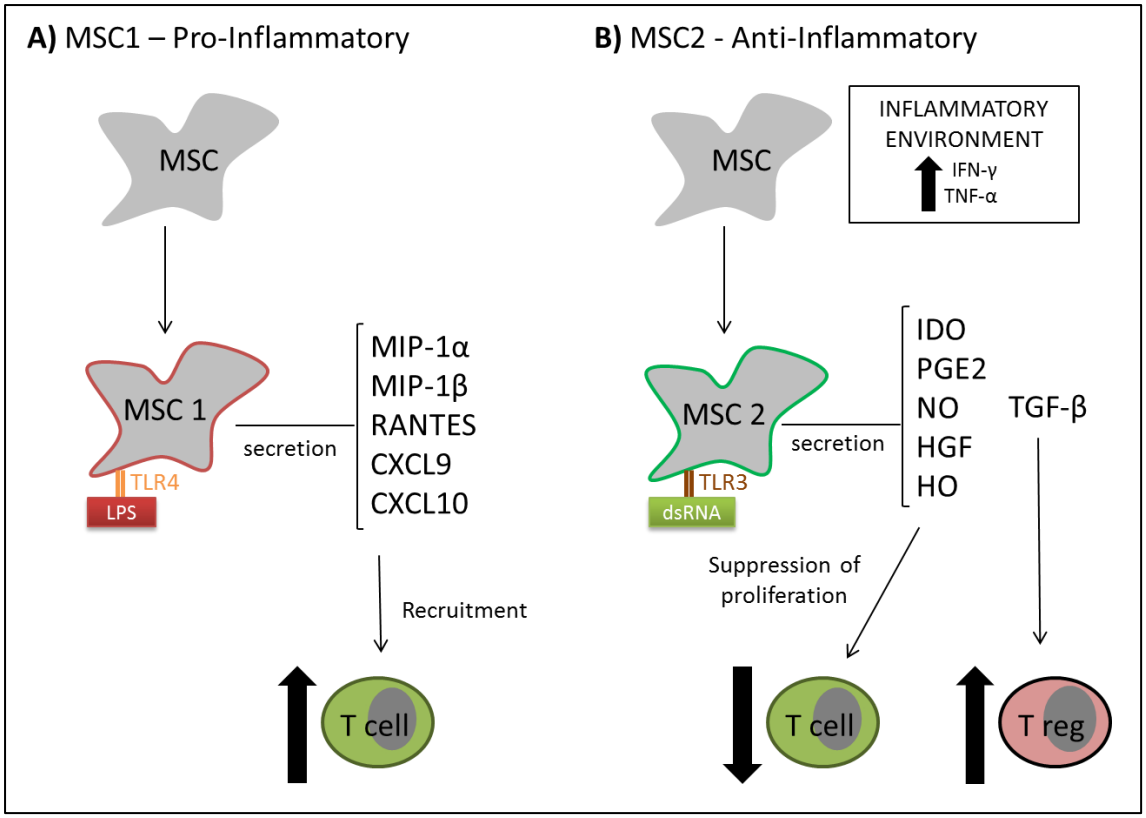

Figure 4. Hypothesis of MSC phenotypes proposed by Waterman and colleagues (2010). MSC can present two phenotypes: **(A)** MSC1 or pro-inflammatory phenotype: In a niche without the presence of inflammatory cytokines, or the presence of LPS from bacteria, MSC secrete different chemokines, which recruit T cells. **(B)** MSC2 or anti-inflammatory phenotype: in the presence of inflammatory mediators or dsRNA from virus, MSC secrete different factors to suppress T cell proliferation and increase the presence of regulatory T cells **[**Adapted from Bernardo et al, 2013].

hMSC have different effects on cells of the immune system, which depend on the microenvironment and the inflammatory molecules that activate hMSC (Table 3).

Neutrophils

Neutrophils are recruited to sites of injury within minutes and are important in acute inflammation [Kolaczkowska et al 2013]. Neutrophils eliminate pathogens through phagocytosis, extracellular traps and secretion of bactericidal molecules, among others [Le Blanc et al 2012; Kolaczkowska et al 2013; Brinkmann et al 2004]. MSC suppress neutrophil

apoptosis through IL-6, IFN-β and GM-CSF secretion [Raffaghello et al 2008; Cassatella et al 2011]. Moreover, MSC secret IL-8 and MIF to recruit neutrophils [Brandau et al 2010; Romieu-Mourez et al 2009; Yu et al 2016; Isakova et al 2016]. It is speculated that MSC may play an important role on the mantainance of the storage pool of neutrophils in the bone marrow, and the neutrophil migration to injured sites [Brandau et al 2014]. Nevertheless, opposite results have also been found. Some studies reported that MSC inhibited neutrophil activation and recruitment, and prevented neutrophil extracellular trap formation [Jiang et al 2016; Munir et al 2016].

Macrophages

Macrophages play a key role in the innate immune system [Wynn et al 2016]. Its main function is to destroy cellular debris, foreign bodies, infected cells and others. Macrophages can be polarized into a pro-inflammatory phenotype (called M1) or an immunomodulatory phenotype (M2) depending on the specific micro-environment. M1 macrophages secrete inflammatory cytokines and chemokines, whereas M2 macrophages release IL-10 and other factors to enhance tissue repair and resolve inflammation [Glass et al 2016].

The *in vitro* co-culture of macrophages and MSC facilitates the generation of M2 macrophages [Zhang et al 2010; Cho et al 2014; Francois et al 2012; Selleri et al 2016; Maggini et al, 2010; Németh et al, 2009; English et al, 2009; Choi et al, 2011]. This effect is produced by cell-cell contact and the release of different soluble factors, such as PGE2 or TGF-β, which act through EP2 and EP4 receptors on macrophages [Nemeth et al 2009; Song et al 2015].

Natural killer cells

Natural killer (NK) cells participate in the innate immunity inducing direct cytotoxicity of infected or carcinogenic cells or by the production of pro-inflammatory cytokines [Childs et al 2015; Fauriat et al 2010]. MSC can suppress NK proliferation, cytokine production and cytotoxicity [Aggarwal et al 2005; Spaggiari et al 2006; Sotiropoulou et al 2006; Spaggiari et al 2008; Qu et al 2015; Michelo et al 2016] through direct cell-cell contact [Michelo et

al 2016; Lu et al 2015 or through the secretion of different soluble factors, such as PGE2, IDO and HLA-5 Spaggiari et al 2008; Selmani et al 2008. Nevertheless, several studies have shown opposite effects, where MSC stimulated NK progenitor's proliferation Boissel et al 2008, or enhanced NK secretion of IFN-γ when stimulated by IL-12/IL-18 Thomas et al 2014. It has been also shown that NK cells can recruit MSC through the secretion of CCL5 and CXCL7 Almeida et al 2016, and can lyse MSC Gotherstrom et al 2011; Sotiropoulou et al 2006. The relation between MSC and NK cells depends on the stimuli received by both cells, the microenvironment and their ratios Sotiropoulou et al 2006; Li et al 2017.

Dendritic cells

Dendritic cells (DC) main function is to present antigens, and thus, to direct the responses of the adaptive immune system Le Blanc et al 2012. MSC can suppress DC by direct contact or through soluble factors Li et al 2017. MSC can impair DC migration, endocytosis, maturation and inflammatory cytokine secretion ability, such that DC become less efficient in T cell activation Zhang et al 2004; Jiang et al 2005; Consentius et al 2015; English et al 2008; Chiesa et al 2011; Li H et al 2008. The soluble factors secreted by MSC involved on DC suppression are still under study, but IL-6, M-CSF and PGE2 seem to be involved Li YP et al 2008; Nauta et al 2006; Zhang Y et al 2014; Djouead et al 2007; Deng et al 2014; Spaggiari et al 2009. Direct cell-cell contact is also important for DC suppression by MSC, which is mediated by activation of Notch signalling in DC Li YP et al 2008.

T cells

T cells play a key role in adaptive immunity, secreting granzymes, perforins and cytokines to kill infected cells (cytotoxic T cells) and cytokines that regulate the adaptive response (T helper cells) Kaech et al 2012. These cells protect the body from infections but also mediate different autoimmune diseases Dimeloe et al 2016. The interaction between MSC and T cells has been widely studied. MSC inhibit T cell proliferation by the secretion of TGF-β and HGF, which lead to arrest of T cell

proliferation in the G1 phase (by decreasing cyclin D2 and increasing $p27^{kip1}$ expression in T cells) [Di Nicola et al 2002; Glennie et al 2005]. IDO is an enzyme that depletes tryptophan and is the main known molecule involved in human MSC immunosuppressive properties [Su et al 2014; Munn et al 2012; Meisel et al 2004]. Accumulation of tryptophan metabolites (kyneurine) induces T cell apoptosis [Plumas et al 2005], which also inhibits the growth and function of other immune cells [Jarvinen et al 2008]. MSC can also affect T cell activation and differentiation of T cells, and induce the generation of Tregs [Luz-Crawford et al 2013; Selmani et al 2008].

As previously said, for MSC to be able to suppress T cells, and other cells, they need a pre-stimulation with inflammatory cytokines (IFN-γ itself or in combination with TNF-α or IL-1α or IL-1β) [Krampera et al, 2011; Ren et al 2008, 2010]. When MSC are primed, expression of iNOS and COX-2 is upregulated, which induces the production of immunosuppressive molecules, such as PGE2 [Ren et al 2008; Crop et al 2010]. Moreover, MSC start producing chemokines and adhesion molecules (CXCR3, CCR5, ICAM-1, VCAM-1) important for lymphocyte recruitment to injured tissues [Wang et al 2014; Ren et al 2008, 2010; Ma et al 2014].

Nonetheless, when the presence of inflammatory cytokines is low, MSC are not primed and do not excert immunosuppressive functions, thus, sometimes MSC are unable to suppress T cell responses, and can even promote an immune response [Li et al 2012].

B cells

B cells are also important in the adaptive immune system. After the recognition of specific antigens, B cells proliferate and differentiate into antibody-producing cells and memory cells [Pieper et al 2013; O'Connor et al 2006; De Silva et al 2015; Depoil et al 2009], which play a key role in the elimination of pathogens and provide long-term protection to the host [Hayakawa et al 1985]. When MSC are primed with sufficient inflammatory stimulus, they suppress proliferation, differentiation and

activation of B cells [Schena et al 2010; Li et al, 2017]. The co-culture of B cells with MSC results in cell cycle arrest, impaired plasma cell generation and reduced chemotactic and immune-globulin-secreting properties of B cells [Corcione et al 2006; Tabera et al 2008; Che et al 2012; Asari et al 2009]. CCL2 secreted by MSC is one of the main factors involved in these actions, increasing PAX5 expression on B cells, which suppresses immunoglobulin synthesis [Che et al 2012; Rafei et al 2008]. Cell-cell contact is also important for MSC immunosuppressive properties on B cells [Schena et al 2010]. Another effect of MSC on B cells is the induction of Bregs. Bregs secrete IL-10, an anti-inflammatory cytokine, and MSC decrease immune responses through them [Franquesa et al 2015]. This induction of Bregs by MSC is involved in the treatment of graft-versus-host disease in mouse models [Franquesa et al 2015; Peng et al 2015; Guo et al 2013; Park et al 2015]. As in T cells, if the presence of inflammatory factors is not sufficient to prime MSC, MSC suppression of B-cell proliferation and differentiation is low, or even MSC can increase the number of antibody-secreting B cells, inducing an immunostimulation [Che et al 2014; Rasmusson et al 2007, Traggiari et al 2008, Maby et al 2009].

Table 3. Effect that MSCs have on different cells of the immune system in an inflammatory niche or an early-inflammatory niche.

AFFECTED CELL	EFFECT OF hMSC			
	IMMUNOSUPPRESSIVE ROLE (inflammatory niche)	Factor secreted by MSC	IMMUNOSTIMULATORY ROLE (early-stage inflammation)	Factor secreted by MSC
Neutrophils	- Inhibition of neutrophil activation and recruitment Jiang et al 2016; Munir et al 2016		- Recruitment of neutrophils Brandau et al, 2010	IL-6, IL-8, GM-CSF, MIF
			- TLR-3 activated MSC promote in vitro survival of neutrophils Cassatella et al, 2011; Raffaghello et al, 2008	IL-6, IFB-β, GM-CSF
Monocytes Macrophages	- Polarization into an anti-inflammatory phenotype (M2) Maggini et al, 2010; Németh et al, 2009; François 2 et al, 2012; English et al, 2009; Choi et al, 2011	IDO, TSG6, PGE2 , TGF-β	- Recruitment and activation of monocytes Merino-Gonzalez et al, 2016	
	- MSC stimulated with TNFα recruit more monocytes/macrophages Ren et al, 2012		- Polarization into a pro-inflammatory phenotype (M1) Merino-Gonzalez et al, 2016	
Natural killer cells	- Impair NK cell activity Spaggiari et al, 2008, Sotiropoulou et al, 2006 - Proliferation suppression of IL-2 or IL-15 induced NK cells. Spaggiari et al, 2006 - Reduction of IFN-γ and TNF-α secretion by IL-2-activated NK cells Sotiropoulou et al, 2006	IDO, TGF-β, PGE2, HLA-5	- Protection from apoptosis Chan et al, 2006 - Stimulation of NK progenitor's proliferation Boissel et al 2008 - Promotion of degranulation and stimulation of NK IFN-γ secretion in presence of IL-12 and IL-18. Cui et al, 2016; Almeida CR et al, 2012	CCL2, MCP1
Dendritic cells	- Impaire their maturation into antigen-presenting cells (APC) Ramasamy 1 et al, 2007, Charbonnier et al, 2007, Tipnis et al, 2010, Sheng et al, 2008 - Impair DC efficacy to activate T cells (affecting DC migration, endocytosis, maturation and cytokine secretion ability) Consentius et al 2015; English et al 2008; Chiesa et al 2011; Li YP et al 2008 - DC acquire tolerogenic abilities: high secretion of IL-10 and reduced secretion of IL-12 Aggarwal et al, 2005	IL-6, M-CSF, PGE2		
T cells	- Inhibition of proliferation, cytokine secretion and cytotoxicity Di Nicola et al, 2002, Ren et al, 2009, Nasef et al, 2008, Gieseke et al, 2010, Lepelletier et al, 2010, Najar et al, 2009, Tipnis et al, 2010, Sheng et al, 2008 - Arresting activated T cells in G0/G1 by inhibiting cyclin D2 expression (reversible inhibition) Glennie et al, 2005, Di Nicola et al, 2002	IDO, TGF-β, HGF, PD-L1/2, Semaphorin -3A, B7-H4, LIF, IL-6, Galectins	- Recruitment of T cells Ren et al, 2008	CXCL-9, CXCL-10, CXCL-11
	- Induction of T cell apoptosis Plumas et al 2005	IDO, FasL		
	- Recruitment of T cells with regulatory phenotype (naive and memory T cells) Di Lanni et al, 2008		- Protection from apoptosis Mougiakakos et al, 2011	
	- Stimulation of Treg expansion Casiraghi et al, 2008; Parekkadan et al, 2011; Nemeth et al, 2010,; Tasso et al, 2012; Chabannes et al 2007; Patel et al, 2010; English et al, 2009	HO-1, TGF-β, PGE2 , HLA-G, IDO		
	- Inhibition of T-cell responses, decreasing Th17 cell differentiation Beyth et al, 2005; Rasmusson et al, 2005; Qu et al, 2012	IL-10		
B cells	- Inhibition of *in vitro* human B-cell proliferation (arrest in G0/G1 phase) Corcione et al, 2006 - Inhibition of differentiation to antibody secreting cells Corcione et al, 2006 - Induction of B regs (which secrete the ani-inflammtory cytokine IL-10) Franquesa et al 2015. - Down regulation of chemotactic receptors (CXCR4, CXCR5, CXCR7) Corcione et al, 2006	IDO, CCL2	- Protection from apoptosis Maby et al, 2009 - Promotion of proliferation and differentiation into immunoglobulin-secreting cells Traggiari et al, 2008	

Although the basics of MSC immunoregulatory functions are well defined, the lack of standard immunomodulatory assays has generated contradictory and poorly comparable results. MSC immunomodulatory properties depend on MSC species, donor, tissue origin, culture conditions, priming and cryopreservation. Moreover, the immune cells used to study MSC immunomodulation can also induce variation into the assays [Ménard et al, 2013]. The International Society for Cellular Therapy (ISCT) opened a discussion on 2013 to try to standardize the immunomodulatory studies that should be done to evaluate MSC properties. They suggested that GMP-grade MSC immune properties should be evaluated using resting and primed cells (with IFN-γ or a combination of IFN-γ and TNFα). They also suggested to use flow cytometry to evaluate different cell-surface markers, such as MHCI and MHCII, which are expressed differently on primed or resting MSCs; cytokine/chemokine receptors (CXCR3, CXCR4, CXCR5, CCR7, CD119, INF-γ receptor); adhesion molecules (CD54, CD106)… [Krampera et al, 2013].

1.4. MSC homing: engraftment to sites of injury or inflammation

As already mentioned, hMSC are attracted to inflamed tissues. The term 'homing' refers to the delivery of cells to a site of injury [Sohni et al, 2013]. This property is very interesting for hMSC therapies, and it has been shown that most MSC that are administered systemically are attracted and accumulated at sites of tissue damage and inflammation [Mahmood et al, 2003; Ortiz et al, 2003; Wu et al, 2008]. Once hMSC arrive to a damaged tissue, they exert beneficial effects, replacing the damaged cells and releasing different soluble factors to promote tissue repair (such as PDGF, TGF, EGF, FGF, VEGF, HGF, IGF, SDF-1 and Ang-1) [Crisostomo et al, 2008; Aggarwal et al, 2005].

MSCs are recruited by inflammatory cells, such as macrophages, NK cells and Dendritic Cells [Almeida et al, 2012; Silva et al, 2014]. Different chemokines, adhesion

molecules and matrix metalloproteinases are involved in MSC homing [Belema-Bedada et al, 2008]. These molecules are up-regulated by inflammatory cytokines, such as TNF-α and IL-1 [Shi et al, 2012; Ren et al, 2010]. Sialyl Lewis(x) is a molecule that promotes leukocyte migration in an inflamed tissue. MSCs that present this molecule show higher efficiency in homing to inflamed tissues [Kocher et al, 2001]. In case of injury, more MSCs are present in the circulating blood [Kocher et al, 2001; Wang et al, 2008].

Chemokine receptors (CXCR1, CXCR2, CXCR4, CCR1, CCR2), vascular endothelial growth factor receptor 1 (Flt-1), PDGFR-α and –β and their ligands (IL-8, SDF-1,MIP-1α, MCP-1, PIGF and PDGF) are important in MSC homing [Ringe et al, 2007; Sordi et al, 2005; Forte et al, 2006; Neuss et al, 2004, Ponte et al, 2007; Lee JM et al 2012]. Extensive passaging of MSCs can affect both their immunosuppressive properties and also the expression of surface receptors, affecting their chemotaxis [reviewed at Karp et al, 2009]. A study evaluating homing efficiency after MSC infusion showed that freshly isolated murine MSC had a better homing efficiency to bone marrow and spleen when compared with cultured cells. Homing efficiency was reduced to 10-65% after 24 hours in culture, and almost to 0% after 48 hours [Rombouts et al, 2003]. CXCR4 is a receptor present on MSCs surface that decreases or disappears after culture. The ligand of this receptor is SDF-1 (also called CXCL12), a molecule present in the bone marrow and ischemic tissues. However, if MSCs are cultured in hypoxic conditions or with cytokines (such as HGF, SCF, IL-3, and IL-6), CXCR4 expression is re-established [Shi M et al, 2007]. Furthermore, other chemokines and respective receptors are also involved in MSC homing, such as NAP-2 and RANTES, the former being produced by NK cells and the later by both NK cells [Almeida et al, 2016] and macrophages [Anton et al, 2012].

Integrins and matrix metalloproteinase (MMPs) are also important for MSC migration. Integrins are cell surface receptors that attach cells to the extracellular matrix. Upon ligand binding, different pathways related with cell

cycle regulation, cytoplasmic kinases activity, growth factor receptors, ion channels, and intracellular actin cytoskeleton organization are activated [Giancotti et al, 1999]. Some important ligands of integrins are vitronectin, fibronectin, collagen and laminin [Elices et al, 1991]. MMP are a family of enzymes that can degrade different extracellular matrix proteins, such as collagen or gelatins [Visse et al, 2003]. They are involved in different aspects of cell behaviour depending on the component of the extracellular matrix they degrade, thus they can be involved in cell proliferation, apoptosis, migration, differentiation and angiogenesis, among others [McCawley et al, 2001]. MMP expression is influenced by hypoxia and high confluent cultures and can be enhanced by inflammatory cytokines (such as TGF-β, IL-1β and TNF-α) [De Becker et al, 2007; Annabi B et al, 2003; Ries et al, 2007].

2. MSCs in clinics

Stem cells are a promising tool for the treatment of inflammatory and autoimmune diseases, as well as for cell therapy and regenerative medicine. Embryonic stem cells (ESC) and induced-pluripotent stem cells (iPSC) present ethical issues because of their origin. Furthermore, they have a tendency to induce allogeneic rejection and teratomas, properties that MSCs do not present. MSC present different properties interesting for therapeutic applications and can be easy isolated from almost all tissues, including adipose tissue, bone marrow, amniotic fluid, umbilical cord, placenta and menstrual blood [Ma et al, 2014].

Thanks to their multipotent capacity, MSC can be used to reconstruct damaged tissues or replace non-functional cells in some diseases, such as diabetes [Karnieli et al, 2007; Rice et al, 2008]. This property is also very interesting for promotion of bone repair or regeneration of cartilage [Veronesi et al, 2013; Vilquin et al, 2006]. The immunomodulatory properties, including the MSC capacity to produce cytokines and growth factors able to suppress immune responses, promote neovascularization and inhibit cell death, are one of the main attractiveness of MSC [Ankrum et al, 2014]. It has been shown that MSC can be used to reverse graft-versus-host-disease (GvHd) [Le Blanc et al, 2006]. Other applications on study use MSC to avoid or treat kidney transplant rejection, ischemic cardiomyopathy and progressive multiple sclerosis, among others [Portmann-Lanz et al, 2006; Casiraghi et al, 2016; Golpanian et al, 2016; Dulamea, 2015].

Many of the MSC beneficial effects on the treatment of different diseases are due to growth factors and cytokines, in a paracrine manner. Thus, MSC therapy can be done not only by the transplantation of MSC, but also through the injection of MSC conditioned medium, including exosomes and other soluble factors. MSC exosomes have similar MSC properties, such as immunomodulation and tissue damage repair. Lai and colleagues were the first

to use MSC-derived exosomes in a mouse model of myocardial ischemia/reperfusion injury in 2010 [Lai et al 2010]. MSC conditioned medium is under study to treat cardiovascular diseases, such as infarct [Timmers et al 2007]; and myocardial ischemia and reperfusion injury [Lai et al 2010; acute kidney injury [Lin et al 2005; Bi et al 2007]; and autoimmune diseases [Mokarizadeh et al 2012], GVHD [Zhang B et al 2014]. The use of MSC conditioned medium might be safer than the use of MSC, but nonetheless, as well as MSC themselves, it might suppress or promote tumour progression *in vivo* [Zhu et al 2011,2012; Lee JK et al 2013].

2.1. hMSC transplantation

It has been reported that MSCs express low levels of MHC class I, and do not express co-stimulator molecules (CD80, CD86, CD40) or MHC class II, being immune privileged and enabling allogeneic transplants [Le Blanc 1 et al, 2003; Rasmusson et al, 2006]. Almost all the *in vitro* data reported show that MSCs are immunosuppressive agents, even though, some studies pointed out that allogeneic MSCs can be immunogenic and can express MHC class I and class II after being exposed to IFN-γ [Le Blanc 1 et al, 2003]. Nevertheless, the immunogenic responses observed are not as serious as the ones observed after allogeneic fibroblasts transplantation, which provokes a rapid rejection in immunocompetent patients. The hMSCs immune rejection seems to depend on the balance between immunosuppressive and immunostimulatory agents present in the niche [Ankrum et al, 2014; Chan et al, 2006; François et al, 2009; Stagg et al, 2006]. For these reasons, Ankrum et al (2014) suggest that MSCs should be considered as immune evasive, and not immune privileged.

In almost all clinical studies already performed, the patients received allogenic hMSCs, assuming that no rejection would happen. However, some studies showed that after injection of MSCs in humans or other animals, the presence

of the injected hMSCs was limited to 48h [Toma et al, 2009; Lee et al, 2009; Kidd et al, 2009]. Although this fact can happen with allogeneic and autologous MSCs, because of the stress that cells suffer after transplantation [Muschler et al, 2004], it is probable that allogeneic MSCs suffer an immunological response by the cells of the host [Ankrum et al, 2014].

The efficacy of an MSC therapy is affected by the route and place of administration, the number of cells administered and the stage of the disease to treat [Walczak et al, 2008; Muschler et al, 2004; Chang et al, 2011; Castelo-Branco et al, 2012; Chan et al, 2007; Duijvestein et al, 2011]. It is very important to select the proper conditions to treat each specific disease. When MSCs are injected intravenously, they migrate to specific inflamed tissues [Mahmood et al, 2003; Horwitz et al, 2002], though many of the administered MSCs are trapped in the lungs [Barbash et al, 2003; Lee et al, 2009]. When possible, a local intra-arterial injection can improve accumulation of MSCs in the injured tissue.

In addition to systemic delivery, MSC can be delivered together with natural or synthetic biomaterials scaffolds. Differentiated or undifferentiated MSCs can be loaded into scaffolds before their implantation into damaged tissue sites [Ohgushi et al, 2005]. MSCs can help to regulate the inflammatory response and lead to successful implantation of biomaterials. Improvements in MSCs culture, delivery vehicles and the scaffolds used will help in the prosperous application of MSCs in clinics [Wei et al, 2013].

