Universidade de Aveiro Secção Autónoma das Ciências da Saúde
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Daniela Filipa Alves Ferreira

Influência da composição de substratos na imunomodulação por MSCs

Influence of substrates composition on immunomodulation by MSCs

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Doutor Mário Grãos, Investigador Auxiliar da Unidade de Biologia Celular do Biocant/Centro de Neurociências de Coimbra e da Professora Ana Gabriela Henriques, Professora Auxiliar Convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro

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palavras-chave

MSCs, Wharton's Jelly, Mecanotransducção, Imunomodulação

resumo

As células estaminais mesenquimais (MSCs) são células não-hematopoéticas, multipotentes, capazes de se auto-renovar e de diferenciar em diferentes tipos celulares. As MSCs estão presentes em tecidos mesenquimais e de tecidos extra embrionários, tais como a matriz do cordão umbilical/Wharton's Jelly(WJ). Estes últimos constituem uma boa fonte de de MSCs, sendo estas mais naive e tendo um maior potencial de proliferação do que as MSCs obtidas de tecidos adultos, como a medula óssea, tornando as MSCs da matriz do cordão umbilical/Wharton's Jelly sejam mais apelativas para uso clínico.

As MSCs possuem a capacidade de modularem tanto o sistema imune inato como o adquirido e os seus efeitos são vastos, afectando todas as células do sistema imune. Esta capacidade é bastante vantajosa para o uso terapêutico destas células em doenças do sistema imunitário.

A mecanotransducção é por definição o mecanismos pelo qual as células convertem estímulos mecânicos em uma resposta bioquímica e com mudanças na sua morfologia. Apartir destas observações colocámos a hipotese de que mantendo MSCs *in vitro* em diferentes substratos poderia influencia a sua capacidade imunomoduladora.

Com este trabalho, demonstrámos que ao plaquear MSCs em diferentes substratos de PDMS, estas mostram uma tendência para secretar quantidades diferentes de vários factores soluveis analisados, relativamente a MSCs mantidas em cultura em plataformas convencionais (placas de cultura de células - TCP). Para além disto, foi também observado que MSCs plaqueadas em substratos de PDMS aparentavam possuir uma maior capacidade imunomoduladora quando comparadas com MSCs mantidas em condições convencionais.

Em conjunto todos os resultados obtidos sugerem que elementos relacionados com a mecanotransdução parecem influenciar a capacidade imunomoduladora de MSCs e a sua secreção de factores solúveis. Deste modo, estudos futuros poderão elucidar os mecanismos responsáveis por estas observações, de modo a permitir que se possa constitutuir melhores estratégias de cultura de MSCs para futuro uso terapêutico dirigido a doenças do sistema imunitário.

keywords MSCs, Wharton's Jelly, Mechanotransduction, Immunomodulation

abstract

Mesenchymal stem cells (MSCs) are non-hematopoietic multipotent stem cells capable to self-renew and differentiate along different cell lineages. MSCs can be found in adult tissues and extra embryonic tissues like the umbilical cord matrix/Wharton's Jelly (WJ). The latter constitute a good source of MSCs, being more naïve and having a higher proliferative potential than MSCs from adult tissues like the bone marrow, turning them more appealing for clinical use.

It is clear that MSCs modulate both innate and adaptive immune responses and its immunodulatory effects are wide, extending to T cells and dendritic cells, being therapeutically useful for treatment of immune system disorders.

Mechanotransduction is by definition the mechanism by which cells transform mechanical signals translating that information into biochemical and morphological changes. Here, we hypothesize that by culturing WJ-MSCs on distinct substrates with different stiffness and biochemical composition, may influence the immunomodulatory capacity of the cells.

Here, we showed that WJ-MSCs cultured on distinct PDMS substrates presented different secretory profiles from cells cultured on regular tissue culture polystyrene plates (TCP), showing higher secretion of several cytokines analysed. Moreover, it was also shown that WJ-MSCs cultured on PDMS substrates seems to possess higher immunomodulatory capabilities and to differentially regulate the functional compartments of T cells when compared to MSCs maintained on TCP.

Taken together, our results suggest that elements of mechanotransduction seem to be influencing the immunomodulatory ability of MSCs, as well as their secretory profile. Thus, future strategies will be further explored to better understand these observation and to envisage new *in vitro* culture conditions for MSCs aiming at distinct therapeutic approaches, namely for immune-mediated disorders.

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List of Abbreviations

A	Alpha-MEM	Alpha modified Eagle's medium
	APTMS	Aminopropyltrimethoxisilane
	APC	Antigen-presenting cells
	APC	Allophycocyanin
	APCh7	Allophycocyanin-hilite 7
	AF	Amniotic Fluid
B	BM	Bone Marrow
	BM-MSCs	Bone marrow mesenchymal stem cells
C	CD	Cluster of differentiation
	CFU-F	Colony forming unit-fibroblast capacity
	CM	Central memory
	CM	Conditioned media
	COX	Cyclooxygenase
	CTLs	Cytotoxic T cells
	CXCL	C-X-C chemokine ligand
	CXCR	C-X-C chemokine receptor
	CDP	Cumulative population doubling

D	dsRNA	Double-stranded ribonucleic acid
	DCs	Dendritic Cells
	DMSO	Dimethyl sulfoxide
	DMEM	Dulbeco's modified Eagle's medium
E	ESCs	Embryonic stem cells
	E	Elastic modulus
	ECM	Extracellular matrix
	EtOH	Ethanol
	EM	Effector memory
F	FoxP3	Forkhead box P3
	FAs	Focal adhesions
	FBS	Fetal Bovine Serum
	FN	Fibronectin
	FAK	Focal adhesion kinase
	FITC	Fluorecein isothiocyanate
G	*G*	Shear modulus
	GT	Generation time
H	HLA	Human leukocyte antigen

	HLA-G6	Human leukocyte antigen G6
	HGF	Hepatocyte growth factor
	HO-1	Heme oxygenase1
	HUVEC	Human umbilical vein endothelial cells
	hWJ	Human Wharton's Jelly
I	INF-α	Interferon gamma
	iDC	Immature dendritic cells
	IDO	Indoleamine 2,3-dioxygenase
	ICAM	Intracellular adhesion molecule
	IL	Interleukin
	ISCT	International Society for Cellular Therapy
K	KO	Krome orange
	kPA	Kilopascal
L	LPS	Lipopolysaccharide
M	MSCs	Mesenchymal Stem Cells
	mAb	Monoclonal antibody
	MFI	Mean Fluorescence Intensity
	mDC	Mature Dendritic cells

	MLC	Mixed lymphocyte culture
	MNCs	Mononuclear cells
	MMPs	Metalloproteinases
	MHC	Major complex of histocompability
	MPa	Megapascal
N	NK	Natural Killer
	NFkB	Nuclear factor-kappa B
	NO	Nitric oxide
O	Oct4	Octamer-binding transcription factor 4
P	PD-1	Programmed death-1
	PE	Phycoerythrin
	PECy7	Phycoerythrin-cyanine 7
	PerCPCy 5.5	Phycoerythrin-cyanine 5
	PD	Population Doubling
	PBS	Phosphate buffered saline
	PDMS	Polydimethylsiloxane
	PMA	Phorbol myristate acetate
	PacB	Pacific Blue
	Pax	Paxilin

	PGE-2	Prostaglandin E2
	PD-L1	Programmed death ligand-1
R	RT	Room Temperature
S	SA – PE	Streptavidin-phycoerythrin
	STAT	Signal transducer and activator of transcription
	SSEA	Stage-specific embryonic antigen
	sHLA-G5	Soluble human leukocyte antigen G5
T	Tregs	Regulatory T cells
	TCP	Tissue culture plates
	Th	T helper
	TGF-β	Transforming growth factor- β
	TLR	Toll-like receptor
	TNF	Tumour necrosis factor
	TNC	Total number of cells
	TSG-6	Tumour necrosis factor stimulated gene 6
U	UC	Umbilical cord
	UV	Ultraviolet
	UCB	Umbilical cord blood

	UCM	Umbilical cord matrix
V	VCAM	Vascular cell adhesion molecule
	Vin	Vinculin
	VASP	Vasodilator stimulating phosphoprotein
	VEGF	Vascular endothelial growth factor
W	WJ	Wharton's jelly
	WJ-MSCs	Wharton's jelly mesenchymal stem cells

Chapter 1 – Introduction

1. Introduction

1.1. Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs), or mesenchymal stromal cells[1] are non-hematopoietic multipotent adult stem cell, that can be found mostly in perivascular niches and are responsible for the homing of hematopoietic stem cells (HSCs)[2,3]. These cells are able to self-renew and differentiate *in vitro* in to different mesodermal cell types, such as osteocytes, adipocytes and chondrocytes, but also cardiomyocyte and neural-like cells[4,5,6], demonstrating a putative plasticity potential.

Alexander Friedenstein and colleagues, in 1970, were the first to describe MSCs in the bone marrow (BM) as fibroblast-like precursors from the bone marrow[7,8] and describe them as plastic-adherent, with a fibroblast-like morphology , high proliferative capacity *in vitro* and with the capacity to form fibroblast colony-forming units (CFU-Fs)[7, 8,9].

According to the *Mesenchymal and Tissue Stem Cell Committee* of the *International Society for Cellular Therapy* (ISCT) there are tree minimal criteria to define human MSCs: (i) to be adherent to plastic in standard culture conditions; (ii) MSCs must express Cluster of Differentiation (CD) 105, CD73 and CD90 and lack expression of CD34 (endothelial or primitive hematopoietic), CD45 (leukocytes), CD14 or CD11b (monocytes and macrophages), CD79a or CD19 (B cell) and human leukocyte antigen (HLA-DR or HLA class II) surface molecules [(unless stimulated by interferon-γ (IFN-γ)]; and (iii) must differentiate to osteocytes, adipocytes and chondrocytes *in vitro*[10, 11]. These criteria established by the ISCT have standardized human MSCs isolation but may not apply completely to other species, like murine.

MSCs secrete several growth factors, extracellular matrix molecules and cytokines that play an important role in the regulation of angiogenesis, haematopoiesis and in immune and inflammatory response[3,12].

1.2. Sources and characteristics of Mesenchymal Stem Cells

Since mesenchymal stromal/stem cells (MSCs) were first described in 1970 in the bone marrow (BM), it has been proven the presence of MSCs in other tissues such as adipose tissue, skeletal muscle, connective tissue, dental pulp, synovium, periosteum, spleen, lung and extra embryonic tissues like placenta, amniotic fluid (AF), umbilical cord blood (UCB) and umbilical cord matrix/Wharton's jelly (UCM/WJ)[2,13,14].

Most of the knowledge concerning MSCs comes from the BM studies, which has been considered as one of the main sources[15]. However, BM-MSCs have limitations in terms of cell numbers (for regenerative medicine only approximately 0.001% to 0.01% of the cell are useful)[16],[17] and decrease significantly with donors age in terms of proliferation and differentiation capacity[2,18]. Moreover, the procedure to obtain these cells is invasive and painful[19] for the patient and can be followed by risk of infection[5] , bleeding and chronic pain[15] and due to these limitations describe above, alternative MSCs sources have been explored.

Extra-embryonic tissues are a good alternative source of MSCs because they have a higher proliferative potential than adult MSCs and immunoprivileged characteristics. Moreover, they seem to be more naive than MSCs isolated from adult mesenchymal tissues[2] and adult MSCs may be less-responsive than extra-embryonic MSCs in clinical applications[19]. MSC from different sources express embryonic stem cell markers namely GATA-4, Rex-1, Nanog, SSEA-1[20].

To circumvent the limitations of BM, the umbilical cord (UC) has been used as an alternative source of MSCs. The procedure to collect these cells is safe, non-invasive and simple compared to BM aspirate[2] (UC is routinely discard at birth)[19] and it may be stored cryogenically with minimal loss of potency and subsequently thawed[15] due to the high potential clinical applications of MSCs[2,21]. Umbilical Cord raises no major ethical, technical or legal[22] issues for scientific research or clinical applications[2].

MSCs from the UC exhibit a fibroblast-like morphology and share commonly the typical MSC immunophenotypic markers, immunogenic and immunoregulatory characteristics[19,23] and differentiation potential similar to BM-MSCs (Table I.1)[2]. Thus they fulfil the minimal criteria proposed by ISCT for MSCs described above. Four populations of

MSCs can been identified in the umbilical cord: (1) Wharton's Jelly (WJ), (2) MSCs surrounding the umbilical vessels , (3) umbilical cord blood (UCB) and (4) MSCs from the subendothelium of umbilical vein[24].

Table 1.1 : Surface Markers expressed by MSCs from UC and BM (Adapted from Malgieri et al., 2010)

Surface markers	hUC	BM
CD13	++++	++++
CD14	-	-
CD29	++++	++++
CD31	-	-
CD34	-	-
CD38	-	-
CD44	++++	++++
CD45	-	-
CD73	++++	++++
CD90	++++	++++
CD105	++++	++++
CD106	+	+++
CD146	++++	+++
HLA-ABC	+++	++++
HLA-DR	-	-

1.3. Umbilical Cord Matrix/Wharton's Jelly Mesenchymal Stem Cells

The UC is an extra-embryonic tissue that constitutes the imperative link between fetus and mother during pregrancy[17]. This tissue contain two arteries and one vein, which are surrounded by gelatinous connective tissue, known as the Wharton's Jelly (WJ)[16,25], and is covered by amniotic epithelium. This tissue was first described in 1656 by Thomas Wharton and the first isolation report was in 1991 by McElreavey et al.[16,26] .

The WJ is constituted by extracellular matrix proteins, namely collagens fibres, proteoglycans, glycosaminoglycans (hyaluronic acid is the most abundant[25]) and have two main cellular types, fibroblast-like and myofibroblast cells[2,16,17].

MSCs from WJ (MSC-WJ) can be isolated from three regions: (1) the perivascular regions[16], (2) intervascular regions and (3) the subamnion[24]. There are two methods to isolate MSCs from WJ , enzymatic digestion and explant culture[23]. In both cases primary populations can be successfully expanded *ex vivo* and further differentiated and characterized.

WJ-MSCs fit the minimal criteria for described mesenchymal stem cells and in addition, these cells express CD200 in greatest proportions and, differently to BM-MSCs, CD68 (macrophage-specific antigen) and CD14[12]. Furthermore, the mesenchymal features of WJ have been confirmed by the expression of specific markers from cytoskeletal lineage, such as nestin[27], vimentin (express mainly in mesenchymal lineage cells[12]) and SMA[16] and ESC markers such as Oct-4, SOX-2, Nanog, c-Kit and SSEA4 have also been described[22,28].

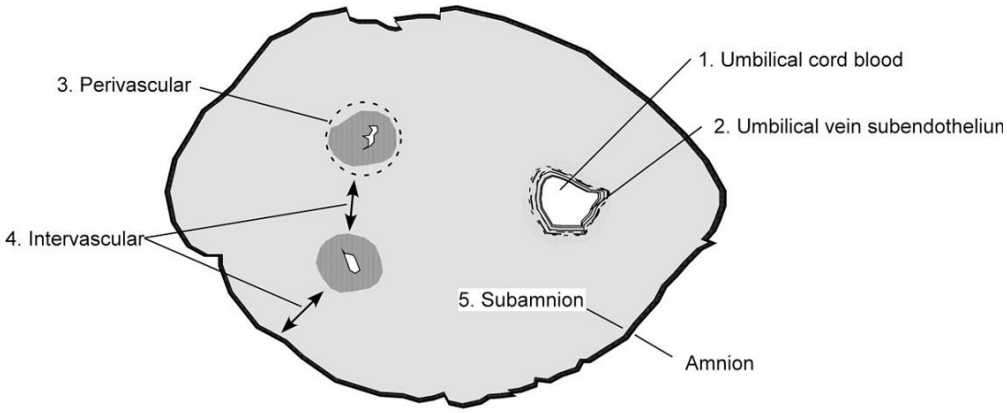

Figure 1.1: Anatomical compartments within the umbilical cord. Umbilical cord contains a vein and two arteries surrounded by a gelatinous connective tissue, the Wharton's Jelly.(Adapted from Troyer et al., 2007)

Fetuses express high levels of HLA-G which provides protection against maternal immune attack and subsequent rejection[29] and induces the expansion of regulatory T cells (Tregs)[16]. WJ-MSCs exhibit higher intracellular concentrations of leukocyte antigen G6 (HLA-G6)[14,21], than BM-MSCs, which suggests a role in immune tolerance during pregnancy by avoiding a maternal immune response against the fetus and even inducing the expansion of regulatory T cells[29,30].

1.4. Immunomodulation by MSC

MSCs have multi-differentiation potential and plasticity and possess unique immunological properties. Regardless of the source from which MSCs are isolated from, they have been shown to be hypo-immunogenic and have potent immunosuppressive activity, both *in vitro* and *in vivo*[16,31]. This phenomenon has been in a focus of interest since 2002 after Bartholomew and colleagues reported the immunosuppressive capacities of allogeneic MSCs and demonstration of the ability of MSCs from baboons to suppress

lymphocyte proliferation in a mixed culture *in vitro* and prevent the rejection of a skin allograft *in vivo*[32,33]. Furthermore, other studies have demonstrated that MSCs are immunosuppressive both *in vitro* and *in vivo* in other animal models and human studies[33]. Therefore, immunomodulation is considered as a promising feature of MSCs populations for clinical application.

The expression of major histocompability complex (MHC) on all cells of the organism allows the immune systems to distinguish self from non-self. The hypo-immunogenicity of MSCs can be attributed to the lack of MHC-Class II and co-stimulatory ligands (CD80,CD86, CD40[14],CD40-L[34], B7-DC) implicated in lymphocytes activation and even express low amounts of MHC-Class I[23,29]. Nevertheless, Western blot analysis of cell lysates shows that cells contain intracellular deposits of class II alloantigens - despite the fact that of not being detectable on the cell surface. Although they can be induced to express MHC-Class I and II, and Fas ligand by IFN-γ treatment[35], but even in these conditions MSCs do not stimulate immunological response[34]. The expression of low levels of MHC-Class I could be a mechanism to protect MSC from alloreactive Natural Killer (NK) cell-mediated lysis[29,36]. MSCs can migrate and home to organs and tissues in response to cytokines, chemokines, adhesion molecules and growth factors and, therein, mediate immunomodulatory actions[37,38]. These characteristics allow the use of mismatched MSCs *in vivo* and lack of T cell response in an allogeneic mixed-lymphocyte reaction *in vitro*[39]. These characteristics are the most intriguing aspects of MSCs biology and offer great therapeutic promise in areas like tissue regeneration, treatment of immune diseases, cell vehicles for gene therapy and enhancement of hematopoietic stem cell engraftment[7].

MSCs immunomodulation is a process involving several steps (1) MSCs responsiveness to inflammation and migration to the site of injury, (2) licensing/activation of MSCs, (3) promotion of pathogen clearance if required and (4) modulation of inflammation[40,41]. *In vitro*, MSCs can be activated by biologically-active metabolites of activated immune cells, called “priming” or “pro-inflammatory activation”, the most well defined mechanism until today[41] namely IFN-γ in the presence of other cytokines including TNF, IL-1α or IL-1β[33,41], mimic an inflammatory environment. IFN- γ remains the first key “priming” agent for MSCs suppressor function. Beyond inflammatory cytokines other

factors might also be involve in "licensing" this ability of MSCs, like Toll-like receptors (TLRs)[42,43]. TLRs are a family of pattern recognition receptors that recognize conserved structures that recognize infection or tissue damage - by the release of molecules (e.g., endotoxin, lipopolysaccharide –LPS-, dsRNA and heat shock proteins)[44] and promote the activation of immune cells[45]. MSC are among the cells that express in their surface TLRs, such as TLR-3 and TLR-4[11,45]. TLR3 and TLR4 differentially license MSC; with TLR3 "priming" inducing anti-inflammatory MSCs and TLR4 "priming" inducing a pro-inflammatory phenotype[40].

MSCs can sense local microenvironment signals and in balance with them promote optimal immune response, which can be either inhibitory or stimulatory depending on current specific state of an organism[40,46,47].

MSCs have been shown to possess a broad spectrum of immunoregulatory capabilities, affecting both innate immune system (DCs, NKs, monocytes and neutrophils) and adaptive immune system (B and T cells)[48] inhibiting proliferation, reducing immune cell cytokine secretion and alter immune cell subtypes[23] and promoting the generation of Tregs[3,36,49] (Figure I.2).

In vitro, the behaviour of MSCs depend on diverse factors, such as the source of the MSCs, the type of immune cells present in the cell culture, the state of activation and differentiation of the T cells and the type of stimuli used.

1.4.1. Mechanisms of Immunomodulation by MSCs

The immunomodulatory actions of MSCs are not fully understood. Several issues are under debate and the literature is filled with contradicting ideas, however, most experimental studies support a non-specific anti-proliferative action of these cells over immune system cells by means of paracrine effects[4,43] and/or by cell-cell contact-dependent mechanisms, which create an immunosuppressive microenvironment (Fig.1.2.)[3,50]. Some discrepancies in the literature may be due to the use of different of experimental designs, distinct cells used *in vitro* and by the individual research groups[47,51].

The cell-cell contacts haves been less investigated than the soluble factors in immunosuppression. In several studies transwell systems were used showing indirectly

that cell-cell contact is required, since MSCs-mediated effects were diminished or abolished when effector and target cells were separated by a membrane[5]. In 2003, Tse et al. reported that close proximity to MSCs was important in suppressing T cell responsiveness and suggested that direct contact between lymphocytes and MSCs was more important than soluble factors in the inducing immunosuppressive function[52]. A number of contact-dependent mechanisms have been reported in MSC immunomodulation, namely Fas/FasL, programmed death-1/programmed death ligand-1 (PD-1/PD-L1), galectins[51], Notch signalling, expression of the adhesion molecules vascular cell adhesion molecule I (VCAM-I), CD72, CD58[34], intracellular adhesion molecule (ICAM-I and ICAM-2)[34,40] and integrins (alpha1, alpha2, alpha3, alpha5, alpha6, alphav, beta2, beta3 and beta4)[34].

The paracrine effects are caused by the release of various soluble immunomodulators. *In vivo* and *in vitro*, various soluble immunosuppressive factors have been reported to be produced constitutively by MSCs or released following cross-talk with target cells such as interleukin (IL)-1, IL-6, IL-8, IL-10[53], tumour necrosis factor stimulated gene 6 protein (TSG-6)[54], heme oxygenase-1 (HO-1)[51], indoleamine 2,3-dioxygenase (IDO) in humans or nitric oxide (NO) in mice[55], transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF)[56], prostaglandin E2 (PGE-2), platelet-derived growth factor[42], matrix metalloproteinases (MMPs)[39], cyclooxygenase-1 (COX-1) and COX-2[46] and soluble HLA-G5[57,58]. It is clear that none of these soluble factors has an exclusive role and that MSC-mediated immunoregulation is a redundant systems that is mediated by several molecules[57]. In relation to the signal transduction pathways involved in MSCs mediated immunoregulation, various intracellular transcription factors have been reported. Signal transducer and activator of transcription 3 (STAT-3) factor has an increased activity in MSCs and antigen presenting cells (APC) upon co-culture. Transcription factor STAT-5 was shown to be diminished in activated T cells in the presence of MSCs. Another transcription factor, nuclear factor-kappa B (NFkB), has also been suggested to play a role in immunomodulation by MSCs[53].

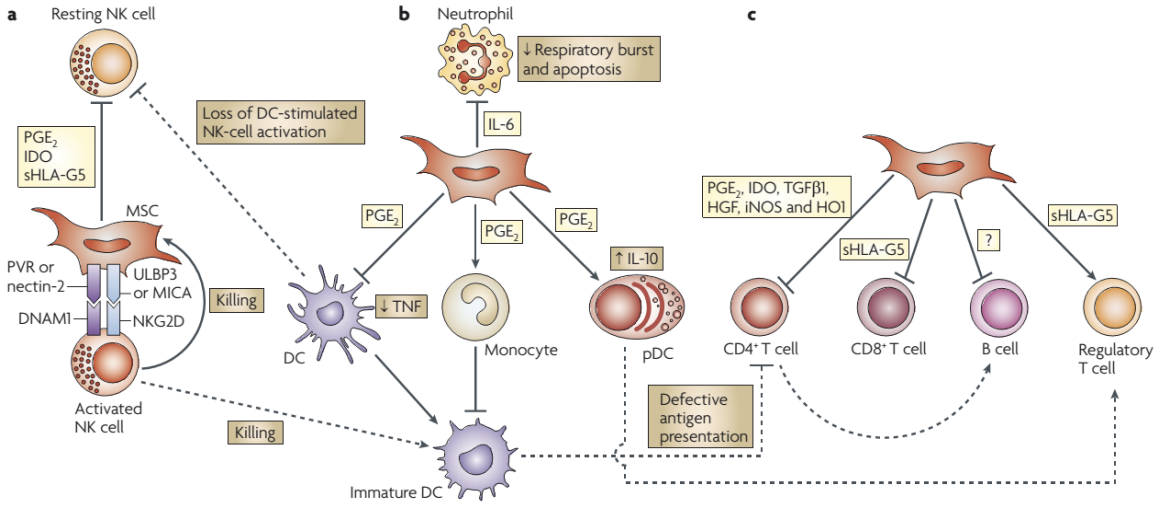

Figure 1.2: Schematic representation of interactions between mesenchymal stem cells (MSCs) and immune cells of the innate and adaptive immune system. A| MSCs supress resting NK cell proliferation, reduce their cytotoxic potential against target cells and cytokine production *in vitro*. **B|** MSCs inhibit differentiation of monocytes into mature dendritic cells (iDC) and their further maturation into mature DC (mDC), skew mature DCs to an immature DC state. The immature DCs are susceptible to killing by activated NK cells. The effect of MSCs on DCs impairs the stimulatory effect of mature DCs on resting NK cells and compromises antigen presentation to T cells, which cannot then undergo clonal expansion. **C|** MSCs inhibit proliferation and differentiation of B cells into plasma cells, reducing antibody formation. (Adapted from (Uccelli et al. 2008)

The immune regulatory mechanisms are composed by a complex interconnected networks that combines cytokines, signalling pathways and micro-environment, and MSCs may exert their effect as several levels, as further detailed below.

1.4.2. The effects of MSCs on immune cells

1.4.2.1. MSCs and T Cells

T lymphocytes (T cells) recognize antigens and are essential for the adaptive immune system, being involved in the maintenance of self-tolerance, activation of other lymphocytes, lysis of infected cells and interaction with cells of the innate immune system[59]. T cells are divided into two main lineages, $CD4^+$ T helper (Th) cells that modulate the other immune cells and $CD8^+$ cytotoxic T cells (CTLs) that induce death of target cells, both of which can be subgrouped into different effector subsets (naïve, central memory, effector memory and effector cells)[60,61]. Currently, an increasing number of studies have reported the inhibitory effect of MSCs over immune cells, the majority of which are focused on T cell but less is described concerning the effect of MSCs over distinct T cell functional

compartments. Data is scarce and there are some contradictory results regarding the influence of MSCs on the naïve-effector T cell differentiation process[62]. This contradictory data may be due to different experimental approaches.

The immunosuppressive effect exerted by MSCs over T cells comprises inhibition of T cells in terms of proliferation, activation, differentiation into effector cells and effector function, and by altering their cytokine profile and impairing the cytolytic activity of CD8+ cells[62] caused by MSC-released soluble factors and cell-cell contact. This suppression may occur directly, or indirectly via immunomodulatory effects on antigen-presenting cells (APC) such as DCs, resulting in altered cytokine expression and impaired antigen presentation[63].

T cells proliferation stimulated by mitogens or allogeneic cells[57] is inhibit by MSCs by preventing their entry into the S phase of cell cycle by arresting irreversibly the G0/G1 phase through the inhibition of cyclin D2 expression[59,60], which results in the induction of T cell anergy[61,64,65]. Anergic T cells are characterized by a lack of cytokine production and proliferation in response to antigenic stimulation, as a result of insufficient co-stimulation by CD40, B7-1 and B7-2, molecules that MSC lack[38,50]. This anti-proliferative effect is associated with the survival of T cells in a state of quiescence that can be partially reversed by IL-2 stimulation[57,60].

Along with the inhibitory action over T cells, there is also a decreased expression of the cytokine IFN-γ, TNF-α and IL-2 both *in vivo* and *in vitro*[60,62,64]. It has also been reported an increased production of IL-10 and IL-4 by Th2 cells, which indicates a shift in T cells phenotype, from a pro-inflammatory (IFN-γ production) state to an anti-inflammatory/tolerant state (IL-4 production)[57] and promote the generation of Tregs[42,61]. The decreased expression of this cytokines inhibit the differentiation of naïve $CD8^+$ T cells into cytotoxic effector cells[20]. MSCs supress the cytotoxicity of $CD8^+$ if added at the beginning of the mixed lymphocyte culture (MLC) but when added in the cytotoxic stage the cytotoxicity is not affected[66]. Therefore, when $CD8^+$ T cells are activated, MSCs are not effective in their immunosuppressive action.

The decrease expression of these cytokines varies within the different T cell compartments, showing distinct inhibitory patterns. The inhibition of IFN-γ in naïve T cells

is reported to be only among $CD8^+$ T cells and no inhibitory effect is detected over naïve $CD4^+$ T cells. Besides, in the central memory (CM), effector memory (EM) and effector compartment a more pronounced effect was observed. Concerning the inhibition of TNF-α, CM and effector $CD4^+$ T cells and EM $CD8^+$ T cells were the functional compartments with a higher decrease of expression of this cytokine. The inhibition of IL-2 is more pronounced among CM, EM and effector $CD4^+$ T cells and CM $CD8^+$ T cells[35,62,67].

The mechanisms used by MSCs to suppress T cells are cell-cell contact mediated by expression of the cell death ligand PD-L1 and by soluble factors such as PGE2, IDO, COX1 and 2, HGF, TGF-β, HLA-G5 and others[19,48].

1.4.2.1.1. Regulatory T cells

Tregs suppress the proliferation and cytokine production of T cells, mediating peripheral tolerance by addressing autoreactive T cells that escape thymic deletion. This regulatory population, may be identify by the co-expression of CD4, CD25 and the transcription factor FoxP3 (CD4+CD25+FOXP3+)[64].

MSCs were shown to upregulate Tregs suggesting that MSCs constitute a suitable niche for Tregs, playing a role in their recruitment, regulation and maintenance of phenotype and function. This induction has been reported to be mediated by PGE2, TGF-β1 and by direct cell-cell contact between MSCs and CD4+T cells[51]. The favoured activation of Tregs by MSCs may represent one of the important mechanisms of the immunosuppressive properties of MSCS, since Tregs have a regulatory function. MSCs upregulate the expansion of existing Tregs but do not stimulate the formation of new regulatory cell from naïve T cells[68].

1.4.2.2. MSCs and B Cells

Similar to the effects on T cells, the molecular mechanisms of action of MSCs on B cells are related to both cell-cell contact and secretion of soluble factors[69].

MSCs suppress B-cell activation, proliferation, development into plasma cells[61] and IgG secretion (even after stimulation of B-cells) through an arrest at the G0/G1 phase of the cell cycle[70].

A study from Rasmusson *et al*. described that MSCs downregulate the expression of the chemokine receptors CXCR4, CXCR5 and CCR7B and the chemotaxis to CXCL12, CXCR4 ligand, CXCL13 and CXCR5 ligand, revealing that MSCs affect the chemotactic properties of B cells significantly[38,50,70].

1.4.2.3. MSCs and NK Cells

Natural Killers (NK) cells are considered the major effector cells of the innate immune system. NKs are mostly involved in the destruction of tumour cells and virus-infected cells, since they kill without MHC class I restrictions through the release of perforin and granzyme from cytotoxic granules[71,72]. MSCs express low levels of MHC class I, so the lethality of KN cells is substantially reduced if MSCs are primed by IFN-γ, as they would be in an inflammatory environment[73]. There are two subsets of NK cells, which are $CD56^{dim}$ NK cells (cytotoxic NK cells) and $CD56^{bright}$ NK cells (a subset that has the capacity to produce abundant cytokines after activation but has a low natural cytotoxicity)[3,59].

MSCs can strongly inhibit the proliferation of resting NK cells and alter cytokine release, decreasing their capacity of secreting IFN-γ, TGF-α and IL-10[73]. Similarly to $CD8^+$ T cells, activated NK cells cannot be suppressed by MSCs, and have the ability to kill allogeneic MSCs. In response to IFN-γ, activating receptors of NK cells such as NKp30, NK44p and NKG2D[60] were downregulated when in co-culture with MSCs[71], which in turn upregulate MHC avoiding being killed by NK cells[60,72].

There is growing evidence that soluble factors such as IDO, PGE2, TGF-β and sHLA-G5 are involved in this immunosuppressive effect[72].

1.4.2.4. MSCs and DC Cells

Dendritic cells (DC) are derived from monocytes and are specialized, phagocytic professional antigen presenting cells (APC) that serve as a connection between the innate and adaptive immune system in mammals by helping to activate T and B cells[4,72]. The life cycle of this APC consists of an immature stage followed by a mature stage characterized by high efficiency in antigen uptake and processing and later on by potent antigen presentation[61]. DCs uptake antigen and during maturation and activation up-regulate MHCs, increase the expression of co-stimulatory molecules and migrate to secondary lymphoid organs and present antigen to T cells, leading to a primary adaptive immune response producing cytokines that affect downstream T cell effector functions, during T cell priming. MSCs have been shown to affect most of these processes[60,74,75].

MSCs are able to strongly inhibit DC generation from both monocytes and $CD34^+$ cell precursors[73], affecting all major stages of their life cycle, differentiation, maturation and activation[61], leading to a reduction of the expression of co-stimulatory molecules and debilitating the ability to stimulate naïve T cell proliferation[48,65]. Additionally, DCs cultured with MSCs have been shown to induce indirect expansion of Tregs.

Monocytes cultured under DC-differentiating conditions in presence of MSCs fail to proliferate and remain at the G0 phase of the cell cycle[7,60].

MSCs reduced DC secretion of pro-inflammatory cytokines such as IFN-γ, IL-12 and TNF-α[70], inhibit the regulation of APC-related molecules such as HLA-DR, CD1a, CD14, CD40, CD80, CD83 and CD86 antigens on their surface during the maturation stage[50] and increased IL-10 leading to the inhibition of DCs maturation and the capacity to activate alloreactive T cells resulting in a state of an immunologic tolerance[51].

Cell contact via surface ligands involving activation of the Notch signalling pathway[51] and soluble factors enhanced the efficiency of this supression[59]. IL-6, macrophage-CSF and PGE2 are described to be involved in MSCs-mediated immunosuppression of DC differentiation from monocytes[73].

1.5. Mechanotransduction

The extracellular environment is an essential mediator of cell survival providing both biochemical and mechanical stimuli that influence cell behavior[76]. The composition and mechanical properties of the extracellular matrix (ECM) are essential for cellular processes such as proliferation, differentiation and other cell fate decisions[77,78]. The microenvironment can influence cells by the presence of not only biochemical stimuli but also by mechanical and physical stimuli[79,80]. Cell adhesion, actin flow, retraction forces or gene expression are also cellular processes influenced by substrate rigidity[79,81].

Mechanotransduction is by definition the mechanism by which cells transform mechanical signals by mechanosensitive receptors or structures that sense and convert them into biochemical responses (Figure 1.3)[82,83]. This can occur as a result of changes in the cell cytoskeleton or through a series of biochemical signalling cascades[78].

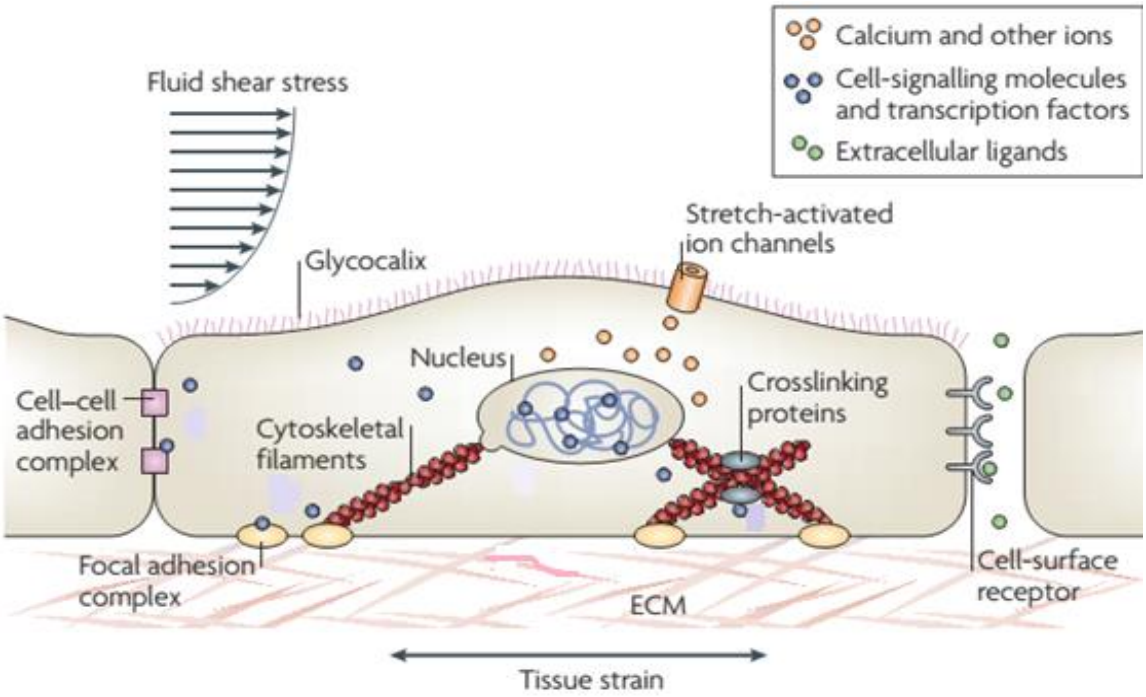

Figure 1.3: Schematic showing mechanotransduction in a Cell-ECM unit. Biophysical signals in the stem cell niche include matrix rigidity and topography, flow shear stress, strain forces, tensile forces actin through the ECM, and other mechanical forces exerted by adjacent support cells. Stem cells sense these signals through mechanosensors such as ion channels, focal adhesions (FA), cell surface receptors, and actin cytoskeleton and cell-cell adhesions. (Adapted from Jaalouk et al., 2009)

Biophysical stimuli in the stem cells niche include matrix rigidity and topography, shear forces associated with fluid flow over the cell, tensile forces acting through the ECM, and contractile forces generated by motor protein and cytoskeleton complexes[84]. These biophysical stimuli can be sensed by stem cells through mechanosensors such as ion channels, cell surface receptors, focal adhesions (FA), actin cytoskeleton and cell-cell adhesions[85]. The variety of mechanosensors in stem cells suggests that they have a robust ability to interact with their mechanical environment. MSCs can sense the rigidity of the ECM through generating contractile forces through adhesion complexes that connect intracellular structures to ECM[86].

Engler et al., provide a new approach to direct stem cell fate, demonstrating that matrix elasticity is enough to direct commitment of adult stem cells toward different lineages[78,80]. In 2010, a study from Gilbert et al., showed that the regenerative capacity of stem cells *in vivo* can be strongly influenced by the mechanical properties of the substrate where cells were cultured *in vitro*[87] . A recent study from Yang and colleagues, using MSCs cultured on hydrogels with distinct degrees of stiffness, demonstrated that stem cells have mechanical memory and that YAP/TAZ (transcription factor) behave as an intracellular mechasensor[78], by exiting the nucleus when cells were kept on soft environments and entering the nucleus when on stiff environments.

1.5.1. Mechanisms of Mechanotransduction

A dynamic relationship exists which allows the ECM to induce signals from the extracellular environment inside the cell and, conversely, signalling from inside the cell that eventually resulting in remodelling of the ECM. This dynamics is largely mediated by integrin binding to ECM proteins[78]. Integrins are transmembrane proteins heterodimers composed of an α- and a β-chain, which mediate the adhesion of cells to a diversity of ECM proteins ligands, including fibronectin, collagen, laminin and vitronectin[79]. Cells sense extracellular stiffness using integrins to attach to the ECM and by generating traction forces via actomyosin contractility, hence sensing the corresponding reaction force generated by the environment. Currently it is known that the integrin receptor itself switches to a high-affinity state in response to force[88].