2.2. Risks of MSC transplantation

Safety data has been gathered from various hMSC clinical trials. Until now, only few secondary effects have been reported [Ucceli et al, 2008; Wyong et al 2011; Parekkadan et al 2010]. The Food and Drug Administration (FDA) states that MSC transplantation is safe [reviewed at Parekkadan et al 2010]. However, although it has been shown that MSC can be expanded *in vitro* up to 15 population doublings with minor spontaneous

differentiation [Pittenger et al 1999; Digirolamo et al 1999], the use of stem cells, with high proliferative potential, always presents a risk of tumour formation [Rice et al, 2008]. Thus, FDA recommends minimal manipulation of hMSC for clinical uses to reduce the risk of tumorigenesis [Bartmann et al, 2007].

MSC transplantation present the risks of maldifferentiation, increase of malignant tumour growth and immunosuppression [Parekkadan et al 2010; Wyong et al 2011]. Malignant transformation of MSC can occur during *in vitro* expansion, after genetic manipulation of MSC or upon the interaction with the tumour stroma [Kunter et al 2007; Aguilar et al 2007; Fiorina et al 2009; Wyong et al 2011]. Although the most common is that MSC suppress tumour growth [Ohlsson et al 2003; Ramasamy 2 et al 2007; Zhu et al 2009; Tian et al 2010], some studies have shown that MSC can contribute to tumour protection via tumour progression, metastasis, antiapoptotic effect or drug-resistance of cancer cells [Ramasamy 2 et al 2007; Matushansky et al 2007; Iwamoto et al 2007; Williams et al 2007; Dierks et al 2007; Ning et al 2008; Patel et al 2010; Wang XF et al 2010; Konopleva et al 2002; Karnoub et al 2007; Lin et al 2006; Kurtova et al 2009; Wei et al 2009; Kucerova et al 2010; Vianello et al 2008, 2010; Li et al 2011]. Moreover, as MSC can suppress the immune system, their use on patients with cancer can enhance tumour growth and metastasis [Klopp et al 2007; Karnoub et al 2007; Shinagawa et al 2010]. Additionally, immunosuppression via MSC enhances the risk of infections [Sundin et al 2006; Parekkadan et al 2010].

As previously explained, MSC are considered immune privileged, but, MSCs cultured in Fetal Bovine Serum (FBS) containing medium can induce immune reactions to patients. FBS based medium is the most commonly used to expand MSC and other cell types. It has been demonstrated that some bovine proteins become associated to MSCs, which will be a cause of immune rejection by patients that receive repeated administrations of MSC [Spees et al, 2004]. For this reason, and others explained in the next section, FBS must be avoided in the MSC culture for human therapies.

3. MSC expansion and culture

Normally, for a human therapy, hMSCs are obtained from an unrelated donor or from the same patient, but, the number of isolated cells (from bone marrow, 10^7-10^8 MSC are usually isolated [reviewed at Ikebe et al 2014]) is not sufficient to perform a therapy (usually 1-2 x10^6 MSC per Kg body weight are injected [Schallmoser et al 2008]). Thus, hMSC need to be expanded *in vitro* before their transplantation to the patient. The niche directly influences hMSCs properties, thus it is very important to establish the best cell culture conditions [Krampera et al, 2013]. As cryopreserved hMSCs are the most used, is it important to remark that hMSCs should not be used before 24 hours have passed upon thawing them, as they may present reduced immune-suppressive properties [François 1 et al, 2012].

As previously explained, conventional media used to expand hMSC is supplemented with FBS. FBS is used to provide the necessary growth and proliferation factors, and adhesion proteins important for cell expansion. Although commonly used, FBS presents several disadvantages: it has never been fully described, its composition varies from batch-to-batch and its availability is limited [Dimasi et al, 2011]. Also, although different safety steps are performed in the process of FBS production, there is a risk of zoonosis and prion transmission because of its animal non-human origin. Moreover, ethical concerns regarding the method of production are also into consideration [Gstraunthaler et al, 2013; Tekkatte et al, 2011; Even et al, 2006; Van der Valk et al, 2010]. In regenerative medicine, there are concerns regarding the xenogeneic immune reaction that a patient can suffer if animal proteins become adhered to the in vitro expanded human cells [Spees et al, 2004]. Thus, the use of FBS has been discouraged by regulatory authorities [European Medicines Agency, 2013].

3.1. Xeno-free alternatives for MSC culture

Different alternatives are being studied to replace FBS in human cell culture media; the most used are serum-free and chemically defined media, and human serum and platelet lysate supplemented media [Jayme et al, 2000; Jung et al, 2012]. It was found that each medium had a different impact on hMSC properties, and thus, media for hMSC expansion should be chosen depending on the characteristics needed for a specific therapy [Bobis-Wozowicz et al 2017].

Serum free medium and Chemically defined medium

As the name says, serum-free medium does not contain serum, but it may contain proteins derived from animals (hormones, attachment factors, albuminin, etc). Serum free medium is an interesting option to overcome the FBS problems already mentioned, but it may not be xeno-free, thus it is convenient to ensure that any of the factors included to the medium are not from animal non-human origin.

Chemically defined medium contains proteins obtained from recombinant bacteria or chemically synthesized. The concentration of each component is known, not presenting batch-to-batch variation and being entirely xeno-free [Jayme et al, 2000; Shenoy et al, 2007]. Although this type of medium would be the ideal, most of the chemically defined media available present important limitations, supporting only expansion for a single-passage or at a low proliferation rate [Lennon et al, 1995; Parker et al, 2007]. A promising chemically-defined medium was approved by FDA (StemPro MSC SFM, Invitrogen) to isolate and expand hMSC. However, some drawbacks are coming out, such as reduction of the hMSC differentiation potential and expression of some proteins when comparing with FBS supplemented medium [Jung et al, 2012]. Nowadays different serum-free media are available and, as shown by Bersenev and colleagues, it is necessary to optimize each medium and coating matrices for each MSC cell type and cell culture conditions [Bersenev 2015].

Supplementation with human autologous serum

The development of xeno-free medium supplemented with human derivatives is showing better results. Medium supplementation with human autologous serum is promising, being able to support MSC isolation and expansion [Stute et al 2004]. However, problems regarding its availability and donor variability would be faced [Jung et al 2012; Tunaitis et al 2011].

Platelet lysate

Platelet lysate (PL) is one of the main xeno-free options used nowadays. PL is obtained from human blood platelets after different freeze/thaw cycles that lyse the cells, releasing a large quantity of growth factors important for cell culture [Schallmoser et al 2009]. Many studies on the use of human PL have shown promising results on hMSC culture and expansion [Schallmoser et al 2007, 2009; Mojica-Henshaw et al 2013; Kocaoemer et al 2007; Flemming et al 2011; Doucet et al 2005; Chieregato et al 2011; Capelli et al 2007; Astori et al 2016]. PL can be easily obtained in the lab but, again, problems of donor variability would be faced [Lohmann et al, 2012]. Thus, commercially available PL made from large pools of donations would be more recommendable. The impact of PL on the immunomodulatory properties of hMSC is controversial, with some studies suggesting that it dampens the immunossupressive capabilities of the cells [Abdelrazik et al 2011; Copland et al 2013]. Additionally, some studies also showed that PL can affect the expression of adipogenic and osteogenic markers [Lange et al 2007; Gruber et al 2004].

Human plasma derived supplement (SCC)

With the objective of expanding hMSC in xeno-free conditions, Grifols has developed a human cell culture supplement (Supplement for Cell Culture, SCC) obtained from the fractionation of human plasma [Diez et al, 2015]. SCC solves the above FBS mentioned problems: it is of human origin, produced under good manufacturing practices (GMP and lot-to-lot variability is not expected (due to

the large size of the plasma fractionation pool and the number of different donations which contribute to each pool, which is from over 1000 different donors). Moreover, to ensure SCC safety, different methods to eliminate pathogens are performed during SCC production [Díez et al, 2015].

SCC is a product obtained in a pharmaceutical company, at a large scale. As a starting material, human plasma from healthy donors is collected through plasmapheresis and sodium citrate is added to avoid coagulation. After that, plasma samples are frozen and each plasma donation is analysed for viral infection markers (e.g. HIV, hepatitis B and C, and other human pathogenic viruses). Different logistic steps are used to ensure that all the donations that enter the factory are virus-free [Jorquera et al, 2012]. Once all the samples are analysed and it is ensured their safety, 1000-4000 plasma units are thawed and mixed (plasma pool). The plasma pool undergoes a cold-ethanol fractionation where fibrinogen and immunoglobulins are precipitated and eliminated [Cohn et al, 1946]. The supernatant obtained is cleaned from ethanol and concentrated and lyophilised to obtain the final SCC product. SCC can be stored for a long period of time at 2-8ºC [Jorquera et al, 2012].

SCC is still under study, but it has been already demonstrated to be useful to culture and expand not only hMSC, but also ESC, iPSC, Chinese hamster ovarian cells, Vero cells and mouse BALB/C myeloma cells [Diez et al, 2012; Rodriguez-Piza et al, 2010]. More studies need to be done to ensure that SCC can be used to expand hMSC without affecting their properties and genetic stability. As SCC doesn't contain selenium, and FBS does, the addition of this trace element in the medium is encouraged. Moreover, ethanolamine can be added to enhance formation of cell membranes. In the patent on the use of SCC, the addition of insulin is also recommended [Díez et al, 2014]. Optionally, antibiotics such as Penicillin/Streptomycin or Gentamicin/Amphotericin can be used to avoid contaminations [Diez et al, 2014].

In this present thesis, three different xeno-free composition media were used (Table 4). The base of all the media was DMEM or DMEM F12 supplemented with 15% SCC and insulin, sodium selenite and ethanolamine, and we called it as 'SCC-Medium'. 'SCC.FGF-Medium' is obtained by the addition of fibroblast growth factor to SCC Medium. And 'SCC.PL-Medium' is obtained by supplementation with platelet lysate of this last medium.

Table 4. SCC based xeno-free medium compositions used in the experiments of this present thesis (15% Supplement for cell culture (SCC), 10 mg/L Insulin, 6.7 µg/L Sodium Selenite, 2 µL/L Ethanolamine, 20 ng/mL Fibroblast growth factor, 10% Platelet lysate, 10 g/L Penicillin/Streptomycin).

Compound	SCC-Medium	SCC.FGF-Medium	SCC.PL-Medium
DMEM / DMEM F12	X	X	X
SCC	X	X	X
Insulin, Selenium, Ethanolamine	X	X	X
Fibroblast growth factor		X	X
Platelet lysate			X

Therefore, the main goal of this present thesis is to evaluate the suitability of SCC for hMSC expansion, and its influence on important hMSC properties for cell therapies, such as immunomodulation and chemotaxis.

Chapter 2. Effect of a human plasma derivative (SCC) on hMSC defining properties

[Adaptation from the article published at Stem Cell Research & Therapy]

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HUMAN MESENCHYMAL STEM CELLS MAINTAIN THEIR PHENOTYPE, MULTIPOTENTIALITY, AND GENETIC STABILITY WHEN CULTURED USING A XENO-FREE HUMAN PLASMA FRACTION

Arantxa Blázquez-Prunera, José María Díez, Rodrigo Gajardo, Salvador Grancha

Abstract

Human mesenchymal stem/stromal cells (hMSCs) show promising characteristics for their use in advanced therapy medicinal products. However, there are some unresolved concerns, such as the use of animal components for their expansion. In this study, we assessed the suitability of a xenofree supplement for cell culture (SCC) derived from human plasma, to culture and expand hMSCs.

hMSCs from different origins (adipose tissue [AT], bone marrow [BM] and umbilical cord [UC]) and supplier sources (commercial/non-commercial) were used. After hMSCs expansion in a xeno-free medium, classical hMSCs markers were studied by immunocytochemistry, and genetic stability was tested by classic karyotyping. The capacity of hMSCs to differentiate into adipogenic, osteogenic, and chondrogenic cells in differentiation media was assessed using different stainings. Different lots of SCC were used to assure consistency between batches.

Results showed that all hMSCs tested maintained their morphology and adherence to plastic during their expansion, and preserved their genetic stability, phenotype and differentiation potential. No differences were observed when using different lots of SCC. Moreover, the proliferation rate, evaluated as population doubling time (PDT) of commercial BM and AT hMSCs, was higher in the xeno-free medium than in the control media provided by the suppliers of the cells (PDT of 4.6 for BM-hMSC and 6.4 for AT-hMSC in xeno-free medium, and 7.0 and 14.7 respectively in the commercial media). UC-hMSCs PDT was similar in all the media tested. When using non-commercial BM-hMSCs, PDT was lower in the xeno-free medium, but reverted to the control level with the addition of growth factors.

In conclusion, SCC-containing medium can be a feasible xeno-free alternative to expand hMSCs for advanced therapies.

1. Introduction

Mesenchymal stem/stromal cells (MSCs) are a subset of non-hematopoietic adult multipotent cells originating from the mesoderm that can be isolated from almost all tissues and expanded in vitro [Singer et al, 2011]. MSCs are defined by their multipotentiality, with the ability to differentiate into adipocytes, osteoblasts and chondrocytes [Horwitz et al, 2005].

Human MSCs (hMSCs) are a promising tool in regenerative medicine and as a treatment for immune-mediated diseases [Singec et al, 2007]. However, there are limitations that need to be addressed to determine the safety of use in humans. One of the main concerns is related to the use of Fetal Bovine Serum (FBS) as a supplement in cell culture medium to expand hMSCs. The introduction of animal derivatives into human cell cultures is not recommended since animal proteins can become associated with hMSCs, and thus induce an immune rejection to the host [European Medicines Agency, 2013]. Another drawback is the lack of lot-to-lot consistency of the FBS and its limited source [Aldahmash et al, 2011; Bieback et al, 2009; Spees et al, 2004]. For these reasons, the presence of animal components is discouraged in the culture media used to expand hMSC for therapy [European Medicines Agency, 2013].

The most commonly used FBS-free options are the Serum-free media, Chemically defined media, and Human serum and Platelet lysate supplemented media [Jayme and Smith, 2000; Jung et al, 2012]. Serum-free media does not contain serum; however, it may contain proteins derived from animals, such as albumin, hormones, and attachment factors. Thus, it may not be a xeno-free medium. In the chemically defined media, all the components and concentrations are known; proteins are obtained from recombinant bacteria or are chemically synthesized, being entirely free of animal-derived components. These media do not present batch-to-batch variations [Jayme and Smith, 2000; Shenoy et al, 2007]. Although

different attempts have been made to develop a succesful chemically defined media, most of them have limitations, such as only supporting expansion for a single-passage or at a low proliferation rate [Lennon et al, 1995; Parker et al, 2007].

Some promising chemically-defined media are already on the market. One is the FDA-approved StemPro MSC SFM from Invitrogen, which allows hMSCs isolation and expansion. However, some drawbacks regarding the use of this medium are evident, such as different expression levels of some molecules and differentiation potential, when compared with hMSCs expanded in an FBS supplemented medium [Jung et al, 2012]. Other commercially available xeno-free media also present controversial results regarding the expansion of hMSCs from different origins, hMSCs attachment or proliferation rates [Lindroos et al, 2009; Miwa et al, 2012; Schallmoser et al, 2008; Liu et al, 2007; Hartmann et al, 2010; Oikonomopoulos et al, 2015; Chase et al, 2012; Corotchi et al, 2013; Patrikoski et al, 2013; Simoes et al, 2013; Gstraunthaler et al, 2003; Li et al, 2015]. Among the different xeno-free/serum-free options that are currently in use, the most common ones are the human platelet lysates and the chemically-defined serum-free media [Miwa et al, 2012; Schallmoser et al, 2007,2008; Cohn et al, 1946; Lange et al, 2007]. Furthermore, the use of supplement for cell culture (SCC) derived from human plasma showed promising results in bone marrow (BM) hMSCs expansion, preserving not only hMSC typical characteristics and multipotentiality [Díez et al, 2015], but also hMSC immunomodulatory properties and chemotaxis [Blázquez-Prunera et al 2017].

In this study, SCC was used to culture and expand hMSCs isolated from different origins (BM, umbilical cord [UC], and adipose tissue [AT]) and supplier sources (commercial/noncommercial). To assess the suitability of SCC to expand hMSCs, cell proliferation, adherence, genetic stability, typical markers and multipotentiality were evaluated.

2. Materials and methods

2.1. Objective and study design

The objective of this study was to assess whether xeno-free SCC is suitable for culture and expansion of hMSCs isolated from BM, AT and UC, using different lots of SCC to confirm consistency between batches. Characteristics of viable cultured hMSCs such as genetic stability (normal karyotype), phenotype (typical surface markers expression) and multipotentiality (adipogenic, osteogenic and chondrogenic differentiation potential) have been qualitatively evaluated (Figure 5).

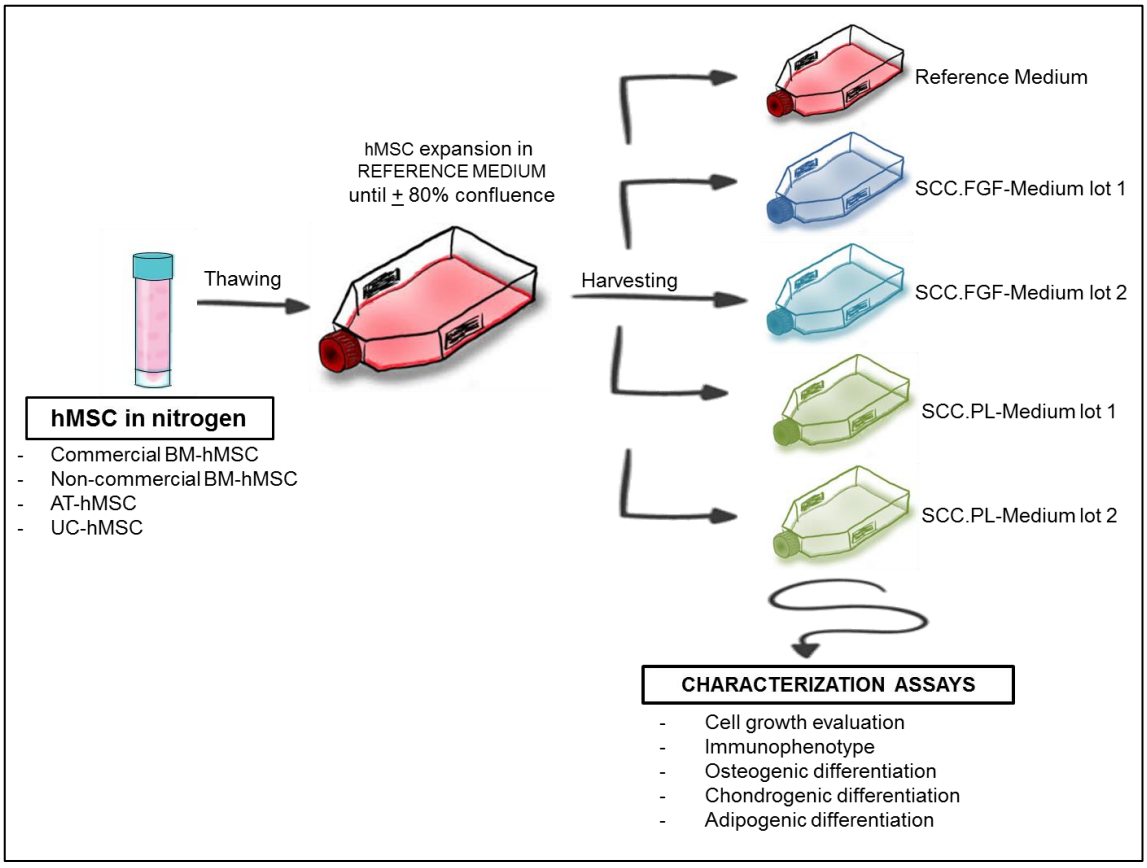

Figure 5. Diagram of the study design. AT-hMSC, UC-hMSC and BM-hMSC (Commercial and non-commercial) are thawed in Reference-Medium (Lonza, Promocell or FBS-Medium). When cells are at 80% confluence, they are harvested and splitted into different flasks with the different media tested. After expansion, different characterization assays are performed.

2.2. hMSCs used

Different hMSC lines obtained from BM, UC and AT were acquired from two different suppliers (Lonza and Promocell). Ethics approval was not required for use of commercial cells. Furthermore, non-commercial BM-hMSCs were kindly supplied by Inbiobank and the Instituto Nacional de Engenheria Biomedica (INEB, Porto, Portugal) (Table 5). Inbiobank isolated the cells following manufacturing procedures based on ISO9001:2000 under GMP conditions. INEB cells were obtained from discarded bone tissues of 2 different patients at the Hospital São João, Porto, who provided written consent. Confidentiality of donors' information was guaranteed. hMSCs were isolated using DMEM supplemented with FBS. 10% DMSO was added to the isolated hMSCs, which were frozen at -80ºC overnight before being transferred to a liquid nitrogen tank. All hMSCs stocks were kept in a liquid nitrogen tank until their use.

Table 5. Summary of the different hMSC cell lines used.

ORIGIN	SUPPLIER SOURCE	DONOR	
		Age (years)	Sex
Bone Marrow	Commercial	20	Female
		21	Female
		22	Female
		64	Male
	Non-commercial	28	Male
		52	Female
		56	Female
Adipose Tissue	Commercial	47	Male
Umbilical Cord	Commercial	Newborn	Male

2.3. Xeno-free medium

As a xeno-free substitute of FBS, a human plasma derivative (Supplement for cell culture, SCC, Grifols) was used. This product is derived from human plasma specifically collected as starting material for the industrial production of different plasma therapeutic proteins. SCC is obtained through cold-ethanol industrial plasma fractionation, and it contains a stable and defined fraction of human proteins from plasma pools which contain samples from at least 1000 different healthy donors [Díez et al, 2015]. Due to the large number of donors in each pool, a high consistency among lots is expected. SCC is manufactured following GMP procedures. Each plasma donation is tested for the presence of pathogen agents (transfusion transmisible). Furthermore, a dedicated step with viral inactivation capacity (gamma irradiation) is included in the manufacturing process, in addition to the various steps taken to eliminate/remove pathogens [Díez et al, 2015].

The xeno-free medium used consists of Dulbecco's modification of Eagle's medium F12 (ref. 21221, Life Technologies-Life Technologies) supplemented with SCC and other growth factors from platelet lysate, as described elsewhere [SCC.PL-Medium] [Díez et al, 2015]. The platelet lysate used was obtained through the freeze-thaw method [Schallmoser et al, 2009]. In this study, a variation without the addition of platelet lysate was also used [SCC.FGF-Medium].

2.4. hMSC cell culture and growth evaluation

All hMSCs were thawed in Reference Medium; for commercial hMSCs, MSC basal medium (ref. PT3238, Lonza) or MSC cell growth medium (ref. C-28010, PromoCell) were used depending on the hMSCs commercial supplier. Non-commercial hMSCs were thawed in FBS-Medium, composed of DMEM low glucose (ref. 21969, Life Technologies-Life Technologies) supplemented with

10% MSC-qualified FBS (ref. 10500, Life Technologies-Life Technologies), 1% L-Glutamine (ref. 25030, Life Technologies-Life Technologies) and 1% Penicilin/Streptomicin (ref. 15140, Life Technologies-Life Technologies). hMSCs were cultured at 37ºC and 8% CO_2. Medium was replaced every 3-4 days until arriving at 80% confluence, when cells were detached using xeno-free trypsin (Tryple Express, ref. 12604, Life Technologies-Life Technologies). hMSCs were split and seeded at a cell density of 5,000 - 6,000 cells/cm2 using the different media (Reference Medium, SCC.FGF-Medium lot 1 and lot 2, SCC.PL-Medium lot 1 and lot 2). After each passage, population doubling time (PDT) was calculated using the formula $PDT = 1 / [3.32 (\log N_H - \log N_1) / (t_2 - t_1)]$ [Díez et al, 2015]. Statistical analysis was performed using GraphPad Prism v5.01 and applying the non-parametric Kruskal Wallis test followed by Dunns multiple comparison test.

2.5. hMSC genetic stability under culture

To determine if xeno-free-expanded hMSCs were genetically stable, hMSCs cultures at early and late passages were compared. UC-hMSCs and AT-hMSCs were thawed in Reference Medium and seeded in 3 flasks at a cell density of 5,000 cells/cm2. After 24 hours the medium was replaced by new Reference Medium, SCC.FGF-Medium or SCC.PL-Medium. After 3-4 passages, a new vial of the same cell line and lot was thawed following the same protocol. When cultures were around 60% confluence, flasks were analysed by GTGbanding. Chromosomes were identified and analysed according to ECA Cytoteogenetic Guidelines (Figure 6).

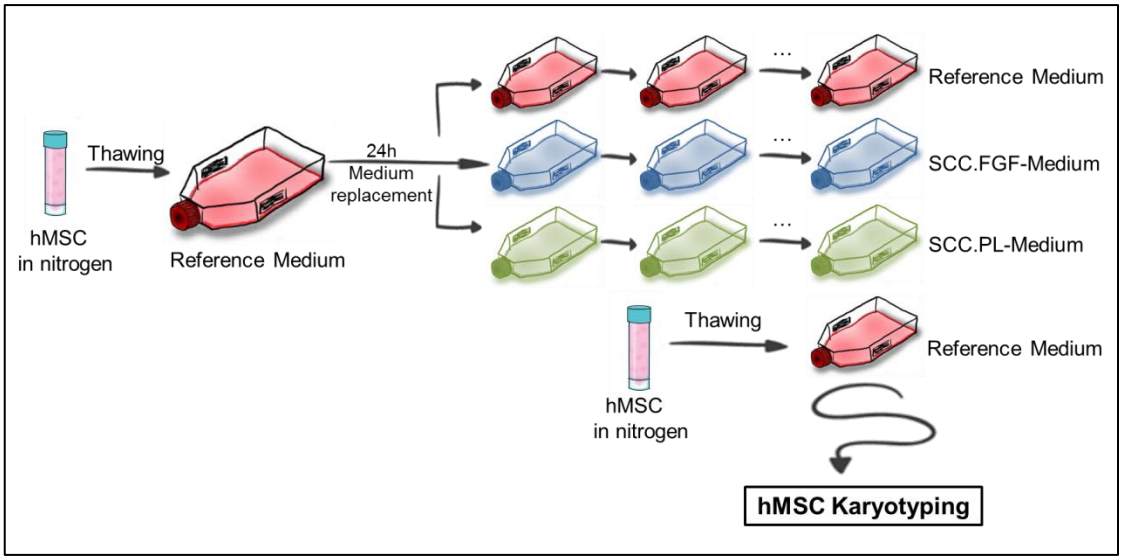

Figure 6. **Diagram of the genetic stability assay**. Different vials of frozen hMSC are thawed in Reference-Medium (Lonza, Promocell or FBS-Medium). After 24 hours the medium is replaced for the different tested media. Cells are cultured for different passages in the different media. In the last passage, a new vial of cells is thawed in reference medium. After expansion, cells are karyotyped.