Mechanotransduction initiates at focal adhesions (FA), a dynamic structure that binds to the ECM, which is formed by coordinated recruitment of intracellular adaptor and catalytic proteins linked to ECM proteins through integrins (Figure 1.4 and figure 1.5)[79,85]. In response to a mechanical stimulus, many enzymes change their kinetics, like focal adhesion kinase (FAK), whose activity increases with mechanical stimuli[79]. This enzyme interacts with integrins and phosphorylates tyrosine residues of intracellular proteins, promoting their recruitment to FA.

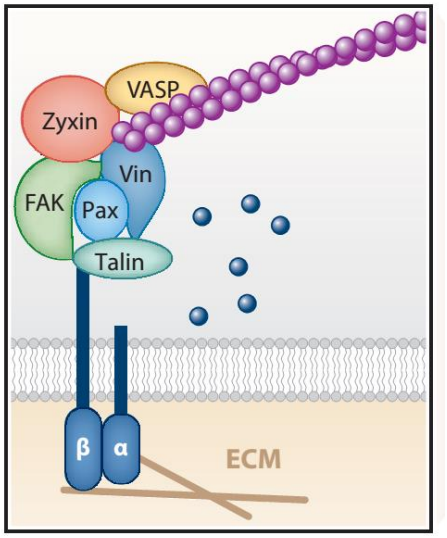

Figure 1.4: Focal Adhesion structure. Schematic representation of focal adhesion structure that is formed by integrin, talin, paxilin (Pax), vinculin (Vin), focal adhesion kinase (FAK), zynix and vasodilator-stimulated phosphoprotein (VASP) and the actin cytoskeleton (in purple) bond to the FA. These molecules provide a direct physical link to the actin cytoskeleton. Talin binds to the cytoplasmic domain of the β integrin subunit, thereby triggering the transition of the entire α integrin –β integrin dimer from an inactive to an active conformation that is capable of high affinity interactions with ECM ligands. (Adapted from Sun et al., 2012)

After FA formation initiates, cells exert force on the substrate through actomyosin contraction, which results in movement of actin fibers. Depending on the rigidity of the substrate, talin may become stretched or not. On a stiff substrate, actomyosin induces tension, talin becomes stretched – hence revealing further domains for the binding of adapter proteins - resulting in reinforcement of early FAs, though the recruitment of more vinculin and other FA adapter proteins[79]. In case of a soft substrate, talin does not become stretched, since the extracellular matrix deforms in response to the force exerted by the cell[79].

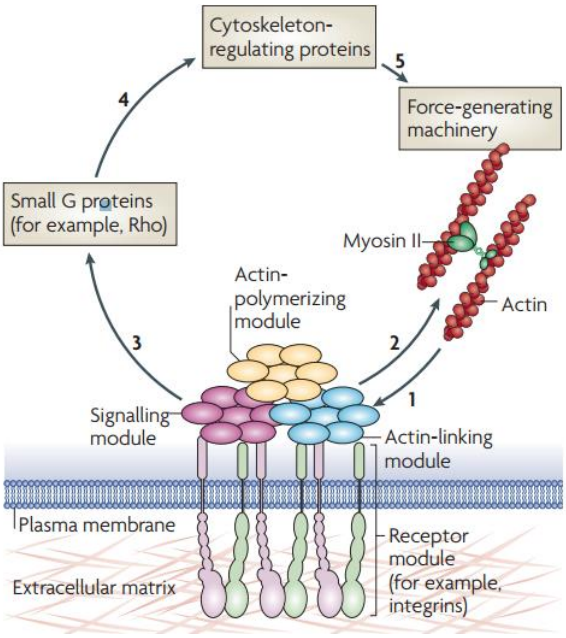

Figure 1.5: Schematic actin cytoskeleton – FA interaction. (Step 1) Generated forces by actin polymerization and myosin II-dependent contractility affect specific proteins mechanosensors in the actin-linking module (such as talin or vinculin), the receptor module (integrins) and co-receptors, the associated actin-polymerizing (zynx) and the signalling module (FAK and p130CAS). These interacting molecules form a mechanoresponsive network. (Step2) The effect on the actin cytoskeleton depends on the integrated response of the entire system to interactions with the matrix and to applied mechanical forces. (Step 3) Stimulation of signalling module leads to activation of guanine nucleotide-exchange factors and GTPase-activating proteins, which leads to activation of small G proteins (namely Rho and Rac). (Step 4) Rho affects actin polymerization and actomyosin contractility through cytoskeleton-regulating proteins, thereby (step 5) modulating the force generating machinery. (Adapted from Geiger et al., 2009)

Once focal adhesion kinase (FAK) becomes activated as a result of integrin activation, it could lead to the activation of MAP kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signalling pathways, regulating diverse cellular processes, such as proliferation, migration and differentiation[89]. Beyond that, FAK can also activate the RhoA pathway, which is involved in actin cytoskeleton tension. When RhoA is activated, recruit myosin II to bind actin cytoskeleton, that will increase cytoskeleton tension leading to a reinforcement of FAs[88]. The activity of RhoA seems to be altered by stiffness, altering the degree of myosin activation and the cellular contractility[84].

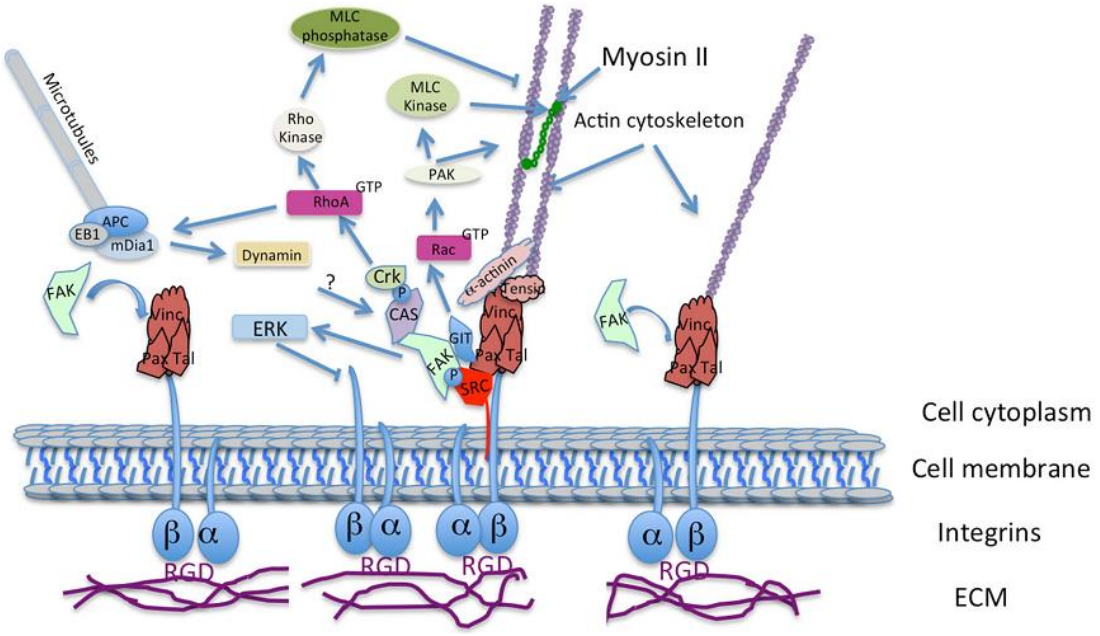

Figure 1.6: Proteins involved in integrin-mediated rigidity sensing. After the formation of the FA, RhoA is activated by FAK and recruits myosin to bind actin cytoskeleton, increasing the cytoskeleton tension and resulting in the focal adhesion reinforcement. (Adapted from McMurray et al., 2014)

In summary, the process of mechanotransduction is composed by several mechanism that permit cells to sense their physical environment and respond accordingly.

1.5.2. ECM Stifness and Biomaterials

Cells generally show better in vitro behaviour when cultured on materials with stiffness similar to that their microenvironment, so the rigidity preferences of cells generally reflect their native enviroments[79]. In the human body, the elastic modulus of adult mammalian tissues ranges several orders, from < 1 kilopaascal (kPa) - or 1 nN/µm^2 - for brain to MPa (Megapascal) for bone (Figure 1.7)[76,90]. The stiffness of the microenvironment has an important consequence in cellular processes such as spreading, morphology and function[81]. Stem cells can sense and respond to alterations of the elastic modulus of the ECM by modulating their endogenous cytoskeleton contractility, balanced by resistant forces which are generated by the deformation of the ECM[85]. MSCs sense and respond to substrate rigidity by exerting traction forces upon the binding between integrins and integrin-specific ligands that are present on the substrate surface. The stiffness of biomaterials can be measured based on the force that is required to deform the matrix[79].

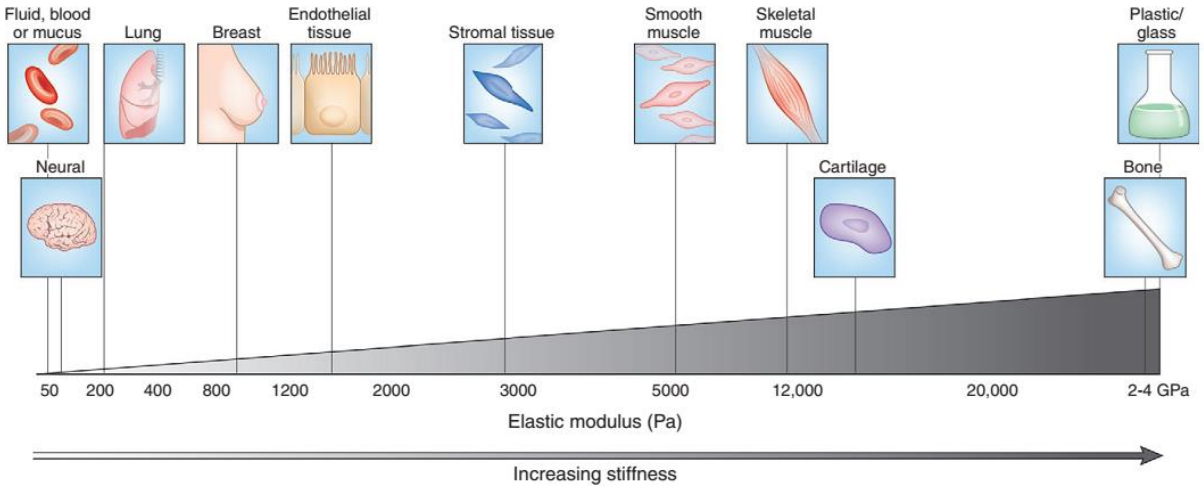

Figure 1.7: Variations in tissue stiffness: tissues exhibit a range of stiffness, measured by the elastic (Young's) modulus, *E*. (Adapted from Cox et al., 2011)

In a biological context, elasticity or stiffness is often referred to as Young's modulus (also known as Elastic modulus) *E,* that consists of the amount of force per unit area needed to deform the material by a given fractional amount without any permanent deformation, being a high elastic modulus corresponding to high stiffness and low deformability. To determine the elastic modulus, the force is applied perpendicular to the material's surface, whereas for the shear modulus, *(-G)*, the force is applied parallel to the surface (Figure 1.8).

The Young's modulus (*E*) can be calculated from the shear modulus,(both representing the amount of force per unit of area[84]) using the following equation : $E = 2G(1+\mathbf{v})$, where **v** is the Poisson ratio.

In case of materials that do not change volume under stretch, like most rubber-like elastic materials, the Poisson ratio equals 0.5 and the elastic modulus will be three times its shear modulus, $E=3G$. The units for rigidity are force per area being the SI unit the Pascal[79,84].

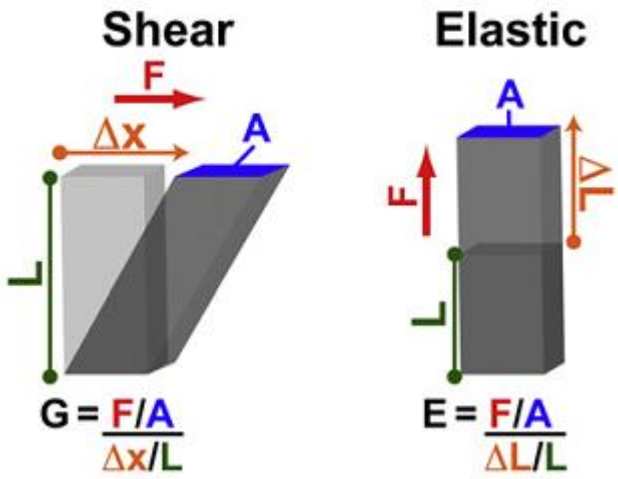

Figure 1.8: Rigidity moduli. Stress is the amount of force that is applied per area (F/A) and the strain is the displacement in the direction of applied force relative to initial length (Δx/L or ΔL/L). Elastic and shear moduli are the ratio of stress over strain, the difference is in the direction of the applied force. (Adapted from Moore et al., 2010)

Chapter 2 – Aims

2. Aims

The aim of this thesis is to understand if elements related to mechanotransduction influence immunomodulation induced by MSCs. Namely, we were interested to elucidate if MSCs derived from different substrates with distinct mechanical and biochemical properties possess different immunosuppressive capabilities and wether their action varies with the immune cell type.

Any positive modulation of the immunomodulation capacity by MSCs from different substrates would represent an important contribution to the field.

Chapter 3 – Materials and Methods

3. Materials and Methods

3.1. Isolation and culture of mesenchymal stem cells (MSCs) from human Wharton's Jelly (WJ)

All the procedures concerning cell culture and manipulation of umbilical cord were performed under aseptic conditions using a class II vertical laminar airflow cabinet (HeraSafe HS-18, Heraeus).

Fresh human umbilical cord were obtained after birth, provided by Crioestaminal S.A and stored in a sterile 50mL conical tube (VWR International) for 12 to 48 hours before tissue processing (as described[27]. In detail, each umbilical cord was cut into sections about 5cm long and then washed 2 or 3 times with sterile phosphate buffered saline (PBS, Life Technologies), to remove the blood. Subsequently, the vein and arteries were removed to avoid endothelial cell contamination and the Wharton's jelly (WJ) was cut into fragments of 2-5mm with the help of a scalpel and forceps. Groups of 15 to 30 fragments were transferred to 21cm^2 tissue culture plates (TCP, Corning-Costar) or to 21cm^2 dishes with collagen-I (Merck) and Fibronectin (Merck) functionalized PDMS substrates and left to dry (30 minutes for TCP and 1 hour for the PDMS substrates) to promote the attachment of the fragments to the plastic or PDMS. Then, the MSC proliferation medium [Alpha-MEM (Life Technologies) supplemented with 10% volume/volume (v/v) MSC-qualified Fetal Bovine Serum (FBS, Hyclone), 10 U/ml Penicillin, 10µg/ml Streptomycin and 2,5 µg/mL Amphotericin B (all from Life Technologies)], pre-warmed in a 37°C water bath, was slowly added to the attached fragments until all the fragments were totally immersed. These fragments were maintained in culture for 15 to 20 days in an incubator at 37°C, in an aseptic environment with 5% CO_2 and humidified atmosphere, until MSCs were migrating out of the hWJ fragments and forming well defined colonies, regularly adding the necessary volume medium (as needed) to keep them immersed. The cells that migrated out of hWJ were detached from the plastic and gel surface after removing the fragments and washing cells with PBS), using Trypsin (500µg/ml) – EDTA (200 µg/ml) solution (Life Technologies) during 5 minutes, at 37°C. The trypsin was inactivated with α-MEM with 10% (v/v) FBS (Life

Technologies) and then cells were re-suspended using a 5 ml serological pipette (Corning-costar) for complete cell dissociation. Next, cells were centrifuged (290 x *g* 5 min), the pellet was re-suspended in MSC proliferation medium using a serological pipette in MSC proliferation medium, counted and plated in new TCP and PDMS dishes. For the passaging procedure cells were washed twice with PBS and then dissociated using Trypsin-EDTA solution (as described above) when reached around 70-80% of confluence. Cells were passaged until a maximum of passage 8 (P8).

MSC identify was confirmed by immunophenotype characterization and colony-forming unit-fibroblast as described by Dominici et al.[10].

3.1.1. Cryopreservation of MSCs

When cells reached around 80-90% of confluence were dissociated using Trypsin-EDTA solution, then re-suspended with MSC proliferation medium and collected to a conical tube for centrifugation (290 x *g*, 5min). The supernatant was removed and the pellet was re-suspended in 1mL of freezing medium, constituted by FBS (Life Technologies) with 10% (v/v) cell-culture tested DMSO (Sigma) and transferred into a cryopreservation vial (Nunc). The vial was frozen at -80°C overnight in an isopropanol-based cryo-cooler (VWR), to promote a gradual freezing (-1°C/min) and then transferred into a nitrogen cryotank, correctly identified with the cell type, passage number, corresponding thawing culture area, substrate type and freezing date.

3.1.2. Proliferation kinetics of MSCs

MSC isolated from WJ were continuously cultured from P2 to P8 and counted once they reached 70-80% confluence at each passage. The population doubling (PD) rate was determined ate each passage using the following equation[91]:

$$X = \frac{log10\ (NH) - log10\ (N1)}{log10\ (2)} \qquad \text{Equation 3.1}$$

NH represents the harvested cell number and N1 the plated cell number. The PD for each passage was calculated and added to the PD of the previous passage to generate data

for cumulative population doubling (CPD). The generation time (GT) (average time between two cells doublings) was also calculated, using the following equation[91]:

$$X = \frac{log10\ (2) \times \Delta t}{log10\ (NH) - log10\ (N1)} \quad \text{Equation 3.2}$$

The total number of cells (TNC) designates the theoretical number of cells that could be obtained if no cells were discarded between each passage. The TNC was determined at each passage by cumulative counting of the cells using the following formula:

$$X = \frac{NH \times B}{N1} \quad \text{Equation 3.3}$$

B represents the total number of cells of the previous passage. The TNC designates the theoretical number of cells that could be obtained if no cells were discarded between passages.

3.1.3. Colony-forming unit-fibroblasts assay

The colony-forming unit-fibroblast (CFU-F) assay was determined at P2 and P8. Cells were seeded at 10 cells/cm^2 on 55cm^2 tissue culture plates in MSC proliferation medium and cultured for 15 days, one third of the medium was replaced twice a week. Then, cells were fixed with 4% PFA for 20 minutes at RT and stained with Giemsa solution (Sigma). Individual colonies were counted manually.

3.1.4. Immunophenotypic study of hWJ-MSCs

The immunophenotypic characterization of MSCs was performed in collaboration with Centro Hospitalar da Universidade de Coimbra – Unidade de Gestão Operacional de Citometria and prior to co-cultures experiments. Cells were detached with Accutase® (LifeTechnologies), washed with PBS and the cell pellet was stained with monoclonal antibody (mAb) for surface protein antigens and, after an incubation period of 10 minutes in the dark at room temperature (RT), washed with PBS. Then, cells were ressuspended in 250 µL of PBS and immediately acquired in a FACSCanto II (BD) flow cytometer. The mAb

used were conjugated with the following fluorochromes: fluorescein isothiocyanate (FITC), allophycocyanin (APC), phycoerythrin (PE), phycoerythrin-cyanine 7 (PECy7), krome orange (KO) and phycoerythrin-cyanine 5 (PerCPCy 5.5). The following monoclonal antibodies were used for the labelling: CD105 FITC (clone 2H6F11, Immunostep), CD90 APC (clone 5E10, BD Pharminogen), CD73 PE (clone AD2, BD Pharminogen), CD13 PECy7 (clone Immu103.44, Beckman Coulter), CD45 KO (clone J.33, Beckman Coulter) and CD34 PerCPCy 5.5 (clone 581, BD Biosciences).

3.2. Preparation and Functionalization of Polydimethylsiloxane (PDMS) for cell culture

3.2.1. Preparation of PDMS

Polydimethylsiloxane (PDMS) is a biocompatible and nontoxic silicone elastomer. The elastic properties of this material can be easily tuned by changing the based/curing agent ratio to cover a wide range of physiologically-relevant elastic modulus for moduli for mechanobiological studies.

PDMS monomer (Sylgard 184 Silicone Elastomer Kit, Dow Corning) was homogeneously mixed with its curing agent in the volume ratio of 10:1 and 40:1 (hereafter referred to as 1:10 and 1:40 PDMS substrates) followed by casting onto a polystyrene dish. This kit is a heat curable PDMS supplied as a two-part kit consisting of pre-polymer (base) and cross-linker (curing agent) components. The mixture was then degassed during 1 hour to remove all of the air bubbles and cured at 70°C for 4 hours in a heating incubator (Binder). The substrate with 1:10 volume ratio has a stiffness around 1000 kPa and 1:40 was a stiffness approximately around 80 kPa – the stiffness of TCP is around 1 gigapascal (GPa) [92] .

3.2.2. Treatment of Polydimethylsiloxane (PDMS) substrates for cell culture

PDMS is hydrophobic and provides extremely low adhesiveness to cells, making it necessary to chemically treat it in order to turn it more hydrophilic and to subsequently allow the covalent binding of ECM proteins (providing a stable bond between the substrate and the protein) providing an adequate environment for mammalian cell culture.

For the hydrophilic treatment and functionalization the surface of PDMS substrates, the 1:10 substrates were detached from the casting dish and reversed in order to treat the smoother side (bottom part) of the substrate. The reason for this procedure was to avoid the roughness of the "upper side" of the substrate, which interfered with the cell culture, as also described in the literature[93]. In case of the 1:40 substrates, these were not detached and were treated in the casting dish, since the surface roughness of these PDMS substrates seemed to be lower and did not cause problems during MSC cell culture. The procedure for treatment of PDMS is summarized in Figure 3.1[93].

The hydrophilic treatment of PDMS was performed using a solution constituted by miliQ Water (H_2O mQ)/ hydrochloric acid (HCl, Fluka)/hydrogen peroxide (H_2O_2, Sigma) in a volumetric proportion of (5:1:1) for the 1:40substrate and (3:1:1) for the 1:10 substrate. The solutions were prepared fresh and added to the PDMS surfaces, allowing the reaction to occur during 5minutes at RT, creating this way silanol groups on the PDMS surface. A silanol is a functional group in silicon chemistry with the connectivity Si–O–H. After this reaction, PDMS was washed three times with H_2O mQ. In order to have a chemical functionalization of PDMS, a solution of 10% (v/v) 3-aminopropyltrimethoxisilane (APTMS) in 96% (v/v) ethanol (EtOH, Merck) was added to the substrates during 30 minutes at RT, followed by three abundant washes with H_2O mQ 10 minutes each with agitation, in order to remove the excess of APTMS that otherwise would avoid the reaction between PDMS-bond APTMS and glutaraldehyde (which was added on the following step).Then, a solution of 3% (v/v) glutaraldehyde in PBS 1x was added to the substrates and incubated for 20 minutes at RT. After that, substrates were washed three times with abundant H_2O mQ 5 minutes each, with agitation. The chemical functionalization of PDMS with the crosslink of APTMS and glutaraldehyde leave one free aldehyde group that covalently binds to ECM protein, and leaving the PDMS substrate functionalized. The substrates 1:10 were cut and

placed in new-cell culture dishes. Afterwards, the substrate-containing dishes were exposed to ultraviolet (UV) light during 30 minutes in PBS and washed one time with PBS. Finally, a solution of 2920 µg/mL Collagen Type I (Merck) was added to 1:40 substrates and 1000 µg/mL Fibronectin (Merck) plus 2920µg/mL Collagen Type I was added to 1:10 substrates and then incubated for 2hours and 3 hours, respectively. The ECM protein composition described for each PDMS substrate was optimized for each condition.

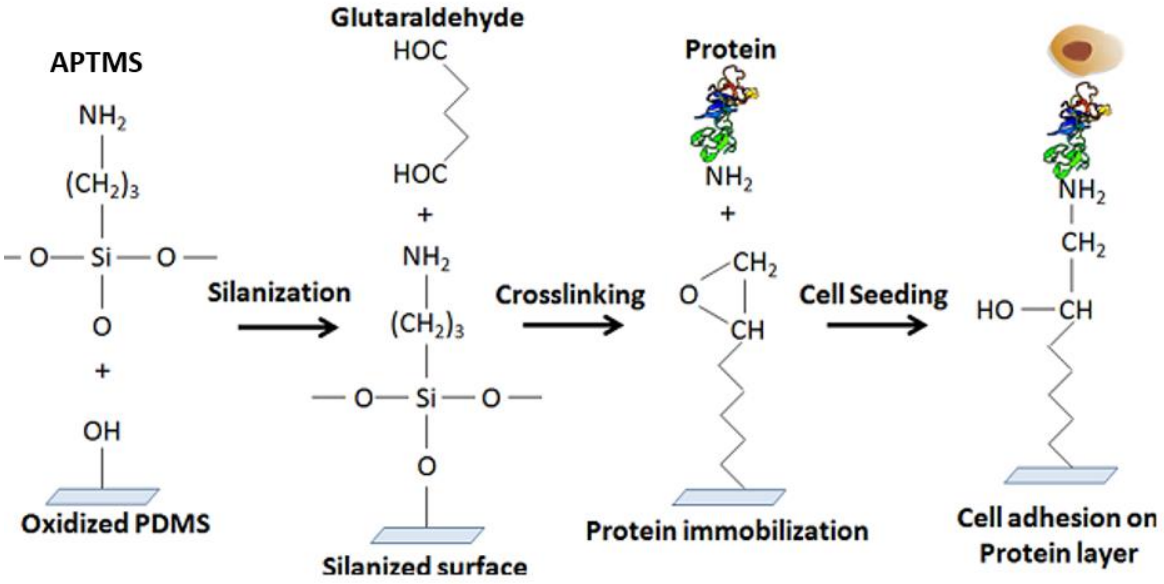

Figure 3. 1 Schematics of PDMS surface chemical modification. PDMS underwent a hydrophilic treatment using a H2O/HCl/H2O2 solution and then, the crosslink between APTMS and glutaraldehyde allows the covalent binding of ECM protein to the PDMS surface. (Adapted from Kuddannaya et al., 2013)

3.2.3. Cell Culture on TCP and PDMS substrates

After the isolation and expansion of MSCs on tissue culture plates (TCP), cells were split and re-plated on dthe distinct substrates (TCP, 1:10, 1:40 PDMS substrates) at $5x10^3$ cells/cm^2 and maintained for 5 days in culture before being harvested for co-culture experiments with mononuclear cells (MNCs). Culture media were changed 24 hours before the co-culture experiments to serum-free α-MEM (supplemented with antibiotics), to obtain MSCs-conditioned media (CM). The distinct CM were then centrifuged (2900 x *g*, 5 mint) and collected for multiplex cytokine analysis and for experiments using MNCs in presence of MSCs-conditioned media.

3.3. Multiplex Cytokine Analysis

In order to analyse concentration of cytokines conditioned culture medium was collected from MSCs after24h with medium serum free and conditioned culture medium after 24h in direct contact with mononuclear cells, was also collected.

The concentration of cytokines on conditioned cell culture medium were evaluated using a Bio-Plex Pro 8-Plex Panel Assay (Bio-Rad, Hercules), according to the manufacturer's instructions and as detailed below, using a Bio-Plex 200 system (Bio-Rad). The 8-plex panel evaluated the presence of the following analytes: GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α. The procedure for multiplex cytokine analysis is summarized in Figure 3.2[93].

Samples were, thawed on ice and homogenized using a microplate agitator (Asal 715) and then processed as follows (according to the kit's instructions). Samples were incubated at RT with agitation with the antibody-conjugated beads for 60 minutes and then washed three times using the kit wash buffer following vacuum aspiration. The incubation with detection antibodies was performed for 30 min (RT) with agitation, followed by three washed as before. Finally, the incubation with the antibody conjugate streptavidin-phycoerythrin (SA-PE) was performed for 10 min (RT, with agitation), followed by three washes. The beads were re-suspended with assay buffer and data was acquired using a Bio-Plex 200 system. Acquisition and analysis was performed using the software Bio-Plex Manager 5 (Bio-Rad).

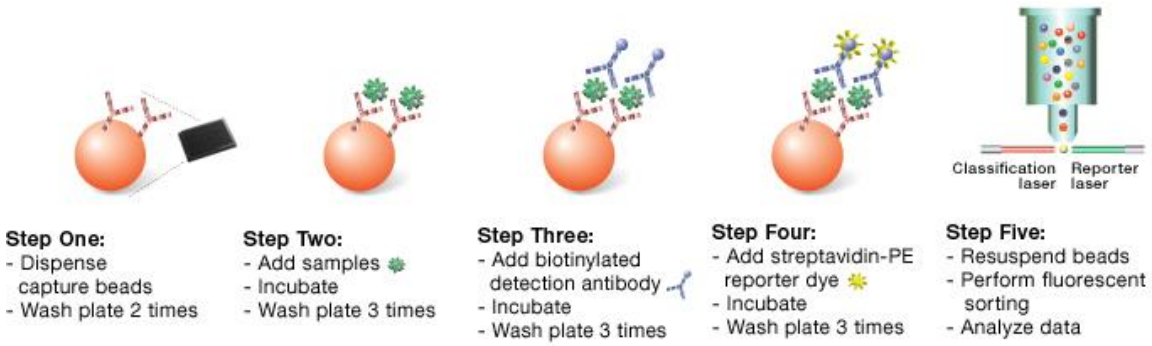

Figure 3. 2. Schematics summarizing the Bio-plex assays workflow. (Adapted from Biorad)

3.4. Immunophenotypic study of MSCs with Peripheral Blood Mononuclear Cells (MNCs)

3.4.1. Collection and Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood samples were obtained from a healthy donor (male, 38 years old), collected in heparin at the Instituto Português do Sangue e da Transplantação - Área de Transplantação (Portugal).

Peripheral blood mononuclear cells (MNCs) were isolated by Histopaque (Sigma-Aldrich) density gradient centrifugation (400 x *g* for 20 minutes) and then, washed in Hank's Balanced Salt Solution (HBSS, Life Technologies). The MNCs pellet was re-suspended in RPMI 1640 with L-glutamine (Life Technologies) and antibiotic-antimycotic and then, counted in a hematology analyser (Coulter Ac-T diff2, Beckman Coulter) and subsequently plated $4x10^5$ cells in 2 or 3 replicate wells per condition of 24 well tissue culture plates (Corning-costar). The cell culture and stimulation protocols are detailed below, in "Co-culture of MSC and peripheral blood (MNC) and *in vitro stimulation*" section.

3.4.2. Co-culture of MSCs and peripheral blood (MNCs) and In vitro stimulation

The co-culture of MSCs and peripheral blood (MNC) and in vitro stimulation was made in collaboration with Instituto Português do Sangue e da Transplantação - Área de Transplantação (Portugal).

All the procedures concerning cell culture and manipulation were performed under sterile conditions using a class-II vertical air-flow cabinet.

In 3 wells of 24 well tissue culture plates (Corning-costar) $4x10^5$ MNCs were plated in 500 µL of RPMI 1640 (Invitrogen, Life Technologies) and 500µL of α-MEM with antibiotic-antimycotic (Life Technologies), and in 6 others wells of tissue culture plates (Corning-Costar) $2x10^5$ MSCs + $4x10^5$ MNCs (each well) were plated in a final volume of 1 mL (1:1 – RPMI:α-MEM) , establishing a ratio of 1:2 (MSCs:MNCs). Additionally, a parallel assay using MSCs-conditioned medium (CM obtained from MSCs during 24h, as detailed above) was also performed, adding 500 µL of CM and to the MNC cells. Cells were cultured for 20-24 hours at 37°C, in an aseptic environment with 5% CO_2 and humidified atmosphere. All

assays were performed using MSCs between passage P3 and P5. Control cultures consisted of MNCs in the absence of MSCs with or without stimulation.

3.4.2.1. *In vitro* stimulation with Phorbol myristate acetate (PMA) plus ionomycin

Phorbol myristate acetate (PMA) is a small organic compound that diffuses through the cell membrane into the cytoplasma, where it directly activates protein kinase C (PKC) omitting the "need" of surface receptor stimulation. Ionomycin, calcium ionophor, is used in addition to trigger calcium release which is needed for NFAT signalling.

After the incubation period (detailed in material an methods section above), 50 ng/mL PMA plus 1 µg/ml ionomycin (Sigma-Aldrich and Boehringer, respectively) were added to the wells with MNCs and MNCs+MSCs. Brefeldin A (10 µg/mL) from *Penicillium brefeldiamun* (Sigma-Aldrich), is a protein transport inhibitor, was added to the wells to prevent the secretion of *de novo* produced cytokines outside the cells by MNCs, blocking the transport processes during cell activation leading to accumulation of most cytokines at the Golgi complex/Endoplasmatic reticulum. Then, proceeded to an incubation at 37°C, in an aseptic environment with 5% CO_2 and humidified atmosphere, for 4 hours. These samples were used to study cytokine expression on T cells compartments (naïve, central memory, effector memory and effector cells) by flow cytometry.

3.4.2.2. *In vitro* stimulation with Lipopolysaccharide (LPS) plus interferon- gamma (IFN-γ)

Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and is one of the most potent stimuli for monocytes.

After the incubation period, 100 ng/ml lipopolysaccharide (LPS) plus 100 U/mL IFN-γ were added to the wells with MNCs and MNCs+MSCs. Brefeldin A (10 µg/mL) from *Penicillium brefeldiamun* (Sigma-Aldrich) was added to the wells to prevent the secretion of *de novo* produced cytokines outside the cells. Then, the samples were incubated at 37°C, in an aseptic environment with 5% CO_2 and humidified atmosphere, for 6 hours. These samples were used to study cytokine expression on monocytes by flow cytometry.

3.4.3. Immunophenotypic analysis of MNCs

After the incubation period for *in vitro* stimulation, the content of each well under different experimental condition was transferred to a 12x75mm polysterene cytometer tube, centrifuged (540 x *g* for 5 minutes). Immunophenotypic study of peripheral blood T cells and monocytes was perfomed using 8-color mAb combinations, as detailed in Table 3.1. Cell pellet were stained with mAB for surface proteins antigens. For intracellular staining, Intraprep Permeabilization Reagent (Beckamn Coulter) was used, according to the manufacter's instructions and in parallel with the mAb, for T cells IL-2 and TNF-α (tube 1), and for monocytes TNF-α (tube 2). After washing twice with PBS, the cell pellet was resuspended in 250µL of PBS and immediately acquired.

Table 3. 1. Panel of mAb reagents (with clones and commercial sources) used for the immunophenotypic characterization

Tube	Fluorochromes								
	PacB	**KO**	**FITC**	**PE**	**PerCPCy 5.5 or PECy5**	**PECy7**	**APC**	**APCH7**	**V500**
1	**CD3** UCHT1 BD Pharmingen	-	**IL-2** MQ1-17H12 BD Pharmingen	**TNF-α** Mab11 BD Pharmingen	**CD27** 1A4CD27 Beckman Coulter	**CD56** N901 Beckman Coulter	**CD45RA** HI100 BD Pharmingen	**CD4** SK3 BD Biosciences	**CD8** RPA-T8 BD Horizon
2	**HLA-DR** L243 BD Pharmingen	**CD45** J.33 Beckman Coulter	**CD35** E11 BD Parmingen	**TNF- α** Mab11 BD Pharmingen	**CD11C** B-ly6 BD Biosciences	**CD33** D3HL60.251 Beckman Coulter	**IREM-2** UP-H2 Immunostep	**CD14** MϕP9 BD Biosciences	-

Abbreviations: mAb, monoclonal antibody; PacB, pacific blue; KO, krome orange; FITC, fluorescein isothiocyanate; PE, phycoerythrin, PerCPCy 5.5, peridin chrophyll protein cyanine 5.5; PECy5, phycoerythrin-cyanine 5 ; PECy7, phycoerythrin-cyanine 7; APC, allophycocyanin; APCH7, allophycocyanin-hilite 7. Comercial sources: BD Pharmingen (San Diego,CA,USA); BD Bioscience (Becton Dickinson Biosciences, San Jose, CA,USA); Beckman Coulter (Miami, FL, USA).

3.5. Data Acquisition and analysis

Data acquisition were perfomed in a FACsCAnto™II (BD) flow cytometer equipped with the FACSDiva software (v6.1.2; BD) at Centro Hospital da Universidade de Coimbra – Unidade de Gestão Operacional de Citometria. For the immunophenotypic studies, the whole sample from each tube was acquired and stored. For data analysis, the Infinicyt (version 1.7) software (Cytognos SL, Salamanca, Spain) was used.

3.6. Statistical analysis

Statistical analysis was performed using the Graph Pad Prism 6 software. Kruskal-Wallis test followed by Dunn's multiple comparison test was performed when data was not normal (non-parametric analysis). When appropriate *t*-test was used, to assess the normality of the data, Shapiro-Wilk's test was performed. Values represent mean ± SEM and statistically significant differences were considered when *p* value was lower than 0.05.

Chapter 4 - Results

4. Results

4.1. Isolation and of mesenchymal stem cells (MSCs) from the Wharton's jelly (WJ)

In the first part of this work, the objective was to isolate mesenchymal stem cells (MSCs) from the WJ in polydimethylsiloxane (PDMS) substrates polymerized on cell culture dishes with different formulations and in tissue culture plates made of polystyrene (TCP). Human MSCs were isolated from, as described in the Materials and methods section 3.1. At the end of 10-15 days in culture, several WJ fragments were still attached to the culture dishes and showed cells migrating from the tissue. After cells migration from the tissue, formed colonies of cells displaying an MSC-like phenotype, with spindle-shaped morphology and could be identify by phase-contrast microscopy (Fig.4.1).

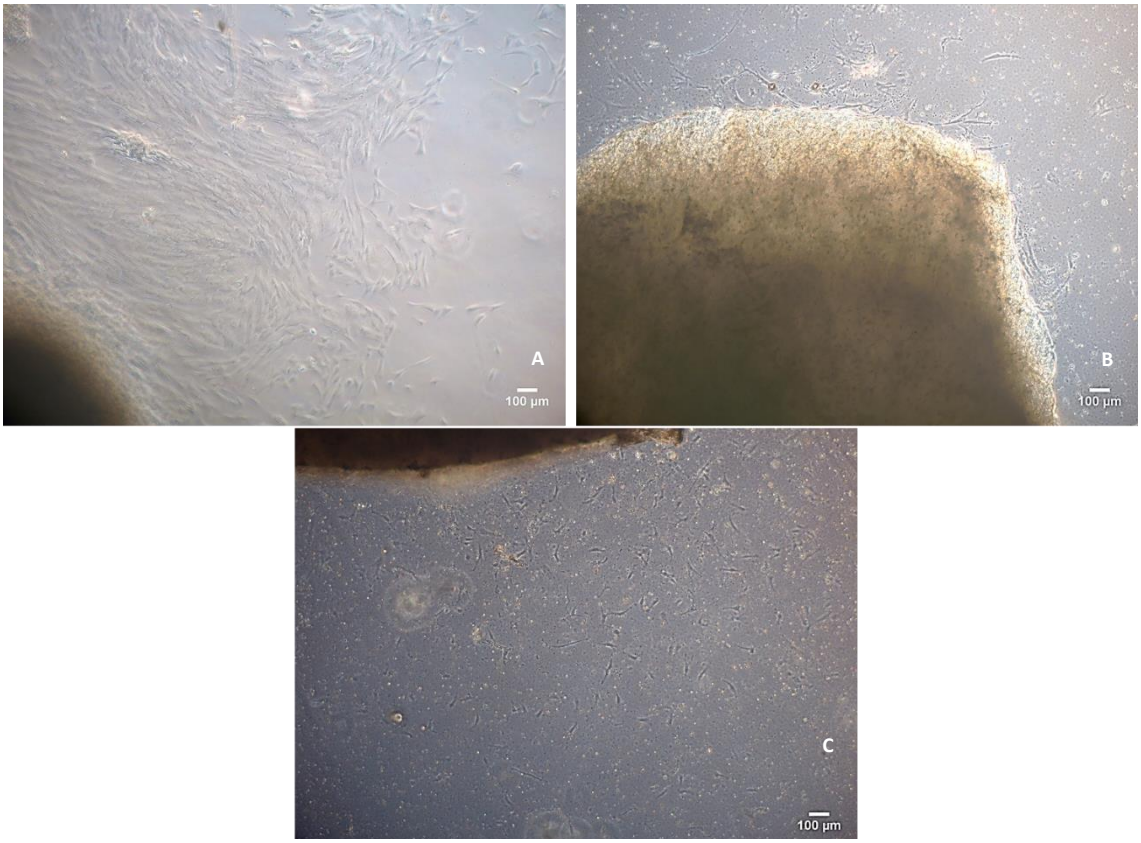

Figure 4. 1. MSCs like cells migrating from Wharton's jelly fragments in proliferation medium, passage 0. A) Isolation in tissue culture plates (TCPs); **B)** Isolation in 1:10 PDMS platform; **C)** Isolation in 1:40 PDMS platform

Despite the fact that isolation in PDMS was successful, there were some difficulties to proliferate the cells in the different substrates, further optimization is needed. Put that, for the aim of this project, WJ-MSCs were isolated in TCP, proliferate and then, plated at the different substrates 5 days, as described in materials and methods section 3.2.4.