2.6. hMSC phenotypic characterization

The xeno-free-expanded hMSCs phenotype was evaluated by immunofluorescent staining of typical hMSCs surface markers as previously described [Díez et al, 2015]. Two negative markers - CD14 (ref. MAB1219, Merck Millipore) and CD19 (ref. MAB1794, Merck Millipore) – and seven positive markers – CD29 (ref. 303002, Biolegend) , CD44 (ref. CBL154, Merck Millipore), CD73 (ref. 344004, Biolegend), CD90 (ref. CBL415, Merck Millipore), CD105 (ref. MABT117, Merck Millipore), CD166 (ref. 343902, Biolegend), Stro-1 (ref. MAB4315, Merck Millipore) - were studied.

Cells were fixed with IC Fixation buffer (ref. FB001, Invitrogen-Life Technologies) and incubated at room temperature (RT) with blocking solution. Then cells were incubated overnight at 4ºC with the primary antibodies (a 1:100 dilution in blocking solution was used for CD14, CD19, CD29, CD44, Stro-1; a 1:50 for CD90 and 1:20 for CD73, CD105, CD166). After incubation, cells were washed twice

with PBS and twice with blocking solution. After the incubation at RT with blocking solution, the secondary antibodies were added to the plate (donkey anti-mouse IgG conjugated with FITC (ref. AP192F, Merck Millipore) and goat anti-mouse IgM conjugated with Cy3 (ref. AP128C, Merck Millipore). After washing the cells with PBS, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI, ref. D3571, Invitrogen-Life Technologies) and visualized under a fluorescent microscope (Axiobserver LD Plan-Neofluar objective; Carl Zeiss).

2.7. Osteogenic differentiation

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs were expanded in the different media. After reaching 80% confluence, cells were harvested and 6 x 10^4/well hMSCs were seeded in a 24 well plate. Osteogenesis was induced by commercial osteogenic medium (ref. C-28013, Promocell). After 7 days of incubation, some of the samples were fixed and the alkaline phosphatase activity was stained with nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyl phosphate salt and nitroblue tetrazolium chloride (BCIP/NBT tablet, ref. B5655, Sigma-Aldrich). After 21 days of incubation, the presence of extracellular calcium deposits was assessed by its specific staining with Alizarin Red S (ref. A5533, Sigma-Aldrich) to determine osteogenic differentiation (Figure 7).

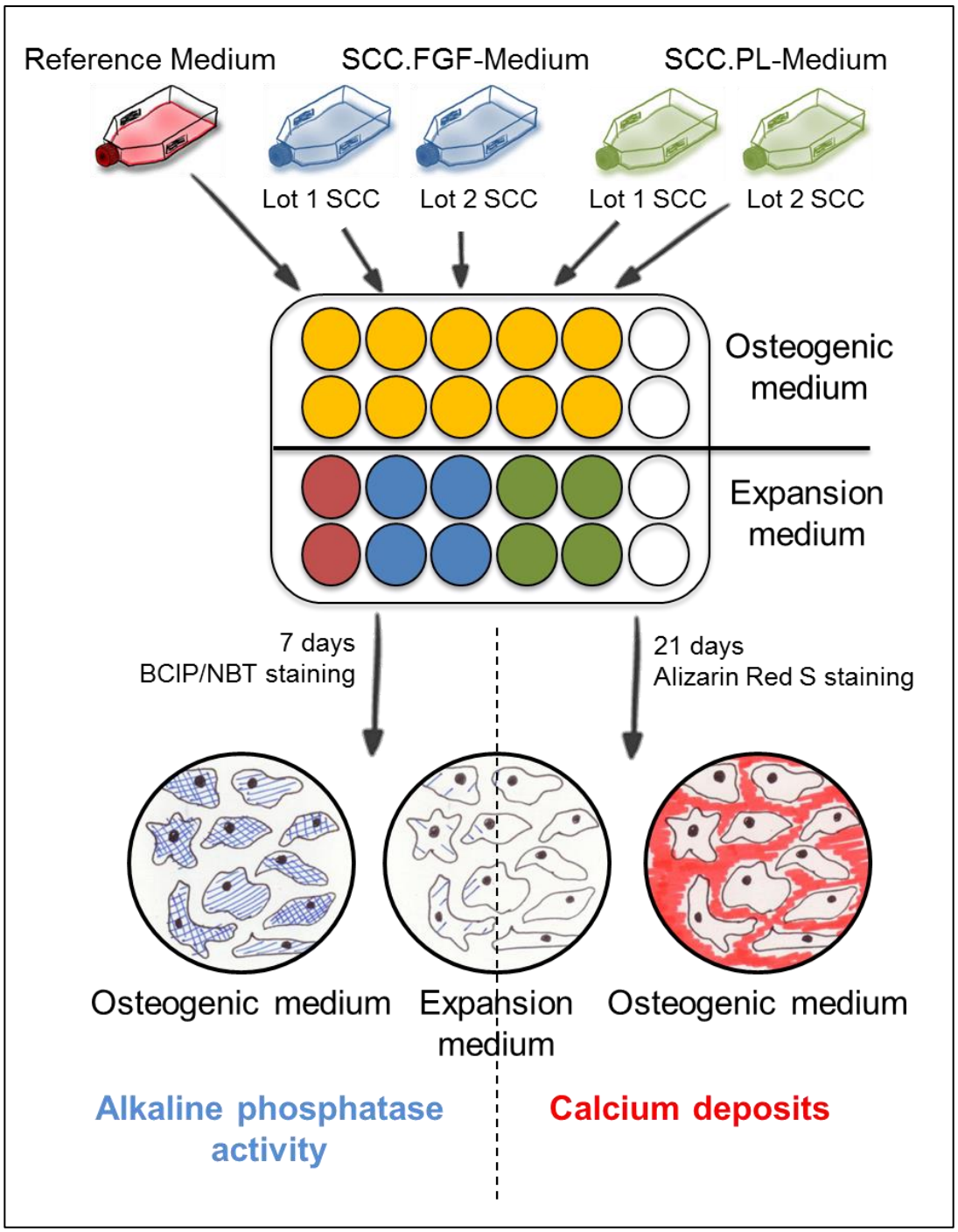

Figure 7. Diagram of the osteogenic differentiation assay and the expected results. hMSC are expanded in the different media. When cell cultures are around 80% confluence, cells are harvested and seeded in a 24 well plate. hMSC are cultured in osteogenic medium or the expandion media. After 7 days, the alkaline phosphatase activity is stained. Ater 21 days calcium deposits are stained with alizarin red S.

2.8. Chondrogenic differentiation

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs were expanded in the different media. After reaching 80% confluence, 3 x 10^5 cells were placed in a 15 mL conical tube. A first centrifugation at 150 g over 15 minutes was done in the expansion media. After that, the media were replaced by commercial chondrogenic differentiation medium (ref. C-28012, Promocell) and tubes were centrifuged at 150 g for 5 minutes. Without disturbing the pellets, tubes were incubated at 37 ºC 5% CO_2 and spheres were allowed to form. After 21 days in culture, spheres were fixed with IC Fixation buffer for 1 hour and cartilage was stained with Alcian Blue (ref. A3157, Sigma-Aldrich) to determine chondrogenic differentiation (Figure 8).

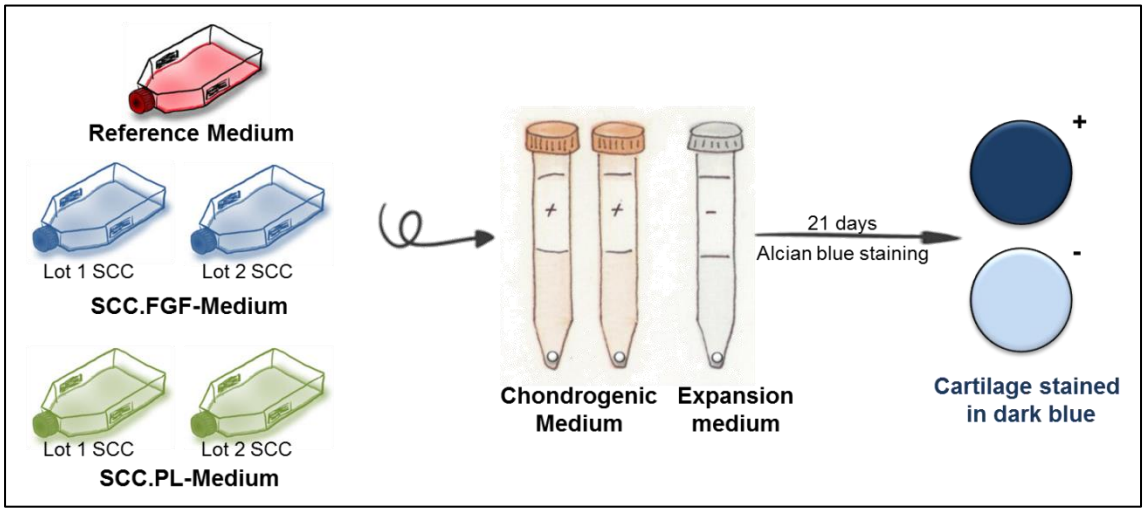

Figure 8. Diagram of the chondrogenic differentiation assay and the expected results. hMSC are expanded in the different media. When hMSC are aroung 80% confluence, cells are harvested and a sphere of cells is made by centrifugation. After 21 days spheroids are stained with alcian blue. Spheroids with high content of cartilage present a dark blue staining.

2.9. Adipogenic differentiation

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs were expanded in the different media. After reaching 80% confluence, cells were harvested and 6 x 10^4/well hMSCs were seeded in a 24 well plate. Adipogenesis was induced by commercial adipogenic medium (ref. C-28011, Promocell). After 14 days incubation, samples were fixed and lipid droplets were stained with Oil Red O (ref. O0625, Sigma-Aldrich) to determine adipogenic differentiation (Figure 9).

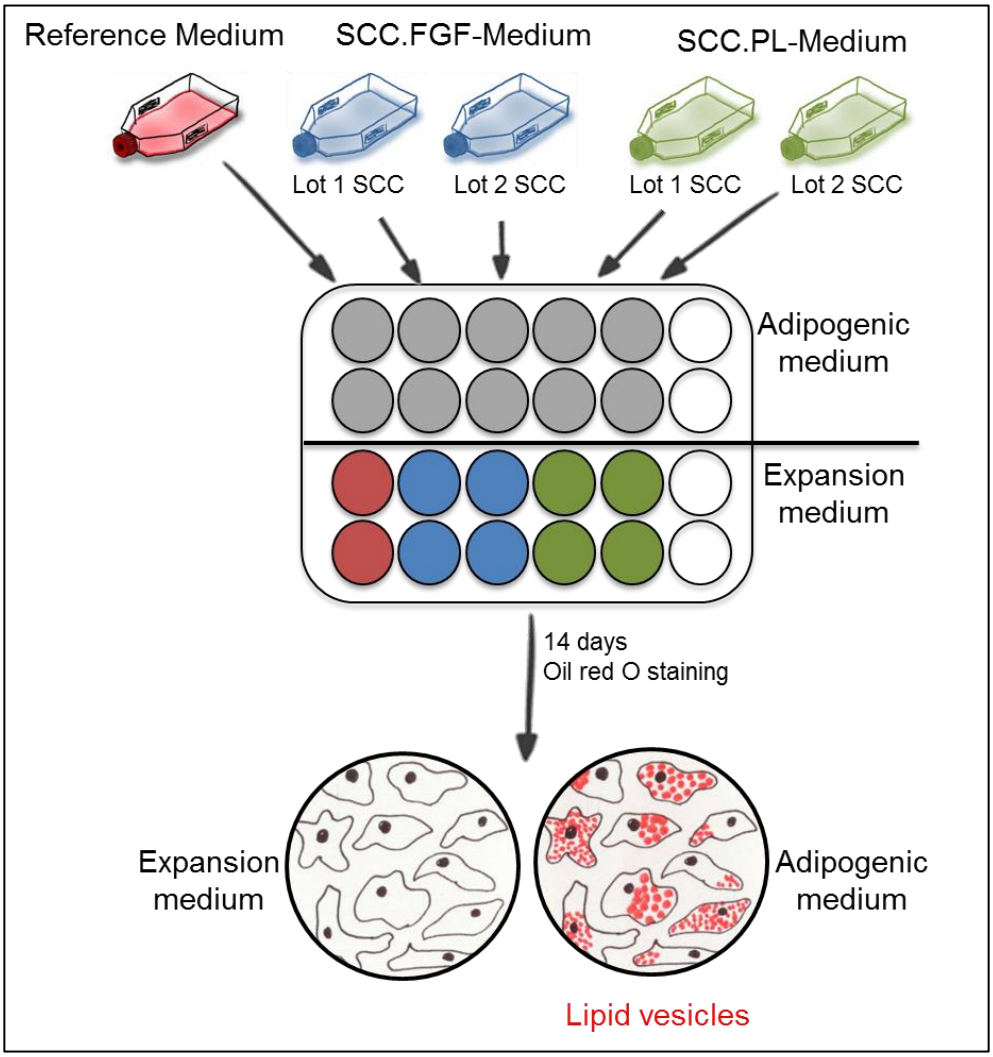

Figure 9. Diagram of the adipogenic differentiation assay and the expected results. hMSC are expanded in the different media. When cell cultures are around 80% confluence, cells are harvested and seeded in a 24 well plate. hMSC are cultured in adipogenic medium or the expandion media. After 14 days, the presence of lipid vesicles is evaluated using oil red O staining.

3. Results

3.1. hMSC growth in xeno-free conditions

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs could be expanded in xeno-free medium, with (SCC.PL-Medium) and without (SCC.FGF-Medium) platelet lysate. All hMSCs tested adhered to the plastic surface of the culture flask without additional supplementation with attachment factors or surface coating. No differences were observed among the different lots of SCC used. As seen in Figure 10, commercial BM-hMSCs showed a tendency to grow faster in xeno-free medium (PDT mean= 4.6) than in Reference Medium (PDT mean= 7). AT-hMSCs grew faster in xeno-free medium (PDT mean= 6.4) than in the commercial Reference Medium (PDT mean= 14.7). The addition of platelet lysate into the xeno-free medium did not produce significant changes in the PDT of the commercial hMSCs lines (BM-hMSC= 4.8, AT-hMSC= 6.2, UC-hMSC=1.9). The cell replicative capacity of UC-hMSCs was similar in all tested media (XF= 2.5, PL= 1.9, Reference= 2). Non-commercial BM-hMSCs grew slower in SCC.FGF-Medium (PDT mean= 8.3) than in the Reference Medium (FBS-Medium, PDT mean= 5.5); however, the cell replicative capacity was equivalent to the control in Reference Medium when growth factors from platelet lysate were added into the xeno-free medium (PDT mean= 5.5).

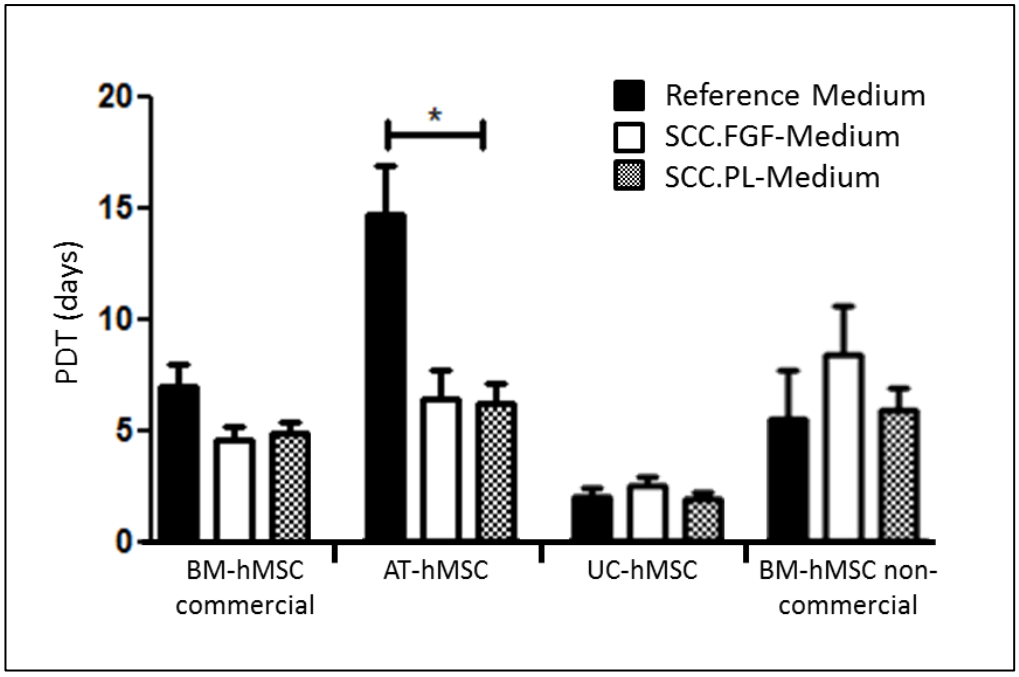

Figure 10. Population doubling time of different hMSC in Reference medium (Commercial medium 1, Commercial Medium 2, FBS-Medium), SCC.FGF-Medium and SCC.PL-Medium (n=3-22, *=p <0.05).

3.2. hMSC genetic stability

To determine whether the faster hMSCs growth rate in xeno-free medium fails to induce chromosomal aberrations, hMSCs were cultured througout different passages and their genomic stability was assessed using conventional cytogenetic analysis. All the karyotypes obtained showed a normal diploid karyotype. hMSCs were genetically stable after 3 and 4 passages in xeno-free medium culture (with and without growth factors from platelet lysate) (Figure 11).

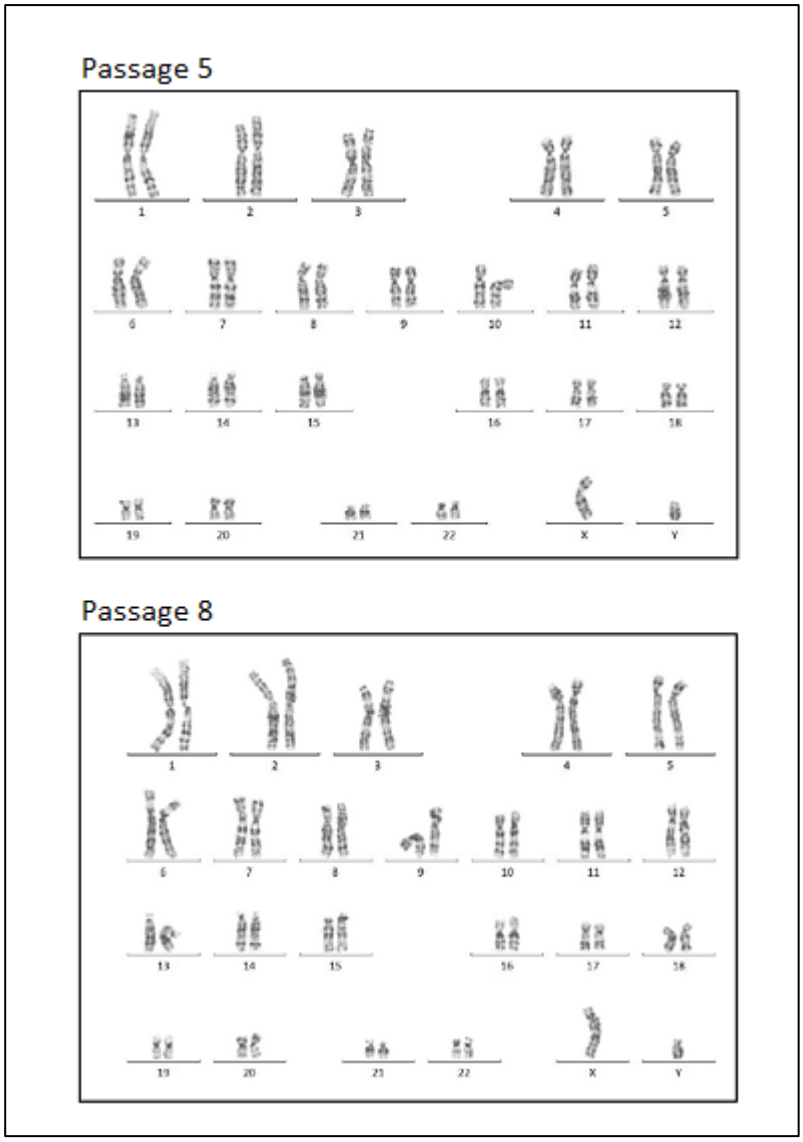

Figure 11. Genetic stability analysis of xeno-free expanded hMSC after long term culture. Representative karyotypes of just thawed UC-hMSC in Reference Medium (Passage 5) and long term culture in xeno-free medium (Passage 8).

3.3. Expression of typical hMSC surface markers

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs expanded in the different xeno-free media (SCC.FGF-Medium lot 1, SCC.FGF-Medium lot 2, SCC.PL-Medium lot 1, SCC.PL-Medium lot 2) presented the normal hMSC phenotype, being negative for CD14, CD19 and positive for CD29, CD44, CD73, CD90, CD105, CD166 and Stro-1 (Figure 12).

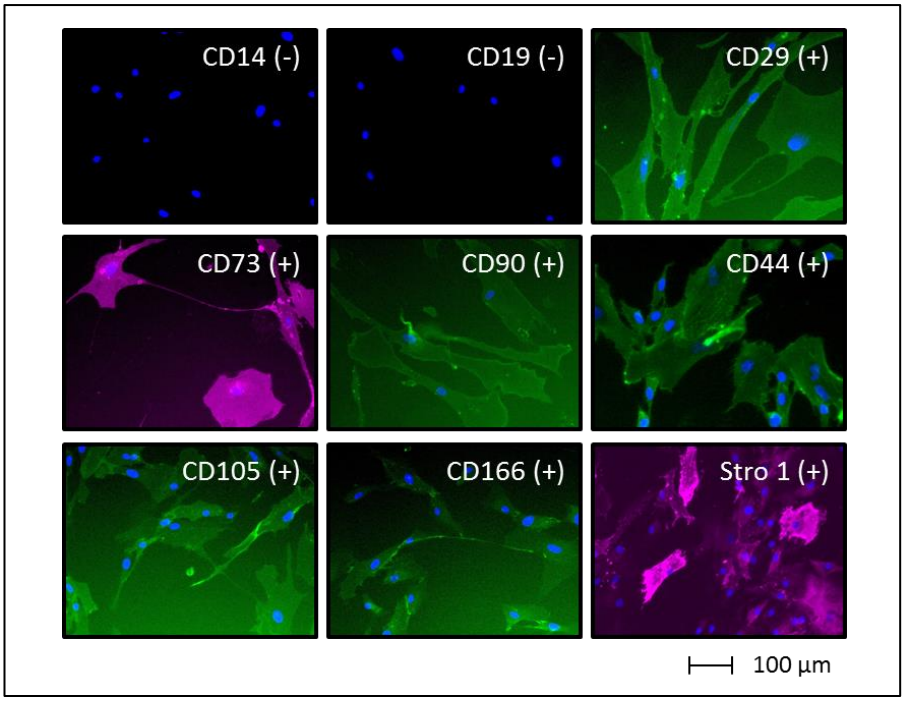

Figure 12. Phenotypic characterization. Expression of the typical hMSC surface markers by xeno-free expanded hMSC as determined by immunofluorescence staining (Representative images).

3.4. Multipotenciality of xeno-free-expanded hMSC

3.4.1. Osteogenic differentiation

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs expanded in the different xeno-free media (SCC.FGF-Medium lot 1, SCC.FGF-Medium lot 2, SCC.PL-Medium lot 1, SCC.PL-Medium lot 2) could be differentiated into osteoblasts (Figure 13). After 21 days in osteogenic medium, hMSCs showed the typical cuboidal and flattened osteoblastic morphology. Extracellular calcium deposits and elevated alkaline phosphatase activity were observed in the differentiated hMSCs. Some differences could be observed when comparing the different cell lines; non-commercial BM-hMSCs presented the highest level of osteogenic differentiation, and UC-hMSCs presented the lowest level of differentiation. No substantial differences were observed regarding the media used to expand the hMSCs or the lots of SCC tested.

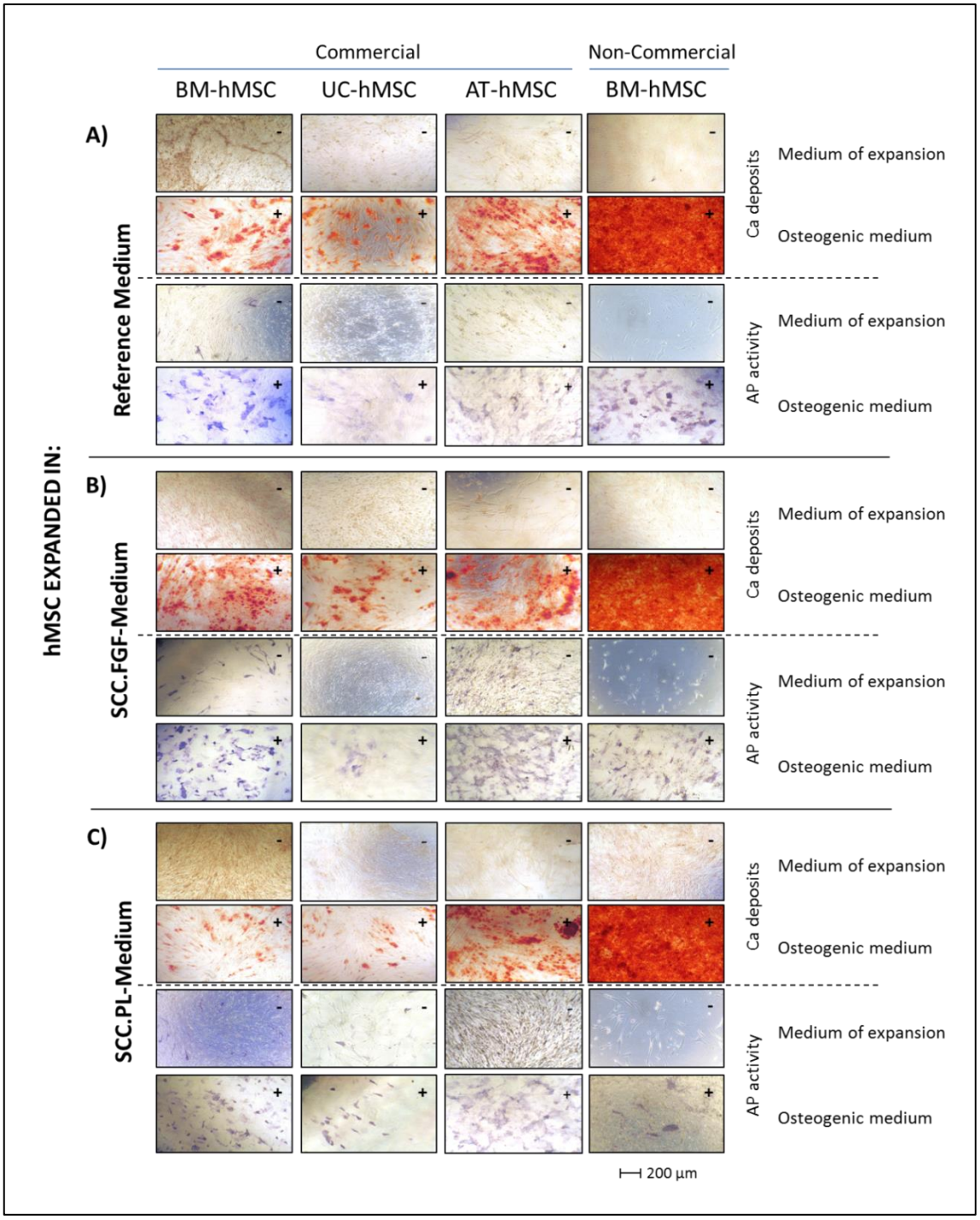

Figure 13. Osteogenic differentiation. Representative images of commercial and noncommercial bone marrow (BM)-, adipose tissue (AT)- and umbilical cord (UC)-derived human mesenchymal stem/stromal cells (hMSCs) expanded in Reference Medium, and xeno-free medium with and without the growth factors from platelet lysate (SCC.PL-Medium, SCC.FGF-Medium). hMSC were cultured for 21 days in osteogenic differentiation medium or the medium of expansion. After 7 days, the alkaline phosphatase (AP) activity was stained in blue with BCIP/NBT. After 21 days, the extracellular calcium depostis was stained with Alizarin red S in red.

3.4.2. Chondrogenic differentiation

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs expanded in the different xeno-free media (SCC.FGF-Medium lot 1, SCC.FGF-Medium lot 2, SCC.PL-Medium lot 1, SCC.PL-Medium lot 2) preserved their potential of differentiation into cartilage (Figure 14). After 21 days in chondrogenic medium, the typical glycosaminaglycans present in cartilage could be stained with Alcian blue. In all commercial hMSCs types and origins, expanded in the different media and lots of SCC, chondrogenic differentiation was observed at a similar level. Non-commercial BM-hMSCs presented a higher presence of glycosaminaglycans than the commercial hMSCs.

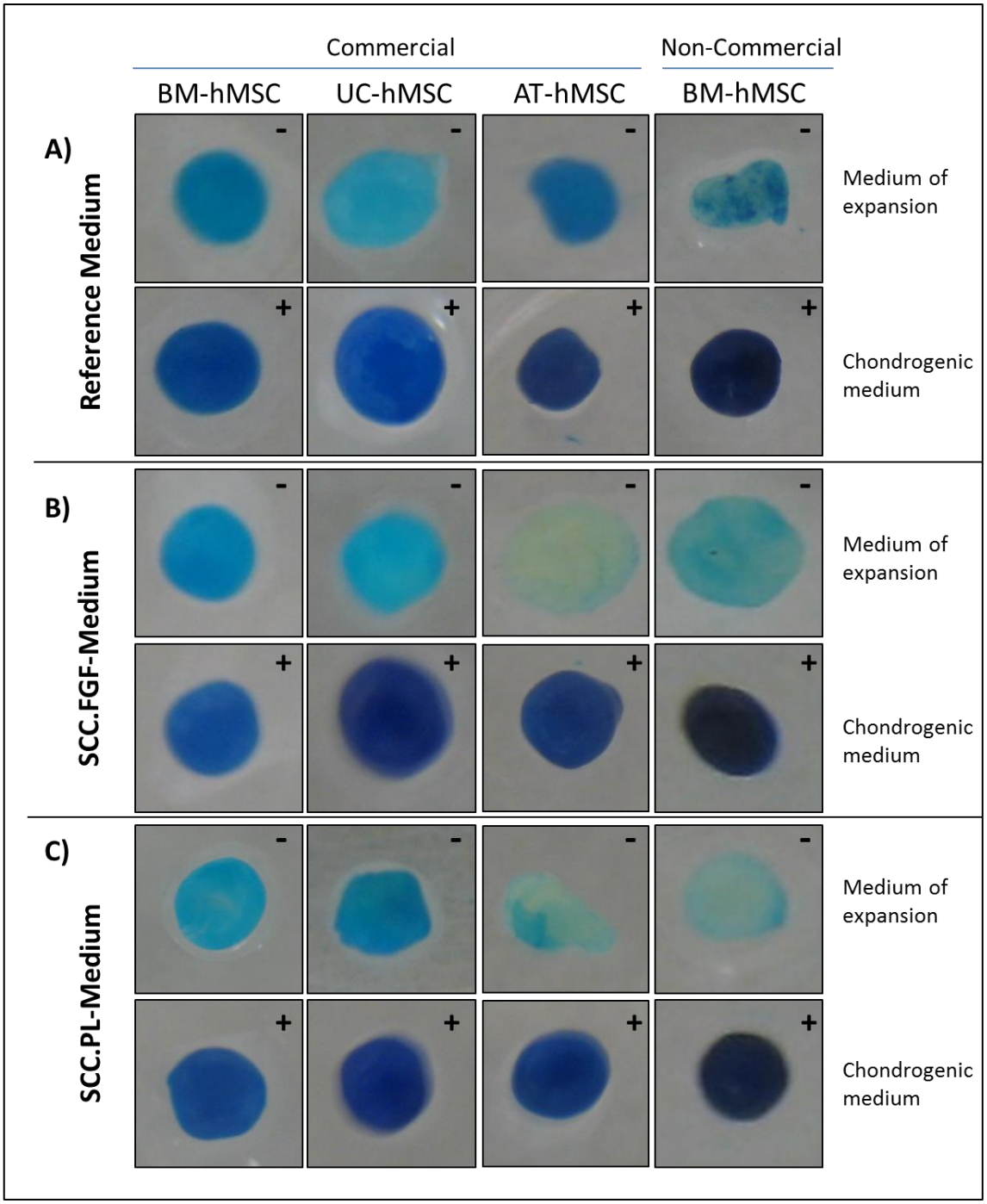

Figure 14. Chondrogenic differentiation. Representative images of commercial and noncommercial bone marrow (BM)-, adipose tissue (AT)- and umbilical cord (UC)-derived human mesenchymal stem/stromal cells (hMSCs) expanded in Reference Medium, and xeno-free medium with and without the growth factors from platelet lysate (SCC.PL-Medium, SCC.FGF-Medium). hMSC were cultured for 21 days in chondrogenic differentiation medium or the medium of expansion. Alcian blue was used to stain the extracellular cartilage matrix in dark blue.

3.4.3. Adipogenic differentiation

BM-hMSCs (commercial and non-commercial), AT-hMSCs and UC-hMSCs expanded in the different xeno-free media (SCC.FGF-Medium lot 1, SCC.FGF-Medium lot 2, SCC.PL-Medium lot 1, SCC.PL-Medium lot 2) could be differentiated into adipocytes (Figure 15). After 14 days in adipogenic medium, characteristic lipid droplets could be stained with Oil Red O. No differences were observed among the adipogenic differentiation of the tested cell types and origins, media or lots of SCC.

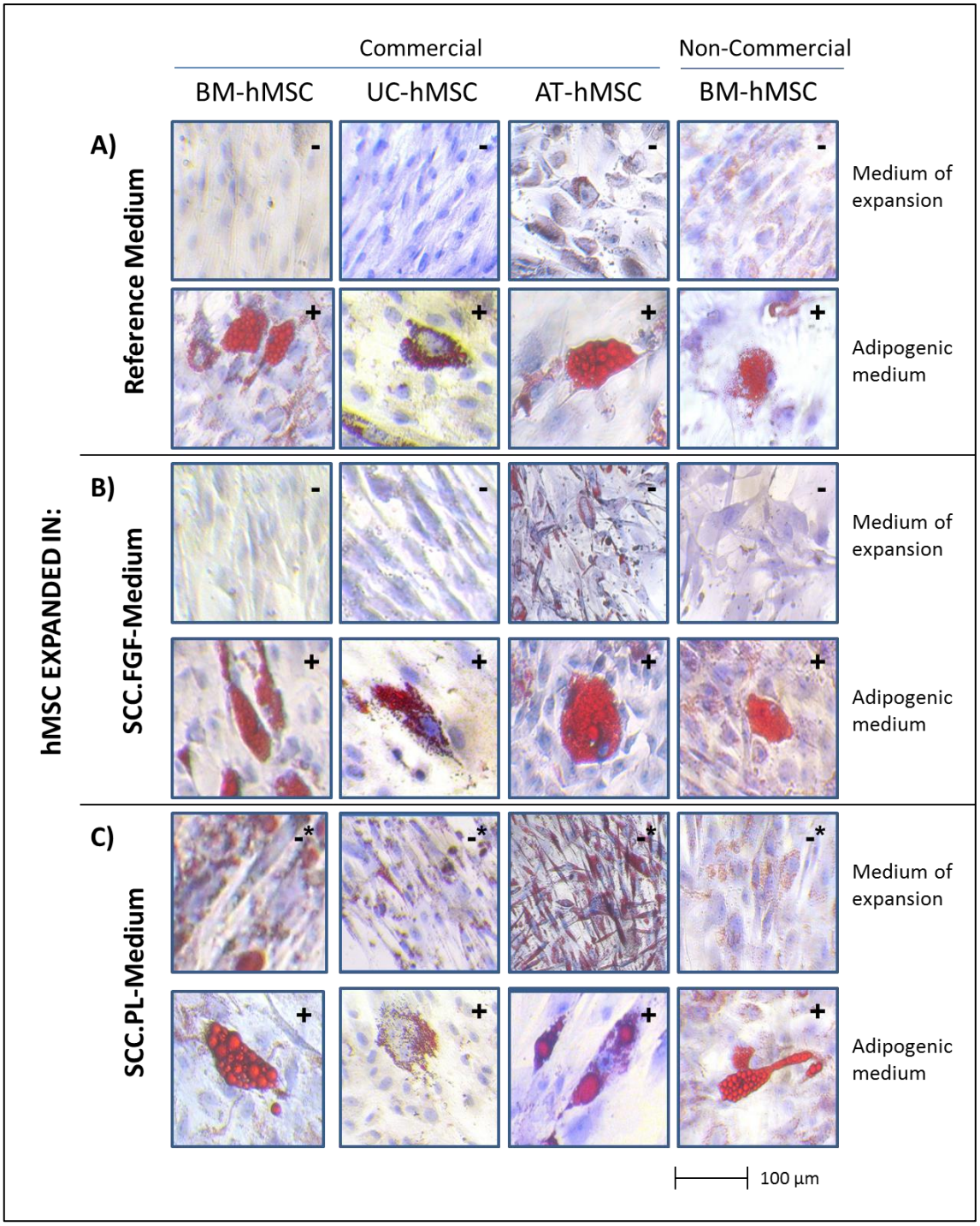

Figure 15. Adipogenic differentiation. Representative images of commercial and non-commercial bone marrow (BM)-, adipose tissue (AT)- and umbilical cord (UC)-derived human mesenchymal stem/stromal cells (hMSCs) expanded in Reference Medium, and xeno-free medium with and without platelet lysate (SCC.PL-Medium, SCC.FGF-Medium). hMSC were cultured for 14 days in adipogenic differentiation medium or the medium of expansion. Lipid vesicles in adipocytes were stained with Oil Red O and are seen in red.

4. Discussion

hMSCs therapies are of great interest, not only for their multipotential characteristics, but also for their use in treating immune diseases [Singec et al, 2007]. In regenerative medicine, there are concerns regarding the immune rejection that a patient can suffer if animal proteins become adhered to the in vitro expanded human cells [European Medicines Agency, 2013; Spees et al, 2004]. Thus, the replacement of the commonly used FBS is a necessity, and different strategies are being developed. In a previous report, we described that commercial BM-hMSCs could be expanded in SCC-containing medium supplemented with growth factors from platelet lysate (SCC.PL-Medium) [Díez et al, 2015]. Moreover, hMSC expanded in SCC-based medium have been shown to preserve their immunomodulatory and chemotactic properties [Blázquez-Prunera et al, 2017]. In this study, we extended the results for non-commercial BM-hMSCs and commercial AT-hMSCs and UChMSCs. Our results indicated that the use of platelet lysate as a supplement in SCC containing medium is optional. In addition, we showed that SCC can be used to culture and expand hMSCs under xeno-free conditions, mantaining their phenotype, genetic stability and multipotentiality.

Xeno-free media supplemented with human autologous serum has been shown to support hMSCs expansion; however, it would be challenging to obtain an adequate amount of serum to expand hMSCs sufficiently, and the age of the donor would influence its properties [Jayme ans Smith, 2000; Tunaitis et al, 2011]. There is controversy on the use of this medium, and contradictory results have been published [Dahl et al, 2008; Mizuno et al, 2006; Stute et al,2004; Shahdadfar et al, 2005; Tateishi et al, 2008; Kuznetsov et al, 2000; Poloni et al, 2009; Le Blanc et al, 2007; Kocaoemer et al, 2007].

There are no established protocols for the isolation and culture of hMSCs; thus, different laboratories use different strategies, making it difficult to compare the outcomes of different studies that try to develop a xeno-free medium. Usually,

the strategies consist of growing hMSCs in the media in the pipeline and checking the International Society for Celluler Therapy hMSCs defining characteristics (hMSCs adherence to plastic, phenotype, and differentiation into osteoblasts, chondroblasts and adipocytes) [Dominici et al, 2006].

SCC is an industrial GMP-product, obtained from a large quantity of plasma sourced from more than 1000 donors, which considerably reduces the lot-to-lot variability. Moreover, no supply problems are expected. In our study, all hMSCs tested were able to adhere and be expanded in SCC.FGF-Medium and SCC.PL-Medium. The proliferation rate of the commercial BM- and AT- hMSCs was higher than in the Reference Medium, as observed in the previous studies [Dominici et al, 2006]. As a high proliferation rate could induce chromosomic aberrations, the genetic stability of the cultures was assesed. hMSCs were passaged for 3 and 4 runs in SCC.FGF-Medium, SCC.PL-Medium and Reference Medium. Our results showed that hMSCs expanded using the different media were genetically stable, showing a normal diploid karyotype. Other studies also showed a higher proliferation of hMSCs in other xeno-free and serum-free media than in FBS-supplemented media; however, usually the genetic stability was not studied and only hMSCs from one origin were used [Parker et al, 2007; Lindroos et al, 2009; Kocaoemer et al, 2007].

After verifying that the cells could be expanded in the xeno-free media, we tested whether the defining hMSCs characteristics were maintained. All hMSCs used presented the typical hMSC phenotype, being positive for CD29, CD44, CD73, CD90, CD105, CD166 and Stro- 1, and negative for CD14, CD19. The multipotentiality of the cells was also studied; all the cells tested could be differentiated into osteoblasts, chondrocytes and adipocytes. Although the assays performed were qualitative, some differences in the differentiation levels could be observed when using the different cell types. Non-commercial BM-MSCs showed a higher chondrogenic and osteogenic differentiation than

the other cell types, an observation that was made in other studies [Li et al, 2015]. On the other hand, UC-hMSCs showed a lower osteogenic differentiation, when compared with the other cells, which was also observed by other researchers [Zeddou et al, 2014; Nagamura-Inoue et al, 2014].

Although platelet lysate have shown promising characteristics for hMSC culture and expansion and is being used in many studies, some drawbacks are coming out, such as donor variability [Lohmann et al, 2012], decreased expression of adipogenic and osteogenic differentiation markers [Lange et al, 2012; Gruber et al, 2004], and interaction with immunomodulatory properties of MSCs [Abdelrazik et al, 2011; Copland et al, 2013]. Due to these concerns, in this study we tested the use of SCC without the addition of platelet lysate. Our results showed that the addition of platelet lysate to SCC-containing Medium is optional. All the hMSCs defining characteristics were mantained in SCC.FGF-Medium without the addition of platelet lysate. The only difference observed was in the proliferation rate of non-commercial BM-hMSCs, which was higher when introducing platelet lysate to the medium. Thus, due to the potential negative effects, as mentioned before, we recommend avoiding the supplementation with platelet lysate in SCC supplemented media.

It has been previously shown that SCC can be used to culture not only hMSCs, but also chinese hamster ovarian cells, Vero cells, and mouse BALB/c myeloma cells [Díez et al, 2015]. All the studies with the commercial cell lines were done in parallel using different lots of human plasma fraction. In this study, hMSCs behaved similarly regardless of the SCC lot used, which indicates that the batch selected does not present significant variations regarding the other batches, as expected from its origin (large plasma pools from over 1000 donors).

5. Conclusion

SCC is a highly robust human plasma fraction. BM-hMSCs (commercial and noncommercial), AT-hMSCs and UC-hMSCs could be expanded in a xeno-free media containing SCC as a supplement while maintaining their genetic stability and typical hMSCs phenotype and multipotentiality. Moreover, our results suggest that the platelet-lysate-free composition is suitable for culture and expansion of hMSCs in xeno-free conditions for human cell therapies.

Chapter 3. Effect of SCC on hMSC immunomodulatory and chemotactic properties

[Adaptation from the article published at Stem Cells International]

HUMAN BONE MARROW MESENCHYMAL STEM/STROMAL CELLS PRESERVE THEIR IMMUNOMODULATORY AND CHEMOTACTIC PROPERTIES WHEN EXPANDED IN A HUMAN PLASMA DERIVED XENO-FREE MEDIUM

Arantxa Blázquez-Prunera, Catarina R. Almeida, Mario A. Barbosa

Abstract

Due to their immunomodulatory and chemotactic properties, hMSC are being explored to treat immune-related diseases. For their use in human therapies, it is necessary to culture hMSC in xeno-free conditions. In this study, the impact that a xeno-free medium based on a human plasma derivative has on these properties was analysed. Bone marrow derived hMSC preserved their immunosuppressive and immunostimulatory properties, as observed with *in vitro* assays with hMSCs co-cultured with Mixed Leukocyte Reactions or with mitogen-stimulated leukocytes. Moreover, hMSC expanded in xeno-free medium were recruited by macrophages in both migration and invasion assays, which indicates that the cells maintained their chemotactic properties. These data suggest that xeno-free expanded hMSC preserved their immunomodulatory and chemotactic properties, indicating that the described xeno-free media composition is a potential candidate to culture and expand hMSC for human cell therapies.

1. Introduction

Human mesenchymal stem/stromal cells (hMSC) are a promising tool in regenerative medicine and for treatment of immune-mediated diseases [Shi et al, 2010]. There are currently more than 600 clinical trials evaluating the use of hMSC for different therapies (search of term 'Mesenchymal Stem/Stromal Cell') [ClinicalTrials]. Some of these therapies are related with the differentiation capacity of hMSC, to promote bone repair or to regenerate cartilage [Veronesi et al 2013; Vilquin et al, 2006], while others are related with the immunomodulatory properties, the capacity to secrete trophic factors, promote vascularization and inhibit cell death, such as in graft-versus-host-disease (GvHd), kidney transplant rejection, ischemic cardiomyopathy and progressive multiple sclerosis amongst other applications [Portmann-Lanz et al 2006; Casiraghi et al 2016; Golpanian et al 2016; Dulamea et al 2015].

hMSC exert immunomodulatory effects on cells of both the adaptive and innate immune systems, in a way that depends on the source of hMSC, number of passages in culture, the specific niche where hMSC are [Ma et al 2014], the type of culture (in suspension or attached) [Gornostaeva et al, 2011, 2013] and the confluence of the culture [Lee MW et al 2013; Kim et al 2014]. The balance between the stimuli received by hMSC determines the acquisition of an immunosuppressive or an immunostimulatory behaviour [Mounayar et al 2015]. The mechanism through which hMSC exert their immunomodulatory action is not fully understood but it is known that hMSC must be primed to produce immunosuppressive mediators. Pro-inflammatory factors secreted by immune cells, such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ), can prime hMSC, inducing synthesis of prostaglandin E2 (PGE2) and activating production of indoleamine 2,3-dioxygenase (IDO). These two molecules play an important role in hMSC immunosuppressive properties [Meisel et al 2004; Krampera et al, 2006; Sotiropoulou et al, 2006; Ren et al 2008.

Many of the currently explored clinical strategies take advantage of the hMSC capacity of homing to an injury or inflammation site. And indeed, recruitment of MSC to a bone injury has been correlated with its repair [Kumar et al 2012; Granero-Molto et al 2009]. Some studies have tackled which mediators, including cytokines, stimulate and regulate this recruitment [Ponte et al 2007; Anton et al 2012]. And it has become clear that inflammatory mediators lead to increased MSC migration, by directly recruiting the cells, or by stimulating production of matrix degrading enzymes [Tondreau et al 2009] or even promoting expression of homing related molecules by the MSC [Ren et al 2010]. Thus, immune cells that produce these mediators, such as NK cells, macrophages and T cells, can attract MSC [Almeida et al 2012, 2016; Spees et al 2004; Anton et al 2012]. Macrophages are one of the most abundant cell types in an injury area and are particularly strong recruiters of hMSC, through their secretion of soluble mediators [Anton et al 2012; Caires et al 2016].

An important point to consider regarding application of hMSC in clinical therapies is their expansion under xeno-free conditions. hMSC are usually cultured in media supplemented with Foetal Bovine Serum (FBS), which is inherently risky, as cells are exposed to bovine immunogenic proteins. Furthermore, FBS presents high variability between lots and its availability is limited [Kuznetsov et al 2000]. Different human-derived supplements, such as human serum or human platelet lysate, are thus being developed to substitute the use of FBS in the culture of hMSC [Mojica-Henshaw et al 2013; Prata et al 2012; Miwa et al 2012; Chieregato et al 2011; Mimura et al 2011; Astori et al 2016]. Supplementation with human autologous serum is an interesting approach, but that nevertheless presents some disadvantages such as donor variability and its availability [Jung et al 2012; Tunaitis et al 2011]. The use of platelet lysate has led to promising results on hMSC expansion [Capelli et al 2007], but, again with variability depending on the donor, if the supplement is made from one single or a low number of donations [Lohmann et al 2012]. Moreover, the impact of platelet lysate on the immunomodulatory properties of hMSC is controversial, with some studies suggesting that it dampens the immunossupressive

capabilities of the cells [Abdelrazik et al 2011; Copland et al 2013], besides affecting expression of adipogenic and osteogenic markers [Lange et al 2007; Gruber et al 2004]. A promising chemically-defined medium was approved by FDA (StemPro MSC SFM, Invitrogen) to isolate and expand hMSC. Different studies were done to show its suitability to culture hMSC [Jung et al 2012; Wuchter et al 2016], but due to the lack of standardization of the protocols, the results obtained are difficult to compare. Some studies reported a reduction on the potential for hMSC differentiation and on expression of some proteins, when compared with FBS supplemented medium [Jung et al 2012]. Bobis-Wozowicz and colleagues made a comparative study of different commercially available xeno-free media for hMSC expansion [Bobis-Wozowicz et al 2017]. It was found that each medium had a different impact on hMSC properties, and thus, media for hMSC expansion should be chosen depending on the characteristics needed for a specific therapy [Bobis-Wozowicz et al 2017].

With the objective of expanding hMSC for human cell therapies, the pharmaceutical company Grifols has developed a Supplement for Cell Culture (SCC) [Jorquera et al 2012]. SCC is derived from human plasma specifically collected for the production of plasma-derived therapeutic products, following GMP rules. Plasma pools from over 1,000 healthy donors undergo a cold-ethanol industrial fractionation to obtain different drugs, including SCC [Cohn et al 1946]. Previous reports have shown that medium supplemented with SCC can be successfully used to culture hMSC, iPSC, ES and other mammalian cell lines [Rodríguez-Pizà et al 2010; Díez et al 2012, 2015]. It has been shown that hMSC cultured with SCC remain undifferentiated in culture and preserve their adipogenic, osteogenic and chondrogenic differentiation potential [Díez et al 2015], but the impact on immunomodulation and chemotaxis remains unknown.

hMSC expanded in SCC containing medium maintain their adherence to plastic, phenotype and multipotentiality [Díez et al 2015]. Here, we went further to investigate the influence that SCC has on two properties of hMSC that are

important for cell therapy. First of all, we investigated the immunomodulatory properties of xeno-free expanded hMSC. hMSC can prime or suppress different cells of the immune system to modulate an immune response and facilitate tissue recovery [Cassatella et al 2011; Bernardo et al 2013]. To investigate hMSC immunomodulation, proliferation of resting or stimulated leukocytes was quantified when cultured with hMSC. Secondly, the chemotactic properties of xeno-free expanded hMSC were also addressed. If hMSC are used for cell therapy it is important that the hMSC injected go to the site of injury, thus, hMSC need to be recruited by cells and cytokines present in the inflamed tissue. Here, hMSC motility and chemotaxis promoted by macrophages were analysed by time lapse microscopy and using transwell systems.