4.2. Proliferation kinetics

A proliferation kinetics study was made with the purpose of characterizing the proliferation and evaluate the expansion capacity of isolated MSCs, since MSCs are cells with a higher proliferative capacity[9]. Cells were isolated from 4 distinct human Wharton's jelly samples (UC#819, UC#841, UC#874 and UC#875). The population doubling (PD) and cumulative population doubling (CPD), generation time (GT) and total number of cells (TNC) of 4 independent samples were calculated from passages 2 to 8, as described in materials and methods section 3.1.3.

As shown in figure 4.2, after 8 passages, the MSCs analysed had doubled their population by 18.77 ± 1.77 times (Fig.4.2-B), during which the observed mean generation time (GT) was between 1.02 ± 1.46 and 1.66 ± 0.48 days and no statistically significant differences were found in GT from passages 2 to 8 (Figure 4.2-C), as previously seen[27]. The total number of cells obtained (if no cells had been discarded until that point) during these passages was $2.17 \times 10^{12} \pm 1.25 \times 10^{12}$ cells (Fig.3.2- D). Hence, the MSCs isolated show high proliferative capacity, maintaining a short generation time from passages 2 to 8.

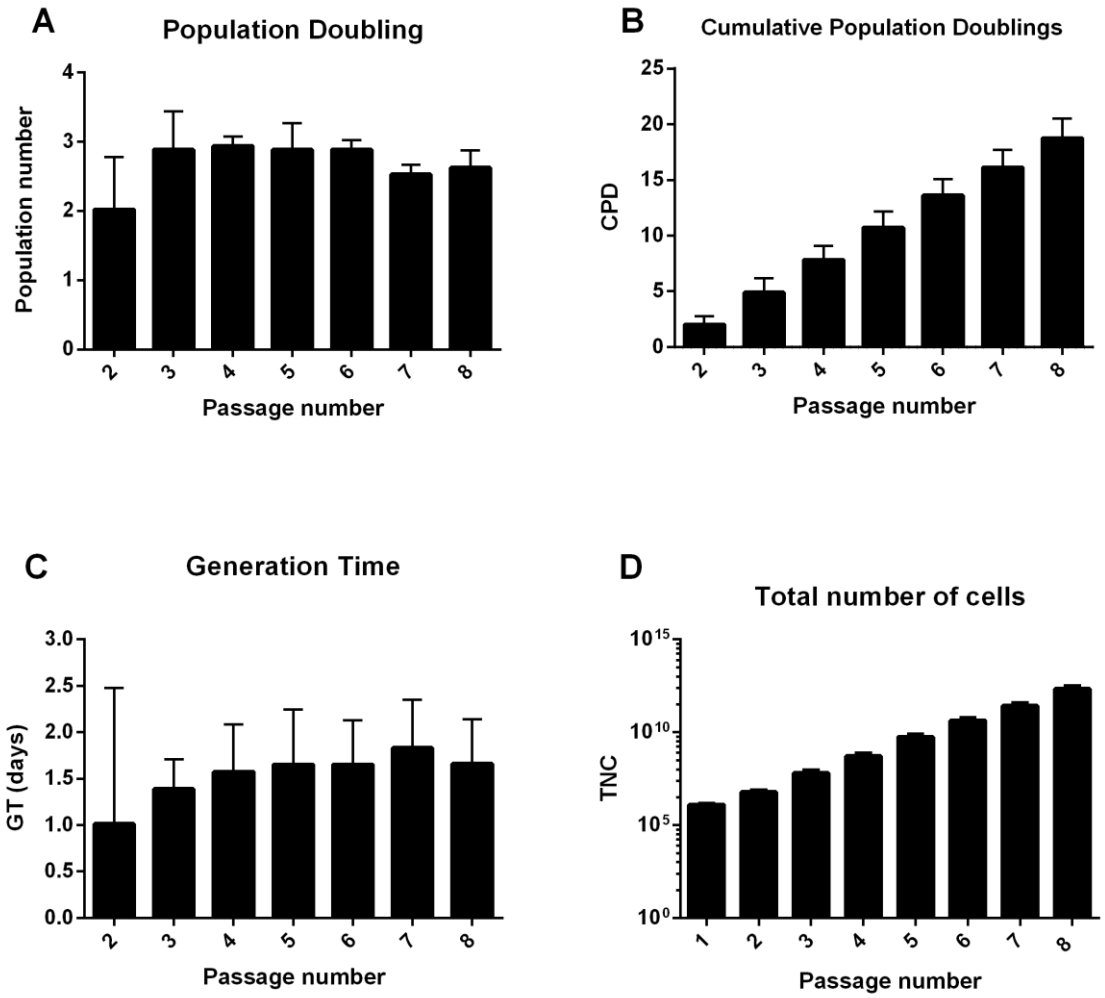

Figure 4. 2. Proliferation kinetics study of Wharton's Jelly-mesenchymal stem cells (WJ-MSCs) *in vitro*. From passage 2 (P2) onwards (inclusively), cells were plated at a fixed density of 4000 cells/cm^2, allowed to proliferate until 70-80% confluence and re-plated the same way. **(A)** The population doubling (PD) was determined using the formula X= [log10 (NH)-log10 (N1)/log10 (2). **(B)** Cumulative population doubling (CPD) was determined by adding the calculated PD to the PD levels of the previous passage. **(C)** The generation of time (GT) was calculated at passages 2 to 8, using the formula X= (log2 x Δt/ log10 (NH)-logq10 (N1). **(D)** The total number of cells (TNC) was determined at each passage (P1-P8) by cumulative counting the cells once they reached confluence of 70-80%. For each assay, 4 independent samples were used. Bars represent mean **±** SEM.

4.3. Colony forming unit-fibroblasts (CFU-F) capacity

One of these cells' characteristics have is the ability to form colonies, when seeded at low density to eliminate cell-cell interactions. In order to assess this ability, cells were seeded at a very low density, 10 cell/cm^{2,} at passages 2 and 8 and cultured for 15 days, as described in the materials and methods section 3.1.4.

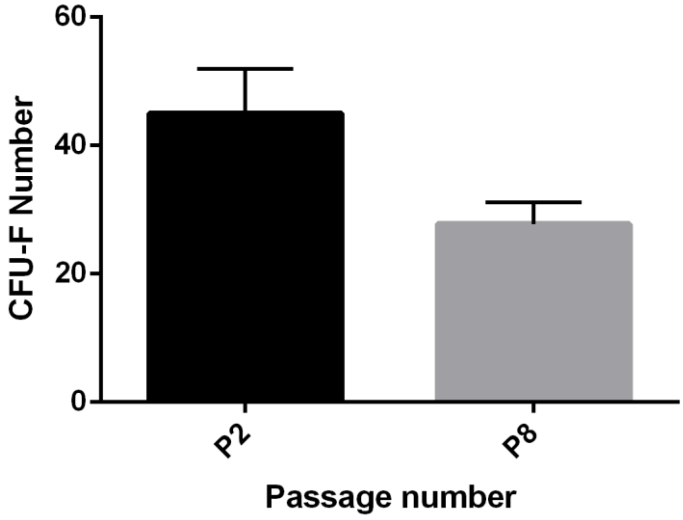

Figure 4. 3. CFU-F capacity of WJ-MSCs. The CFU-F capacity was determined by counting manually the number of colonies formed after 15 days in culture at low density of cells (10 cells/cm^2) obtained at passages 2 and 8. Results are representative of 4 independent samples. No significant differences were found performing two-tailed Mann-Whitney test. Bars represent mean ± SEM.

As shown in figure 4.3, the four samples have great ability of forming colonies at passages 2 and 8 (45 ± 6.96 and 28 ± 3.43). In all samples the CFU-F capacity decreases at passage 8, although no statistically significant differences were found.

4.4. Immunophenotypic characterization of WJ-MSCs

In order to determine if the cells isolated from Wharton's jelly were *bona fide* MSCs, the expression of cell surface antigen was evaluated on 4 independent samples before the co-culture assay. The cells were cultured until subconfluency, dissociated using accutase and labelled with antibodies against cell surface markers typically used for the characterization of MSCs and analysed by flow cytometry (Table 4.1).

Table 4. 1. Summary of the flow cytometry analysis of WJ-MSCs

Positive markers	Negative markers
CD13	CD34
CD73	CD45
CD90	
CD105	

Flow cytometry analysis showed that the cells isolated from WJ were positive for CD13, CD73, CD90 and CD105 (Fig 4.4). In contrast, the cells did not expressed CD34 (hematopoietic lineage marker) and CD45 (leukocyte marker) (Fig.4.4). This analysis (Table 4.1 and Fig.4.4) show that this phenotypic profile was consistent with the MSC phenotype previously described[10,27]. CD105 marker it's not strongly positive due to the chosen fluorochrome, a different one should have been used, for example CD105 PE.

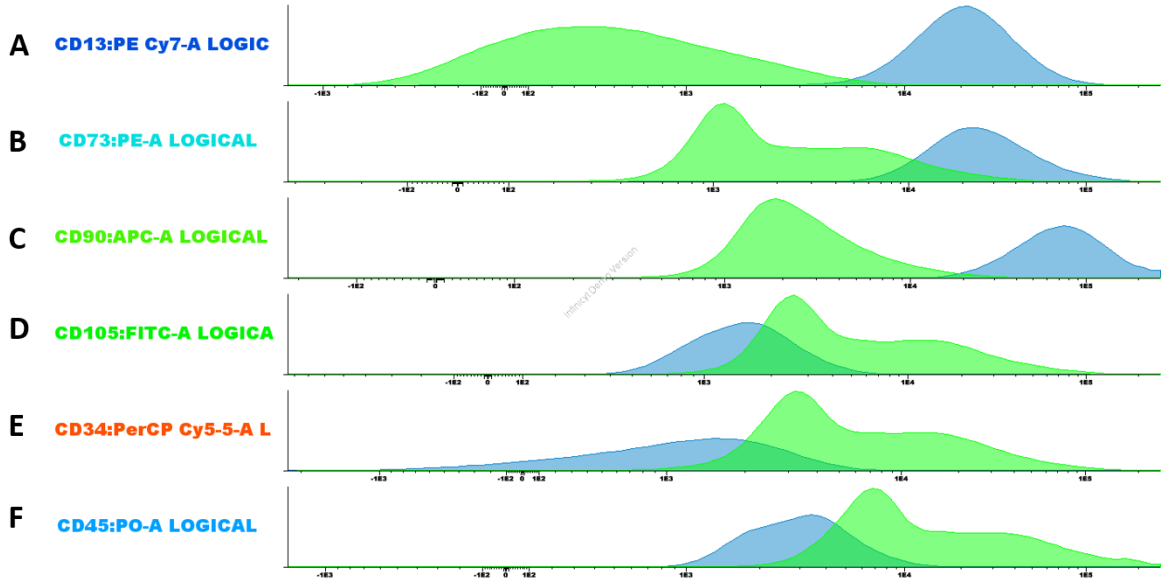

Figure 4. 4. Illustrative immunophenotypic characterization of hWJ-MSCs. Cells were dissociated using accutase, labelled with antibodies against the indicated antigens and analysed by flow cytometry. Cells were positive for CD13 (A), CD73 (B), CD90 (C) and CD105 (D) and negative for CD34 (E) and CD45 (F) (blue lines) when compared with unlabelled MSCs (green lines), as depicted in the histograms. Histograms were obtained from one sample at passage 3 and are representative of 4 independent samples at P3.

The cells obtained from the hWJ behave as MSCs, in other words, they are adherent to plastic, have high proliferative potential and CFU-F capacity, and typical immunophenotype signature of MSCs.

4.5. Multiplex cytokine analysis

MSCs produce a myriad of cytokines and in order to assess the impact of substrate stiffness on the secretory prolife of MSCs, conditioned culture medium was collected and assayed for a 8-plex panel evaluating the presence of the following analytes: GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α. The conditioned culture medium from co-culture of MSCs with MNCs after 24h in direct contact was also assayed for this same 8-plex panel.

As shown in figure 4.5 and 4.6, WJ-MSCs differentially secrete soluble factors in response to matrix compliance. It notable that MSCs isolated from hWJ secrete in high concentration IL-6 and IL-8 (as described in the literature[94,95]) and at a very low concentration IL-10 (which is also described in the literature that it is secreted by MSCs)[53,64]. MSCs showed a tendency to secret higher concentrations of IL-2, IL-4, IL-6 and IL-8 when cultured on 1:40 PDMS and 1:10 PDMS substrate than when maintained on standard tissue culture plate (TCP). Statistically significance differences were found for IL-2, IL-4 and IL-6 in cells cultured on 1:40 PDMS substrates when compared with TCP (but not between 1.10 PDMS and TCP conditions) – Figure 4.5. Despite the fact that it is described that MSCs do not secrete IL-2 or IL-4, they could be detected at a very low concentration using a BioPlex assay, which is particularly sensitive (pg/ml range).

To rule out any putative effect caused by a possibly distinct number of cells that could be contributing to the overall cytokine secretion due to eventually distinct proliferation rates on the distinct substrates, cytokine concentration was normalized to the final number of cells present in each condition, and the results obtained (Figure 4.6) were similar to the raw data, except that for IL-6 the *p* value when comparing 1.40 PDMS with TCP was equal to 0.05 and not lower (as for the raw values – Figure 4.5)

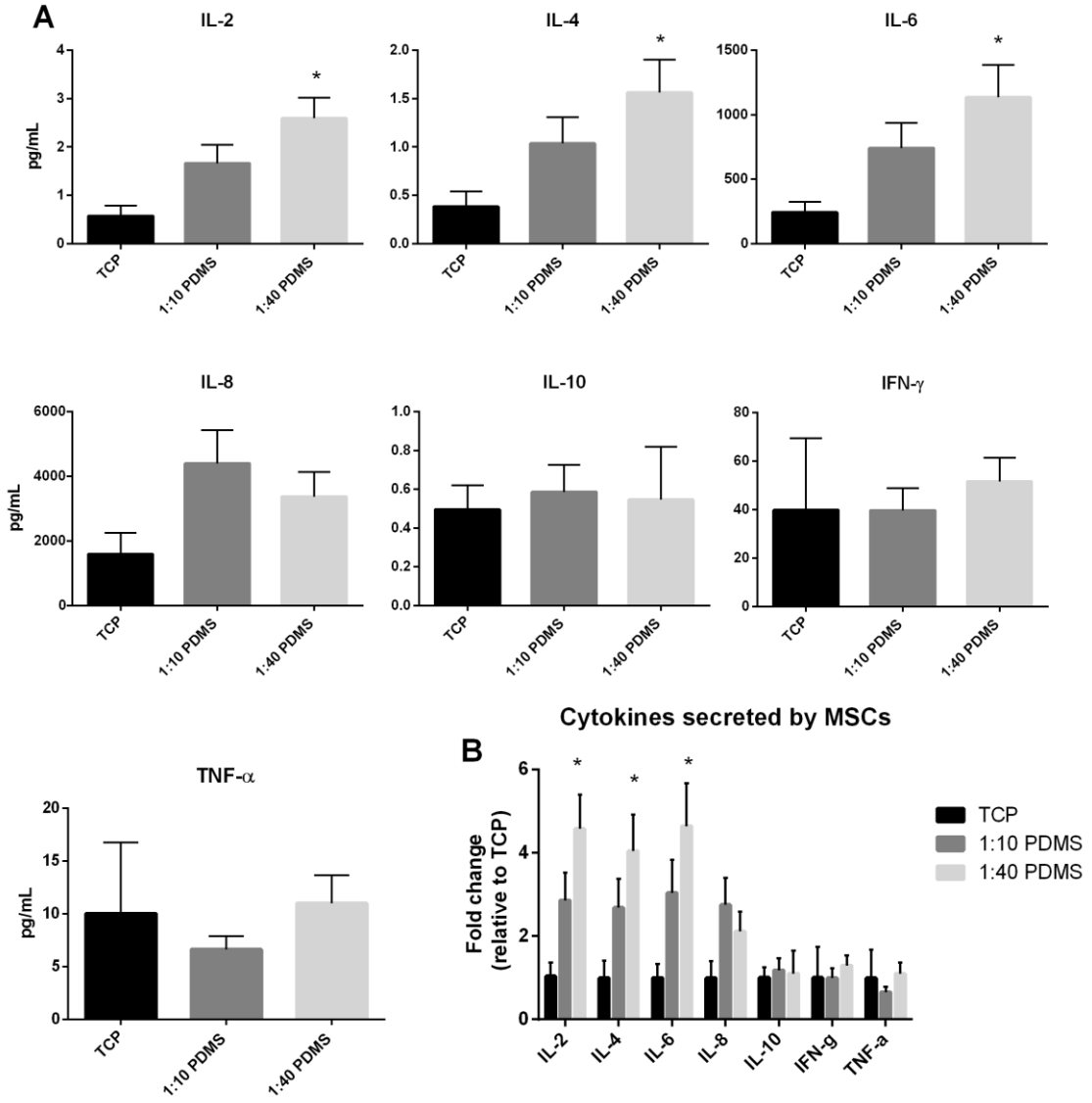

Figure 4. 5. Cytokines produced by Mesenchymal stem cells (MSCs) cultured on different substrates. Cells were plated (5 x 10^4 cells/cm^2) on respective substrates and medium was conditioned for 24h, and then collected and analyzed. **(A)** Concentration of cytokines secreted by MSC. **(B)** Fold change of concentration compared to concentration of TCP. Bars represent mean ± SEM. Results are representative of 4 independent samples for TCP and 1:40 PDMS and 3 independent samples for 1:10 PDMS. Statistically analysis was performed by Kruskal-Wallis one way ANOVA followed by Dunn's Multiple Comparison test (*< *P* 0.05). MSC, mesenchymal stem cells; IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon γ; TCP, tissue culture plate.