2. Material and methods

2.1. Xeno-free Medium Preparation

The xeno-free medium used for this study was similar to the one used by Diez et al 2015, but in this present study, platelet lysate and basic fibroblast growth factor were not added. Thus, the final composition used was: DMEM (Life Technologies) supplemented with 15% Supplement for Cell Culture (SCC, Grifols), 10 mg/L Insulin (Life Technologies), 6.7 µg/L Sodium Selenite (Sigma-Aldrich), 2 µL/L Ethanolamine (Sigma-Aldrich-Aldrich) [ISE] and 10 g/L Penicillin/Streptomycin (Gibco) – 'SCC-Medium' [Diez et al 2015]. SCC is supplied in a freeze-dried format, which was reconstituted in 50 mL DMEM. After reconstitution, medium was filtered with 0.4 µm and 0.2 µm filters. Control medium was the commonly used to expand hMSC: DMEM supplemented with 10% hMSC-qualified FBS (Hyclone) and 1% Penicillin/Streptomycin (Gibco) – 'FBS-Medium'.

2.2. Origin and culture of hMSC

hMSC were isolated from human bone marrow by density gradient centrifugation and selection of adherent cells as in Almeida CR, et al. 2012. Bone marrow collection was approved by "Comissão de Ética do Centro Hospitalar de S. João". After written consent, bone marrow was obtained from discarded bone tissues of 3 different patients (females, 40, 52 and 56 years old) undergoing total hip arthroplasty at the Hospital São João (Porto), whom did not present known inflammatory diseases. After isolation and expansion in FBS-Medium for two passages, cells were re-suspended in media with 10% DMSO and placed in a Mr. Frosty freezing container which was placed at -80 ºC for approximately 24 hr, before transferring the cells to liquid nitrogen. Before performing any assay and due to practical reasons, cells were moved to a -80 ºC freezer, where they stayed for a maximum period of one month.

To perform the following studies, frozen aliquots of hMSC were thawed and firstly cultured in FBS-Medium for one passage: hMSC were grown at 37 ºC and 5% CO_2 until reaching 80% confluence, when they were detached by treatment with xeno-free trypsin (TrypLE^{TM} Express, Life Technologies) and counted using Trypan Blue. hMSC were then seeded in T-150 flasks at 3,000 cells/cm^2 in either FBS-Medium, as control, or SCC-Medium, and expanded between one and four passages before any assay was performed. Assays were performed with hMSC in passages ranging from 5 to 10.

2.3. Cell growth kinetics - Popultion Doubling Time

To calculate population doubling time (PDT), 5x10^4 hMSC were seeded in T-25 flasks and passaged every 4 days for two passages in SCC-Medium or FBS-Medium. At each passage, hMSC were detached using xeno-free trypsin and counted by Trypan Blue exclusion. Briefly, cells were washed with PBS and incubated with 1 mL trypsin for 2 minutes at 37 ºC. Then, 1 mL of medium was added to stop the reaction and cells were centrifuged. PDT was determined by the formula PDT = 1/[3.32 (log NH – log N1)/(t2 – t1)], where N1 is the inoculated cell number, NH is the cell number at harvest, t1 the time at seeding and t2 the time at harvesting [McAteer et al 2001].

2.4. Immunomodulatory properties

2.4.1. Immunoregulation assay - MLR

An Immunosuppression Assay was implemented to study hMSC capacity of immunomodulation after expansion in SCC-Medium (Figure 16). Each Mixed Leukocyte Reaction (MLR) was performed with peripheral blood mononuclear cells (PBMCs). PBMCs from one randomly selected donor were used as

Responder cells, whose proliferation was evaluated. As Stimulator cells we used PBMCs from 5 different donors, to increase the likelihood to induce an immune response by the Responder cells. The stimulator cells were treated with a mitosis inhibitor to avoid their proliferation and thus interference in the analysis of proliferating responder cells [Tanaka et al 2012; Bromelow et al 2001; Bartholomew et al 2002]. PBMCs were isolated from buffy coat residues of unrelated healthy volunteers (following the approval and recommendations of the Ethics Committee for Health from Centro Hospitalar S. João (Porto – References 259/11 and 260/11)) by density-gradient centrifugation (LymphoprepTM; Axis Shield). Briefly, buffy coats were diluted in PBS and overlaid carefully on Lymphoprep at a 2:1 ratio. Tubes were then centrifuged during 30 min at 800 g with no break and lowest acceleration at room temperature. The PBMC layer was collected and washed 3 times by adding cold PBS up to 40 mL and centrifuging at 300 g during 10 min. Finally cells were resuspended in 10 mL PBS (Figure 17).

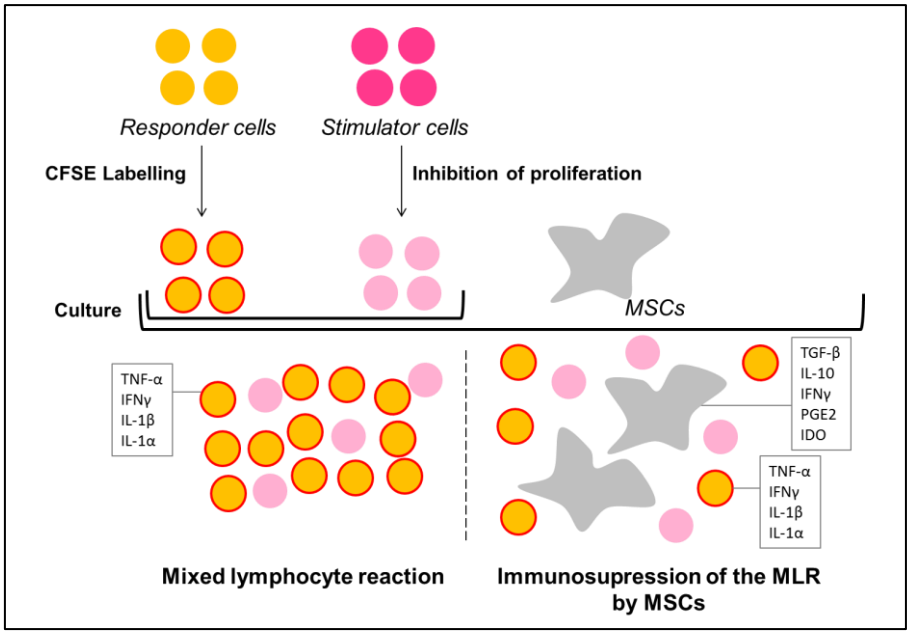

Figure 16. Schematic representation of an Immunosuppression Assay. CFSE-Labelled Responder Lymphocytes are co-cultured with Mitomycin-inhibited Stimulator Cells from un-related donors. In a MLR, Responder Cells recognize the allogenic Stimulator Cells, secreting cytokines and stimulating their proliferation. When Responder Cells and Stimulator Cells are co-cultured together with hMSC, the cytokines produced by the Responder Cells prime hMSC. Primed-hMSC act as immunosuppressors, inhibiting Responder's proliferation.

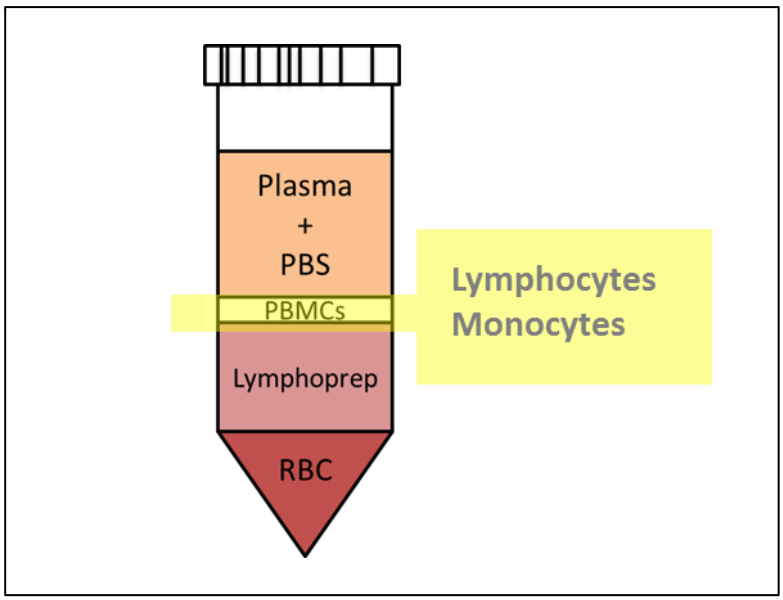

Figure 17. PBMC isolation. Layers formed after buffy coat centrifugation overlaid on Lymphoprep. The PBMC layer was collected.

To obtain a stock of Stimulator Cells, PBMCs from 5 different donors were mixed, with the same quantity of cells from each donor, at a final concentration of 5×10^8 cells/mL in PBS and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich-Aldrich). Aliquots were frozen and stored at -80 ºC until required. For the MLR, proliferation of Stimulator cells was inhibited with Mitomycin C (Sigma-Aldrich). First, the Stimulator Stock vial was thawed and washed and 5×10^7 cells were resuspended in 1 mL PBS. Mitomycin C was then added at a final concentration of 50 µg/mL and incubated for 20 min at 37 ºC. After the incubation time, an excess of RPMI supplemented with 5% FBS was added. The suspension was washed in this medium 3 times to ensure that there was no remaining Mitomycin C in the sample. Finally cells were resuspended in serum-free RPMI (GIBCO, RPMI Medium 1640 (1x) + GlutaMax; Life technologies). Freshly isolated PBMCs from another donor were used as Responder Cells, which were labelled with CFSE (CellTraceTM CFSE; Life Technologies) by incubating 10^7 cells/mL in PBS with 0.5 µM CFSE for 15 min at 37 ºC (concentration optimized

in our laboratory). After washing twice with PBS supplemented with 20% FBS, cells were resuspended in 1 mL RPMI.

To study hMSC immunomodulatory properties, 5 x 10^4 hMSC were seeded in a 24-well flat-bottom tissue-culture plate in SCC-Medium or FBS-Medium and cultured overnight. Then, 5 x 10^5 CFSE-labelled PBMCs (Responder Cells, R) and 5 x 10^5 PBMCs (Stimulator Cells, S) were added to hMSC containing wells, or to new wells, in RPMI-1670 with 2mM L-glutamine (Life Technologies) supplemented with 10% FBS or 15% SCC (depending on the media used for hMSC expansion). After 6 days of culture at 37 ºC and 5% CO_2, supernatants were kept at -80 ºC for future quantification of cytokines. Cells were harvested with trypsin and fixed using a 4% solution of paraformaldehyde (PFA, Sigma-Aldrich). Fixed samples were filtered using a 100 µm pore size nylon membrane and analysed by Flow Cytometry in a FACS Calibur instrument. Data obtained were analysed using FLowJo software.

2.4.2. Immunoregulation assay - Mitogenic stimulation (PHA)

The immunomodulatory properties of hMSC on mitogen-stimulated Responder Cells were also studied. The protocol used was similar to the MLR: hMSC were seeded and incubated overnight and the day after, Responder Cells were added. When indicated, 5 µg/mL phytohemagglutinin (PHA, Sigma-Aldrich) were added to the medium. In this way, the hMSC immunosuppressive properties were studied towards mitogen stimulated Responder Cells, whereas the immunostimulatory properties were studied upon co-culture with non-stimulated Responder Cells.

2.4.3. Cytokine quantification

The medium of the different conditions was collected at the end of the assay and centrifuged at 400 g for 10 min. Supernatants were collected and stored at -80ºC until ELISA analysis. TNF-α was measured with a Human TNF-alpha ELISA Kit (RayBio). Immunoassays were performed according to manufacturer's instructions. Absorbance of ELISA plates was read on a microplate reader (Sunrise™ Tecan).

2.5. Monocyte isolation and differentiation to macrophages

Monocyte-enriched populations were obtained from human buffy coats from 4 different healthy donors (kindly provided from Centro Hospitalar S. João), by negative selection using a Tetrameric Antibody Complexes kit (RosetteSep, StemCell Technologies) as in Almeida et al 2012. Macrophages were obtained by allowing *in vitro* differentiation of the isolated monocytes: $2x10^5$ monocytes/well were plated in 24-well companion plates (Falcon) and cultured at 37 ºC, 5% CO_2 during 10 days in RPMI medium supplemented with 10% FBS. The purity of macrophages was determined examining cell morphology under the microscope and by analysis of CD14 and human leukocyte antigen (HLA)-DR expression by flow cytometry, which was found to be 70-85%.

2.6. Migration and invasion assay

Recruitment assays were performed in 24-well plates using Inserts with a membrane with 8 µm pores (BD Biosciences). Membranes were incubated before the assay with 100 µL of bovine gelatine (0.1% in PBS) for 1h at 37 ºC and washed with PBS. Invasion assays were performed with an insert with the same characteristics, but Matrigel-coated (Corning® BioCoat™ Matrigel™).

These membranes were incubated with DMEM for 1h at 37ºC. After the incubation time, the lower compartments of the chambers were filled with 750 µl DMEM, to evaluate basal motility, DMEM with 30 % FBS, which contains a mix of soluble stimuli that recruit different cells types, as a positive control, or DMEM with macrophages (approximately $2x10^5$ cells/well), as an inflammatory stimuli. Macrophages were not harvested, with the recruitment assay being performed in the same plate where monocytes were platted and allowed to differentiate. Then, $4x10^4$ hMSC previously expanded in SCC-Medium and FBS-Medium were seeded into the upper compartment in 500 µL DMEM, obtaining a 1:5 hMSC:Macrophage ratio. hMSC from 3 different donors were used.

Chambers were incubated at 37 ºC, 5% CO_2 for 7 hours, in the case of migration assays, and 24 hours for invasion assays. After incubation, membranes were washed with PBS and cells were fixed with 4% PFA for 15 min at RT, followed by a washing step with PBS. Inserts were kept in PBS at 4 ºC until analysis. For analysis, cells on the top part of the membrane were removed with a cotton swab, and the membrane was cut and mounted on a slide with 4 µL Vectashield mounting medium with DAPI (Vector Laboratories). Cells that migrated were counted on an inverted fluorescence microscope at x200 fields of view. An average of the number of cells in 10 fields of view was calculated for each membrane. Chemotactic indexes were calculated by dividing the average number of hMSC that crossed the membrane in the experimental condition by the average number of hMSC that crossed the membrane in the negative control.

2.7. Time lapse assay

To study the influence that SCC had on hMSC motility, 5,000 hMSC from three different donors expanded in SCC-Medium or FBS-Medium were seeded in a 24 well plate in serum-free DMEM medium (without SCC, FBS or ISE). After

overnight incubation, image acquisition was performed every 10 minutes during 12 hours at 37 ºC and 5% CO_2 using the IN Cell Analyser 2000 (GE Healthcare). Images from four randomized positions per well were obtained in brightfield with a 10x objective. The percentage of motile cells was calculated by dividing the number of motile cells by the total number of cells that appeared in each video. Cell velocity of three randomly selected cells in each video was measured using Fiji program.

2.8. Morphological evaluation

Morphometric analysis of hMSC in the different media was performed from 50x microscope images. Cells in contact with the border were discarded. The area and 'circularity' of each cell were determined with ImageJ software. Circularity is calculated by ImageJ software using the formula 4pi(area/perimeter^2). A circularity value of 1 indicates a perfect circle and as it approaches 0, it indicates an increasingly elongated polygon [ImageJ 2016].

2.9. Statistical analysis

Statistical analysis was performed using GaphPad Prism software, v5.01. Normal distribution of populations was verified with D'Agostino-Pearson omnibus test. The comparison of two populations was performed using the parametric paired or unpaired T-test or Mann-Whitney test (unpaired test). The comparison of three or more samples was done using the non-parametric Kruskal Wallis test, followed by Dunn's multiple comparison test. Differences between samples were considered statistically significant when *P* values were < 0.05 (*), < 0.01 (**) and < 0.001 (***).

3. Results

3.1. Xeno-free hMSC preserve their immunomodulatory properties

In order to determine the impact of SCC-medium on MSC immunomodulatory properties, cells were expanded in medium containing SCC, similar to the one used by Diez et al [Diez et al 2015]. However, here, basic fibroblast growth factor and platelet lysate were not added as the later might impact on MSC immunomodulation. The Population Doubling Time (PDT) of cells expanded in this medium was calculated with hMSC from two different donors. The average PDT was 2.7 days for cells in FBS-Medium and 7.4 days for cells in SCC-Medium, indicating that hMSC can be expanded in the xeno-free conditions defined here, even though taking 4 to 5 days more to duplicate their population when compared to the control.

The immunomodulatory capability of hMSC was then analysed by quantifying inhibition of proliferation of alloreactive T-lymphocytes in Mixed Leukocyte Reactions (MLR; Figure 16). The results obtained were diverse, but two distinct patterns could be observed (Figure 18A), corresponding to the two MSC types defined by Waterman *et al*: MSC1, for immunostimulatory MSC, and MSC2 for immunosuppressive MSC [Waterman et al 2010]. Generally, even though differences were not statistically significant, when the percentage of proliferative Responder cells was higher than 30% in the control MLR (without hMSC), adding hMSC resulted in suppression of this proliferation (43.71$\pm$21.23 % reduction of proliferative cell number) (Figure 18A, i). On the other hand, when the percentage of proliferative cells was lower than 30% in the control MLR, hMSC did not affect T cell proliferation, or acted as immunostimulatory agents and led to an increase in the percentage of proliferative cells (208.2$\pm$451.2 %) (Figure 18A, ii). This trend was observed both for hMSC expanded in SCC-Medium and hMSC expanded in FBS-Medium (Figure 18A).

As the MLR did not always lead to high proliferation of Responder Cells, and in order to obtain more reproducible data, the capacity of hMSC to suppress proliferation was studied against mitogen-stimulated PBMCs, as performed elsewhere [Krampera et al 2006; Carrade et al 2012]. As shown in Figure 18B, hMSC cultured in xeno-free conditions were able to inhibit proliferation of mitogen-stimulated PBMCs. When cells were stimulated with 5 µg/mL PHA, the mean percentage of proliferative cells was 70±21 % (medium containing FBS) and 76±16 % (medium containing SCC) and the presence of MSC led to a mean reduction of 43±13 % for cells expanded in SCC-Medium, and 46±23 % when using FBS-Medium (no statistically significant differences, paired t-test). On the other hand, immunostimulation was observed when culturing resting PBMCs with hMSC ((Figure 18C). The presence of xeno-free hMSC increased 260±153 % the number of proliferative PBMCs, and the control hMSC increased 280±167 % (no statistically significant differences, paired t-test).

We further analysed the levels of TNF-α in these cultures ((Figure 18D). TNF-α levels were low in resting PBMCs (R), independently of their co-culture with hMSC and medium used. Mitogen-stimulated PBMCs secreted higher levels of TNF-α when compared with resting PBMCs ($p<0.05$, $n=4$, Kruskal-Wallis test followed by Dunns multiple comparison test). In the presence of hMSC, the levels of TNF-α were no longer different from the unstimulated control (R). And although it is not statistically significant, there was a tendency of hMSC to decrease the production of TNF-α by mitogen-stimulated PBMCs (19±12 % reduction in SCC-hMSC and 40±29 % in FBS-hMSC). Generally, TNF-α levels were lower in SCC-Medium than in FBS-Medium.

Taken together, these data indicate that expansion of hMSC in SCC-Medium did not interfere with the immunosuppressive and immunostimulatory properties of the cells.

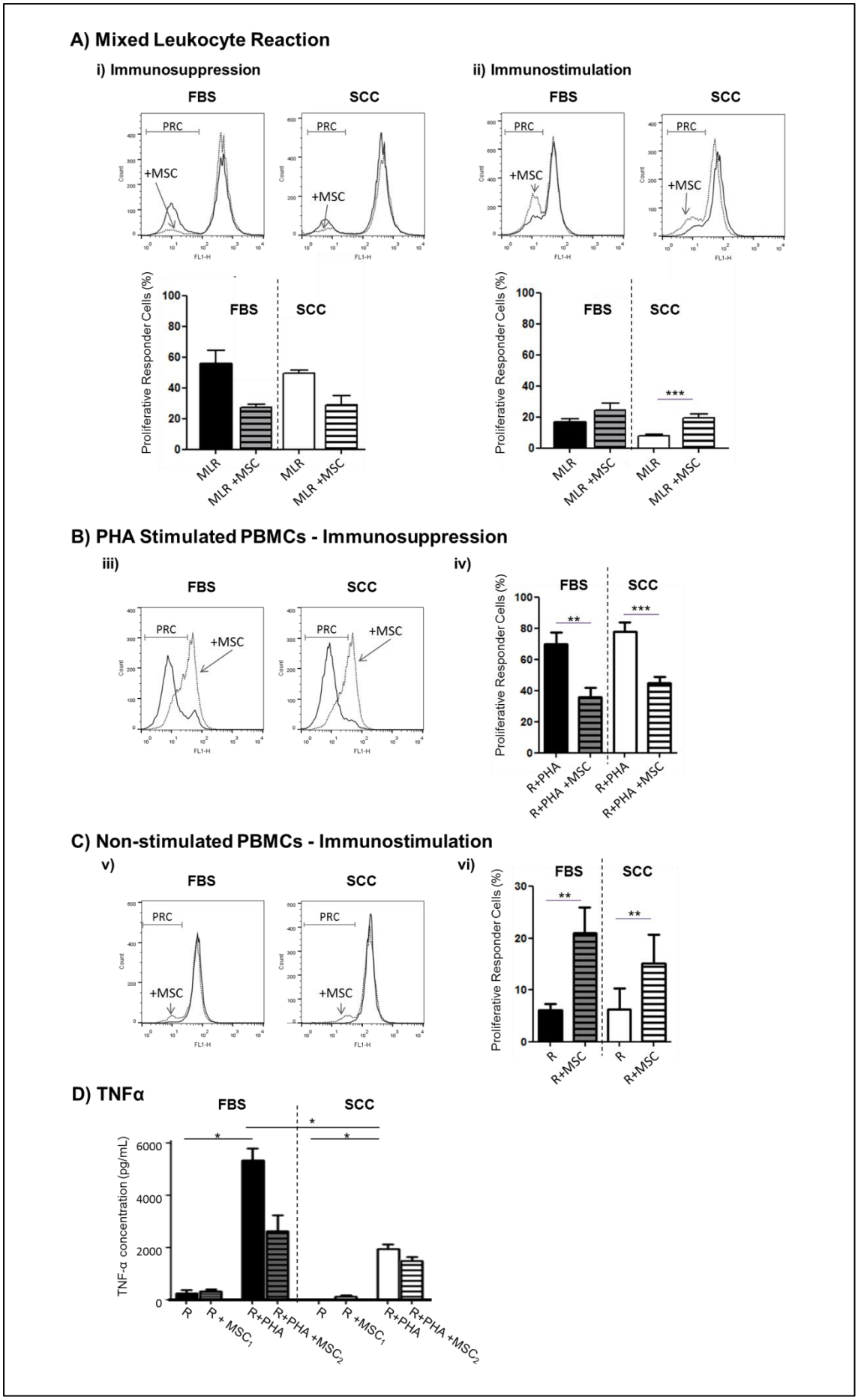
A) Mixed Leukocyte Reaction
i) Immunosuppression
ii) Immunostimulation
FBS
SCC
PRC
+MSC
Count
FL1-H
Proliferative Responder Cells (%)
MLR
MLR +MSC
***
B) PHA Stimulated PBMCs - Immunosuppression
iii)
iv)
**
R+PHA
R+PHA +MSC
C) Non-stimulated PBMCs - Immunostimulation
v)
vi)
R
R+MSC
D) TNFα
TNF-α concentration (pg/mL)
R + MSC1
R+PHA +MSC2
R +MSC1
R+PHA +MSC2

Figure 18. Immunomodulation by xeno-free hMSC. (A) Analysis of the percentage of Proliferative responder cells (PRC) present in a Mixed Leukocyte Reaction (MLR) in the absence (continuous line) or in the presence of MSC (discontinuous line, indicated by "+MSC") (n=10, with 3 different hMSC donors). Representative comparative histograms (top) and bar graphs (bottom) of Immunosuppression (i) and Immunostimulation (ii) (n=4-18, with 3 different hMSC donors; paired T-test). (B) Immunosuppression - Proliferation of R cells was stimulated with PHA. iii) Histograms show examples of hMSC immunosuppression (expanded in FBS- or SCC- Medium). iv) Summary of the collected data - The percentage of Proliferative R is indicated (n=8-9, with 3 different hMSC donors; paired T-test). (C) Immunostimulation - Proliferation rate of resting PBMCs compared with the one obtained when co-culturing the same cells with hMSC. v) Histograms of these two conditions with hMSC expanded in FBS-Medium or SCC-Medium. (vi) Summary of the collected data - The percentage of Proliferative R is indicated (n=8, with 3 different hMSC donors, paired T-test). (D) TNF-α production in different cultures. Supernatants of resting PBMCs (Responder Cells, R) or PBMCS stimulated with 5 µg/mL PHA (R+PHA) in co-culture or not with hMSC were collected and analysed by ELISA. Assays were done in FBS containing medium when hMSC were expanded in FBS-Medium or in SCC containing medium for hMSC expanded in SCC-Medium. (MSC1: hMSC with immunostimulatory properties; MSC2: hMSC with immunosuppressive properties)(n=4, Kruskal-Wallis test followed by Dunns multiple comparison test).

3.2. Xeno-free expanded hMSC are attracted by macrophages

The capacity of hMSC to be recruited to an inflammation area is important for many of its clinical applications. It has been shown that hMSC can be recruited by immune cells, such as macrophages, NK cells and T cells [Almeida et al 2012, 2016; Spees et al 2004; Anton et al 2012]. To study whether hMSC expanded under xeno-free conditions can be recruited by macrophages, a migration and an invasion assays were performed. These assays are based on the Boyden chamber principle, with hMSC cultured on the top chamber and macrophages on the bottom chamber (Figure 19A). The transwell system used for the invasion assay was coated with Matrigel, giving us information about both the migratory capacity and also the capacity of hMSC to degrade and invade through extracellular matrix. This invasion ability of hMSC is important for the cells to reach the place of injury and/or inflammation. In migration assays, membranes are coated with gelatine

at a very low concentration, so that adhesion is promoted but a matrix is not formed, giving us a measure of cell migration alone.

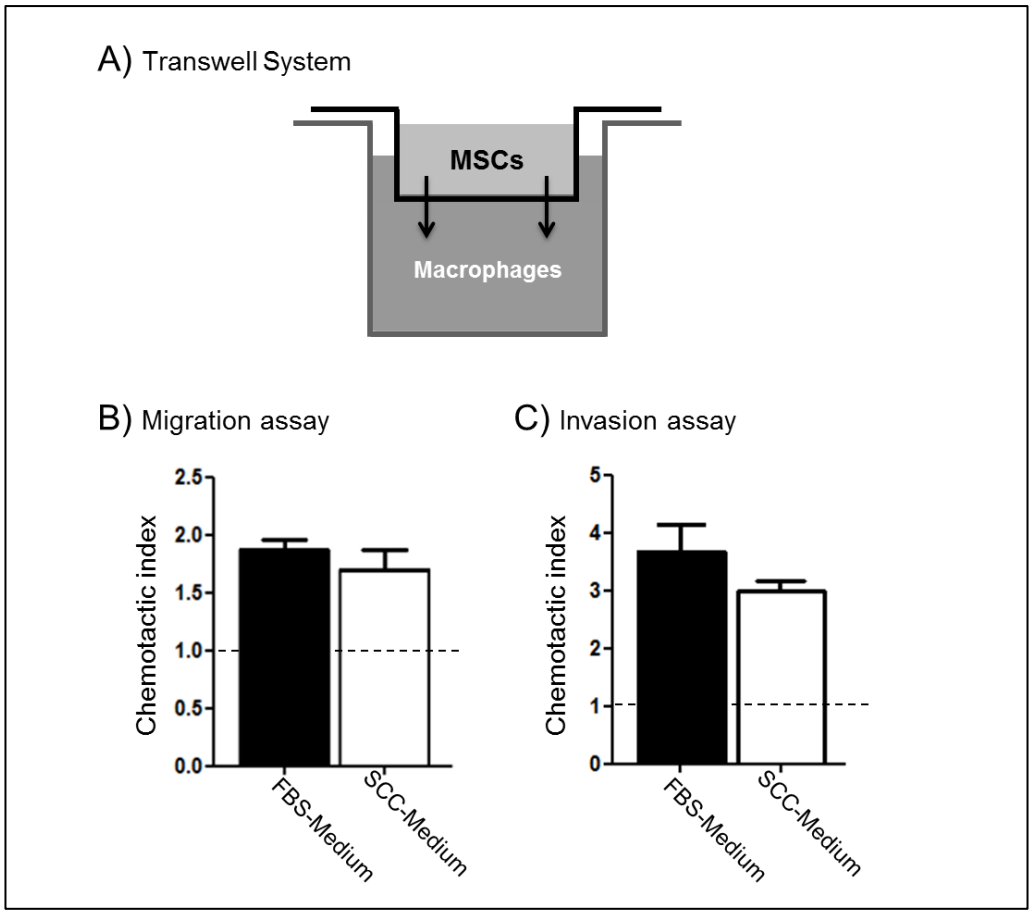

Figure 19. hMSC expanded in SCC-Medium maintain their chemotactic properties towards macrophages. Recruitment assays were performed in 24-well plates using a transwell chamber (A). The lower compartment was filled with serum-free medium (Negative control) or serum-free medium with macrophages. hMSC expanded in FBS-Medium or SCC-Medium were added to the upper compartment and allowed to migrate for 7 hours in the migration assay (B) or for 24 hours in the invasion assay performed with the membrane coated with Matrigel (C). No statistical differences were observed among the different media used (n=4, Mann-Whitney test). The dashed line indicates the chemotactic index in the negative control (1).

Macrophages were able to recruit hMSC cultured in SCC-Medium in both migration and invasion assays, showing that xeno-free expanded hMSC maintain their chemotactic and matrix remodelling properties (Figure 19B-C). No significant differences were observed between the use of SCC-Medium or FBS-Medium to expand hMSC (Man-Whitney test). Although hMSC expanded

under xeno-free conditions showed a chemotactic response towards macrophages, the number of cells that crossed the membranes in both the negative control and the experimental condition was reduced (69-90% reduction in the migration assay, and 54-74% reduction in the invasion assay), which was also verified with a positive control (Figure 20A-B). To clarify whether xeno-free hMSC present an impaired motility, we performed time lapse microscopy analysis by imaging for 12 hour three different hMSC donors. Quantification of the number of motile cells and the cells velocity showed impairment in the motility of hMSC expanded in SCC-Medium (Figure 20D-E). Careful observation of the movies indicated that the front of hMSC expanded in SCC-Medium could not adhere to the substrate and thus cells did not move in a certain direction (Figure 20C).

Careful observation suggested that the morphology of cells cultured in this medium was slightly different from the one observed in FBS-Medium (Figure 21A-B). It was observed that cells in FBS-Medium presented a more elongated shape with sharp ramifications, while cells in SCC-Medium were more heterogeneous, including cells with elongated shape but also polygonal and circular cells. This observation was corroborated by morphological quantification: 94% of the hMSC cultured in FBS-Medium presented a circularity factor lower than 0.5, while hMSC cultured in SCC-Medium presented a more heterogeneous morphology, only 50% of the cells presenting a circularity factor lower than 0.5 (Figure 21C). This change in morphology could be related with differences on cell polarization, which might be linked with differences on hMSC motility. Therefore, xeno-free expanded hMSC maintained their chemotactic and immunoregulatory properties but showed impaired motility in comparison with hMSC expanded in FBS.

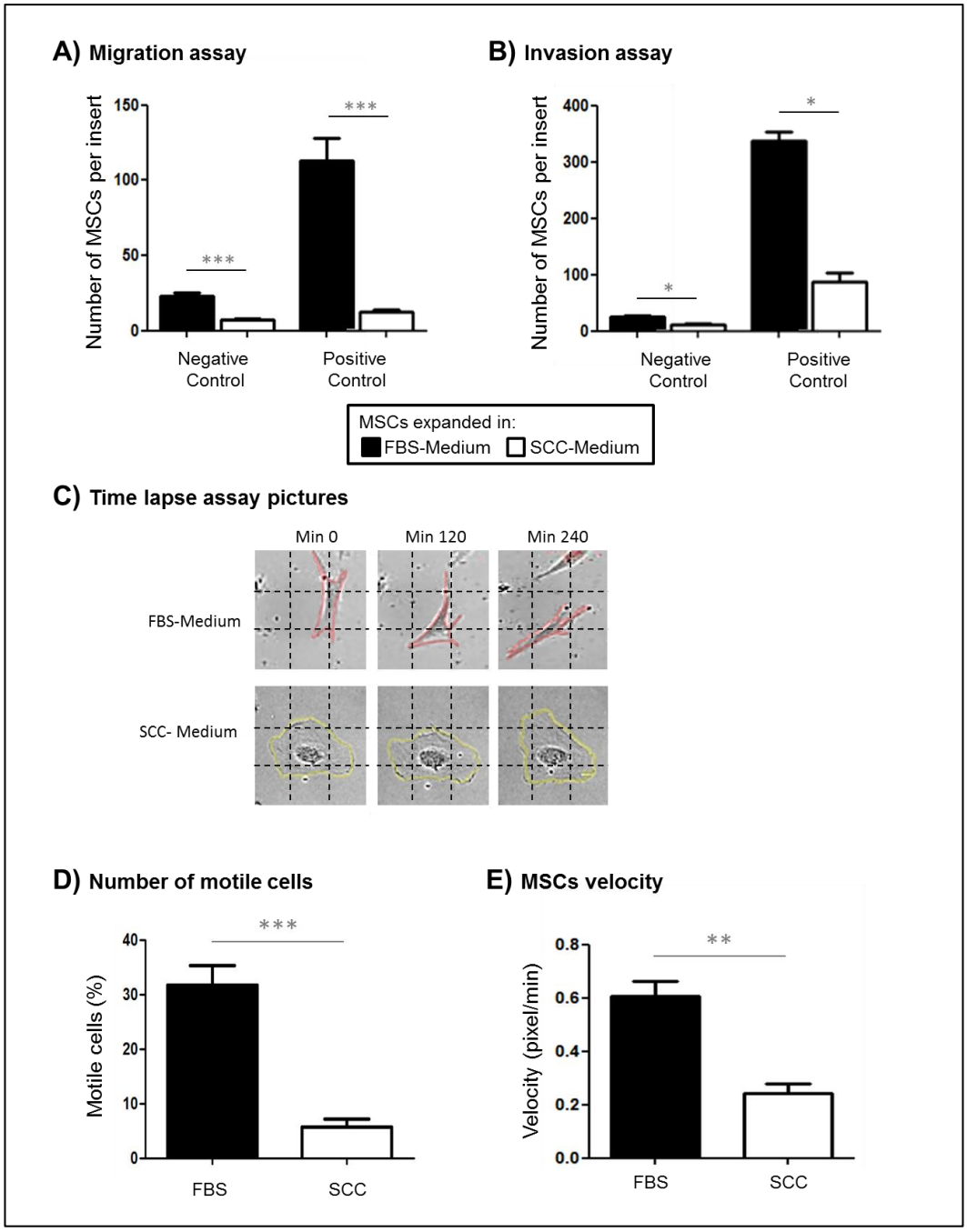

Figure 20. Motility of hMSC cultured in SCC-Medium. (A-B) Recruitment assays - The lower compartment was filled with serum-free medium (negative control) or with medium supplemented with 30% FBS (positive control); hMSC were expanded in SCC-Medium or FBS-Medium (n=4, Mann-Whitney test) (C-E) Time lapse analysis - hMSC expanded in FBS-Medium or SCC-Medium were incubated overnight in DMEM without supplements. Snapshots were taken every 10 minutes for 12 hours (C). The percentage of motile cells was calculated counting the number of motile cells and the total number of cells that appear in each video. (n=10; unpaired t-test) (D). The velocity of 3 randomly selected cells in each video was calculated using Fiji program. The average velocity from hMSC expanded in FBS-Medium and SCC-Medium is shown (n=36; Mann-Whitney test) (E).

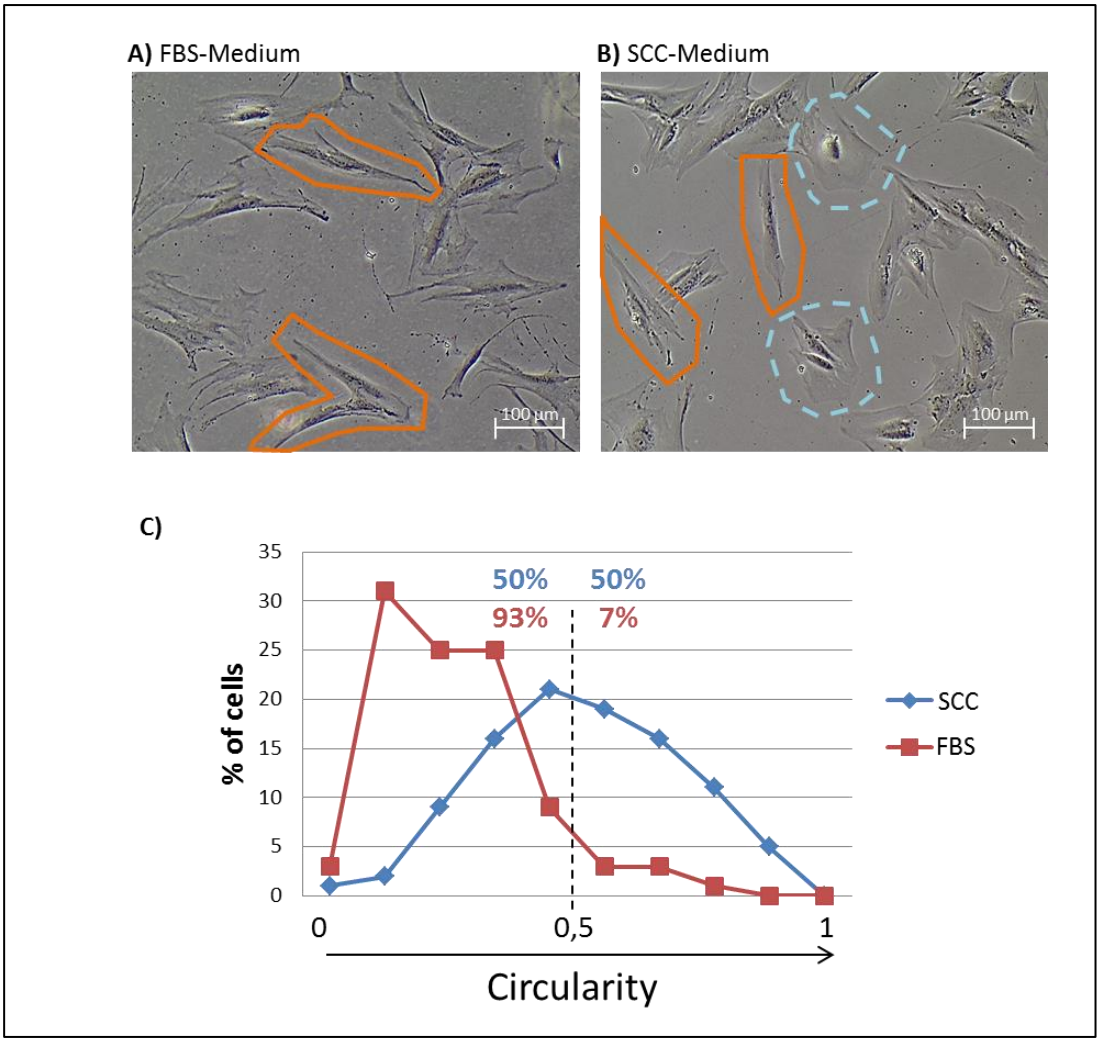

Figure 21. h**MSC morphology.** Brightfield images of hMSC cultured in control (FBS-Medium) (A) and SCC-Medium (B) in passage 5 after 6 days in culture. Elongated cells with sharp ramifications are highlighted in orange (continuous line) and cells with a more polygonal and rounded shape are highlighted in blue (discontinuous line). C) Frequency distribution of hMSC circularity of cells expanded in FBS- or SCC-Medium (3 samples, 2 fields per sample, 13-20 cells per field counted; n=100).

4. Discussion

There is a need for developing new animal-free media to expand hMSC for use in human therapies. Previous studies have shown that a human plasma derived cell culture supplement (SCC) is a potential candidate to substitute FBS. Díez et al showed that this product can be used to expand hMSC, maintaining their phenotype and multipotentiality [Diez et al 2015]. In this present study we have shown that SCC does not interfere with human hMSC chemotactic and immunomodulatory properties.

The immunomodulatory properties of hMSC are of high interest to treat immune-related diseases. Other studies performed in xeno-free conditions only studied hMSC immunosuppression, and showed that hMSC expanded in medium containing platelet lysate have reduced immunosuppressive properties than hMSC expanded in FBS containing medium or serum-free medium [Copland et al 2013; Oikonomopoulos et al 2015; Gottipamula et al 2014]. Here we studied not only the immunosuppressive hMSC properties, but also the immunostimulatory ones. For this, hMSC were co-cultured with resting PBMCs, with mitogen stimulated PBMCs or with a Mixed-Leukocyte Reaction. The results obtained demonstrate that hMSC were able to suppress proliferation of mitogen stimulated PBMCs, independently of their expansion Medium (SCC- or FBS-Medium). Furthermore, xeno-free expanded hMSC and Control-hMSC were able to stimulate proliferation of resting PBMCs, thus maintaining their immunostimulatory properties. The results obtained in the Mixed Leukocyte Reaction were diverse, with hMSC acting as immunosuppressors or immunostimulators depending on the PBMC response. Our results agree with the current view that the immunomodulatory properties of hMSC depend on the niche; in an inflammatory niche, cytokines prime hMSC, so they can act as immunosuppressor agents, but in a niche where no pro-inflammatory cytokines

are present, hMSC act as immunostimulatory agents [Mounayar et al 2015; Meisel et al 2004; Le Blanc 2 et al 2003].

Activation of PBMC proliferation with a mitogen (PHA) led to increased TNF-α level. Introducing hMSC into this pro-inflammatory culture led to a decrease in TNF-α level, both with xeno-free and control-expanded hMSC. These results agree with the idea that activated T lymphocytes secrete TNF-α, which then primes hMSC to act as immunosuppressors and block TNF-α release [Aggarwal et al 2005; Newman et al 2009]. TNF-α levels did not change when PBMCs were resting, suggesting that TNF-α is only related with hMSC immunosuppressive properties but not the immunostimulatory ones. TNF-α levels were overall lower when using SCC as supplement than when using FBS, suggesting that SCC-medium might be affecting cytokine production by the PBMC.

hMSC chemotactic response towards cells of the immune system is crucial in therapies where the hMSC are implanted systemically or far from the site of inflammation. Cells involved in the immune response (such as macrophages and NK cells) are able to attract hMSC [Almeida et al 2012]. Here, xeno-free-expanded hMSC maintained their capacity to be attracted by macrophages. We analysed not only the chemotactic response of xeno-free expanded hMSC (migration assay), but also their ability to degrade the matrix, using matrigel to cover the membrane (invasion assay). MSC showed a higher chemotactic index in the invasion assay than in the migration assay. The chemotactic index is obtained by dividing the number of cells that migrated in the experimental condition by the number of cells that migrated in the negative control. As it is more challenging for the cells to cross the matrigel on the invasion assay, hardly any cell could cross the membrane in the absence of macrophages. We can speculate that the strong effect seen in the presence of macrophages might be due to the fact that besides producing the chemoattractants that are important to stimulate migration, these cells also produce MMPs that contribute to matrix

remodelling. In the case of the migration assay, without any Matrigel, a few more cells can cross the membrane in the negative control, thus, the difference with the number of cells recruited by macrophages is lower, and the resultant chemotactic index is reduced.

Although the chemotactic properties of xeno-free expanded hMSC were preserved, the motility of hMSC was impaired, as found in Time Lapse assays. This impairment in cells motility may be related with the morphological changes observed as the most common motility process used by mammalian cells involves polarized actomyosin-driven shape change [Friedl et al 2010]. It is important that future studies with xeno-free media consider its impact on the cells motility as new formulations should promote both the cells immunomodulatory properties but also their homing capacity.

It has previously been shown that hMSC grown in SCC containing medium maintain their hMSC characteristics, including expression of typical hMSC proteins and differentiation potential into adipocytes, chondrocytes and osteoblasts [Diez et al 2015]. In Diez et al. 2015, commercial bone marrow-derived hMSC were expanded in the same medium used here but with additional platelet lysate and fibroblast growth factor. Platelet lysate supplementation is controversial, and its effect on immunomodulatory MSC properties is not clear, with some studies showing that it reduces hMSC immunosuppressive properties [Abdelrazik et al 2011; Copland et al 2013; Gottipamula et al 2014; Flemming et al 2011].Therefore, in this work we tested the effect of SCC based medium without platelet lysate. The addition of platelet lysate, together with fibroblast growth factor, may have led to a higher proliferation rate in the previous study. Also, this proliferation may have been affected by the age of the MSC donors. Here we opted to use cells from older donors, as it is likely that most people in need of therapies using autologous cells are the elderly. Notwithstanding, and despite these differences, hMSC in SCC-Medium without platelet lysate could be successfully expanded in vitro, while maintaining their immunomodulatory properties.

Apart from preserving hMSC immunomodulatory and chemotactic properties, SCC based xeno-free medium presents some advantages compared with other available xeno-free media. SCC is a GMP product that undergoes different safety steps to avoid the transmission of any disease, and that is produced from more than 10,000 donations, reducing the variability between lots. Platelet lysate would also be an interesting xeno-free supplement, but high variability is expected if the number of donors is low and it might impact on immunomodulation. As Bobis-Wozowicz et al showed, different media can have different effects on each hMSC, thus, it is highly recommended to test which is best for each particular application [Bobis-Wozowicz et al 2017].

5. Conclusion

Here, we report expansion of hMSC in a xeno-free medium without platelet lysate while preserving the cells immunomodulatory and chemotactic properties. As SCC is an industrial GMP product from human origin obtained from a large number of healthy donors, and with an improved safety margin, SCC can be a potential candidate to culture hMSC for human cell therapies.

Chapter 4. Xeno-free expanded hMSC motility

[Article submitted for publication]

FGF IMPROVES THE MOTILITY OF HUMAN MSC EXPANDED IN A HUMAN PLASMA DERIVED XENO-FREE MEDIUM

Arantxa Blázquez-Prunera, Catarina R. Almeida, Mario A. Barbosa

Abstract

Mesenchymal stem/stromal cells (MSC) are being explored for cell therapies targeting varied human diseases. For that, cells are being expanded in vitro, many times with fetal bovine serum (FBS) as the main source of growth factors. However, animal-derived components should not be used to avoid an immune rejection from the patient that receives the hMSC. To solve this issue, different xeno-free media are being developed and an industrial-grade human plasma fraction (Supplement for Cell Culture, SCC) is a promising candidate to substitute FBS. Indeed, hMSC expanded in SCC based medium maintain their phenotype, multipotentiality, genetic stability and immunomodulatory and chemotactic properties. However, the motility of hMSC grown in this medium was reduced when comparing with hMSC motility on FBS based medium. Thus, we tested different factors to improve the motility of bone marrow hMSC expanded in this xeno-free medium.

Time lapse assays and experiments with transwells revealed that supplementation of the xeno-free medium with FGF or PDGF, but not TNF-α or SDF-1, increased hMSC motility. Interestingly, FGF and PDGF supplementation also led to alterations on hMSC morphology to a shape similar to the one observed when using FBS supplemented medium. The mechanism behind the effect of FGF on MSC motility involved the increased expression of the integrin αVβ3. Furthermore, assays with small molecule inhibitors revealed that the signaling molecule p38 MAPK is important for hMSC motility, and that MEK/ERK and PI3K/AKT also have a role on FGF supplemented expanded hMSC. Thus, it was found that FGF supplementation can improve the motility of hMSC expanded in xeno-free medium and that the cells motility is regulated by αVβ3 integrin.

1. Introduction

Human mesenchymal stem/stromal cells (hMSC) are multipotent, non-hematopoietic cells that can differentiate into osteoblasts, chondrocytes and adipocytes[Horwitz et al,2005]. hMSC can be easily isolated from different tissues, such as bone marrow, adipose tissue, peripheral blood and umbilical cord, and can be expanded in culture mantaining their growth and differentiation properties[Singer et al, 2011;Sethe et al, 2006]. Moreover, hMSC are immunomodulatory and immunogenic [Ma, et al 2014; Toubai 2009]. All these characteristics make hMSC interesting for regenerative medicine and the treatment of immune-mediated diseases[Shi 2010]. However, their widespread and safe use in humans has not been achieved yet, due to different concerns.

An important issue that needs to be solved is the medium used for *in vitro* hMSC expansion. Fetal bovine serum (FBS) is ususally used as a cell culture supplement to provide different growth factors and other components that cells need for proper growth. FBS presents different problems, but the most important one is that animal proteins can become associated with cells in culture, and when the cells are transplanted, this might trigger an immune rejection to the host [Spees, 2004]. Thus, the use of FBS has been discouraged by the European Medicines Agency and big efforts are being made to find a safe substitute [European, 2013]. It has been previously shown that an indutrial-GMP human plasma fraction (Supplement for Cell culture, SCC, Grifols) can replace FBS in hMSC cell culture medium [Díez et al, 2015; Blázquez-Prunera et al, 2017].

Efficient hMSC motility is important since the most explored route of administration of hMSC for clinical applications is systemic delivery, requiring proper homing and migration to ensure that enough hMSC arrive to a site of injury. The exact mechanisms of MSC migration are not yet fully understood but it is thought that it may be similar to the leukocyte homing process [reviewed at De Becker and Riet 2016]. And indeed the production of different immune mediators by

immune cell populations such as NK cells or macrophages stimulate recruitment of BM-MSC [Almeida et al, 2016; Caires et al, 2016]. We have previously found that the expansion of hMSC in SCC-based medium (SCC-Medium) leads to a reduced cell motility [Blázquez-Prunera et al, 2017]. Our goal is to improve medium composition for better hMSC migrative properties. Thus supplementation of medium with different factors that have been related with MSC motility, such as FGF, PDGF, TNF-α and SDF-1, was analysed [Sato et al, 2002, Schmidt et al, 2006 ,Naaldijk et al 2015].

2. Methods

2.1. Xeno-free Medium Preparation

The xeno-free medium used is the same used in our previous studies, composed by DMEM F12 (Life Technologies) supplemented with 15% Supplement for Cell Culture (SCC, Grifols), Insulin (Life Technologies), Sodium Selenite (Sigma-Aldrich), and Ethanolamine (Sigma-Aldrich) – 'SCC-Medium' [Blázquez-Prunera et al, 2017].

Addition of different supplements to the SCC-Medium was tested: 20 ng/mL recombinant human FGF-basic (Peprotech), 50 ng/mL recombinant human Platelet-derived growth factor BB (PDGF, Immunotools), 10 ng/mL recombinant human Tumor necrosis factor-alpha (TNFα, Immunotools), or 100 ng/mL recombinant human Stem cell derived factor 1-alpha (SDF-1α, Peprotech).