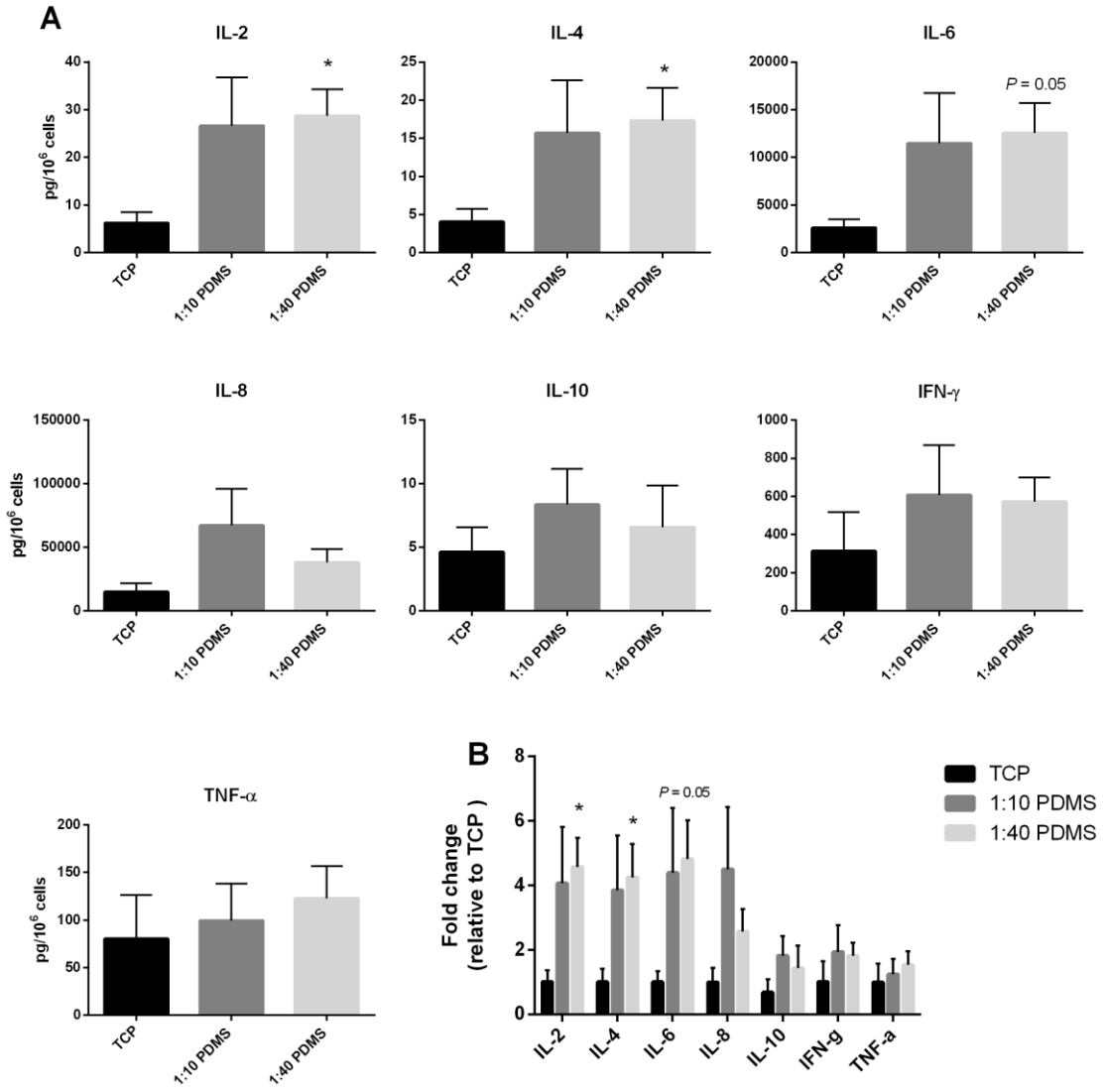

Figure 4. 6. Cytokines produced by Mesenchymal stem cells (MSCs) cultured on different substrates. Cells were plated (5 x 10^4 cells/cm^2) on respective substrates and medium was conditioned for 24h, and then collected and analysed. The amount of culture medium assayed was normalized to final cell number. **(A)** Concentration of cytokines secreted by MSC. **(B)** Fold change of concentration per million of cells compared to concentration per million of cells from TCP. Bars represent mean ± SEM. Results are representative of 4 independent experiments for TCP and 1:40 PDMS and 3 independent experiments for 1:10 PDMS. Statistically analysis was performed by Kruskal-Wallis one way ANOVA followed by Dunn's Multiple Comparison test (* P <0.05). MSC, mesenchymal stem cells; IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon γ; TCP, tissue culture plates.

Since 1:40 PDMS substrate revealed more differences in the cytokines secreted than 1:10 PDMS substrate, only 1:40 PDMS was analysed in co-cultured with MNCs during 24h without any *in vitro* stimulation, as described above in materials and methods section 3.3.

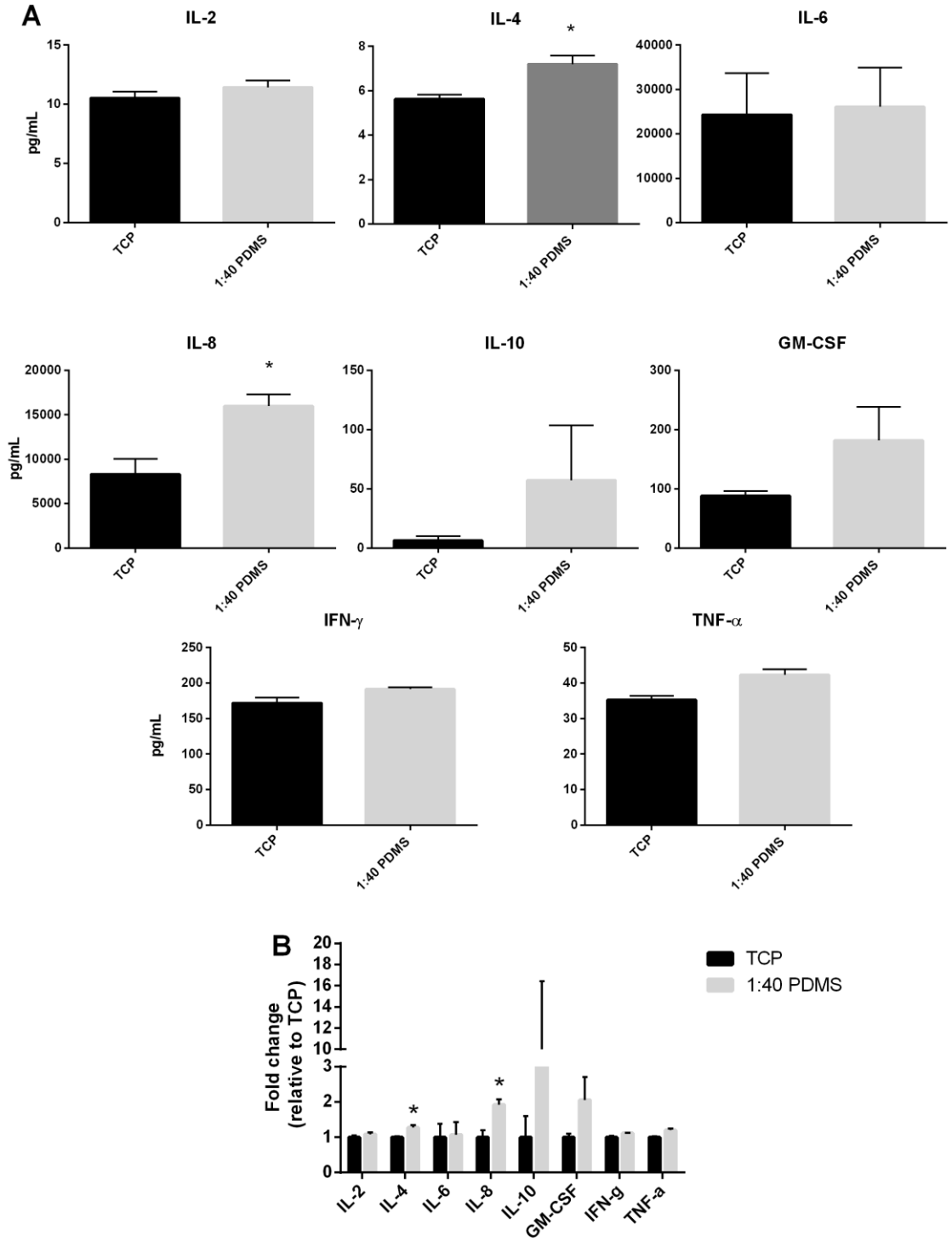

Figure 4. 7. Cytokines produced in co-cultured of mesenchymal stem cells (MSCs) with peripheral blood (MNCs). MSC previously cultured on different substrates (TCP or 1:40 PDMS, as indicated) were co-cultured with MNCs during 24h in direct contact without any stimulation *in vitro.* After 24h in co-cultured, conditioned culture medium was collected and analysed. **(A)** Cytokines produced after 24h in direct contact. **(B)** Fold change of cytokine concentration compared to concentration of control (TCP). Results are representative of 3 independent experiments. Statistically analysis was performed by *t*-test (* $P < 0.05$). MSC, mesenchymal stem cells; IL, interleukin; GM-CSF. Granulocyte macrophage- colony stimulator factor; TNF-α, tumour necrosis factor-α; IFN-γ, interferon γ; TCP, tissue culture plate standard.

MNCs were co-cultured with MSCs in the absence of activating stimuli. It could be observed that when MSCs had been previously maintained on 1:40 PDMS substrates, there was a significant increase of IL-4 and IL-8 secretion when compared to the condition in which MSCs had been cultured on TCP (both in case of raw data – Figure 4.7-A – or fold change relative to control – Figure 4.7-B). A similar tendency was observed for GM-CSF and IL-10 secretion, although in this case no statistically differences were found (Figure 4.7). When data was normalized to the number of cells, the same was observed (Figure 4.8). This normalization according to total cell number allowed comparing the secretory profile of MNCs alone with ttat of MNCs in co-culture with MSCs obtained from TCP or 1:40 PDMS substrates, as represented in Figure 4.9.

As shown in figure 4.9, comparing with MNCs alone when MNCs were co-cultured with WJ-MSCs a notable increased in all cytokine under analysis (except for TNF-α), Statistically significant differences were observed for the concentration of IL-2, IL-4, IL-10 and IFN-γ present in the conditioned medium when WJ-MSCs were previously cultured on 1:40 PDMS (but not TCP) and then co-cultured with MNCs, when compared with MNCs alone. These results suggest that the cytokine context of MNCs alone or in co-culture with MSCs, especially with MSCs previously cultured on 1:40 PDMS substrates is significantly different.

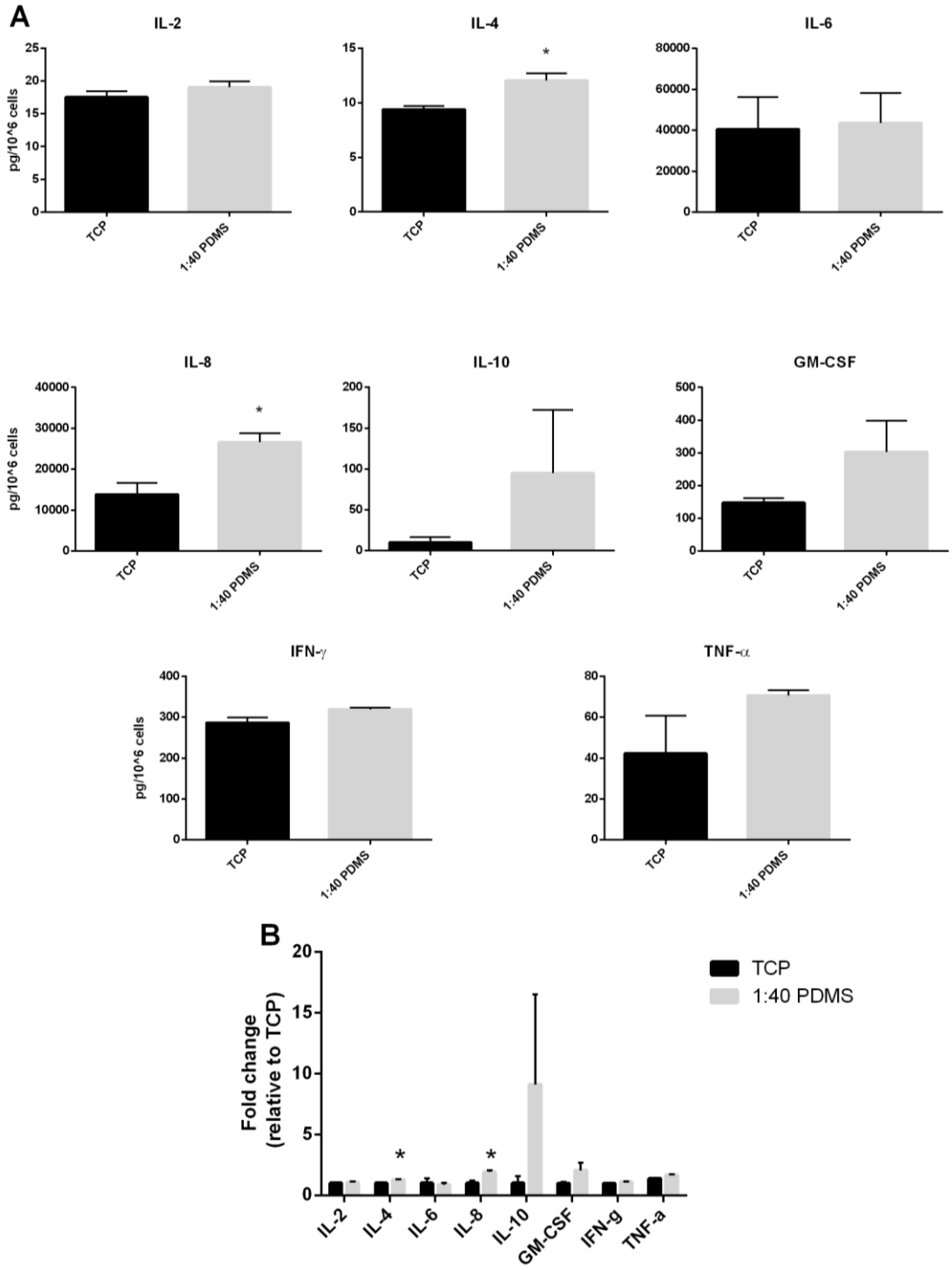

Figure 4. 8. Cytokines produced in co-culture of mesenchymal stem cells (MSCs) with peripheral blood (MNCs). MSC previously cultured on different substrates (TCP or 1:40 PDMS, as indicated) were co-cultured with MNCs during 24h in direct contact (without any stimulation in vitro). After 24h in co-cultured, conditioned culture medium was collected and analysed. The amount of culture medium assayed was normalized to the sum of cell number of MNCs and MSCs plated. **(A)** Cytokines produced after 24h in direct contact. **(B**) Fold change of cytokine concentration per million of cells compared to concentration from TCP. Results are representative of 3 independent experiments. Statistically analysis was performed by t-test (* P< 0.05).MSC, mesenchymal stem cells; MNCs, Mononuclear cells; IL, interleukin; GM-CSF. Granulocyte macrophage- colony stimulator factor; TNF-α, tumour necrosis factor-α; IFN-γ, interferon γ; TCP, tissue culture plate.

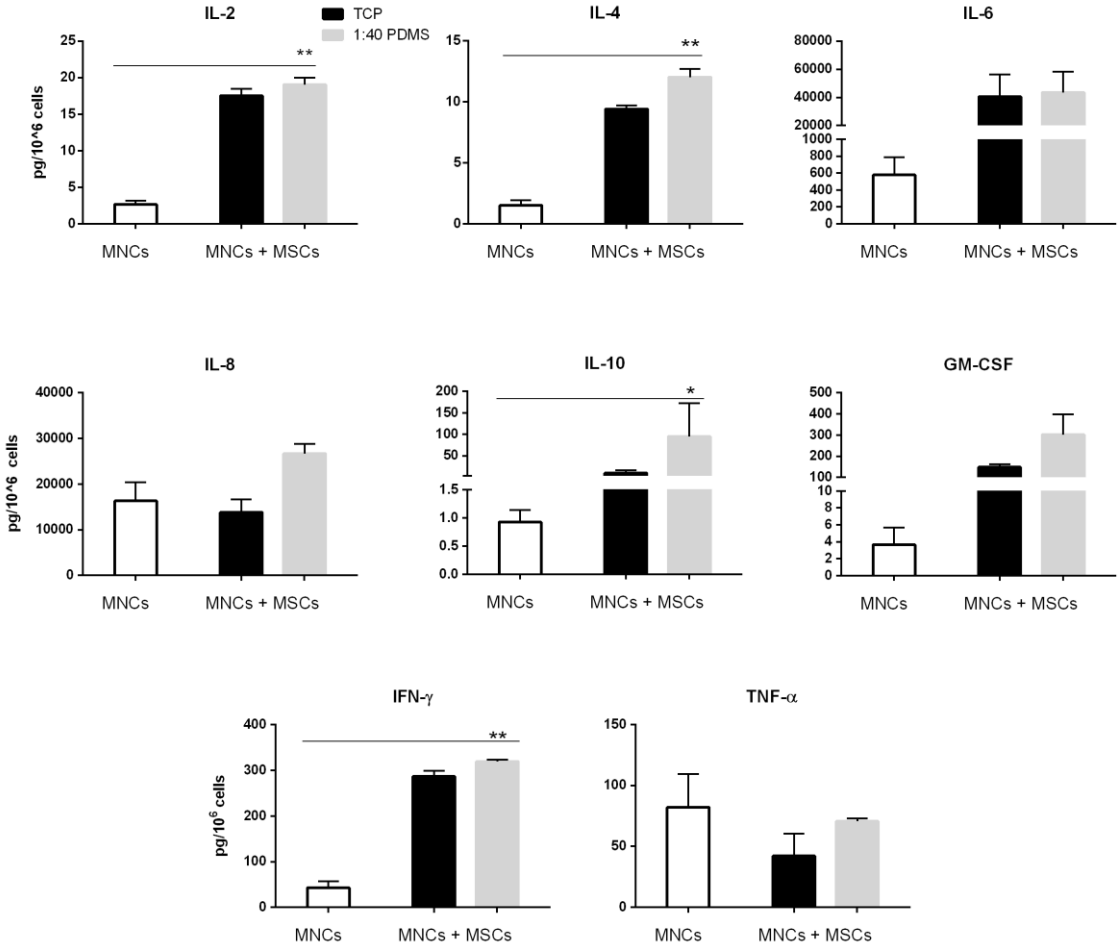

Figure 4. 9. Cytokines expressed in peripheral blood (MNCs) and in co-culture of mesenchymal stem cells (MSCs) with MNCs. MNCs were culture 24h alone without any stimulation *in vitro* and MSC previously cultured from different substrates (TCP or 1:40 PDMS , as previously indicated) were co-cultured with MNCs during 24h in direct contact without any stimulation *in vitro.* After 24h, both conditioned culture medium was collected and analysed. The amount of culture medium assayed was normalized to the sum of cell number of MNCs and MSCs plated. Results are representative of 4 independent experiments to MNCs and 3 independent experiments to MSC in co-cultured with MNCs. Statistically analysis was performed by Kruskal-Wallis one way ANOVA followed by Dunn's Multiple Comparison test (* *P* <0,05; ** P < 0,001). MSC, mesenchymal stem cells; MNCs, Mononuclear cells; IL, interleukin; GM-CSF. Granulocyte macrophage- colony stimulator factor; TNF-α, tumor necrosis factor-α; IFN-γ, interferon γ; TCP, tissue culture plate.

4.6. Co-culture of peripheral blood MNCsand MSCs and *in vitro* with Phorbol myristate acetate (PMA) plus ionomycin

To the best of our knowledge, no study to date investigated and compared the influence of MSC cultured on different substrates over the peripheral blood T cells subpopulations. Several studies have demonstrated that MSCs have immunoregulatory effects on T cells (although few studies have studied the different T cell compartment) and in order to better understand how MSC cultured on different substrates regulate the immune function of the different T cell compartments [naïve, central memory (CM), effector memory (EM) and effector cells]. To investigate that, PMA+ ionomycin-stimulated MNCs were cultured in absence or presence of MSCs previously cultured on the different substrates (TCP, 1:10 PDMS or 1:40 PDMS). The control consisted on stimulated MNCs alone and stimulated MNCs + MSCs cultured on tissue culture plates (TCP). In order to identify the four abovementioned compartments among CD4+ and CD8+ T cells, 8-color mAb combinations was used (described in materials and methods 3.3.3 section) to analysed the protein expression of IL-2 and TNF-α within each cell compartment. The results obtained are preliminary, consisting of a limited number of independent experiments and for that reason, the interpretation of the results will also be based on the tendencies observed so far, with no possible statistical analysis.

The functional compartments of $CD4^+$ and $CD8^+$ T cells (imunophenotypically characterized as $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$, respectively) were identified according to their differential expression of CD45RA and CD27, as follows: naïve T cells were characterized by $CD45RA^+$ $CD27^+$ expression, CM T cells by $CD45RA^-$ $CD27^+$, EM and effector T cells by $CD45RA^-$ $CD27^-$ and $CD45RA^+$ $CD27^-$, respectively.