As control medium, it was used medium commonly used to expand hMSC: DMEM supplemented with 10% hMSC-qualified FBS defined (Hyclone, GE Healthcare Life Sciences) – 'FBS-Medium'.

2.2. Origin and culture of hMSC

Bone marrow hMSC were isolated by density gradient centrifugation and selection of adherent cells as in Almeida CR, et al. 2012. Bone marrow collection was approved by "Comissão de Ética do Centro Hospitalar de S. João". Bone marrow samples used were kindly supplied by Hospital São João (Porto), after written consent. Samples were obtained from discarded bone tissues of total hip arthoplasties of five patients from 50 to 60 years old who did not present known inflammatory diseases. After isolation, BM-hMSC were expanded in FBS-Medium for two passages and resusspended in media with 10% DMSO and frozen in nitrogen.

For these studies, frozen aliquots were firstly thawed and cultured in FBS-Medium for one passage. hMSC were cultured at 37 ºC and 5% CO_2 and when

cell confluence was approximately 80%, cells were harvested using xeno-free trypsin (TrypLE^{TM} Express, Life Technologies) and seeded in the different media to test. hMSC were expanded in SCC-Medium for 1-4 passages before any assay was performed. All assays were performed with hMSC in passages from 4 to 7.

2.3. Time lapse assay

After hMSC expansion in different media, cells were harvested and 5,000 hMSC were seeded in a 24 well plate in serum-free DMEM medium (without any supplementation). After overnight incubation, image acquisition was performed every 10 minutes during 12 hours at 37 ºC and 5% CO_2 using the IN Cell Analyser 2000 (GE Healthcare). Images from four randomized positions per well were obtained in brightfield with a 10x objective. The percentage of motile cells was calculated dividing the number of motile cells by the total number of cells that appeared in each video.

2.4. Cell growth evaluation

hMSC cell growth in the mentioned media was estimated using resazurin (Sigma-Aldrich), a compound that live cells metabolize producing resorufin, a fluorescent intermediate. This assay measures metabolic activity, thus allowing the estimation of cell growth. Cells expanded in FBS, SCC, SCC+FGF and SCC+PDGF media were harvested and 5,000 cells/well were seeded in a 96 well plate in triplicates for each specific medium and incubated overnight. After 24 hours, cell culture supernatants were removed and monolayers were washed carefully with PBS. 200 µL of resazurin diluted into each specific medium at 0.02 mg/mL were added into the wells and incubated for 2 hours. After incubation, supernatants were transferred into 96 well plates to measure fluorescence using a microplate spectrofluorometer (Ex = 540 nm, Em= 590nm) (Fluorimeter

Synergy Mx, BioTek). New medium was added to the wells. The process was repeated at days 3, 5 and 7.

2.5. Transwell assays

Recruitment assays were performed using inserts with a membrane with 8 µm pores (BD Biosciences). To facilitate cell adhesion, the membranes were coated with bovine gelatine (Sigma-Aldrich) (0.1% in PBS). The lower compartments were filled with DMEM, as negative control, or DMEM supplemented with 30% FBS, to stimulate hMSC migration. $4x10^4$ hMSC in 500 µL DMEM were seeded in the upper compartment. Upper and lower compartment were supplemented with FGF or PDGF for cells expanded in SCC+FGF or SCC+PDGF media (at the same final concentration).

hMSC were allowed to migrate at 37 ºC, 5% CO_2 for 6 hours. After incubation, cells were fixed with 4% PFA. hMSC that did not cross the membrane were removed with a cotton swab and the membrane was cut and mounted in a slide with Vectashield mounting medium with DAPI (Vector Laboratories). Cells that crossed the membrane were counted in an inverted fluorescence microscope (Zeiss Axiovert 200M). The number of cells present in 10 fields of view at 200x were counted. Chemotactic indexes were calculated dividing the average number of hMSC that crossed the membrane in the experimental condition by the average number of hMSC that crossed the membrane in the negative control.

2.6. Flow cytometry analysis of integrins (αVβ3 and β1)

Upon harvesting with TrypLE Express, 10^5 hMSC per condition were incubated for 1 hour on ice with anti-αVβ3 (clone 23C6, Biolegend), anti-β1 AlexaFluor 488

conjugated, also called CD29 (clone TS2/16, Biolegend) (1,25 µg/ml), in PBS with 1% BSA and 0.01% sodium azide or the isotype control IgG Alexa Fluor 488 (clone MOPC-21, Biolegend). After washing, cells incubated with anti-αVβ3 were stained for 30 min on ice with AlexaFluor 488-conjugated secondary anti-mouse IgG (colne Poly4053, Biolegend) (1 µg/ml). Cells were washed with PBS and fixed with 4% paraformaldeahyde and kept in PBS at 4ºC until analysis. Before acquisition, samples were filtered using a 100 µm pore size nylon membrane and analyzed by Flow Cytometry in a FACS Calibur instrument. Data obtained was analyzed using FlowJo software. The mean fluorescent intensity (MFI) of FL1 channel was calculated by the software after selecting hMSC cells.

2.7. Immunocytochemistry of αVβ3

hMSCs were seeded on coverslips and incubated at 37 ºC for 3 days in each specific media. After incubation, cells were fixed with 4% paraformaldehyde at room temperature for 10 minutes. For staining, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS at room temperature for 5 minutes. After proper washing, cells were blocked with 5% BSA (Sigma-Aldrich) in PBS for 30 minutes. Anti-αVβ3 was diluted used at 10 µg/ml in PBS containing 1% BSA and incubated with the cells overnight at 4ºC before washing 3 times with PBS with 1% BSA. AlexaFluor 488- conjugated secondary anti-mouse IgG (Invitrogen) was at 2 µg/ml in 1% BSA and incubated with the cells for 45 minutes at 4ºC in the dark. After incubation and gently washing, cells were incubated with Cy3-conjugated anti-Vimentin (10 µg/ml) (clone V9, Sigma-Aldrich) for 1 hour at 4ºC in the dark. Each coverslip was mounted on Vectashield mounting medium with DAPI.

Images were collected on a confocal microscope (Leica TCS SP5) using a 63x oil objective. All images were acquired using the same settings. The presence of

αVβ3 clusters was analysed using the 'analyse particles' function of ImageJ software [Chen et al 2010]. The threshold was set up at a specific level for all images to reduce signal background (Otsu: 0-6355). Particles bigger than 0.85 pixels were counted.

2.8. Blocking of αVβ3 and inhibition of different signaling pathways

hMSC were expanded in different media (FBS- and SCC-Medium with and without FGF or PDGF supplementation). After cell harvesting, cells were platted in a 24 well plate for time lapse analysis as explained before. After overnight incubation, wells were washed with PBS and the cells were pre-treated with DMEM with different inhibitors for 1 hour before image acquisition: MEK/ERK pathway inhibitor (10 µM, PD0325901, Stem Cell Technologies), p38 MAPK inhibitor (14 µM, BIRB-796, Stem Cell Technologies) and PI3K/AKT pathway inhibitor (180 nM, LY294002, Stem Cell Technologies) were used. Moreover the integrin αVβ3 was blocked with the same antibody used before at 10µg/mL in DMEM. Time lapse was performed as previously explained.

2.9. Statistical analysis

Statistical analysis was performed using GaphPad Prism software, v5.01. Normal distribution of populations was verified with D'Agostino-Pearson omnibus test. The comparison of two populations was performed using the parametric paired T-test Comparison of three or more samples was done using ANOVA followed by Bonferronis multiple comparison test, when the data was normally distributed; or Kruskal Wallis test followed by Dunn's multiple comparison test, when data was not normally distributed. Differences between samples were considered statistically significant when *P* values were < 0.05 (*), < 0.01 (**) and < 0.001 (***).

3. Results

3.1. Supplementation of SCC-Medium with FGF or PDGF increase hMSC motility

hMSC motility on SCC-Medium was evaluated by time lapse analysis. Five different BM-hMSC donors were evaluated in 14 different assays. All donors showed a decrease on the percentage of motile cells in SCC-Medium when comparing with the control, in FBS-Medium (25-98% reduction, mean 64%) (Figure 22A).

In order to improve the behavior of hMSC expanded in SCC-Medium, it was studied if supplementation of SCC-Medium with FGF, PDGF, TNFα or SDF-1 could improve hMSC motility. In this case, 3 different hMSC donors were used in 8 different assays. The addition of FGF or PDGF into SCC-Medium led to a significant increase on hMSC motility (258% and 297% respectively, when compared with SCC-Medium). Moreover, the percentage of motile cells was even higher than with cells expanded in FBS-Medium (51% and 67% increase, respectively). Supplementation of SCC-Medium with TNFα or SDF-1 showed a lower increase on hMSC motility (84% and 27% increase comparing with SCC-Medium, with 22% and 46% less than in FBS-Medium) (Figure 22B). Supplementation of FBS-Medium with FGF had no effect on hMSC motility while supplementation with PDGF increased the percentage of motile cells by 150% (Figure 22C).

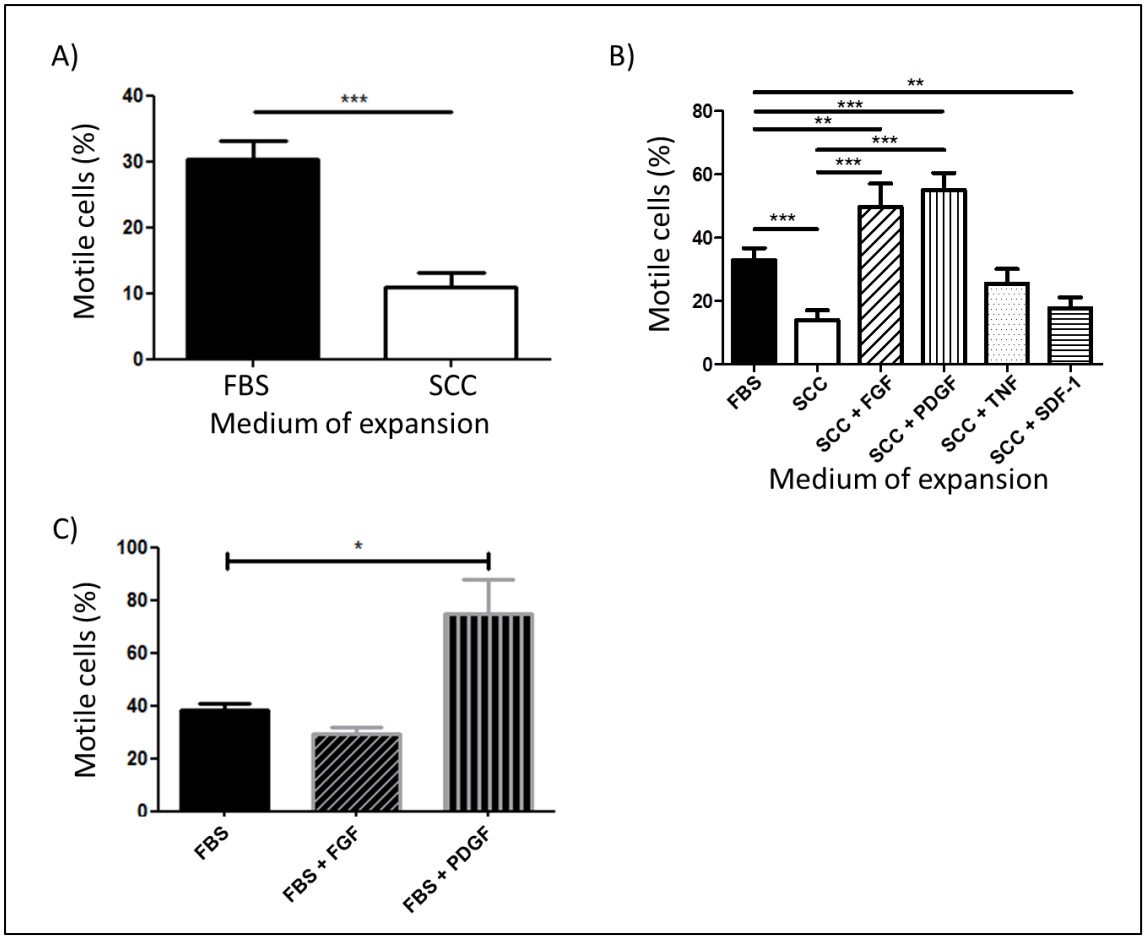

Figure 22. Percentage of motile cells analyzed by time lapse assays. A) BM-hMSC were expanded using FBS-Medium or SCC-Medium (n=14, 5 hMSC donors, paired t-test). **B)** BM-hMSC were expanded in FBS-Medium, SCC-Medium with/without supplementation with FGF, PDGF, TNFα or SDF-1. (n=8, 3 hMSC donors, repeated measurements ANOVA and Bonferronis Multiple Comparison test). **C)** BM-hMSC were expanded in FBS-Medium with/without FGF and PDGF supplementation. (n=4, 1 hMSC donor, repeated measurements ANOVA and Bonferronis Multiple Comparison test).

3.2. FGF and PDGF lead to a change on hMSC morphology and increased cell growth

The morphology of xeno-free expanded hMSC was clearly altered by the addition of FGF or PDGF to the medium (Figure 23A). Cells in SCC-Medium were more polygonal and circular than cells in FBS-Medium, which presented a more elongated shape with sharp ramifications. Supplementation with either FGF or

PDGF into SCC-Medium made cells look more elongated and with the sharp ramifications observed in hMSC cultured in FBS-Medium.

Supplementation of the SCC-Medium with FGF also had an effect on the cell growth kinetics, as estimated by measuring the cells metabolic activity (Figure 23B). After 7 days of culture, hMSC in FBS-Medium triplicated their population. On the other hand, cells in SCC-Medium only increased 50% its population. Supplementation with PDGF did not produce a high difference on cell growth while the addition of FGF promoted cell expansion, with 2.5 times more cells in the 7^{th} day that in day 1.

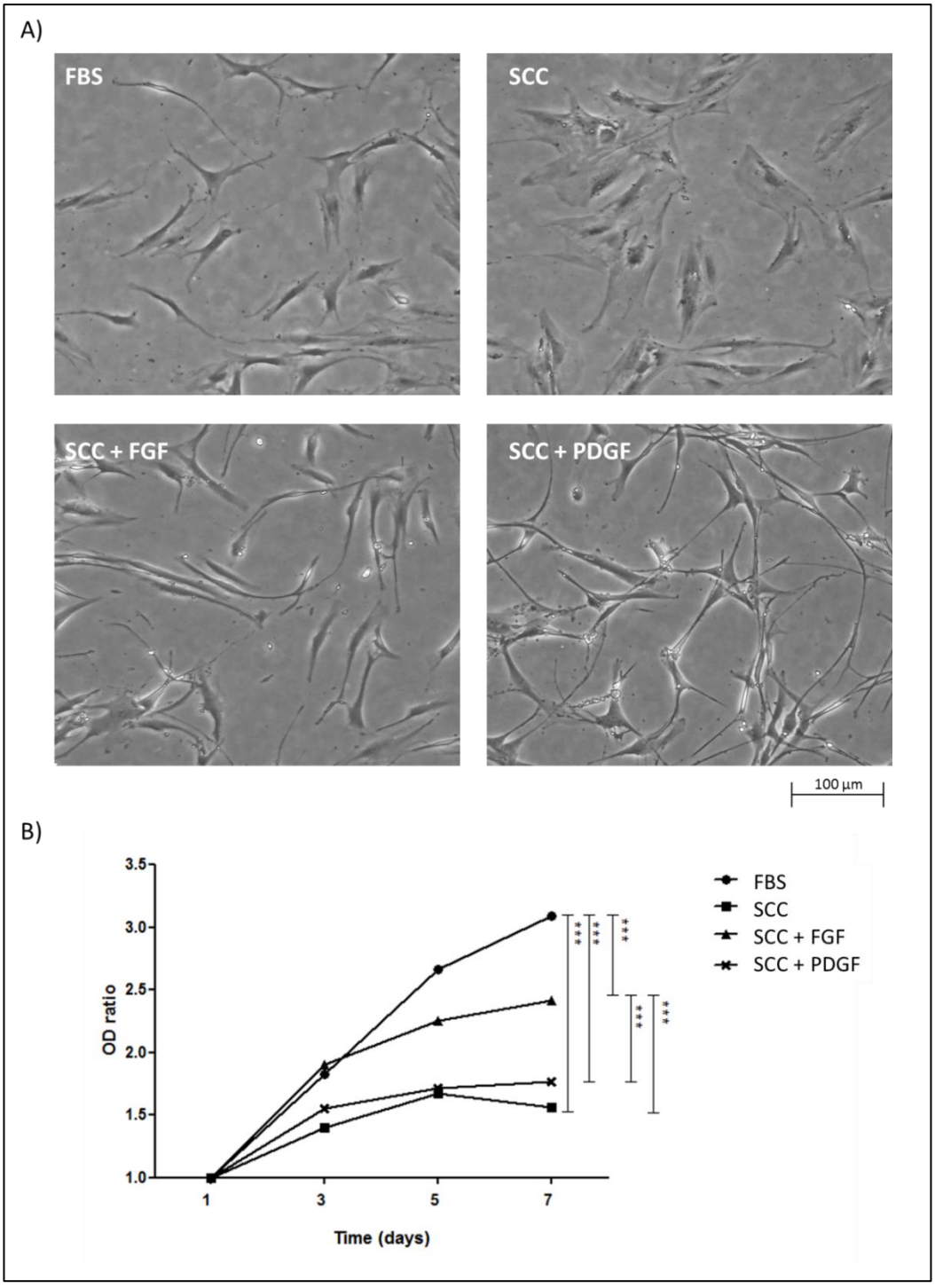

Figure 23. hMSC morphology and proliferation. A) Illustrative brightfield images (objective 5x) of hMSC at passage 6 cultured in FBS, SCC, SCC+FGF and SCC+PDGF medium for 3 days. **B)** Cell growth curves of hMSC expanded in the different media (stats shown for day 7, n=5, 2 hMSC donors; two-way ANOVA followed by Bonferroni test).

3.3. The chemotactic properties of hMSC expanded in SCC-Medium are mantained

The chemotactic properties of hMSC expanded in SCC-Medium supplemented with FGF and PDGF were further studied. Using a migration chamber, motility of hMSC towards FBS was analysed. The chemotactic index was similar in all conditions (Figure 24A). However, differences in the total number of cells that crossed the membrane were found. Cells expanded in SCC-Medium migrated 65% less than cells expanded in FBS-Medium. When SCC-Medium was supplemented with FGF the number of cells that migrated was similar to the ones that migrated in FBS-Medium. Supplementation of the SCC-Medium with PDGF showed a tendency to increase hMSC motility (94% more than SCC-Medium) but to a lower level than FGF (Figure 24B).

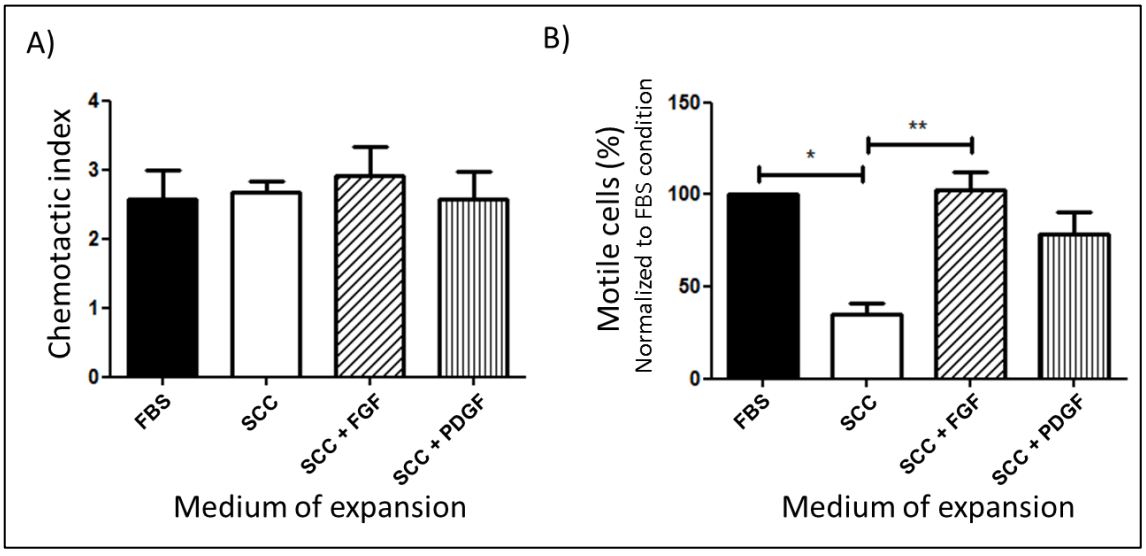

Figure 24. hMSC recruited by FBS. hMSC were expanded in FBS- or SCC-Medium with/without FGF and PDGF supplementation. DMEM supplemented with 30% FBS was used to recruit hMSC. **A)** Chemotactic index (n=6, Kruskal Wallis followed by Dunns multiple comparison test) **B)** Percentage of motile cells recruited by FBS. Normalized to the number of cells expanded in FBS-Medium that crossed the membrane. (n=6, Kruskal Wallis followed by Dunns multiple comparison test)

3.4. FGF supplementation of SCC-Medium increases expression of αVβ3

In order to dissect the mechanism behind the improved motility of cells expanded in SCC medium with supplementation, we started by analyzing expression of integrins that have been described to be regulated by FGF [Enenstein, 1992]. Thus, the surface expression of αVβ3 and β1 integrins was analyzed by flow cytometry. Expression of αVβ3 was similar in hMSC expanded in FBS-Medium, SCC-Medium and SCC-Medium supplemented with PDGF, while hMSC expanded in SCC-Medium supplemented with FGF showed a higher expression of αVβ3 (86% more than in FBS condition) (Figure 25A). No differences were observed on the expression of β1 (Figure 25B).

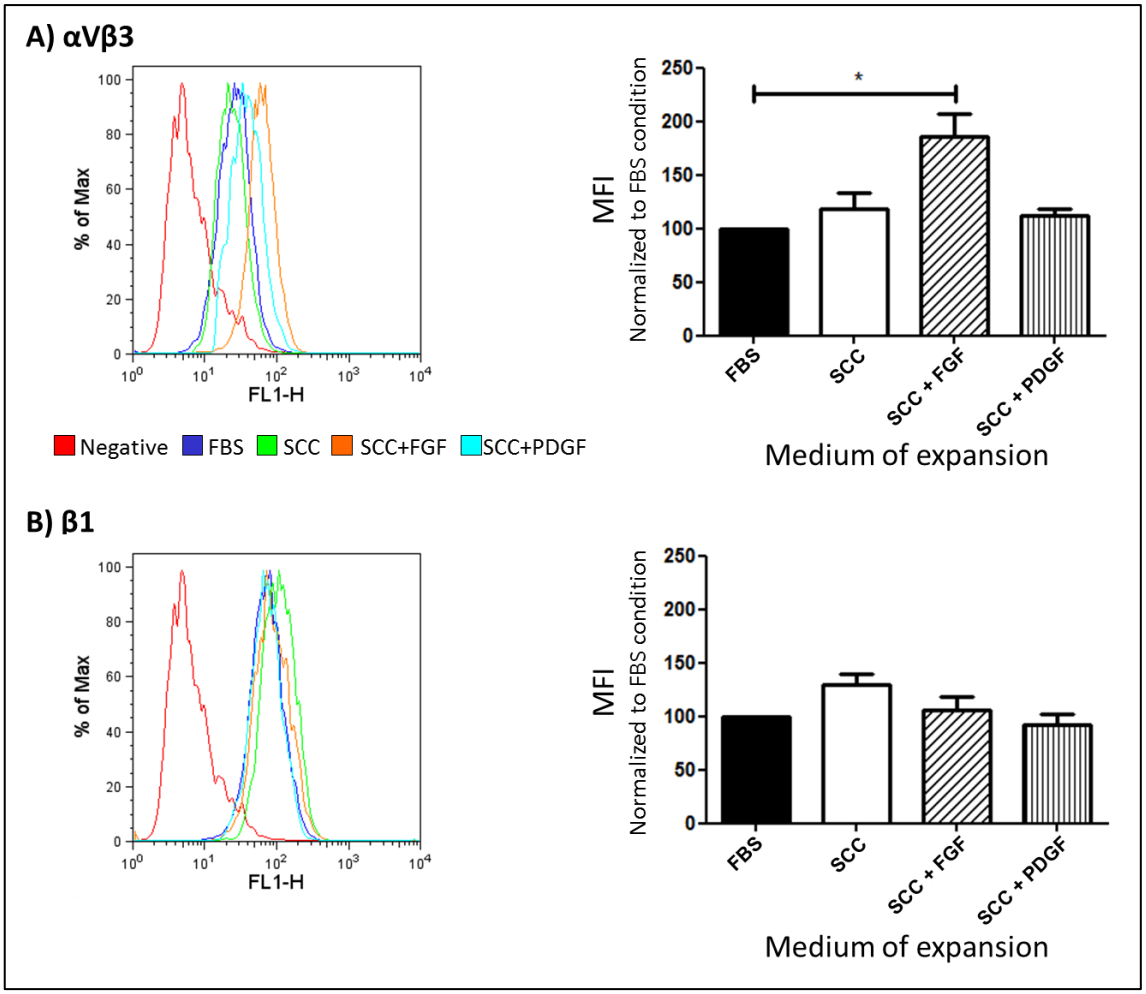

Figure 25. Integrin staining and analysis by flow cytometry of hMSC expanded in FBS-Medium and SCC-Medium with/without FGF or PDGF supplementation. Representative histograms from flow cytometry analysis (Negative control: hMSC stained with isotype control). **A)** Mean fluorescence intensity (MFI) resulting from staining of αVβ3. Data was normalized to the level presented by cells grown in FBS-medium (n=5, Kruskal Wallis followed by Dunns multiple comparison test). **B)** MFI resulting from staining of integrin β1. Data was normalized to the level presented by cells grown in FBS-medium (n=4, Kruskal Wallis followed by Dunns multiple comparison test)

Moreover, expression and distribution of αVβ3 clusters was also analyzed by immunocytochemistry (Figure 26). The percentage of cells expanded in SCC-Medium that presented αVβ3 clusters was 63% lower than the one observed when hMSC were expanded in FBS-Medium. Supplementation of SCC-Medium with FGF or PDGF increased the number of cells with αVβ3 clusters to a

percentage similar to cells grown in FBS-Medium (86-89%) (Figure 26 A, B). Furthermore, hMSC expanded in FBS-Medium showed more clusters per area than cells in any other medium. And, even though the cells morphology and area was different in SCC-medium, it was possible to see that adding FGF or PDGF to SCC-Medium increased the expression of αVβ3 by 4.6 and 7.2 times respectively (Figure 26B).