As shown in figure 4.10 and 4.11, the presence of WJ-MSCs decrease the percentage of both $CD4^+$ and $CD8^+$ T cells expressing IL-2 or TNF-α (after stimulation of the MNCs with PMA + ionomycin) in the distinct compartments analysed. The co-cultures of MNCs with WJ-MSCs derived from distinct substrates led to different levels of decreased percentage of both $CD4^+$ and $CD8^+$ T cells expressing IL-2 (Figure 4.10), but not in case of TNF-α (Figure 4.11), in which the MSCs obtained from distinct substrate appear to produce the same inhibitory effect. In detail, MSCs previously cultured on 1:10 or 1:40 PDMS

substrates seems to have a higher inhibitory effect over the percentage of IL-2 expressing $CD4^+$ or $CD8^+$ cells than MSCs cultured on TCPs (Figure 4.10). As shown in figure 4.10, WJ-MSCs derived from both PDMS substrate (1:10 or 1:40) seem to decrease more the percentage of $CD4^+$ T cells expressing IL-2 in all compartments – showing a higher tendency for immunosuppression, namely in case 1:40 PDMS - except for the naïve functional compartment, where the results are comparable with WJ-MSCs derived from TCP. Regarding $CD8^+$ T cells, WJ-MSCs derived from the PDMS substrates decreased the percentage of cells expressing IL-2 in the naïve, central memory, effector memory and effector functional to a greater extent than MSCs cultured from TCP (Figure 4.11). In the effector functional compartment $CD4^+$ T cells there was no detection of IL-2 or TNF-α positive cells, and for that reason, that functional compartment is not represented (Figure 4.10).

Concerning TNF-α, figure 4. 11 showed that the presence of WJ-MSCS decreased the percentage of cells expressing this cytokines in all functional compartments in both $CD4^+$ and $CD8^+$ T cells.Comparing the effect of MSCs obtained from the distinct susbtrates, all seem to present similar immunosuppression in all compartments and in $CD4^+$ and $CD8^+$.

In order to evaluate the soluble factors (alone) secreted by WJ-MSCs influence MNCs, the latter were culture and then stimulated in the presence of MSC-conditioned medium (as described in materials and methods section 3.4.2). As shown in figure 4.12 no effect seems to have to occured in the percentage of IL-2 producing in $CD4^+$, although a slight decrease was observed in the amount of protein per cell (measured by mean fluorescence of intensity - MFI) except for the naïve functional compartment. Concerning the percentage of IL-2 producing in $CD8^+$ T cells there is no decrease observed in any functional compartment. Only in the amount of protein produced per cells (refelected by IL-2 MFI) a decrease was observed in all compartments except for effector $CD8^+$ T cells. Concerning the percentage of TNF-α, producing cells, a slight decreasedwas observed in all the functional compartment, except for naïve compartment of both $CD4^+$ and $CD8^+$ T cells, with the same trend in terms of amount protein per cells (MFI-) – Figure 4.13.

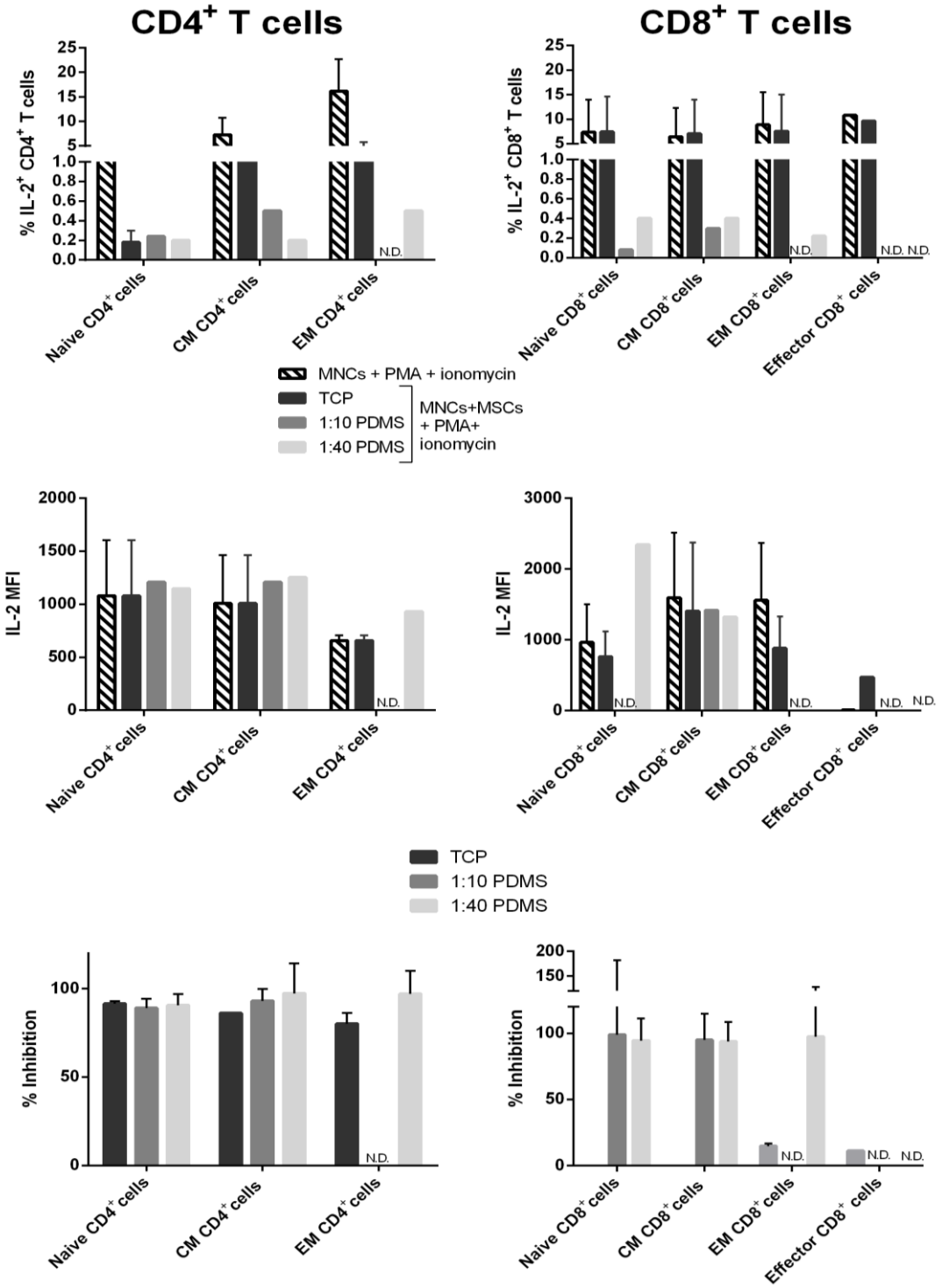

Figure 4. 10. Percentage of CD4⁺ and CD8⁺ T cells expressing Interleukin-2 (IL-2) determined by flow cytometry. MNCs were stimulated with PMA and ionomycin (MNCs + PMA + ionomycin) in the absence or presence of WJ-MSCs derived from different substrates, as indicated. The upper panels show the percentage of CD4⁺ and CD8⁺ T cells, producing IL-2, distributed among their functional compartments. The middle panels depicts the mean fluorescence intensity (MFI) of the signal, correlating with the amount of IL-2 expressed per cell. The lower panel represents the percentage of inhibition induced by WJ-MSCs on the distinct functional compartment of CD4⁺ and CD8⁺ T cell expressing IL-2. Results are representative of 2 independent experiments for TCP and 1:40 PDMS and 1 independent experiment for 1:10 PDMS. Bars represent mean ± SEM. CM, central memory; EM, effector memory; N.D., not determined)

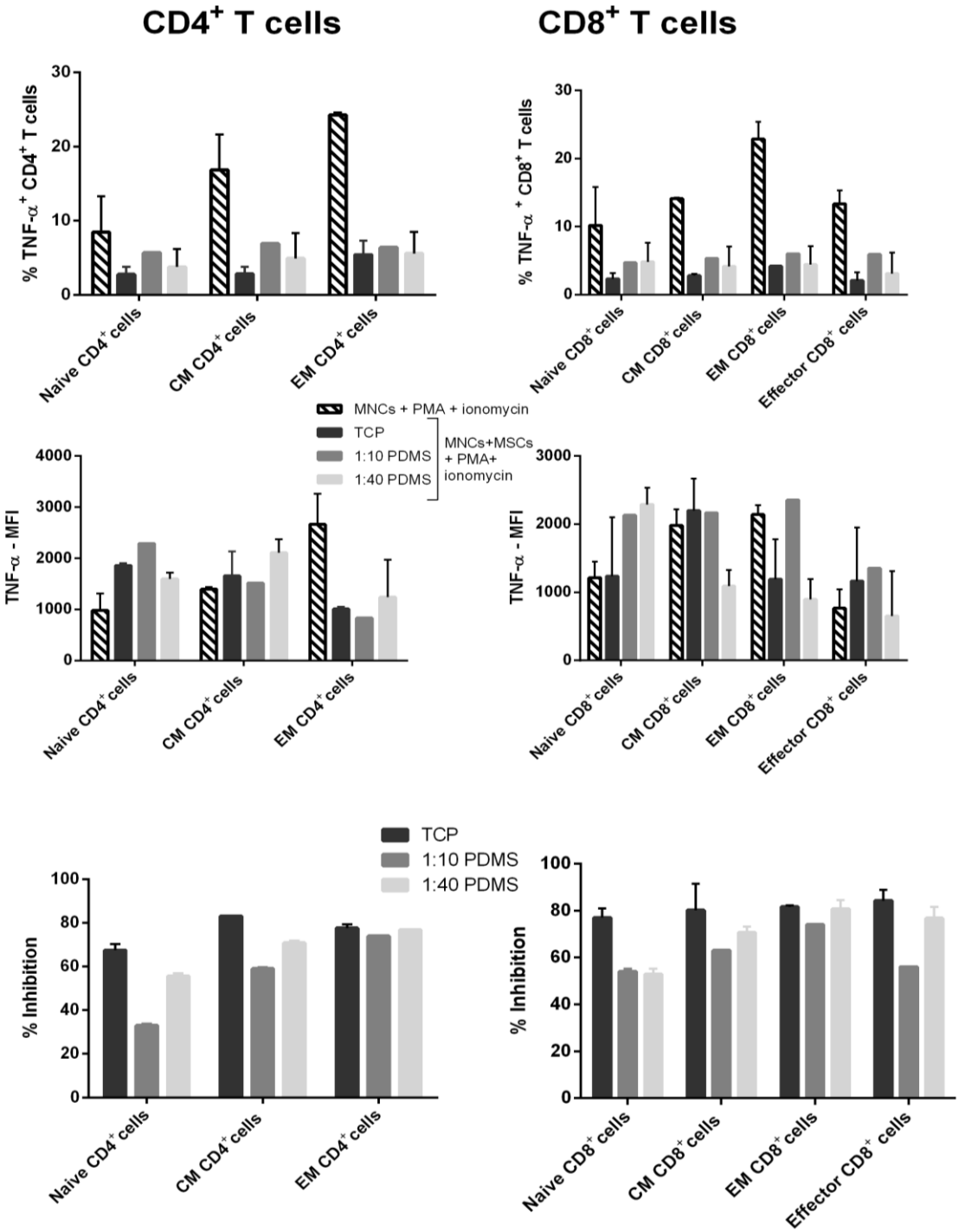

Figure 4. 11. Percentage of CD4⁺ and CD8⁺ T cells expressing Tumour necrosis factor- alpha (TNF-α) determined by flow cytometry. MNCs were stimulated with PMA and ionomycin (MNCs + PMA + ionomycin) in the absence or presence of WJ-MSCs derived from different substrates, as indicated. The upper panels show the percentage of CD4⁺ and CD8⁺ T cells, producing TNF-α, distributed among their functional compartments. The middle panels depicts the mean fluorescence intensity (MFI) of the signal, correlating with the amount of TNF-α expressed per cell. The lower panel represents the percentage of inhibition induced by WJ-MSCs on the distinct functional compartment of CD4⁺ and CD8⁺ T cell expressing TNF-α. Results are representative of 2 independent experiment for TCP and 1:40 PDMS and 1 independent experiment for 1:10 PDMS. Bars represent mean ± SEM. CM, central memory; EM, effector memory; N.D., not determined

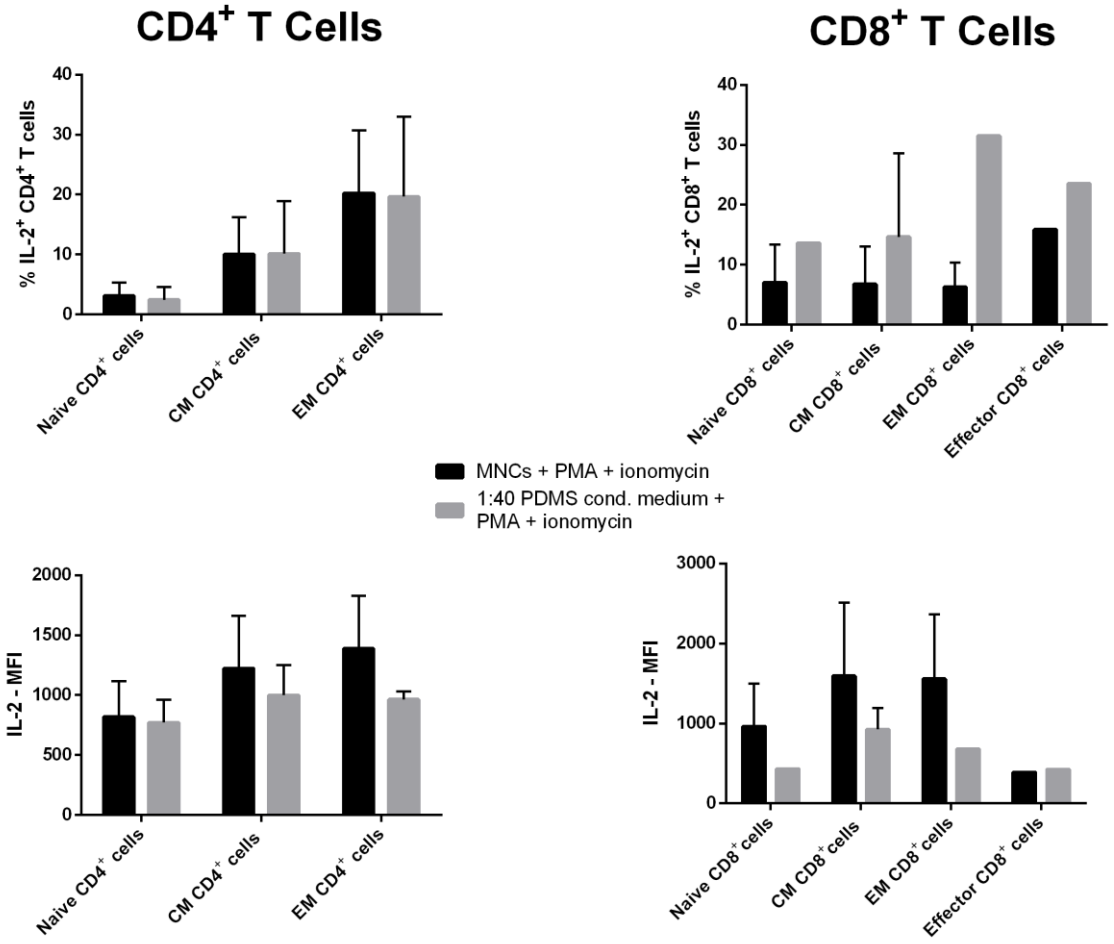

Figure 4. 12. Percentage of CD4$^+$ and CD8$^+$ T cells expressing Interleukin-2 (IL-2) determined by flow cytometry. MNCs were stimulated with PMA and ionomycin (MNCs + PMA + ionomycin) in the absence or presence of MSC-conditioned medium derived from different substrates, as indicated. The upper panels show the percentage of CD4$^+$ and CD8$^+$ T cells, producing IL-2, distributed among their functional compartments. The middle panels depicts the mean fluorescence intensity (MFI) of the signal, correlating with the amount of IL-2 expressed per cell. The lower panel represents the percentage of inhibition induced by MSC-conditioned medium on the distinct functional compartment of CD4$^+$ and CD8$^+$ T cell expressing IL-2. Results are representative of 2 independent experiments. Bars represent mean ± SEM. CM, central memory; EM, effector memory;

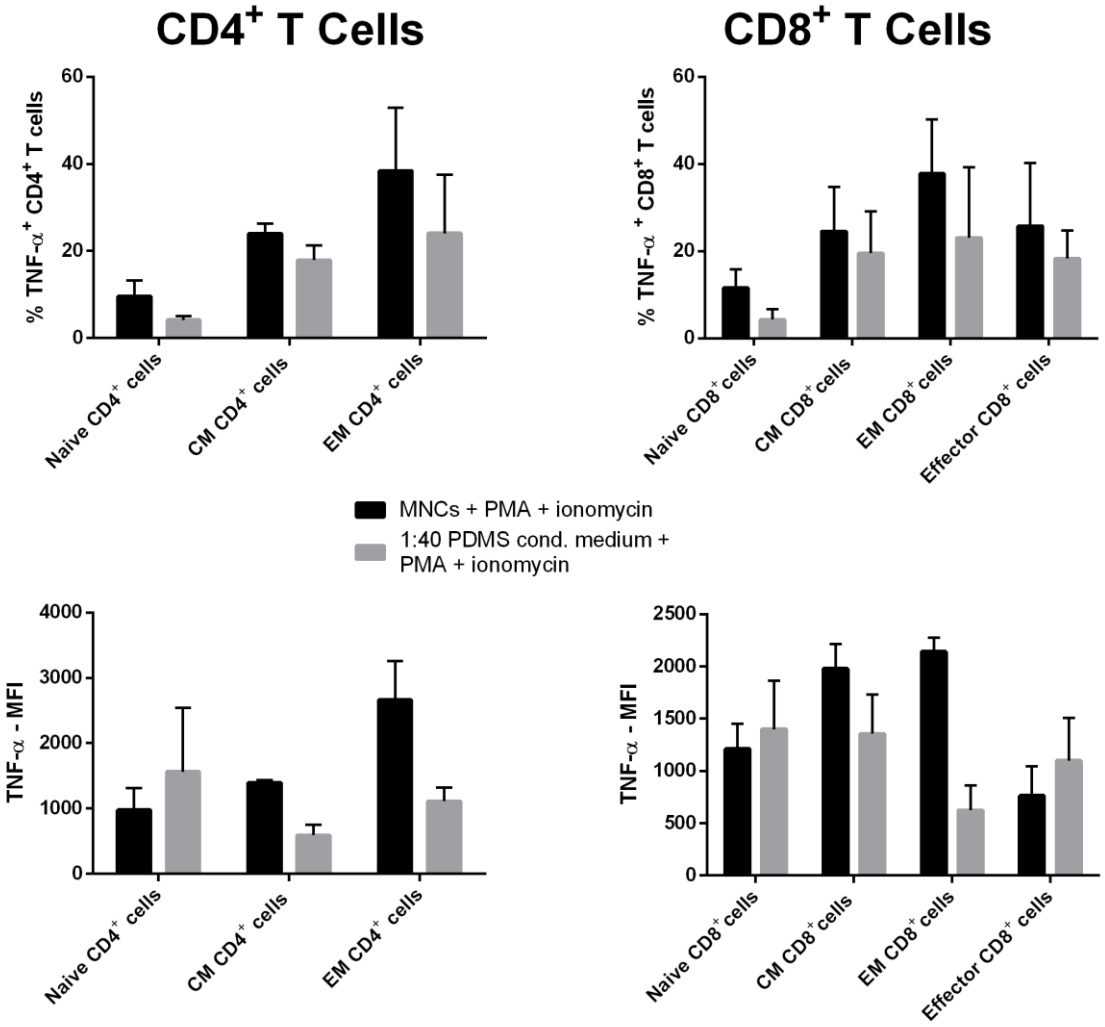

Figure 4. 13. Percentage of CD4⁺ and CD8⁺ T cells expressing Tumor necrosis factor-alpha (TNF-α) determined by flow cytometry. MNCs were stimulated with PMA and ionomycin (MNCs + PMA + ionomycin) in the absence or presence of MSC-conditioned medium derived from different substrates, as indicated. The upper panels show the percentage of CD4⁺ and CD8⁺ T cells, producing TNF-α, distributed among their functional compartments. The middle panels depicts the mean fluorescence intensity (MFI) of the signal, correlating with the amount of TNF-α expressed per cell. The lower panel represents the percentage of inhibition induced by MSC-conditioned medium on the distinct functional compartment of CD4⁺ and CD8⁺ T cell expressing TNF-α. Results are representative of 2 independent experiments. Bars represent mean ± SEM. CM, central memory; EM, effector memory;

4.7. Co-culture of peripheral blood MNCs and MSC *in vitro* with LPS plus IFN-γ

To the best of our knowledge, no study to date investigated and compared the influence of MSC cultured on different substrates over the peripheral blood monocytes population. In order to better understand if MSC cultured on distinct substrates regulated the immune function of monocytes, LPS+IFN-γ-stimulated MNCs were cultured in absence or presence of WJ-MSCs cultured on the distinct substrates (TCP, 1:10 and 1:40 PDMS). The control consisted of stimulated MNCs alone and stimulated MNCs + MSCs previously cultured on tissue culture plated (TCP). An 8-color mAb combinations was used (described in materials and methods 3.3.3 section) to analyse the percentage of TNF-α cells. The results obtained are preliminary, consisting of a limited number of independent experiments and for that reason, the interpretation of the results will also be based on the tendencies observed so far, with no possible statistical analysis.