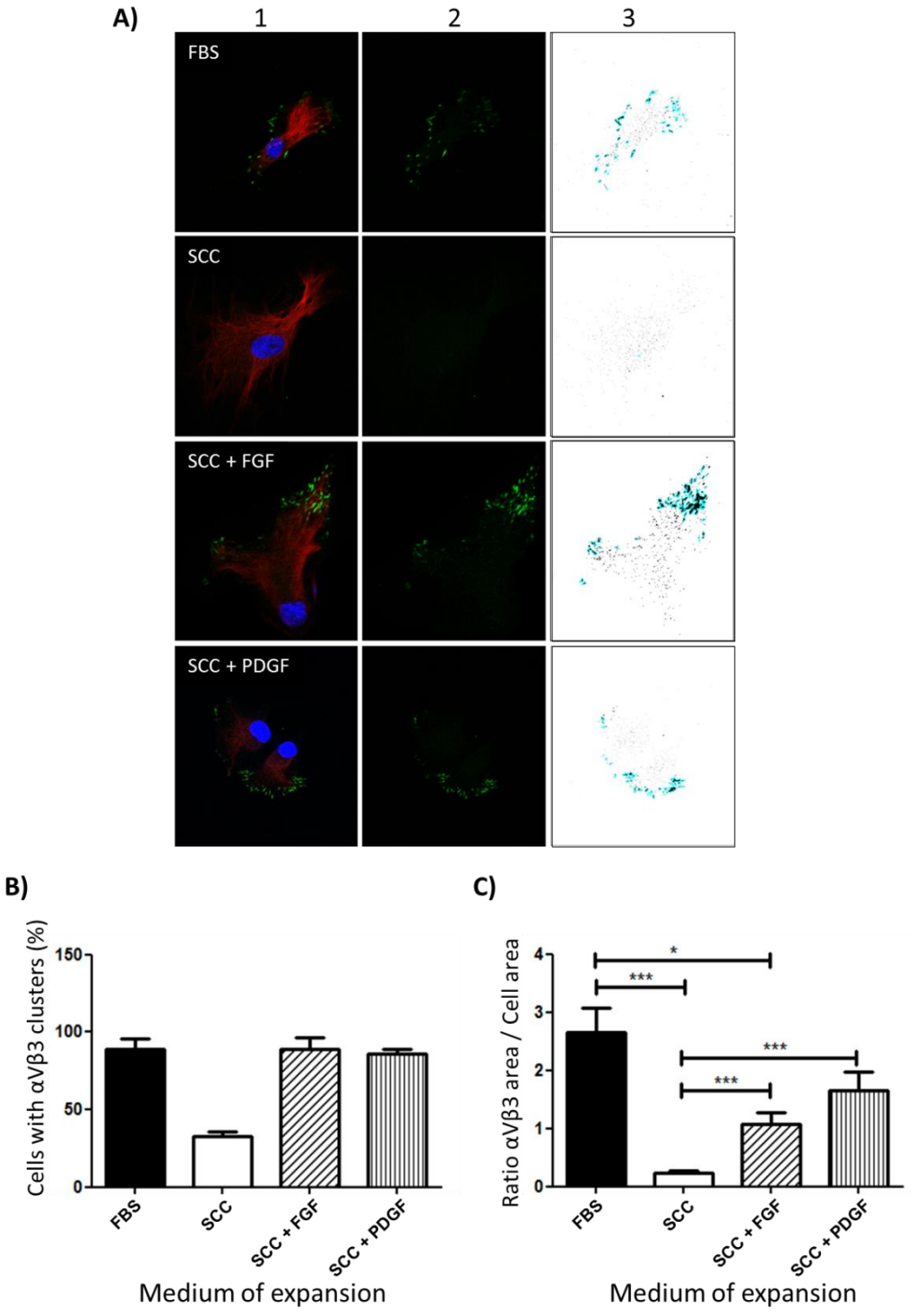

Figure 26. αVβ3 expression analysis by fluorescence microscopy. **A)** Illustration of the image analysis protocol followed. 1) Image of the cell (blue: DAPI, nucleus; red: vimentin; green: αVβ3) 2) Selection of the αVβ3 channel. 3) Application of the threshold to count the number of particles present. **B)** Percentage of cells with αVβ3 staining. **C)** Area of αVβ3 staining per cell area. (n=3 assays, 12 cells counted per assay, Kruskal Wallis followed by Dunns multiple comparison test)

3.5. hMSC motility depends on p38 MAPK signaling

Different inhibitors were used to determine which signaling pathways might be involved in SCC-expanded hMSC motility (Figure 27). Inhibition of both MEK and PI3K reduced cell motility of hMSC expanded in FBS-Medium and FGF supplemented SCC-Medium (FBS: 24% and 30% reduction; FGF: 46% and 50% reduction respectively). The inhibitor of p38 MAPK reduced cell motility in all conditions tested (72-87% reduction). Blocking αVβ3 integrin also had an effect on cells expanded in both FBS-Medium (20%) and FGF supplemented SCC-Medium (39%).

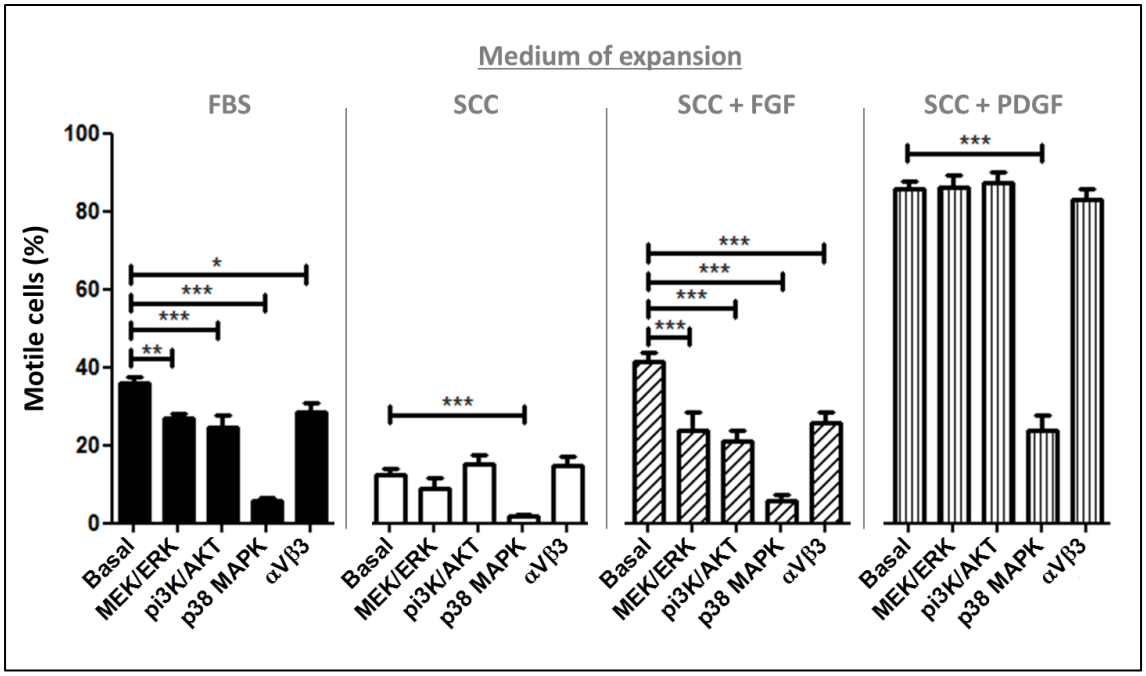

Figure 27. Inhibition of different proteins and blocking of αVβ3 - Percentage of motile cells analyzed by time lapse assay. hMSC expanded in FBS-Medium, SCC-Medium, SCC+FGF Medium and SCC+PDGF Medium were seeded and cultured with inhibitors of MEK, PI3k and p38 MAPK proteins, or with a αVβ3 blocking antibody during image acquisition (n=9, 2 hMSC donor, repeated measurements ANOVA followed by Bonferronis).

4. Discussion

MSC recruitment to sites of inflammation is crucial for therapies where hMSC are implanted far away from the site to treat. It has been already shown that cells of the immune system, such as macrophages or NK cells, can attract hMSC [Almeida et al, 2012; Silva et al, 2014]. We previously showed that BM-hMSC expanded in SCC-based xeno-free medium can also be recruited by macrophages [Blázquez-Prunera et al, 2017]. However, SCC-expanded hMSC showed a mean reduction of motility of 64% when comparing with hMSC expanded in FBS-Medium. Even though Kobayashi et al. (2005) have shown that FGF does not influence motility in hMSC expanded in autologous human serum, here the supplementation of SCC-Medium with FGF significantly improved hMSC motility. An increase in motililty was also observed when supplementing SCC-Medium with PDGF, as well as when adding it to FBS-Medium, as previously reported [Veevers-Lowe et al 2011]. Supplementation of SCC-Medium with TNF-α and SDF-1 showed a tendency to increase hMSC motility, but to a lower extent. This might be because these factors are more involved in recruitment than in motility per se [Xiao Q, 2012; Wynn 2004]. Moreover, it is known that SDF-1 receptor (CXCR-4) decreases after hMSC expansion and passaging, thus we may not see a SDF-1 strong effect because of the loss of its receptor on hMSC surface [reviewed at Karp 2009].

To further analyse which supplements are better to improve MSC motility, recruitment assays were also performed with transwells. Here we used FBS, a common positive control used in these assays, to recruit hMSC [Corning]. All hMSC, expanded in the different media (FBS, SCC, SCC+FGF and SCC+PDGF) were recruited by the growth factors in FBS, and had similar chemotactic indexes. Nonetheless, differences on the total amount of cells that crossed the membrane were found. This assay corroborated what was observed in the time lapse assays: supplementation of SCC-Medium with FGF or PDGF increased cell

motility. However, in this case only FGF increased it to the level observed when using FBS-Medium for cell expansion.

hMSC expanded in SCC-Medium showed a rounder morphology, while supplementation of SCC-Medium with FGF or PDGF had a clear effect on hMSC morphology, with cells more elongated and with sharp ramifications, similar to the morphology observed when using FBS-Medium. This change in morphology might be related with hMSC motility. Moreover, addition of FGF, but not PDGF, led to an increase in cell proliferation of SCC-expanded hMSC. Thus, supplementation with FGF is a good option not only to increase hMSC motility, but also to improve cell growth, which is important for future clinical applications.

Time lapse and recruitment assays performed showed that FGF improves the motility of SCC-expanded hMSC. It was hypothesized that some proteins related with adhesion, such as integrins, were affected. As Rusnati et al, 1997 showed that FGF interacts with αVβ3 in endothelial cells and Mori et al, 2008 showed that the direct binding of FGF to αVβ3 is important for FGF signalling, expression of this integrin was analyzed. Flow cytometry analysis and immunocytochemistry showed that the levels of αVβ3 were increased by FGF supplementation. Moreover, as Enenstein et al (1992) showed that FGF increased the biosynthesis of integrin β1 in endothelial cells, we also analysed its expression by flow cytometry, but no differences were observed among the cells expanded in the different media, indicating that FGF specifically affected αVβ3. And importantly, blocking of αVβ3 integrin led to a reduction on the percentage of motile cells in FBS or SCC+FGF medium, suggesting that this integrin is essential for the improved motility of MSC expanded in FGF supplemented SCC-Medium. Finally, to analyse which signalling pathways were behind the effect of FGF on hMSC motility, assays were performed in the presence of different inhibitors and αVβ3 blocking antibody. Inhibition of the

p38 MAPK pathway decreased hMSC motility of the cells in all conditions tested (FBS, SCC, SCC+FGF and SCC+PDGF Medium). It is known that p38 signalling is an important pathway in cell motility, and that it is activated by many growth factors, such as FGF, PDGF and TNF-α [Ono and Hand,2000]. Thus, this kinase and its pathway are important for cell motility, but not exclusive for stimulation with FGF as its inhibition also affected cells expanded in its absence. Previous studies have shown that the MEK/ERK pathway is also involved in the migration of different cell types in response to growth factors such as VEGF, FGF, EGF and insulin [Shono et al, 2001, Huang et al, 2004]. The activation of the PI3K/AKT pathway through growth factors and integrins has been also related with cell motility. Xue et al, 2013 And Schmidht et al, 2006 showed the importance of this pathway on the effect of FGF on MSC motility. Here, MEK/ERK and PI3K/AKT pathway inhibitors had no effect on hMSC expanded in SCC or SCC+PDGF media, but hMSC motility was reduced when FBS or SCC+FGF media were used for expansion. Thus, having in mind that FBS also contains FGF (Thermo Fisher), these data indicate that the pathways involved in motility of cells grown in FBS and FGF supplemented SCC-Medium are the same, and involve the integrin αVβ3 as well as the signaling molecules p38 MAK and to a lower extent MEK/ERK and pI3K/AKT.

Taking all these data together, we can conclude that supplementation of the xeno-free medium based on a human plasma derivative (SCC) with FGF improves hMSC motility by promoting αVβ3 integrin expression.

Chapter 5. General Discussion and Future Perspectives

MSC present interesting properties for regenerative medicine and to treat immune-mediated diseases. Moreover, their relatively easy obtention and expansion *in vitro* makes them a better choice than other stem cells. Since Lazarus and colleagues injected autologous MSC to successfully treat haematological malignances [Lazarus et al 1995], many clinical trials have been performed to treat a variety of important diseases, such as multiple sclerosis, stroke, myocardial infarction, amyotrophic lateral sclerosis or leukaemia[Portmann-Lanz et al, 2006; Casiraghi et al, 2016; Golpanian et al, 2016; Dulamea, 2015]. Thus, the use of MSC based therapies is becoming closer to reality.

Until now, only few secondary effects have been reported, however, long term followup of the patients should be done to evaluate tumour formation. If the MSC therapy is done in cancer patients, extra care should be taken and the benefits of the therapy must overcome possible adverse effects. Moreover, to minimize the risk of malignant transformation *in vitro*, extra care and safety control steps should be undertaken after MSC expansion or genetic manipulation [Wyong et al 2011]. When MSC transformation to a differentiated cell type is not needed, and the desired effects of MSC are done through growth factors and cytokines [Caplan et al 2006], the use of MSC conditioned medium or exosomes might be a safer option.

Another big issue that needs to be solved for the use of MSC in therapies is the medium used to expand these cells. Normally, for a human therapy, hMSCs are obtained from an unrelated donor or from the same patient, but, the number of isolated cells is not sufficient to perform a therapy. Thus, hMSC need to be expanded *in vitro* before their transplantation to the patient. This expansion is usually done in medium supplemented with fetal bovine serum (FBS), which is inherently risky, present high lot-to-lot variability and availability problems, and can induce an immune rejection to the host [Dimasi et al 2011; Gstraunthaler et al 2013; Tekkatte

et al 2011; Even et al 2006; Van der Valk et al 2010. Thus, it is currently encouraged to culture hMSC in a xeno-free, safe and efficient medium European Medicines Agency 2013.

This present thesis is focused in the study of a new xeno-free medium based on a human plasma derivative (SCC). This project is the result of a collaboration between the pharmaceutical company Grifols, which developed the product (SCC), and academia, INEB, where it was possible to deepen on some scientific aspects of the product. Here we showed that SCC-based medium can be used to expand hMSC while maintaining their defining characteristics (adherence to plastic, phenotype and multipotentiality) and genetical stability. Moreover, BM-hMSC immunomodulatory and chemotactic properties were not affected. Furthermore, it was found that addition of FGF to the xeno-free medium improved BM-hMSC growth and morphology, as well as the cells motility, increasing αVβ3 integrin expression.

Throughout this thesis, hMSC grown in the presence of FBS were used as control, as it is the most common medium used to culture hMSC. However, what happens in an FBS-containing medium should not be considered as the complete truth or image of what happens in the human body (where no FBS is present), but only as a reference. Thus, the goal of a new xeno-free medium for hMSC expansion is not to exactly mimic what happens in an FBS-Medium, but to be an efficient growing medium, where hMSC can be expanded while maintaining their defining and clinically-relevant properties.

During this thesis different supplements have been added to SCC based medium. The addition of insulin, selenium and ethanolamine was crucial to properly expand hMSC. On the other hand, the adition of platelet lysate was beneficial to increase hMSC proliferation rate, but not essential for hMSC culture and expansion. Apart from the increase of the proliferation rate, which was also observed by the supplementation with FGF, the combination of SCC with PL did not show any other important benefits. Moreover, other studies

have already shown that supplementation of basal medium (such as DMEM) with PL itself can be used to culture hMSC [Schallmoser et al 2007, 2009; Mojica-Henshaw et al 2013; Kocaoemer et al 2007; Flemming et al 2011; Doucet et al 2005; Chieregato et al 2011; Capelli et al 2007; Astori et al 2016], thus, the combination of SCC with PL would not introduce such important changes to justify its use, increasing the price of the final medium. Nonetheless, as some studies are showing that PL can negatively affect MSC immunomodulatory properties and differentiation potential [Abdelrazik et al 2011; Copland et al 2013; Lange et al 2007; Gruber et al 2004], the supplementation of SCC Medium with PL is not recommended. On the other hand, the addition of FGF to SCC-Medium not only increased MSC proliferation rate, but also MSC motility. Thus, even though FGF supplementation is not essential for hMSC culture, its adition to the medium could be recommended in some cases.

SCC suitability for the expansion of hMSC was tested on the most used hMSC types. hMSC commercially available, or obtained in the laboratory and isolated from bone marrow, adipose tissue and umbilical cord were expanded using SCC based medium. The results obtained showed that all hMSC tested, independently of their origin or source, maintained their adherence to plastic, phenotype and multipotentiality. hMSC phenotype was evaluated by immunostaining. Ideally, it would have been better to study it by flow cytometry, as the data obtained using this equipment is quantitative and we would have been able to evaluate the percentage of cells positive for each marker as well as the expression levels of each marker. Nonetheless, as the cells that we used had been previoulsy characterized, the immunostaining technique allowed us to analyse whether SCC induced any relevant alteration.

The study of hMSC immunomodulation was one of the objectives of this thesis, as this property is key for many of the hMSC clinical applications under study [Ucceli et al 2008]. MSC immunomodulatory properties vary depending on the niche where cells are, thus, the effect of SCC on BM-hMSC immunomodulation was

assessed. BM-hMSC immunomodulatory properties were firstly studied using a Mixed Leukocyte Reaction. The results obtained on the MLR were diverse, depending on the donor of the leukocytes used as Responder Cells. When the response of these cells was high, added BM-hMSC acted as immunosuppressors, but, when the response of leukocytes was low, BM-hMSC had none or an immunostimulatory effect. This assay had been previously reported by others, [Bartholomew et al, 2002] and allowed us to observe the duality of hMSC immunomodulation in a single assay. In order to obtain more robust data, a mitogen (PHA) was used to stimulate leukocyte proliferation, as previously done by Carrade and colleagues (2012). This assay was indeed more robust and the immunosuppressive properties of BM-hMSC could be more easily evaluated. Moreover, it was possible to study BM-hMSC immunostimulation by co-culturing BM-hMSCs with non-stimulated leukocytes. For our knowledge, this assay was never performed before, and allowed us to observe how the same cells were able to stimulate or suppress leukocyte proliferation depending on the state of the leukocytes and the niche they create. It was possible to measure the levels of TNF-α on the supernatants of these assays but it would have been interesting to also measure IDO, as it is the main known soluble mediator of hMSC immunosuppression [Dazzi et al, 2008; Benvenuto et al, 2007; Mougiakakos et al, 2011; Kemp et al, 2010; Tabera et al, 2008; Prasanna et al, 2010; Krampera et al 2006]. Other interesting molecules that we tried to measure, but unfortunately without success were PGE2 and TGF-β.

As many of the currently explored clinical strategies take advantage of hMSC capacity of homing to an injury or inflammed site, the chemotactic and motility properties of BM-hMSC were also studied. Here we evaluated the ability of BM-hMSC to be recruited by macrophages, as these cells are one of the most abundant cell types in an injury site and actively recruit hMSC [Almeida et al 2012]. SCC-expanded BM-hMSC could be recruited by macrophages, with a chemotactic index similar to FBS-expanded BM-hMSC. However, while performing the

different recruitment assays, it was observed a reduction in the total number of motile cells, indicating there was impairment on the motility of SCC-expanded BM-hMSC. Thus, different supplements were tested for their effect on the motility of BM-hMSC expanded on SCC-Medium. FGF and PDGF induced an important increment of BM-hMSC motility and also led to changes in cell morphology, which became more similar to the one observed when using FBS Medium. Moreover, FGF increased BM-hMSC proliferation rate. The supplementation of FBS-Medium with FGF did not induce an increase on BM-hMSC motility, while PDGF did, suggesting that FGF was the key factor missing in the xeno-free medium. Furthermore, it was hypothesized that differences on integrin expression could be behind this impairment of cell motility. Indeed, analysis of αVβ3 integrin showed that adding FGF lead to an increase on this integrin surface expression. Thus, supplementation of SCC-based medium with FGF is highly recommended to increase not only BM-hMSC cell growth, but also to maintain cell motility.

Nowadays there are different xeno-free options to culture hMSC. Different studies have been published to try to find the best composition medium. However, even though ISCT tried to standardize the procedures that should be done to evaluate a new medium for MSC culture, the reality is that there is no consensus, and it is still difficult to compare the results between different studies [Krampera et al 2013]. One of the most used options used is the supplementation with platelet lysate [Schallmoser et al 2007, 2009; Mojica-Henshaw et al 2013; Kocaoemer et al 2007; Flemming et al 2011; Doucet et al 2005; Chieregato et al 2011; Capelli et al 2007; Astori et al 2016].

Platelet lysate has shown to contain the necessary factors to allow hMSC isolation and proliferation, while maintaining hMSC defining properties and a high proliferation rate [Juhl et al 2016]. PL can be easily obtained in the laboratory, but, if PL from a low number of donors is used, high variability is gonna be faced. Thus, it is more interesting to use commercially available PL, which are made from a large pool of donors, as SCC. The first commercially available PL

was PLTMAX® from Mill Creek Life Sciences in 2010. Different published studies successfully used PL to culture and expand different types of hMSC [Schallmoser et al 2007, 2009; Mojica-Henshaw et al 2013; Kocaoemer et al 2007; Flemming et al 2011; Doucet et al 2005; Chieregato et al 2011; Capelli et al 2007; Astori et al 2016]. However, some problems regarding the effect of PL on MSC immunomodulatory properties and differentiation potential are being published [Abdelrazik et al 2011; Copland et al 2013; Lange et al 2007; Gruber et al 2004]. SCC is a new product that has not been comercialized yet, and thus, the number of published studies is limited [Díez et al 2012, 2015, Rodriguez-Piza 2010]. As well as some commercial PL, it is obtained following GMP and lot-to-lot variability is not expected, as it is obtained from a pool of over 1000 donors. Here we described that hMSC from different origins and sources could be succesfully expanded and no effect on the immunomodulatory or differentiation potential have been observed [Diez et al 2015, Blázquez-Prunera et al 2017].

hMSC used in our studies were isolated on FBS supplemented medium. It was possible to test isolation of BM-hMSC in xeno free conditions, but only once. Although we could isolate the cells, the number of cells obtained was very low, maybe because at the moment of the assay the composition of the medium was not optimized and no insulin, selenium, ethanolamine or FGF were used. In the future it would be important to test hMSC isolation using the complete xeno-free formulation. Moreover, other critical experiments to be performed should be a comparison in parallel with other xeno-free media commercially available, with different hMSC types and origins. In some cell therapies, 3D scaffolds are used to culture hMSC, providing the appropriate environment for the regeneration of tissues and organs [Mencía Castaño et al, 2016]. Thus, the use of SCC on 3D cultures should be evaluated. Furthermore, SCC could be used to expand in a xeno-free medium other human cell types used for cell therapies, such as endothelial cells or neural stem cells.

In conclusion, SCC based medium is a promising option for the xeno-free culture of hMSC for human cell therapies, as it is an industrial-grade product, made following good-manufacturing-practice process, which ensures its safety, lot-to-lot stability and availability.

Personal experience

I do not want to finish without talking about what this project has given to me. Having the opportunity to develop my PhD thesis both in academia and industry was the reason why I enrolled in this program. Being in academia I could be in contact with different scientists from different areas and I could have access to different types of equipment and techniques. Not only had I learnt things about the topic of my thesis, but also from many other areas. Moreover, in academia all my colleagues were in a similar situation to mine. But what makes this thesis special is the time I have spent in the industry (1 year and 4 months). Working in industry exposed me to a totally different environment. There my colleagues were not students, as me. People there were working as a whole team, having each person a specific task of a big mechanism. At Grifols I have learnt to defend my project in front of people that are not experts on the topic. I had to adapt to the way they worked in the laboratory and follow good-laboratory-practice rules (GLP). Moreover, I have learnt how to work in an industrial environment, where there is a fix schedule and rules to follow. Other skills that I have improved in both industry and academia were the organization of my time and tasks, problem identification and definition, experiment design and planning, project management, oral communication skills, data management, statistical analysis, scientific writing and poster and presentation edition.

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