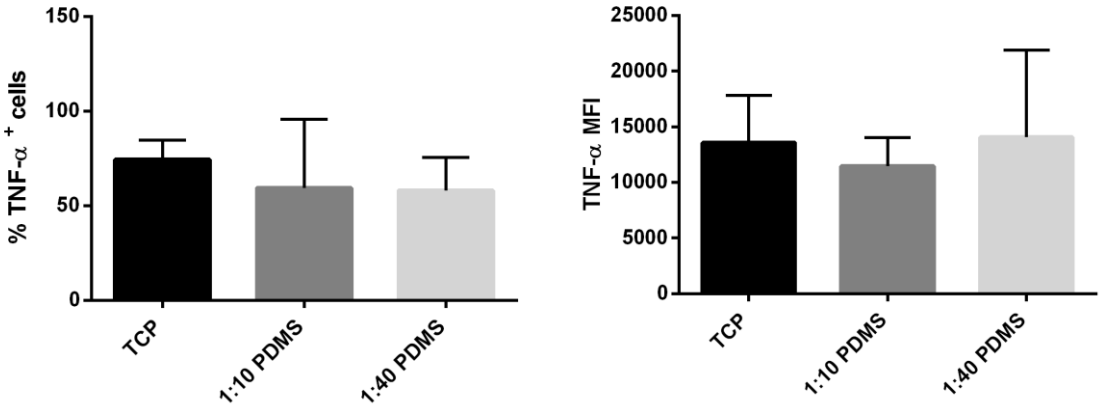

Figure 4. 14. Production of TNF-α by stimulated monocytes with LPS plus IFN-γ, under the following conditions: MSCs derived from different substrates co-cultured with MNCs and *in vitro* stimulation (A) Percentage of cells producing TNF-α for activated monocyte with LPS plus IFN-γ. (B) Amount of protein expressed per cell, measured as mean fluorescence intensity (MFI) for activated monocyte with LPS plus IFN-γ. Results are representative of 3 independent experiments for TCP and 1:40 PDMS substrates and two independent experiments for 1:10 PDMS substrates. No significant differences were found performing Kruskal-Wallis one way ANOVA followed by Dunn's Multiple Comparison test (compared with TCP). Bars represent mean ± SEM.

As shown in figure 4.14, the WJ-MSCs derived from TCP co-cultured with MNCs with in vitro stimulation with LPS + IFN-γ showed a very slight decrease in percentage of cells producing TNF-α (74.4 ± 10.2) comparing with the PDMS substrates - 1:10 PDMS (59.4 ± 39.4) and 1:40 PDMS (58.1 ± 17.5).

Observing the figure 4.15 that corresponds to monocytes cultured and stimulated in presence of MSCs-conditioned medium, the CM obtained from TCP –derived MSCs showed less percentage of cells producing TNF-α (50.2 ± 47.1) compared with the 1:10 PDMS and 1:40 PDMS, (77.7 ± 11.7) and (59.5 ± 25.7), respectively. Observing figure 4.16, monocytes cultured and stimulated in presence of MSCs-conditioned medium appear to not have any differences in the producing of TNF-α.

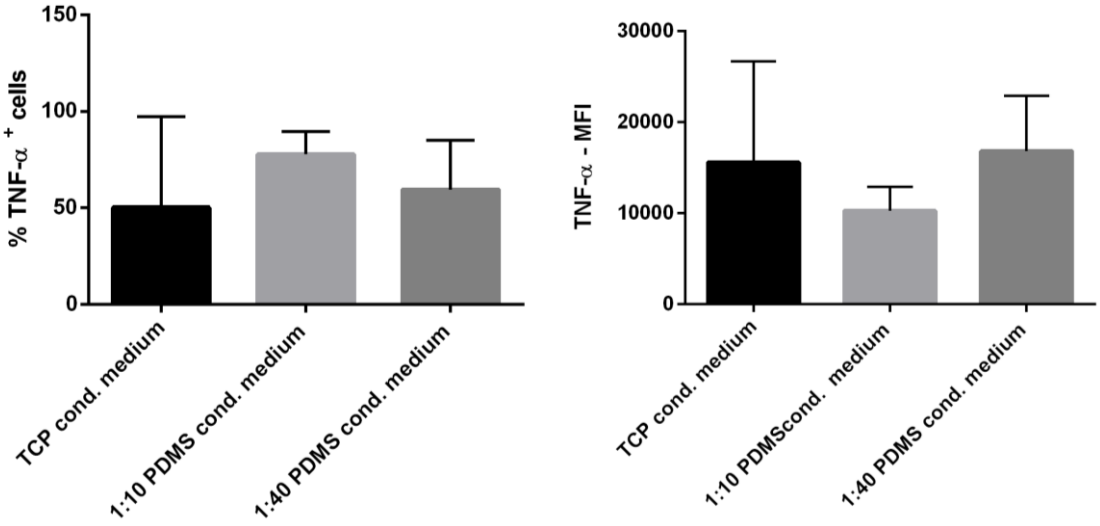

Figure 4. 15. Production of TNF-α by monocytes stimulated with LPS plus IFN-γ, under the following conditions: MSC-conditioned medium derived from different substrates co-cultured with MNCs and *in vitro* stimulation (A) Percentage of cells producing TNF-α for activated monocyte with LPS plus IFN-γ. **(B)** Amount of protein expressed per cell, measured as mean fluorescence intensity (MFI) for activated monocyte with LPS plus IFN-γ. Results are representative of 3 independent experiments for 1:40 PDMS MSC-conditioned medium and 2 independent experiments for TCP MSC-conditioned medium and 1:10 PDMS MSC-conditioned medium. Bars represent mean ± SEM.

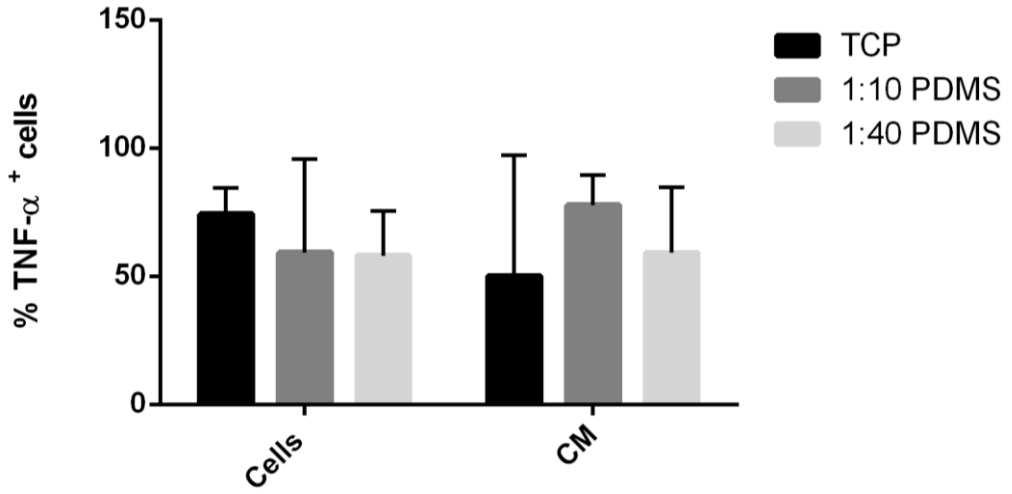

Figure 4. 16. Production of TNF-α by monocytes stimulated with LPS plus IFN-γ co-culture with MSCs derived from different substrates and the respective MSCs-conditioned medium. Percentage of cells producing TNF-α. Results are representative of 3 independent experiments for TCP and 1:40 PDMS substrates and 2 independent experiments for 1:10 PDMS substrates and 3 independent experiments for 1:40 PDMS MSC-conditioned medium substrates and 2 independent experiments for TCP MSC-conditioned medium and 1:10 PDMS MSC-conditioned medium. Bars represent mean ± SEM. CM, Conditioned medium.

Chapter 5- Discussion

5. Discussion

Human Wharton's Jelly mesenchymal stem cells (hWJs-MSCs) were isolated with 100% efficiency and the cells exhibited low generation time and proliferated readily up to at least 8 passages – P8 (Figure 4.2 A-D), reaching a total number of cells over $1x10^9$ at P5. This number of passages could be further lowered to reach this number of cells, which is well above what is commonly considered to be therapeutic dose - of at least 2×10^6 MSCs/kg of body weight for infusion - by increasing the amount of Wharton's Jelly fragments, since only a small part of each umbilical cord sample was processed and used. In terms of clinical applications, it is not possible to use of MSCs without previous *ex vivo* expansion, in order to achieve the necessary therapeutic dose[42,91]. Notably, the generation time did not increase significantly when cells reach P8, corresponding about 22 cumulative population doublings (Figure 4.2- A and C), which indicate that the WJs-MSCs isolated did not reach senescence until this number of duplications, in agreement with literature that revealed that WJ-MSCs could be kept in proliferative conditions *in vitro* until approximately 33 cumulative population doublings (33.7 ± 2.1)[96] before entering replicative senescence. The cells demonstrated also to be able to form colonies with similar frequency with no statistically significant differences at passages 2 and 8 (Figure 4.3), which indicates that the cells were proliferative, maintaining stemness for at least 8 passages. The efficiency of CFU-F of these cells at P2 and P8 was 45 ± 6.96 and 28 ± 3.43 respectively, which is consistent with the efficiency described in the literature by Hou *et al*. for these cells, 35.2 ± 2.69.

The immunophenotypic characterization of WJ-MSCs , together with proliferation kinetics, added to the fact that cells were adherent to plastic, displayed fibroblast-like morphology and presented CFU-F capacity , validated their genuine MSC identity. The immunophenotype analysis showed that the cells were positive for CD13, CD73, CD90 and CD105 (Figure 4.4 A - D) and lacked of expression of CD34 and CD45 (Figures 4.4 E ,F) as expected[10].

MSCs secrete several soluble factors, such as cytokines, growth factors and extracellular matrix molecules that play and important role in the regulation of

haematopoiesis and in immune and inflammatory response. These soluble factors have been proposed as one of the key of MSCs' therapeutic versatility[94,97]. The results obtained by multiplex cytokine analysis revealed a tendency in both conditions analyzed - WJ-MSCs alone and WJ-MSCs co-cultured with MNCs cells without any stimulation *in vitro* - showing that WJ-MSCs cultured on 1:40 PDMS substrates had higher secretion of cytokines than those maintained on tissue culture plates (TCP). WJ-MSCs secretory profile demonstrated significant higher secretion for IL-2, IL-4 and IL-6 comparing with TCP (Fig.4.5 and 4.6). The secretion of IL-6 and IL-8 observed, corroborates with previous studies reporting this feature[53,94]. IL-8 is associated with innate immune responses during which it induces chemotaxis and IL-6 is a multifunctional cytokine that plays a central role in host defense due to its wide range of immune and hematopoietic activities. IL-4 and IL-2 secretion by MSCs were significant higher in WJ-MSCs cultured on 1:40 PDMS comparing with TCP. To our best knowledge, this observation has not yet been described in the literature, which might be due to the sensitive analysis performed which allows detection of these cytokines. Furthermore, to confirm IL-4 secretion by WJ-MSCs an mRNA expression analysis could be done. IL-4 has an important role in regulating antibody production, haematopoiesis and inflammation, and the development of effector T-cell response.

When WJ-MSCs cultured on different substrates were co-cultured with MNCs a tendency was observed, WJ-MSCs previously cultured from 1:40 PDMS substrates co-cultured with MNCs showed a tendency for increased secretion of several cytokines, in particularly a significant increase for IL-4 and IL-8 (Fig.4.7 and 4.8) when compared with co-cultures of MNCs with MSCs kept on TCP.

Additionally, as shown in figure 4.9, by comparing the concentration of cytokines secreted (normalized to the total number of cells), between MNCs alone and MNCs in co-culture with WJ-MSCs, there was notable increase of all cytokines under analysis, with the exception for TNF-α. Statistically significant differences were observed for the concentration of IL-2, IL-4, IL-10 and IFN-γ present in the co-culture medium when WJ-MSCs were previously cultured on 1:40 PDMS (but not TCP) when compared with MNCs alone (Figure 4.9). These results suggest that the cytokine context of MNCs alone or in co-culture with MSCs previously cultured on 1:40 PDMS substrates is significantly different. Although

these experiments did not allow to identify subpopulations of cells (MSCs or MNCs) responsible for these differences, we may speculate (based on existing literature) that the increase observed might be due to the presence of MNCs that could be enhancing the secretion of the cytokines analysed.

MSCs have an immunomodulatory capability and low immunogenicity which make them attractive for the clinical applications, namely for immune system disorders candidates for MSCs cell therapy like of Graft versus host disease, autoimmune disorders, among others. These pathologies are heterogeneous in what concerns to the distribution of T cells among their functional compartments. MSCs are able to inhibit T and B cells and natural killer (NK) cells, induce of regulatory T cells and inhibition of antigen presenting cells. MSCs immunomodulation seems to be mediated by the secretion of soluble factors and direct contact, creating an immunosuppressive microenvironment.

$CD4^+$ and $CD8^+$ T cells have different functional compartments naturally occurring in the peripheral blood of healthy individuals. The central memory preferentially mounts recall responses to antigens. Even though these cells lack immediate effector functions, they rapidly proliferate and differentiate into effector T cells. Effector memory cells provides immediate protection upon antigen challenge through the rapid production of effector cytokines. The functional compartments were identified within $CD4^+$ and $CD8^+$ T cells populations by flow cytometry, based on their differential expression of CD45RA and CD27 and the production of pro-inflammatory cytokines - IL-2 and TNF-α - was evaluated within each cell functional compartment.

Our preliminary results showed that, in co-culture with MNCs (stimulated with PMA + ionomycin), WJ-MSCs induce a generalized decrease of the percentage of $CD4^+$ and $CD8^+$ T cells expressing IL-2 and TNF-α (Figure 4.10). These observations are in agreement with previous studies reporting that MSCs have immunosuppressive abilities and induce a decrease of the percentage of T cells producing these cytokines *in vitro* experiments and *in vivo* (at the mRNA level)[35,98]. Analysing within each T cell functional compartment, it seems that WJ-MSCs previously cultured on distinct substrates induce different levels of T cell inhibition. MSCs obtained from PDMS substrates and then co-cultured with MNCs revealed a higher tendency to downregulate the percentage of IL-2 expressing $CD4^+$ and

$CD8^+$ T cells in all functional compartments as compared with MSCs cultured on TCP, except for naïve functional compartment $CD4^+$ T cells compared with TCP. This decrease is more pronounced among $CD4^+$ T cells, where the effect was the same (Figure 4.10). This decrease was also more pronounced among $CD4^+$ than $CD8^+$ cells (Fig.4.10). WJ-MSCs cultured on 1:40 PDMS, showed a 97% percentage of inhibition in CM and EM memory $CD4^+$ T cells.

Concerning TNF-α expressing T cells, WJ-MSCs induced a decrease of the percentage of $CD4^+$ and $CD8^+$ T cells expressing this cytokine in all functional compartments of both cell types (figure 4.11). In contrast with what was observed for IL-2, MSCs obtained from TCP or PDMS substrates seem to produce a similar immunosuppressive effect in terms of TNF-α expression. These observations suggest that distinct mechanism may govern the modulation of IL-2 and TNF-α positive T cells by MSCs. Overall, our results suggest that MSCs pre-cultured on 1:10 or 1:40 PDMS present an increased immunosuppressive ability towards $CD4^+$ and $CD8^+$ T cells, which is a novel and important observation in the field. Further studies must be performed in order to validate these results and eventually assess the relevance of these observations using an *in vivo* model.

Several in vitro studies have shown that the immunosuppressive effect of MSCs is sustained in trans-well systems, suggesting that soluble factors are responsible for such inhibition, while others studies claimed that a direct cell contact is required, which may be due to the use of different systems by the individual research groups[35,99]. In our experimental setup, the preliminary results obtained from WJ-MSCs conditioned medium from different substrates co-cultured with MNC (stimulated with PMA + ionomycin) did not showed a decrease in the percentage of IL-2 produced by $CD4^+$ and $CD8^+$ T cells (Figure 4.12). Concerning to TNF-α, a very slight decreased was observed in all the functional compartments (except for naïve compartment) of both $CD4^+$ and $CD8^+$ T cells in terms of percentage and amount of protein (MFI) per cells (Figure 4.13). These preliminary results could indicated that a cell-cell contact is important and needed for MSCs immunosuppression, since the conditioned medium seemed to induce only a partial suppression of T cells as observed by a decreased expression of TNF-α, but not IL-2. Further experiments need to be done to elucidate this aspect, namely by performing assays directly comparing the effect between MSC-conditioned medium – in which there was no

communication between MSCs and MNCs – and the trans-well system setup, in which soluble factors are allowed to pass and establish communication between the distinct cell types. In future studies will also be interesting to evaluate mRNA expression in MSCs and purified $CD4^+$ and $CD8^+$ T cells.

The preliminary results obtained with WJ-MSCs cultured on distinct substrates and then co-cultured with MNCS *in vitro* stimulated with LPS + IFN-γ did not revealed differences concerning the producing of TNF-α when comparing between WJ-MSCs obtained from different substrates (Figure 4.14 and 4.16). The same was observed when using only conditioned medium obtained from MSCs cultured on the distinct substrates (Figures 4.15 and 4.16).

Taking together, the results obtained in this study suggest that elements related to mechanotransduction – namely substrate stiffness and ECM-protein composition – seem to influence the profile of cytokines secreted by WJ-MSCs, as well as their immunomodulatory ability towards $CD4^+$ and $CD8^+$ T cells.

Chapter 6- Conclusion

6. Conclusion

Our results reveals that secretory profile of WJ-MSCs cultured on distinct substrates - which include differences at the level of stiffness and ECM-protein composition – is modulated by those biophysical and biochemical elements, leading to a differential expression profile of distinct cytokines. This might have important implications in what concerns the therapeutic efficacy and therapeutic targets of MSCs as an emerging cell therapy. Our preliminary results also indicate that WJ-MSCs cultured on distinct substrates seems to possess different inhibitory patterns towards T cells, among the several T cell functional compartments, which may impact the outcome of MSCs cellular therapy. Overall, our results suggest that the protocols used for MSC expansion may be tuned according to the desired therapeutic application, both at the level of the secretory profile and immunomodulatory approaches. Understanding the impact of micro-environmental cues on WJ-MSCs, such as substrate stiffness and ECM protein composition, is therefore important to maximise their therapeutic effects.

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